POLITÉCNICO DE LEIRIA
ESCOLA SUPERIOR DE TURISMO E TECNOLOGIA DO MAR

Cultivation of the wild seaweed Gracilaria gracilis under laboratory scale: The Effect of Light Intensity and Nutrients on Growth, Pigment and Total Soluble Protein Content

Gonçalo Filipe Pica Cabral Bastos

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Gonçalo Filipe Pica Cabral Bastos

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Masters project carried out under the guidance of Doctor Teresa Margarida Lopes da Silva Mouga and co-supervision of Doctor Clélia Correia Neves Afonso

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For my family,

For Inês,

To the Universe

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Abstract

Abstract

Considered primary sources of bioactive natural compounds with enormous applicability in various scientific-technological sectors, macroalgae have gained some prominence in recent years. The red macroalgae *Gracilaria gracilis,* one of the most dominant in worldwide cultivation systems, is fundamentally characterized not only by its high agar content, but also for its extraordinarily high phycobiliproteins (PBPs) levels endowed with a remarkable importance in the food industry, cosmetics, pharmaceuticals and in the field of bioactivities. The main objectives of this study consisted in manipulate laboratorily certain cultivation conditions of *G. gracilis* from the Lagoa de Óbidos (namely, light intensity and concentration of the macronutrients N and P (in the form of ammonia and phosphate, respectively)) and test their effects on growth, on the content of the R-phycoerythrin (R-PE) and R-phycocyanin (R-PC) PBPs, total soluble protein (TSP) content of the algal tissue and evaluate different methods of PBPs extraction applied to different states and pre-treatments of algal biomass to assess the most effective method.

As to the different extractions techniques used, for oven-dried wild samples, a maximum of 1.186 ± 0.008 mg of R-PE was obtained per g of dry weight with the bead beater, whereas enzymatic hydrolysis with cellulase only provided a maximum pigment content of 0.220 ± 0.001 mg R-PE g^{-1} of dry weight, clearly indicating that the first technique is much more efficient than the last one. For the wet samples, the methodology employing the tissue homogenizer yielded maximum values of 4.568 ± 1.773 mg R-PE g^{-1} WW and 0.099 ± 0.076 mg R-PC g^{-1} WW, proving that this extracting-with-water mechanical methodology is the most effective of the three in the extraction of phycobiliproteins from *G. gracilis*.

One of the first things to note in culture assays is that light intensity and quality play a major role on the quality of the biomass and, thus, depending on the parameters desirable to increase in cultivation - DGR, PBPs or TSPs -, it may be preferable to use one or the other culture lamps tested - Cool White Fluorescent (CWF) or Plant Growth Fluorescent (Gro-Lux). The maximum DGR (16.219 ± 3.84 % day^{-1}) was obtained for Gro-lux (with nutrient increment on the growth medium) while the obtained for CWF did not exceed the 14.936 ± 1.243 %, revealing a greater importance of nutrients (in particular, ammonium), comparatively to light, for this parameter. The TSPs concentration ranged from 18.06 ± 2.027 (for CWF) to 9.71 ± 0.364 % of dry weight (for Gro-lux without nutrient or light increment). As for R-PE and R-PC pigments, extracted with the tissue homogenizer, the highest peaks were obtained for CWF after two weeks of cultivation (9.599 ± 1.722 and 0.156 ± 0.043 mg g^{-1} of wet weight, respectively) - originated by a higher light intensity of the CWF lamp in comparison to the Gro-lux one.

In sum, the similarity between the high levels of PBPs already studied in *Gracilaria* and the considerable levels of mechanically extracted R-PE in this study for *Gracilaria gracilis,* allied with an optimization of cultivation conditions (light and nutrients) to increase DGR, PBPs or TSP content in specific situations, may certainly justify the large-scale cultivation of this macroalgae species.

Keywords: Seaweeds growth; Ammonia; Phosphate; Light intensity; Growth rate; Phycoerythrin; Total Protein

Resumo

Resumo

Consideradas fontes primárias de compostos naturais bioativos com uma enorme aplicabilidade em vários setores científico-tecnológicos, as macroalgas têm ganho, nos últimos anos, algum destaque. A macroalga vermelha *Gracilaria gracilis*, uma das mais dominantes em sistemas de cultivo mundiais, caracteriza-se fundamentalmente não só pelo seu elevado conteúdo em ágar, mas também pelos seus índices extraordinariamente altos de ficobiliproteínas dotadas de uma importância notável na indústria alimentar, cosmética, farmacêutica e no domínio das bioatividades. Os principais objetivos deste estudo consistiram em: - manipular laboratorialmente algumas condições de cultivo de *G. gracilis* proveniente da Lagoa de Óbidos (nomeadamente, intensidade luminosa e concentração dos macronutrientes N e P (sob a forma de amónia e fosfato, respetivamente)) e testar os seus efeitos no crescimento, no conteúdo das FBPs R-ficoeritrina (R-FE) e R-ficocianina (R-FC), no teor de proteínas solúveis totais (PSTs) do tecido algal e avaliar diferentes métodos de extração de FBPs aplicados a diferentes estados e pré-tratamentos de biomassa algal a fim de aferir o método mais efetivo.

Quanto às diferentes técnicas de extração empregues, para amostras selvagens secas em estufa, obteve-se um valor máximo de 1,186 ± 0,008 mg g^{-1} de peso seco para R-FE com o "bead beater" enquanto que a hidrólise enzimática com a celulase apenas permitiu obter um máximo de pigmento de 0,220 ± 0,001 mg g^{-1} de peso seco, indicando claramente que a primeira técnica é muito mais eficiente que a última. Para as amostras frescas, a metodologia que emprega o homogeneizador de tecidos rendeu valores máximos de 4,568 ± 1,773 mg de R-FE por g de peso húmido e 0,099 ± 0,076 mg de R-FC por g de peso húmido, provando que esta metodologia mecânica de extração com água é a mais eficaz das três na extração de ficobiliproteinas a partir de *G. gracilis*.

Um dos primeiros aspetos a salientar nos ensaios de cultivo é que a intensidade de luz e sua qualidade desempenham um papel principal na qualidade da biomassa e, portanto, dependendo dos parâmetros que se pretendem aumentar em cultivo - TCD, FBPs ou PSTs -, poderá ser preferível utilizar uma ou outra das lâmpadas de cultivo testadas - "Luz Branca Fria Fluorescente" (LBF) ou a "Luz Fluorescente de Crescimento Vegetal (Gro-lux)". A TCD máxima (16,219 ± 3,84 % day^{-1}) foi obtida para Gro-lux (com incremento de nutrientes no meio de crescimento) enquanto a obtida com LBF não foi além dos 14,936 ± 1,243 %, revelando uma maior importância dos nutrientes (em especial, amónia), comparativamente à luz, para este parâmetro. A concentração de PSTs variou entre 18,06 ± 2,027 (para LBF) a 9,71 ± 0,364 % de peso seco (para Gro-lux sem incremento de nutrientes). Já para os pigmentos R-FE e R-FC, extraídos com o homogeneizador de tecidos, os picos máximos foram obtidos para a LBF após duas semanas de cultivo (9,599 ± 1,722 e 0,156 ± 0,043 mg g^{-1} de peso húmido, respetivamente) - originados por uma intensidade de luz maior da LBF em relação à Gro-lux.

Em suma, a similaridade entre os elevados índices de FBPs já estudados em *Gracilaria* e os teores consideráveis de R-FE extraídos mecanicamente neste estudo para *Gracilaria gracilis*, aliados a uma otimização das condições de cultivo (luz e nutrientes) para aumentar a TCD ou o conteúdo em FBPs ou PSTs em situações específicas, poderão certamente justificar o cultivo a larga-escala desta espécie de macroalga.

Palavras-chave: Crescimento de algas marinhas; Amónia; Fosfato; Intensidade da luz; Taxa de crescimento; Ficoeritrina; Proteína Total

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Introduction

1. *Introduction*

1.1. Marine Organisms as a Source of Bioactive Metabolites

Containing about 1.36 billion cubic kilometers of water and portraying approximately 71 % of the Earth's surface, oceans gather a number of unusual properties, such as surface tension and high heat capacity, which, besides contributing to the climate stabilization phenomena and waves propagation, also grant them the ability to sustain diverse life forms [1]. From the microscopic cyanobacteria and coccolithophores, part of the phytoplankton composing the basis of most marine trophic chains, to the gigantic and majestic proportions of a blue whale, it is estimated that more than 90 percent of the world's biodiversity resides on the oceans [1]. Giving as an example coral reefs or the ocean depths, two of the Earth's most productive ecosystems, the number of species yet to be discovered is unimaginable, as is the extensive diversity of compounds they produce eventually useful for human development. On the other hand, given the exponential increase in the world's population characterizing modern society nowadays, there is an inherent necessity in finding new sources of biomolecules capable of ensuring all the nutrients required by humans in their physiological activities and, in addition, to increase the amount of matter that the existing ones already produce. Furthermore, the increase in resistance of many pathogenic microorganisms (e.g. through genetic modifications) to drugs until then effective, creates a demand to synthesize novel bioactive molecular structures capable of counteracting and responding to these problems [2].

Whether belonging to Eukarya domain, Bacteria or even Archaea, one has been witnessing to a growing interest for marine organisms, justified by the evidence that there is an eccentricity of metabolic products by them synthesized that can represent an evolution in research areas so vast as nutrition, pharmaceutical industry, cosmetics, agriculture, among others [3,4,5]. These, in turn, are made up of biological molecules endowed of essential physiological and behavioral functions which, although not being involved in the basic machinery of life (primary metabolism) as are proteins and nucleic acids, are able to ensure the aptitude and survival of a certain organism even in the most hostile environments. Commonly produced in response to ecological/environmental stress, many of these secondary metabolites may be originated by certain factors such as competition for space, osmotic and oxidative stress, abrupt temperature fluctuations or even reproductive ability [5,6]. Over the years several biotechnologists have focused their interest and attention on the activities these secondary products ("bio by-products") may hold: within a spectrum of interesting potentialities, antiviral and antitumor agents [7,8], antimutagenic ones [9], immunossupressants [10], cardiac stimulants [11], antifungal and antibacterial agents [12], among others, are strongly highlighted.

Thus, not being able to exist any indifference regarding the rich biota residing in this type of environments, more and more investigations seek to make the most of and profit from all properties derived from marine organisms.

1.1.1. The Role of Macroalgae

Within the massive group of algae, avascular photosynthetic organisms of different shapes, sizes and degrees of cellular organization (uni, multi or pluricellular, procaryotes or eukaryotes) found all over the world predominantly in rivers, lakes, estuaries, seas, oceans, ponds and marshes, macroalgae (also known as seaweeds) possess a quite pertinent role [13]. Integral component of food chains and of the entire marine (or freshwater) ecosystem and providing habitat for a panoply of organisms, they constitute photosynthetic multicellular individuals of macroscopic dimensions lacking highly specialized tissue structures that are present in higher terrestrial plants [13]. Regarding the type of habitats where they thrive, it's possible to establish an intimate relationship between the light conditions of their dwelling areas and the dominant composition of photosynthetic pigments in their tissues, allowing us to classify them into three large groups: green algae (Chlorophyta), brown algae (Phaeophyceae) and red algae (Rhodophyta) [14]. In the chlorophytes, the green color results from the dominant content of chlorophylls *a* and *b*, despite the existence of other pigments such as carotenoids and xanthophylls. Their energy requirements in terms of light intensity are higher (dominance of chlorophyll *a*) for photosynthesis accomplishment [13]. Hence, they generally occupy ecological niches more open in terms of spatial areas, such as coastal areas, rocky shores or the intertidal region, where sunlight strikes more intensively. In the second case, the brown coloration is attributed to the dominance of certain pigments such as fucoxanthin despite the less pronounced presence of chlorophylls *a* and *c* and β-carotenes. Their light energy requirements are somewhat lower than for chlorophytes, and, as such, are more frequent in zones of intermediate depths where light reaches with more difficulty. Finally, for red algae, the prominence of phycobiliproteins such as phycoerythrin (PE) and phycocyanin (PC) mask the presence of other pigments – some classes of chlorophylls, β-carotene and a number of unique xanthophylls [13,14]. Endowed with the lowest light requirements, these algae adapted better to the subtidal depths (≈ 40 m deep) where solar radiation barely arrives, having the ability to occasionally extend up to 250 m deep. Nonetheless, they are also frequent in the intertidal region [14].

Nowadays, it is no novelty the benefits they can bring, nor the applicability that seaweeds appear to demonstrate in various technological and socio-economic sectors (e.g. agricultural domain, cosmetics, food and animal feed, medication field) [13]. Synthesized by macroalgae, as already mentioned, in response to growth in extreme conditions and to all the challenges deriving from it (for instance, defense against herbivores, protection against UV radiation, pathogens, abrupt fluctuations in temperature and salinity, nutrient availability), many of these bioactive phytochemicals possess well-known health benefits, namely, antimicrobial properties, antioxidant action, anticoagulant, antiprotozoal and anti-obesity qualities [5,15,16]. Some of these secondary metabolites can be: terpenes, alkaloids, polyphenols, sterols, terpenoids, ketones, vitamins, halogenated compounds and phycobiliproteins, amongst many others [17].

Contrasting with green and brown, the red algae, comprising nearly 8000 species, are considered the most important sources of many of these active biological metabolites. Among the wide variety of compounds already isolated from their members, one highlights here only a small fraction of their most relevant biological activities: cytotoxic potential, antiviral effect, anthelmintic, anti-inflammatory, free radical scavenger, neurophysiological, insecticidal, antimicrobial and antimalarial activities [15,18].

1.1.2. *Gracilaria gracilis* and its Contribution

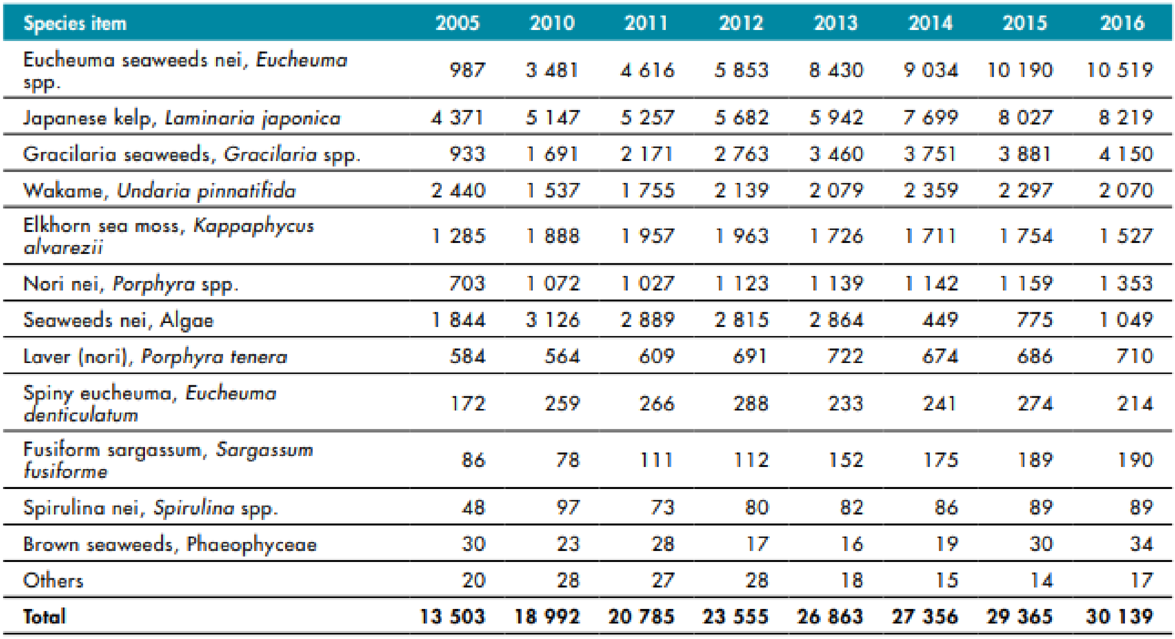
The macroalgae of the genus *Gracilaria* Greville, 1830 (Gracilariales order) comprise a group of more than 100 species with only half of that number already being taxonomically accepted. Two are the main practices in which *Gracilaria* algae are applied: in the extraction of phycocolloids (main source of agar), condition that allows them to be denominated by agarophytes; or directly in human or marine animal feed [19]. Some other important usages have also been inciting a few economic sectors: the paper manufacture, from the solid waste generated by the agar production [20]; biofuel, namely bioethanol, from the high levels of carbohydrates present in *Gracilaria* species (approximately 45 % of their dry weight [21]); nutrient bioremediation capacity, being able to incorporate/extract the inorganic nutrients excreted by the organisms being cultivated (e.g. fish) in Integrated Multi-Trophic Aquaculture (IMTA) systems [22,23]; and as a source of numerous biotechnological multi-products (e.g. lipids and fatty acids, sterols, proteins, phenols, carbohydrates) [24].

A particular species within this genus of macroalgae is the *Gracilaria gracilis* (Stackhouse) M.Steentoft, L.M.Irvine & W.F.Farnham [25] to which, in 2011, was assigned a production of around one million and a half tons per year in countries such as China, Taiwan and other Asian conglomerates [26]. Typically inhabiting in sheltered environments such as bays, estuaries or river mouths or even protected rocky areas on the medium and sublittoral levels (0 to 20 m), it has the peculiarity of supporting a wide range of salinities (15-50 ‰) without significant tissue damage, being thus an euryhaline species. Nonetheless, they grow better at 25-35 ‰ [27,28]. Its optimal temperature for growth falls in the range 19-27 °C [29]. A part of its biotechnological and nutraceutical importance, as well as pharmaceutical applications, can be evidenced by: remarkable ability of nutrient absorption – a useful bioremediator in controlling the risk of eutrophication in a couple of coastal lagoons [22]; the production of bio-oils rich in aromatic compounds, sugars and other high-value chemicals through a microwave (MW) radiation-mediated pyrolysis reaction [30]; attenuation and remediation of progressive chronic mental illnesses such as Alzheimer’s, Parkinson’s and Dementia diseases (extracts) – by inhibiting the activity of the cholinesterase enzyme (responsible for the active hydrolysis of the neurotransmitter acetylcholine), some methanolic extracts of *G. gracilis* have proved to be an alternative approach in the symptomatic treatment of these clinical conditions [31]. Substantial for this study, another focus of interest inherent to *G. gracilis* relates with the possibility of extracting phycobiliproteins (phycoerythrin, phycocyanin or allophycocyanin) from it, endowed with an array of applications associated with its fluorescence abilities (approached in detail in Section 1.3 and 1.4). Finally, the referred seaweed is one of the best candidates for aquaculture cultivation thanks to a rapid growth rate, ease in propagation (asexually and sexually) and good resistance to salinity and temperature oscillation (even though the alga shows suffering signs at temperatures ≥ 28°C) [19].

1.2. Algal Cultivation

According to the Food and Agriculture Organization of United Nations (FAO) [32], the harvest of wild seaweeds in 2006 accounted for values of 1.06 million tonnes, having remained slightly balanced up until 2015 (1.09 million tonnes). Yet, these numbers are still pretty big. While the majority of biomass is being used in the phycocolloid and food industries, agricultural and health care products are, even though crucial, minor destinations [28]. Demands on algae biomass have been multiplying on a global scale since the emergence of more and new scientific evidence about their potential benefits, usefulness and applications in several sectors of human life. However, with the increase of information, awareness and consequent need to have more and more, comes the greed and inability to manage limited natural resources. As such, in order to avoid overharvesting of natural populations, habitat loss and damage to marine ecosystems, a sustainable and prudent exploitation and management of natural algal resources is thus critical, as algae, as primary producers and habitat providers, are fundamental and functional elements of aquatic life. An alternative and more sustainable route capable of replacing considerably an excessive harvesting of wild algae consists in its controlled cultivation through several techniques [32].

Table I.I: World aquaculture production of macro and microalgae (million tons, fresh weight).



Species item	2005	2010	2011	2012	2013	2014	2015	2016
Eucheuma seaweeds nei, *Eucheuma* spp.	987	3 481	4 616	5 853	8 430	9 034	10 190	10 519
Japanese kelp, *Laminaria japonica*	4 371	5 147	5 257	5 682	5 942	7 699	8 027	8 219
Gracilaria seaweeds, *Gracilaria* spp.	933	1 691	2 171	2 763	3 460	3 751	3 881	4 150
Wakame, *Undaria pinnatifida*	2 440	1 537	1 755	2 139	2 079	2 359	2 297	2 070
Elkhorn sea moss, *Kappaphycus alvarezii*	1 285	1 888	1 957	1 963	1 726	1 711	1 754	1 527
Nori nei, *Porphyra* spp.	703	1 072	1 027	1 123	1 139	1 142	1 159	1 353
Seaweeds nei, Algae	1 844	3 126	2 889	2 815	2 864	449	775	1 049
Laver (nori), *Porphyra tenera*	584	564	609	691	722	674	686	710
Spiny eucheuma, *Eucheuma denticulatum*	172	259	266	288	233	241	274	214
Fusiform sargassum, *Sargassum fusiforme*	86	78	111	112	152	175	189	190
Spirulina nei, *Spirulina* spp.	48	97	73	80	82	86	89	89
Brown seaweeds, Phaeophyceae	30	23	28	17	16	19	30	34
Others	20	28	27	28	18	15	14	17
Total	**13 503**	**18 992**	**20 785**	**23 555**	**26 863**	**27 356**	**29 365**	**30 139**

Adapted from [32]

Still according to FAO (2018), in 2005, the total global production of algae (including seaweeds and *Spirulina*) amounted to approximately 13.5 million tons, reaching values of 30 million tons in the following 11 years. Despite its large diversity potentially available, algal cultivation is limited to nearly 40 species, including both freshwater and marine macroalgae (Table I.I). *Gracilaria* seaweeds occupy the third place in the ranking of global production representing almost 14 % of the total amount of seaweeds cultivated through aquaculture in 2016.

1.2.1. Macroalgal Cultivation Systems

As human population grows, seaweed culture will be important to replenish the wild resource. Seaweed aquaculture constitutes a considerable slice (≈ 24 %) in the proportion of organisms cultivated worldwide, with a monetary basis of nearly 5.7 billion U.S. dollars [32]. The dominant species are carrageenophytes (*Kappaphycus* spp. & *Eucheuma* spp.), kelps (*Saccharina japonica* & *Undaria pinnatifida*), the red agarophytes (*Gracilaria* spp.) and nori (*Porphyra* spp.) (Table I:I). High and fast growth rates, providing economic and competitive advantage in macroalgal culture, is considered one of the top features when picking species for commercial farming [32]. Macroalgae can be grown, more commonly, in open-sea coastal or off-shore environments suspended in lines, rafts or nets, or still on land in ponds or in tank-based culture systems [33,34]. Briefly, like the bioextractive organisms they are, seaweeds enjoy the ability to intake the excess of nutrients (nitrogen and phosphorus) and carbon dioxide generated by other aquatic species (heterotrophs) which they use for growth and production of proteins and energy storage products. Any seaweed culture system demands a specific set of prerequisites for a well-succeeded culture. Hence, three of the pivotal ingredients for a good cultivating start are the seawater media (involving the seawater itself and the added nutrients), and the establishment and control of proper water temperature and light conditions [34]. Regarding the first, a reliable supply of clean, filtered and sterilized (almost ever by autoclave in small scale lab-cultivations) natural seawater is essential to establish a successful culture free of any organic or inorganic contaminants. As for the other two, they will be discussed more accurately in the next section.

Nevertheless, despite the cruciality of factors such as these affecting and influencing a successful culture initiation step, the condition/status of the biological material also plays a central role. Hence the extreme importance of establishing from the outset an axenic culture devoid of any other competing organism (e.g. diatoms – the most common and problematic contaminants in a seaweed culture – usually introduced through natural seawater channels or directly attached to the harvested wild material).

Concerning the specimens of the *Gracilaria* genus, these have been cultivated primarily in 4 different ways: open water rope cultivation, near shore bottom cultivation, pond culture and tank cultures [35]. Currently, most of the *Gracilaria* seedstock is still supplemented from the wild, either by harvesting healthy branches from natural stocks or by selecting reproductive plants to collect spores for seeding [34,36]. Two other relevant points to note are: a dependence on the condition of natural stocks may cause some complications, including physiological variations (agar content, growth, lipids content, phycobiliproteins, etc.), in the seedstock of either *Gracilaria* or other seaweeds; the quality of wild *Gracilaria* has been declining in recent years due, in one hand, to increases of algal diseases and, on the other, to decay and abrupt fluctuations in cultivation environments [37].

1.2.1. Main Environmental Parameters Influencing Cultivation

With an ever increasing awareness regarding the benefits of algal products, it arises an attempt to manipulate the culture conditions affecting the already existing cultivation techniques for specific groups, species or strains of algae. By identifying the best environmental parameters controlling the metabolism of a certain alga species (species-specific responses) in cultivation systems, it becomes possible to maximize the production of compounds of interest [33]. Their prosperity deeply depends on the production of these biologically active secondary metabolites. The ultimate objective consists in modifying, based on the adjustment of culture conditions to a particular organism requirements and to the basic biological function(s) of the desired compound, not only levels of produced compounds, but also their composition which is related to their bioactive potential and, in last analysis, with its final application [16,33]. Yet, despite considerable advances, there is still a lack of expertise in identifying optimum conditions/requirements for synthesis of novel compounds in species (or strains). From the main environmental parameters influencing and affecting *Gracilaria* cultivation susceptible to be controlled at laboratory scale, some have been identified: light, temperature, salinity, nutrients and water movement (gas exchanges).

Light is one of the most important environmental factors influencing seaweed growth. Coming directly from the sun or from artificial systems, it constitutes the main energy source for phototrophic algal cells. However, not always much light is synonymous of high photosynthesis rates. In fact, the photosynthetic activity of algal cells increases until light intensity reaches a certain threshold point from which additional increments won’t cause any rising in the photosynthetic rate (a saturation point of light is said to be reached) [38]. Light intensities superiors to the adequate ones (above the threshold) can damage light receptors within cell chloroplasts and decrease the photosynthetic rate – a phenomenon known as photoinhibition [38]. Fluorescent light bulbs (the most common ones) will provide, in laboratory, sufficient light energy for the culture of plants. Constant assessment of the light intensity available for the growing seaweeds is substantial once light requirements change over time. That’s why it’s always crucial to have an interval timer connected to the light source to control photoperiod (a neutral photoperiod of 12 hours of light followed by 12 hours of darkness (12:12, L:D) is commonly used) [34]. Another common complication associated to the said parameter is the mutual shading occurrence – for high density culture, the cells closest to the surface of the media liquid receive the majority of the available light whilst cells below (or in the center of entangled filaments) are left with very little quantities of light. Optimum light levels vary considerably not only according to the type of cultivation (indoor vs. outdoor), but also with the target species, while classic irradiance responses include changes in pigment levels and composition [39].

As for temperature, this factor holds a determinant influence not only on seasonal and geographic distribution, but also in the growth rate and several others physiological processes in algae such as diffusive rates and nutrient uptake [33]. Each algae species possess an optimal temperature range for growth, as well as a lower and upper limit of survival temperature [40]. In order to attend these needs, for small and medium scale cultivations as the present case of study, cultures should be placed, whenever possible, inside a temperature controlled space. Similarly to light, growth rates of macroalgae generally increase exponentially with increasing temperatures until they reach an optimal value. From that, growth rate ceases or declines with further temperature increases. For *Gracilaria* species, this value is around 20 °C or slightly above [41]. For water temperature over 26 °C, the seaweed generally dies. For temperatures lower than 10 °C, the average daily growth rate of *Gracilaria* species becomes negative, but unlike high temperatures, the low ones don't kill the algae until the water freezes [41]. Between both, the upper limit of survival temperature is more crucial to take into account, for example, when considering outdoor culture where the ability to control this parameter is often limited and almost always dictated by environmental temperatures and solar irradiance.

Salinity is another parameter influencing seaweed cultivation. Its fluctuations not only determine the growth of many seaweeds, but may also have an impact on their biochemical composition, inducing the production of specific proteins and enzymes [42]. These impacts are particularly decisive in open pond or cultivation tanks where evaporation is able to cause variations on salinity values – determinant for halophilic species in which production of interest compounds is specifically stimulated under salinity stress [42].

Algae require various inorganic nutrients (e.g. the macronutrients nitrogen, phosphorus, potassium and sodium and the trace elements iron, manganese, copper and zinc) along with vitamins (organic nutrients), hydrogen ions and metal-ion buffers (TRIS, EDTA) in order to produce healthy cultures with high biomass productivity. Although there is still much debate regarding the ideal levels of these nutrients (normally added in excess to prevent growth stagnation by nutrient limitation), or even about which ones to use (in most cultures the macronutrients nitrogen and phosphorus are applied at a ratio of 16 parts of nitrogen to one part of phosphorus (16N:1P) [43]. Being an essential element incorporated in the production of many organic macromolecules (e.g. pigments, nucleic acids), nitrogen supplementation is responsible for the photosynthetic activity augmentation and thus, for the growth of macroalgae [38]. By manipulating and adjusting the levels and ratios of these two nutrients (or different ones) and possibly combining them and interacting with other environmental parameters, it also becomes possible to study the levels of specific compounds produced by algae: total protein (in the case of red algae, it includes the proteinaceous phycobilins bounded to phycocyanin and phycoerythrin proteins), high-value polysaccharides and lipids such as polyunsaturated fatty acids [5,16].

At last, the water movement inside the cultivation vessel allows to accelerate the diffusion rates of gases and nutrients in and out of the seaweed thalli, creating favorable environmental conditions for the rapid growth of seaweeds. Since algal cells are made up of approximately 45-50 % carbon [38], they continue to require, in culture, the incorporation of this chemical element into their tissues under the condition of growth limitation in the absence of such supplementation. It's here that gases diffusion becomes indispensable in a growing system: carbon is often added as CO_2 (usually bubbled through sparging stones or perforated pipes); the occurrence of photosynthesis under cultivation leads to the release of O_2 into the liquid phase when the CO_2 is consumed; however, high concentrations of O_2 are susceptible to cause photo-oxidative damage to chlorophyll [38], surely inhibiting afterwards the photosynthesis process and thus reducing productivity. In systems like open ponds or tanks, this situation doesn't seem to be a problem since a large surface area is present for O_2 mass transfer to occur, as well as for CO_2 dissolution on water. Yet, in closed systems, (e.g. photobioreactors or closed tanks), gas exchange channels are critical to reduce dissolved O_2 levels. Other pertinent aspect to remind is that cultivation systems where maintaining a stable pH is crucial, CO_2 plays an additional role. Upon being absorbed into the culture media, it is converted to carbonic acid, effectively lowering the culture pH [44]. As algae consume the acid, the pH rises. So, by controlling CO_2 levels, the pH can be manipulated very effectively. Back to the phenomenon of mutual shading, this is a common problem in *Gracilaria* specimens since individuals tend to grow entangled over themselves in the form of a woolen ball or "human hair" threads, limiting high cell density culture. Hence, mixing movements (through aeration) ensure that all cells in culture receive an equal amount of light.

1.3. Phycobiliproteins (PBPs) amongst other Algal Proteins

Seaweeds are considered a viable source of protein. Whether in human nutrition, functional foods or animal feed, it's unquestionable, nowadays, that seaweeds have a strong word to say in this domain. Among other types of proteins (with all their tremendous and varied composition of the most diverse amino acids) present on macroalgae and susceptible to be extracted by diverse methods, phycobiliproteins gain prominence in this study.

The phycobiliproteins (PBPs) are the major antennae-protein pigments involved in light harvesting (photosynthesis process) in Cyanobacteria (blue-green algae), Rhodophytes (red algae) and Cryptophyta (Cryptomonads), complementing other pigments such as chlorophylls or carotenoids [38,45]. In the first two groups, four main classes of phycobiliproteins exist based on their color and absorption characteristics: phycoerythrocyanin (PEC, reddish-purple), phycoerythrin (PE, pinkish-red), phycocyanin (PC, blue) and allophycocyanin (APC, bluish-green), having λ_{Amax} = 560-600 nm and λ_{Fmax} = 610-625, λ_{Amax} = 540-570 nm and λ_{Fmax} = 575-590 nm, λ_{Amax} = 610-620 nm and λ_{fmax} = 645-653 nm, λ_{Amax} = 650-655 nm and λ_{Fmax} = 657-660, respectively, and emitting light at 607 nm, 577 nm, 637 nm and 660 nm, respectively (Fig. 1.1) [46,47].

According to the original source of phycobiliproteins, a further differentiation based on subclasses was adopted for PC and PE: C for Cyanobacteria species, R for Rhodophyta and B for Bangiales (the most primitive order of red algae) [48]. An interesting peculiarity of PBPs is that when access to nutrient sources becomes limited, under conditions of nitrogen and/or phosphorus starvation, a degradation/catabolism process of these biomolecules occurs to ensure cell survival, thus decreasing the concentration of PBPs [47]. Such a capacity will, in turn, reduce their light-harvesting ability.

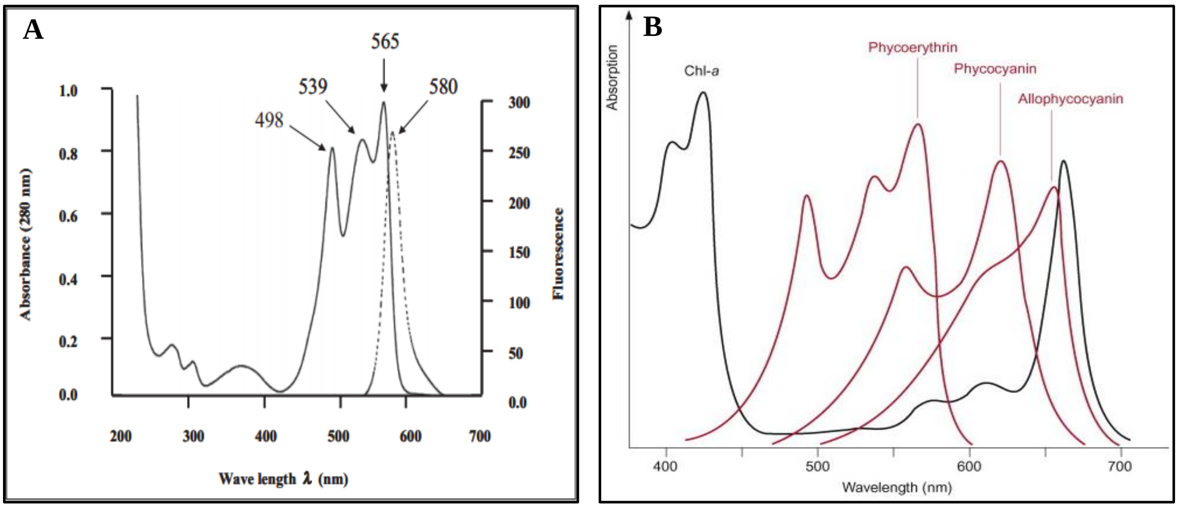


Figure 1.1: Absorption spectra of phycobiliproteins: (**A**) R-phycoerythrin (R-PE); (**B**) R-phycoerythrin (R-PE), R-phycocyanin (R-PC) and Allophycoacyanin (APC). Adapted from [46].

In cyanobacteria and red algae, phycobiliproteins are aggregated and organized in supramolecular light energy-capturing subcellular complexes called phycobilisomes (PBSs), with a diameter ranging from 30 to 60 nm, anchored on the outer surface of the thylakoid membranes, located in the stroma. They are also located near the photosystem II, one of two pigment complexes involved in the photosynthesis mechanism [48]. The PBSs is stabilized and tied up by colorless linker proteins (LPs) in a manner that increases absorption of the light energy and its transfer through the photosystem apparatus [45,48]. PBSs have two morphologically distinct substructures: the rods and the core (Fig. 1.2). The PBSs core is essentially composed by APCs and is bounded by polypeptides responsible for thylakoid connection. As for the core, it consists of three cylinders, each, in turn, constituted by four disks (consisting an APC trimer). The other major biliproteins are confined to the rod substructure, shaped like a stick, whereas each stick is composed by 6 disks which are hexamers of distinct phycobiliproteins and also linked by polypeptides – representing only around 15 % of the proteinic part of the PBSs [48,49]. These linkage polypeptides have as main functions the fixation of the phycobilisome to the thylakoid membrane, the determination of the aggregation state of the phycobiliproteins, thus stabilizing the whole structure, still bearing some influence on different subunit positions and in chromophore conformation [45,48]. Briefly, chromophores constitute groups of atoms and electrons forming the non-proteinous, light-capturing part of an organic molecule that causes it to be coloured [45]. PBSs can transmit the light energy from chromophores absorbing at green wavelengths (such as PEs or PECs) to chromophores absorbing at red ones (such as APCs) (spectral zones where chlorophyll *a* can't absorb light), finally passing it to chlorophyll *a*, the only pigment able to transmit this energy to the photosystem II reactive center [48]. Beyond the main function of phycobilisomes as photon collector, they also act in the photoprotection of the system under conditions of intense light [47].

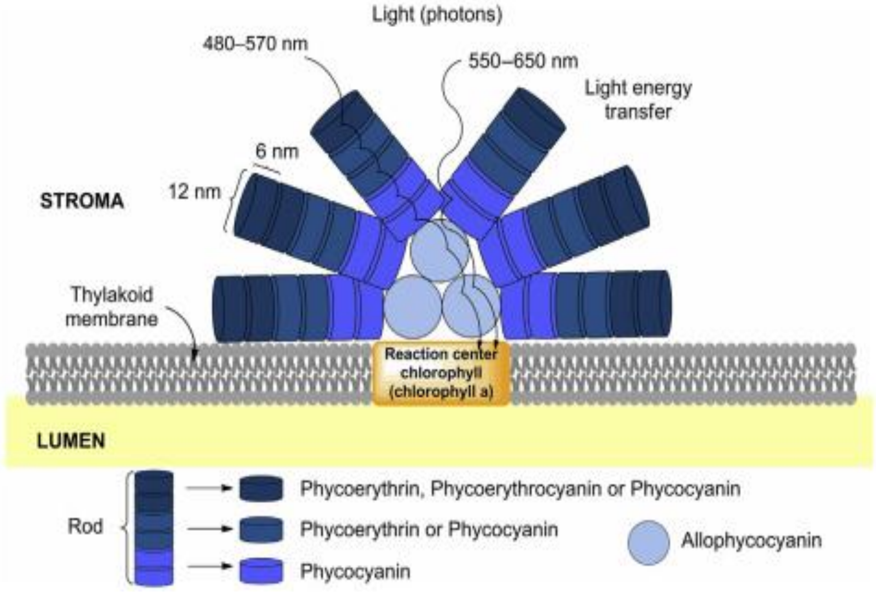

Light (photons)
480–570 nm
550–650 nm
Light energy transfer
6 nm
12 nm
STROMA
Thylakoid membrane
Reaction center chlorophyll (chlorophyll a)
LUMEN
Rod
Phycoerythrin, Phycoerythrocyanin or Phycocyanin
Phycoerythrin or Phycocyanin
Phycocyanin
Allophycocyanin

Figure 1.2: Schematic diagram of the common phycobilisome structure, present in red macroalgae, on the thylakoid membrane – its design is such that excitation energy is delivered from any of the main chromophores to the reaction center. Adapted from [48].

Also constituting water-soluble, brilliantly colored oligomeric proteins, phycobiliproteins are built up from chromophore-bearing polypeptides belonging to two families – α and β, which constitute dissimilar polypeptide chains of approximately 160-180 amino acid residues [49]. All PBPs contain the same basic unit: a heterodimer of these α and β subunits conventionally referred as "monomer". Three of these monomers are associated to form the basic skeleton of phycobilisomes - the $(\alpha\beta)_3$ trimer. The trimers are synthetized and assembled into hexamers – $(\alpha\beta)_6$ with the help of linkage polypeptides (Fig. 1.3) [48]. The spectral properties (and thus color) of the PBPs are originated mainly from covalently bound prosthetic groups that are open-chain (linear, not cyclized, contrary to chlorophylls) tetrapyrrole chromophores bearing A, B, C and D rings named phycobilins. These may be the blue-colored phycocyanobilin (PCB), the red-colored phycoerythrobilin (PEB) (the first ones discovered), the yellow colored phycourobilin (PUB), or the purple-colored phycobiliviolin (PXB) (also named cryptoviolin) (Fig. 1.4) [45]. All four types of PBPs differ on the amino acid sequence, number of chromophores per subunit and type of chromophores (these last two are responsible for their coloration): two bilins are found in allophycocyanin and three bilins each in phycocyanin and phycoerythrocyanin, with phycoerythrin carrying five or six prosthetic groups (Fig. 1.4) [47].

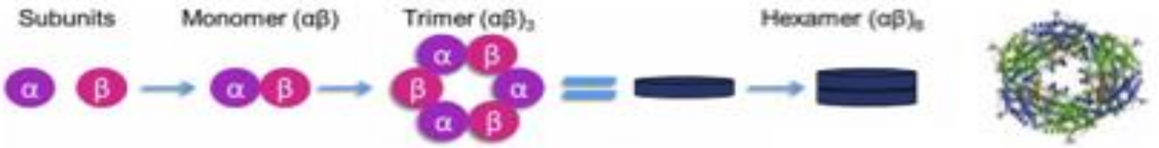

Subunits
Monomer (αβ)
Trimer (αβ)₃
Hexamer (αβ)₆
α
β

Figure 1.3: Diagram of phycobiliprotein subunit assemblage. Adapted from [48].

In sum, the PBPs classification is based on the chemical nature of its chromophores and on the spectral properties of the native proteins. As for phycoerythrocyanin, this pigment is found in few cyanobacteria strains unable to synthesize PE. It appears in trimeric $(\alpha\beta)_3$ or hexameric $(\alpha\beta)_6$ forms. The PXB chromophore, specific to phycoerythrocyanin, is located on the α subunit while the β subunit possesses two PCB chromophores (Fig. 1.4) [47,48]. The allophycocyanin PBP is mostly defined by a 110 kDa molecular weight trimer $(\alpha\beta)_3$ in which both α and β subunits possess one PCB chromophore (Fig. 1.4). Its maximum absorption wavelength was found to be at 650 nm, corresponding approximately to the second maximum of chlorophyll *b*. Being a minor pigment but constituting one of the core constituents near the start of the rod (PEs), it possesses an intermediary function in the energy transfer mechanism through the chlorophyll *a* reaction centers [38]. As regards to phycocyanin, the blue pigment is present in Cyanobacteria, Rhodophyta and Cryptophyta with two types already being described: C-PC (cyanobacterial pigments) and R-PC (red-macroalgal biliproteins), being C-PC more widespread. In this case, both α and β subunits possess a PCB chromophore but the β subunits have two chromophores [47]. The protein and chromophore structures of phycoerythrocyanin and phycocyanin are very similar, except in the α-subunit with the substitution of phycobiliviolin for phycocyanobilin (Fig. 1.4) [48]. Finally, the phycoerythrin is the main pigment found in Rhodophyta but may also arise in Cyanobacteria. According to the algal species, different forms of PE can occur: R-PE (for Rhodophyta), B-PE and b-PE (for Bangiales), and C-PE (for Cyanobacteria). B-PE and R-PE are the most abundant PEs in red algae [50]. Both are composed of $(\alpha\beta)_6\gamma$ hexameric complexes with a molecular weight between 240 and 260 kDa while C-PE and b-PE are $(\alpha\beta)_3$ complexes without any γ subunit (Fig. 1.4). Pertinent for this study, the R-phycoerythrin has a three-peak absorption spectrum in its native state with absorption maxima at 565, 539 and 498 nm (Fig. 1.1) [46].

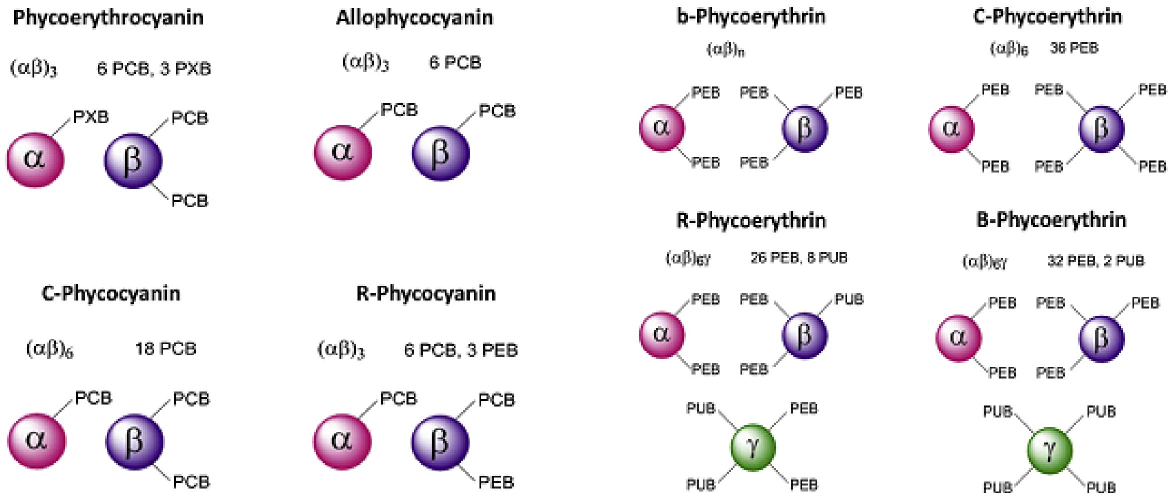

Phycoerythrocyanin
(αβ)3 6 PCB, 3 PXB
α PXB
β PCB PCB
Allophycocyanin
(αβ)3 6 PCB
α PCB
β PCB
b-Phycoerythrin
(αβ)n
α PEB PEB
β PEB PEB PEB
C-Phycoerythrin
(αβ)6 36 PEB
α PEB PEB
β PEB PEB PEB PEB
R-Phycoerythrin
(αβ)6γ 26 PEB, 8 PUB
α PEB PEB
β PEB PEB PUB
γ PUB PEB PUB PEB
B-Phycoerythrin
(αβ)6γ 32 PEB, 2 PUB
α PEB PEB
β PEB PEB PEB
γ PUB PUB PUB PUB
C-Phycocyanin
(αβ)6 18 PCB
α PCB
β PCB PCB
R-Phycocyanin
(αβ)3 6 PCB, 3 PEB
α PCB
β PCB PEB

Figure 1.4: Structural configuration, based on the family of subunits, type and number of chromophores per subunit, of the phycobiliproteins phycoerythrocyanin (PEC), allophycocyanin (APC), the two existing types of phycocyanin (PC) and the four described types of phycoerythrin (PE). Adapted from [48].

1.4. R-Phycoerythrin (R-PE)

1.4.1. Structure and Physiochemical Factors Affecting its Stability

R-Phycoerythrin (R-PE) is a major photosynthetic pigment in red seaweeds [38]. It’s an oligomeric water-soluble chromoprotein of around 240 to 260 kDa, characterized by its absorption spectrum between 400 and 650 nm. The pigment is composed of an open chain tetrapyrrolic (bilin) covalently linked to the apoprotein. The molecular weight of the non-denatured protein structure suggests a $(\alpha\beta)_6\gamma$ polypeptide structure. Highly purified R-PE comprises two major subunits (α, β) with approximately 18-20 and 19.5-21 kDa, and a minor subunit of 30 kDa (γ) [46]. The α-subunit possesses two PEB (phycoerythrobilin) chromophores while the β-subunit owns three chromophores: two containing PEB and one with PUB (phycourobilin). As for the γ-subunit, two PEB and two PUB chromophores are present [47]. This last subunit (absent in other PBPs) is located right in the center of the molecule and links the $(\alpha\beta)_3$ trimers, conferring high stability to the pigment [47].

With the recent modernization of phycobiliproteins extraction and purification techniques, some investigations regarding the manipulation of certain parameters have been arising in order to study and promote a good stability of R-PE. One of the most recent ones is based on a study conducted by Munier *et al*. (2014) in which the stability of R-phycoerythrin extracted from the red macroalgae *Grateloupia turuturu* was investigated for different conditions of pH, exposure time to light, temperature and storage time. In the first case, in relation to the reference spectrum (0 h), successive reductions in absorption and in fluorescence were observed as exposure time to light increased**.** After 48 h, the pigment solution was colorless and the reduction in concentration of R-phycoerythrin was around 70 %. A decrease of 68 % on the emission (fluorescence) spectra, after 48 h, was observed whereas on the absorption spectra the absorbance peaks at 565 and 540 nm decreased slightly more (55 % and 60 %, respectively) than those at 498 nm (39 %) [51]. The authors presented the hypothesis that the stronger stability of PUB (corresponding to the 498 nm peak) was most likely due to the double binding of rings C and D in PUB to β50 and β61 Cys residues, respectively, whereas PEB (540 and 565 nm peaks) was bound to the protein through one Cys residue [52]. R-PE was stable only up for 8 h of exposure to light. For pH, R-PE presented good absorbance and fluorescence stability from a pH of 4 to 10 (no color change). While at pH of 12 the pigment was degraded (its intensity decreased from a pH of 11 to 12 until the solution became colorless), at a pH of 2 (the solution was purplish since pH = 3), there was a reduction of circa 40 %. For the absorption spectra, at pH of 2, the peaks disappeared at 545 and 565 nm while at pH of 12 the spectra of R-PE became distorted due to precipitation and denaturation of the protein [51]. As for the emission spectra, the fluorescence decreased by 97 % at pH of 2 whereas at pH of 12 there was no fluorescence at all. The authors knew that large (extreme) changes in pH could result in the disturbance of electrostatic properties and hydrogen bonds involved in protein association, susceptible to induce changes in the chromophore structure. Liu *et al*. (2009) even mentioned that very low pH levels could cause the dissociation of trimers to monomers, monomers into individual subunits and partial unfolding of subunits - leading to a complete denaturation of PBPs. Regarding temperature, R-PE showed good stability up to 40 °C (no significant modifications on both spectra). At 60 ºC, the reduction in concentration was around 70 % whereas at 100 ºC there were none of both types of spectra at all. It was concluded that, as temperature kept rising, the loss of pigment stability could have been caused by a decrease in the amount of α-helix [51]. PUB showed a better thermal stability than PEB – decrease of 49 % on the absorbance at 540 and 565 nm against a minor reduction for 498 nm (57 %), respectively. In order to determine how long R-PE extracts could be stored before pigment degradation became evident, the stability of PEs during storage at -20 °C and at 4 °C (the conventional method recommended to preserve pure PE (by Sigma) and pigment samples) in darkness was studied. At 4 °C, R-PE was stable up to 48 h whereas after 2 weeks (336 h) its absorption and fluorescence spectra were completely distorted. At -20 ºC, both spectra had only been slightly modified after the 336 hours with absorbance and fluorescence properties being the same as at 0 h.

Shortly, Munier and collaborators (2014) stated that, in order to prevent changes in spectral properties (caused by changes in protein conformation), pigment denaturation and to preserve its functional structure, the best conditions for extracting, purifying and preserving R-phycoerythrin are: temperatures lower than 40 ºC; pH ranging from 4 to 10; absence of light (darkness); storage temperature of -20 ºC. Hence, these conditions could be used as a solid starting point for other studies whenever extracting R-PE is desired.

1.4.2. Importance and Applications

PBPs bring together a set of unique properties that allow them to be used in several economic and industrial sectors. Generally, for the whole set of PBPs, the following attributes stand out: their inherent bright color (different according to the type of PBP), a very intense emission of light, a non-toxic nature of the protein, easy availability (several groups and species of micro- or macroalgae possess them), high solubility in water and the fact of being classified as a natural product (opposing and replacing the huge industry of harmful chemicals endowed with the same effects) [49]. Particularizing for R-phycoerythrin (R-PE) – the main focus pigment to be extracted in this study – this pinkish pigment is of interest due to the following additional characteristics: the protein fluorescence (besides its pinkish color, R-PE can also emit yellow fluorescence), an exceptionally high molar absorption coefficient (ε) (2.4 x 10^6 $cm^{-1}M^{-1}$) (allowing a strong absorbance of light), high quantum efficiency (0.84) (giving the molecule a high sensitivity), a large Stokes shift (gives low background, allowing multicolor detections), a relatively high stability and the possession of excitation and emission bands at visible wavelengths, allowing to be considered the highest quality fluorophore in the market [46,47]. The leading application areas of PBPs are shown in Fig. 1.5 - depending on their purity ratio, PBPs can have different destination fields. Their global market is being estimated at US $50 million with proximate yearly growths of 10 % [53]. Despite their vast and versatile applicability, the primary commercial application appears to be as natural dyes [45,48]. Due to the toxic effect of several synthetic dyes, there is an increasing preference to use natural colors instead in food, pharmaceuticals, cosmetics, textiles and as printing dyes. As such, PBPs deserve some recognition: in the food industry, B-PE and b-PE extracted from *Porphyridium cruentum* and R-PE from the seaweed *Porphya* sp. are used as red colorants especially for jellified desserts and dairy products, whereas PC isolated from *Arthrospira platensis* is widely used as natural pigment in sorbets, ice-creams, soft beverages, desserts, jellies, chewing gum and other dairy products [48].

Additives for functional food constitute another emergent promising field [54]; as for cosmetics, the blue pigment is applied in lipsticks, eyeliners, sun-protecting creams and as vital anti-aging factors [48]. Another application of PBPs (and particularly phycoerythrin for its remarkable fluorescence and remaining features described above) is as fluorescence probes in flow cytometry, fluorescent immunoassays, histometry, fluorescent labelling and fluorescent microscopy (a whole set of fluorescence-based detection systems) for diagnostics and biomedical research [49,55]. Moreover, since R-PE subunits carry chromophore groups characterized by a deep rose color and its subunits have low molecular weight, they can also be used as protein markers (internal markers) for electrophoretic techniques (e.g. SDS-PAGE) and size gel exclusion chromatography [55]. Finally, PE constitutes a very important reagent in proteomics and genomics, forming the basis of the detection system in the DNA microarray technology [45].

Recently, phycobiliproteins have stopped being just mere colored pigments to become globally known valuable bioactive compounds in therapeutics, nutraceuticals and in the pharmaceutical industry. Some of their health-promoting biological activities are, undoubtedly, noteworthy to refer: specifically for R-PE, the immunosuppressive, antitumoral, antiparasitic, antiviral, anti-inflammatory and antihypertensive activities, the anti-oxidative and antidiabetic properties, the neuroprotective effects, the anticancer action, the anti-alzhelmeric potential, among others, stand out [47,49,50]; meanwhile, for phycocyanin (PC), the antioxidant (ability to scavenge the reactive oxygen species alkoxyl, hydroxyl and peroxyl radicals connected to Alzheimer’s or Parkinson’s disease), the antitumoral (reduction of the levels of tumor necrosis factor (TNF-α) in the blood serum of mice-treated with endotoxin) and the anticancer activities (inhibitory effect, in a dose- and time-dependent manner, on the growth of human leukemia K562 cells) are highlighted as well, along with an hypocholesterolemic action or even the potential of inhibiting microsomal lipid peroxidation

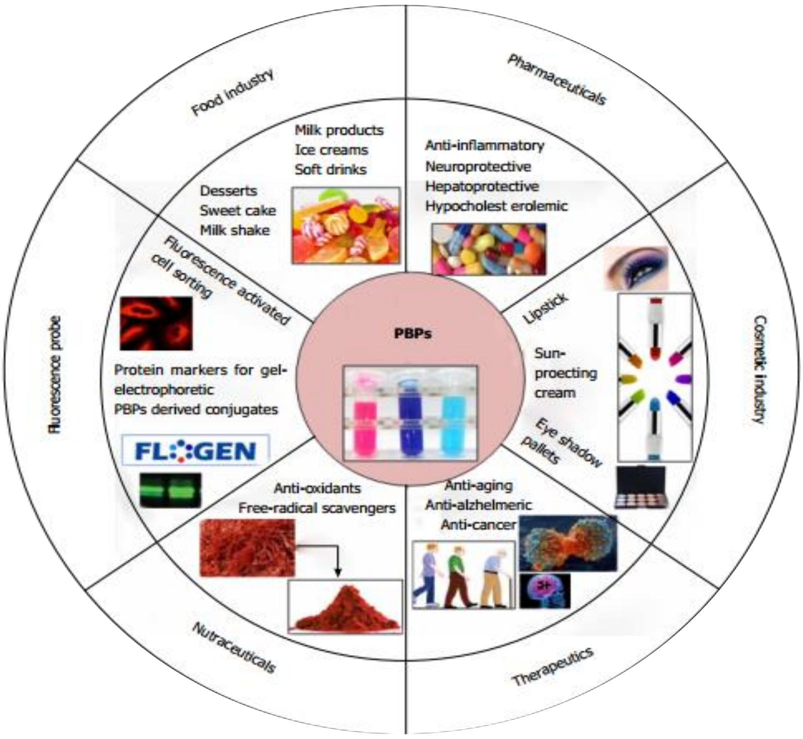

Food industry
Pharmaceuticals
Milk products
Ice creams
Soft drinks
Desserts
Sweet cake
Milk shake
Anti-inflammatory
Neuroprotective
Hepatoprotective
Hypocholest erolemic
Fluorescence activated cell sorting
Lipstick
PBPs
Sun-proecting cream
Protein markers for gel-electrophoretic
PBPs derived conjugates
Eye shadow pallets
Fluorescence probe
Cosmetic industry
Anti-oxidants
Free-radical scavengers
Anti-aging
Anti-alzhelmeric
Anti-cancer
Nutraceuticals
Therapeutics

Figure 1.5: Application of phycobiliproteins (PBPs) in food, cosmetics, pharmaceutical and biomedical industries. Adapted from [49].

[45,47,50].

Therefore, and even though only a handful of studies about the biological activities of R-PE is available yet, the pigment already fulfills many human requirements in diverse areas, leaving even greater opportunities for future research.

1.5. R-Phycoerythrin Conventional Extraction Procedures

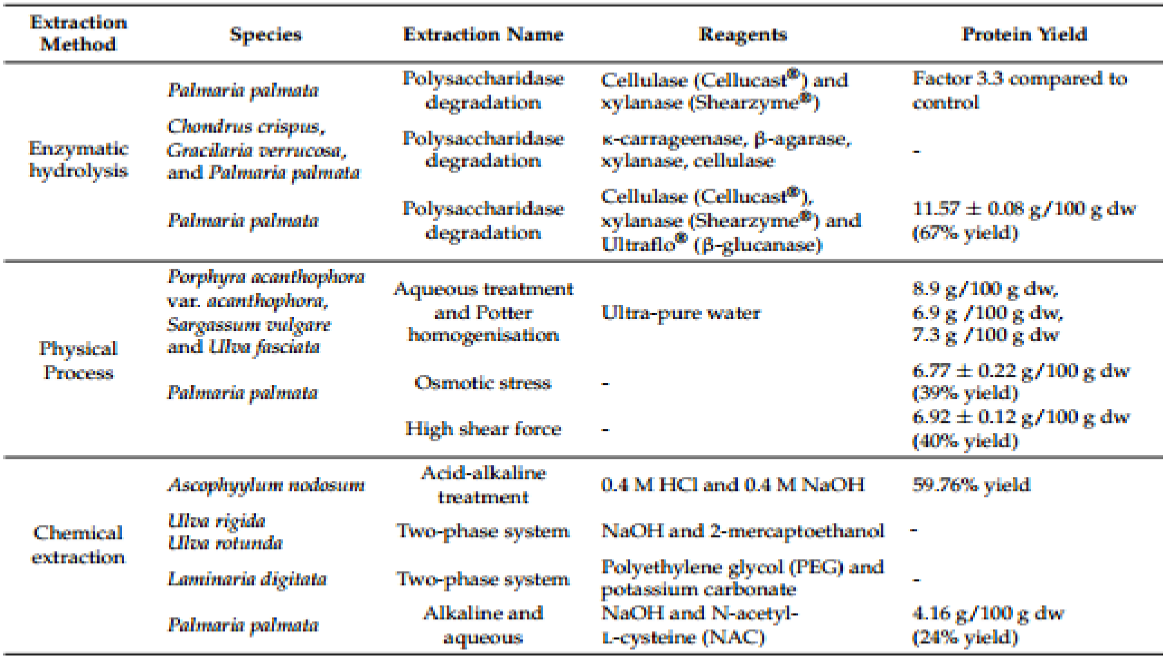
Before the application of any method for extracting phycobiliproteins from macroalgae, there is an extremely important assumption to be taken into account that is capable of conditioning any of the available extraction methods – the fact that seaweed cell walls are constituted by a strongly linked polysaccharides network involving compounds such as cellulose, alginates, xylans, agar or carrageenans (the latter two in greater proportion in rhodophytes), which, allied to a strong covalent bonding between mix-linked xylan and glycoprotein complexes, are capable of conferring a outstandingly tough and cohesive rigidity (even though flexible and permeable as well), providing the cell with an amazing structural support and protection against aggressions from the surrounding environment [13,56]. Ergo, this structure constitutes the biggest obstacle in accessing and extracting PBPs from the macroalgae biomass – the presence of elevated and varied contents of polysaccharides is considered a limiting step, conditioning the contact between the extraction solvent and the protein content, thus significantly reducing the extraction efficiency.

PBPs are generally extracted from phycobilisomes either from fresh biomass or from oven-dried or freeze-dried (dry) biomass (initial condition of the macroalgae) using a gigantic variety of cell disruption methods: going from repeated freezing and thawing cycles, osmotic shocks, sonication, mechanical homogenization to enzymatic treatments, the truth is that choosing the right cell disruption technique is the key to achieve maximum efficiency and levels of product recovery [48,50] There is no such thing as a standard technique to collectively extract PBPs from algal material. Cell wall composition may differ for groups of algae and even from species to species, varying with the seasonality factor as well.

Among the classical (or conventional) extracting processes of PBPs, three main types are highlighted: the physical and the chemical treatments and the enzymatic hydrolysis (Table I.II) [50]. Regarding the physical methods, the following is known: PBPs (phycoerythrin (PE) in this case) can be simply extracted by soaking seaweeds in water for one or several days – phenomenon possible and attributed to the condition of them being water soluble [48]. In this case, by extracting proteins by means of cellular osmotic shock, there are some disadvantages associated to the method as its high slowness and partial degradation of PEs by proteases [48,57]; normally R-PE is classically extracted by maceration of seaweeds in (sodium) phosphate buffer (5-50 mM, pH 7) or in water; a very common and advantageous alternative (but sometimes inefficient and not very profitable on an industrial scale) is the grinding process of macroalgae in liquid nitrogen (increasing the contact surface between the biomass and the solvent) to facilitate the destruction of cell walls, being the resultant powder normally homogenized in phosphate buffer [45,58]. Other methodologies involving the usage of a Potter homogenizer (tissue grinder) followed by immersion in ultra-pure water, high shear force mixers, French presses, mortar and pestle, and ultrasonic probes or baths (sometimes with sand or small-particle silica to further aid the disruption process) are also currently used in the extraction of R-PE (Table I.II) [50]. As for the chemical methods, algal proteins may be extracted by means of aqueous, acidic and alkaline treatments followed by several rounds of centrifugation to recover the pigment [50,58]. It's the example of the two-phase acid and alkali treatments or the aqueous two phase extraction, being this last one employed in the extraction of R-PE from the red seaweed *Gelidium pusillum* [59].

However, the successful extraction process and its efficiency can be greatly influenced by the bioavailability of the protein molecules, which, in turn, can be substantially hindered by high viscosity and anionic cell-wall polysaccharides such as carrageenans in red seaweed [13,60]. Despite very good results and quite a lot promising extraction yields attributed to physical and chemical methods, alternative extraction methods for R-PE and other seaweed proteins were investigated, being the enzymatic hydrolysis of the cell wall suggested as another way of accessing algal protein.

Table I.II: Enumeration and comparison of different conventional pre-treatment cell disruption methods and extraction methods for recovering proteins from distinct species of seaweed. Dry weight; dw.



Extraction Method	Species	Extraction Name	Reagents	Protein Yield
Enzymatic hydrolysis	*Palmaria palmata*	Polysaccharidase degradation	Cellulase (Cellucast$^{®}$) and xylanase (Shearzyme$^{®}$)	Factor 3.3 compared to control
	Chondrus crispus, *Gracilaria verrucosa*, and *Palmaria palmata*	Polysaccharidase degradation	κ-carrageenase, β-agarase, xylanase, cellulase	-
	Palmaria palmata	Polysaccharidase degradation	Cellulase (Cellucast$^{®}$), xylanase (Shearzyme$^{®}$) and Ultraflo$^{®}$ (β-glucanase)	11.57 ± 0.08 g/100 g dw (67% yield)
Physical Process	*Porphyra acanthophora* var. *acanthophora*, *Sargassum vulgare* and *Ulva fasciata*	Aqueous treatment and Potter homogenisation	Ultra-pure water	8.9 g/100 g dw, 6.9 g /100 g dw, 7.3 g /100 g dw
	Palmaria palmata	Osmotic stress	-	6.77 ± 0.22 g/100 g dw (39% yield)
		High shear force	-	6.92 ± 0.12 g/100 g dw (40% yield)
Chemical extraction	*Ascophyylum nodosum*	Acid-alkaline treatment	0.4 M HCl and 0.4 M NaOH	59.76% yield
	Ulva rigida *Ulva rotunda*	Two-phase system	NaOH and 2-mercaptoethanol	-
	Laminaria digitata	Two-phase system	Polyethylene glycol (PEG) and potassium carbonate	-
	Palmaria palmata	Alkaline and aqueous	NaOH and N-acetyl-L-cysteine (NAC)	4.16 g/100 g dw (24% yield)

Adapted from [50].

As such, the use of enzymes such as polysaccharidases (e.g. cellulase, xylanase, κ-carrageenase, β-agarase) could be applied as a cell disruption treatment prior to protein extraction in order to increase protein yield [61,62]. Furthermore, the enzymatic process doesn't appear to be denaturant for the pigment and, in the majority of the cases, demonstrate a much higher PE recovery yield compared to mechanical extraction methods that grind seaweeds [50,62] (Table I.II). Ultimately, the optimization of the enzymatic process is even capable of enhancing very satisfactorily the purity index of PBPs in some situations [63].

Nevertheless, the conventional protein extraction methods (either mechanical or enzymatic) conducted to date in seaweeds possess some constraints: they are limited for commercial use due to concerns with scaling-up processes; may affect the integrity of extracted PBPs due to the release of proteases from cytosolic vacuoles; are laborious and time consuming; the price of enzymes or enzymatic mixtures is a heavy economic conditioner; involve relatively high solvent consumption, etc. [45,50]. Thereat, a necessity is urging in developing advanced and improved methods of cell disruption and extraction of proteins (and, therefore, phycobiliproteins) capable of responding to these limitations and, as such, satisfying the interests of leading industries turned to the giant sphere of marine resources.

A final remark on the R-PE purification methods is almost obligatory due to the high demands in purifying the pigment solutions after extracting the PBPs content. The applying of PBPs in realms such as pharmaceutics, nutraceuticals and therapeutics (fundamentally) requires high levels of purity. Varying with the source (organism), protein stability, degree of purity, supplying company and lab recognition, its prices can normally range between US$ 160-620 mg^{-1}, even reaching values of US$ 1500 mg^{-1} for highly purified molecular markers or crossed-linked pigment solutions (e.g. with antibodies) [53]. Some of the different methodologies proposed to isolate and purify R-PE (having as goal the achievement of better purity ratios) consist fundamentally in the ammonium sulfate precipitation and several chromatographic techniques (e.g. ion exchange chromatography, gel filtration chromatography, hydroxyapatite chromatography, among others) [46,49,58].

1.6. Study Site: Lagoa de Óbidos (Portugal)

The Lagoa de Óbidos (distributed over Caldas da Rainha and Óbidos townships, western region of Portugal) is a coastal lagoon system of enormous ecological importance – the most extensive of the Portuguese coast (6.9 km^2) – situated in a relatively shallow depression (average depth of 2 m), of irregular and very unstable boundaries near the sea, whose natural barrier of separation with the Atlantic Ocean is formed by a chain of coastal dunes composing a part of the locality of Foz do Arelho – where lagoon waters reach the sea. It is possible to identify two distinct arms extending upstream - the Braço do Bom Sucesso, to southwest, and the Braço da Barrosa, to east.

Lagoon systems such as Lagoa de Óbidos constitute some of the richest and most diversified ecosystems in Portugal. Due to the constant influx of nutrients and organic matter of continental origin, the system presents a significant primary productivity [64]. Housing hundreds of fish, mollusks and crustaceans' species and being the habitat of an immense birdlife, the lagoon harbors an explosion of life and biodiversity. Within this sphere, some seaweed species also play an important role as is the case of our species of interest - the red seaweed *Gracilaria gracilis* - that grows and flourishes fixed by a holdfast (fixing organ) to the gravel substrate found in some areas of the lagoon or drifting with the stream in the form of tangled "human hair", normally in zones characterized by greater depths. Sometimes, it can also be found beneath the dense prairies of sea lettuce (*Ulva lactuca*) present in the area.

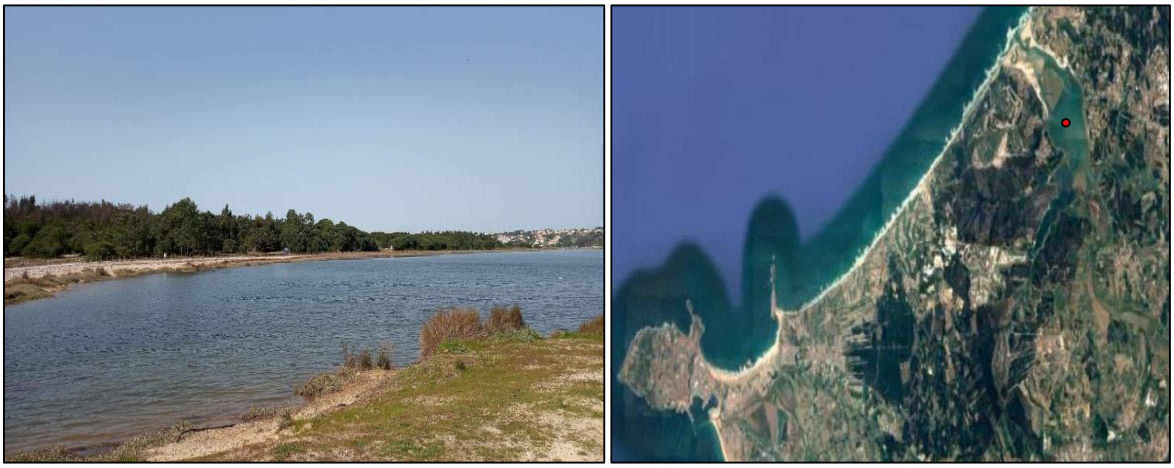


Figure 1.6: On the left, the sampling site of *G. gracilis* - Braço do Bom Sucesso (Lagoa de Óbidos)**.** The seaweed can be found fixed by a holdfast to the gravel substrate characteristic of some areas of this lagoon's arm, or simply loose, drifting with the water stream; on the right, a satellite image of the Lagoa de Óbidos (marked by the red dot) and its tributaries. The peninsula of Peniche is also presented to show close proximity between both locations.

The sampling site where *G. gracilis* was collected in this study is shown in Fig. 1.6. Yet, with the construction of dams along the tributaries and the excess of pollution (contaminating materials coming from two WWTPs (Wastewater Treatment Plants) – Águas Santas and Foz do Arelho -, from ceramics factories, domestic sewage, pesticides industry, etc.) transported to the lagoon, there has been a decrease, in recent years, of freshwater flows compared to salty ones (which tends to cause an alteration of salinity values), with the consequent reduction and/or disappearance of certain species [65,66].

The Lagoa de Óbidos is an ephemeral environment continually shifting its morphology (upstream, on its water channels; downstream, its sandbanks are under constant dynamism, diverting cyclically the opening of the Lagoon to either north or south), with a natural tendency for silting/sedimentation phenomena since a greater transportation of sediment from the sea and rivers to the lagoon, than from the latter to the sea, has always been observed [67]. Downstream, tide and waves action seem to play a significant role as well in its morphology [68]. If its sedimentary regime was to be maintained, the Lagoa de Óbidos, just as the other coastal lagoons, would be transformed, in a natural way and in a medium or long term, into authentic marshes. However, human intervention accelerates the sedimentation process through the constant changes it causes in the watersheds that lead to a greater production of sediment. On the other hand, the population of Lagoa de Óbidos is trying to reverse the situation by intervening through dredging actions carried out to increase the system depth and avoid to close the opening of the lagoon (communication with the sea), thus prolonging the life and avoiding the vanishing of these aquatic environments [65]. The decision to change is in our hands.

Objectives

2. Objectives

Nowadays, in an increasingly technologically advanced world where knowledge is much more easily spread to the four corners of the Earth, it is commonly known that seaweeds and microalgae constitute authentic and rich ponds of proteinaceous compounds [50]. Amongst these, much scientific investigation regarding the extraction of such algal proteins reveals to us that, within the genus *Gracilaria*, many are the species that present high protein levels with special emphasis for the phycobiliproteins [24,69]. Namely, and concerning this study, the R-phycoerythrin pigment [48].

Furthermore, many studies have already focused on the importance of this exclusive type of proteins: from the numerous applications arisen due to their fluorescence properties, passing by their ability to be used as natural colorants, to the varied bioactivities they possess, it's undeniable the benefits that these biomolecules can bring to society [48,49].

Another point to note is that the biomass of *Gracilaria gracilis* in the Lagoa de Óbidos is not inexhaustible. Even though there are extensive prairies of this alga to lose sight of and the brackish water of the lagoon corresponds exactly to its physiological needs for survival, the seaweed in this site is currently threatened by some factors, mainly of human nature: construction works, general pollution, deregulation of freshwater courses arriving at the lagoon, etc.

Therefore, the general challenge inherent to this work is to tackle these environmental and anthropogenic adversities by producing healthy specimens of *G. gracilis* in laboratory, avoiding as far as possible the excessive harvesting and recurrence to these threatened wild reservoirs.

2.1. Specific Objectives

Based on the observations abovementioned, three major objectives will be fulfilled:

1. Application of different methodologies – mechanicals and an enzymatic one – in the extraction of the phycobiliproteins R-phycoerythrin (R-PE) and R-phycocyanin (R-PC) of high commercial value from: the culture samples of *G. gracilis*; and, the lab-dried wild biomass coming directly from the Lagoa de Óbidos - comparison between their efficiencies and yields.

2. Attempt to successfully implement the permanence of healthy tips of *G. gracilis* in laboratory, promoting its growth, avoiding contaminations, and striving to fully simulate some of the main environmental factors conditioning its proliferation and survival in the natural habitat.

3. Study of the influence that specific conditions of light intensity and concentration of essential nutrients – ammonia and phosphate – may have on the growth, total soluble protein content and concentration of phycobiliproteins of the seaweed in cultivation, allowing the manipulation of the quantities of these biological molecules within the algal tissue and, at the same time, the establishment of a lesser dependence on the seasonality factor.

Materials and Methods

3. Materials and Methods

3.1. Sampling and Acclimatization

Specimens of *Gracilaria gracilis* (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 March 2019 during low tide and transported to the laboratory in plastic containers. The seaweed was taxonomically classified according to the online platform *AlgaeBase* [25] and confirmed by proper experts. In the laboratory, each specimen was thoroughly washed with tap seawater and minutely cleaned on trays to remove debris from the lagoon, necrotic, and therefore, unhealthy parts, epiphytes, fouling invertebrates and other organisms from the thalli surface. Seaweeds were kept in constantly aerated seawater (35 ‰) during the week that followed the sampling stage, in a climatic room (24 ± 1ºC) 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 $m^{-2}s^{-1}$).

3.2. Optimization of the Extraction, Quantification and Spectrophotometric Characterization of PBPs

The extraction of phycobiliproteins in this study was conducted according to two naturally different strategies: a mechanical and an enzymatic approach. On the first approach, biomass of *G. gracilis* in two different conditions, dried and fresh, was used for trials. On the second one, only dried biomass was used. Dried material always came from wild individuals whereas fresh had as source the cultivation system. Due to higher availability of wild biomass of *G. gracilis*, this was used to test different methodologies of PBPs extraction. Even though other PBPs beyond R-phycoerythrin and R-phycocyanin may have been also extracted, by manners of interest and simplification, only these two were spectrophotometrically quantified. Appendix A, Figure A.1, shares a better perception of all the extraction planning employed along the study.

3.2.1. By Mechanical Methods

3.2.1.1. Sample Preparation

As aforementioned, in this section both dried biomass and fresh biomass were used to extract the phycobiliproteins content. In the first situation, the preliminary steps of cleaning and adaptation of *G. gracilis* specimens harvested as well from the Lagoa de Óbidos followed the methodology adopted in section 3.1. Sampling and Acclimatization. Afterwards, the seaweed was oven-dried at 25º C for 24 h, being immediately grinded in a commercial blender and stored at room temperature. As for the studies performed with fresh biomass of *G. gracilis*, the biological material came directly or indirectly (accompanied by a freezing step in this last case) from the culture flasks. Due to logistic reasons, non-frozen biomass was used to extract PBPs solely from the assays “Light Increase” and

"Control" of the 3rd Set of Independent Trials, whereas frozen biomass was used to extract PBPs from all assays (Table III.II).

3.2.1.2. Extraction and Quantification

Towards the extraction of PBPs through mechanical ways, two distinctive paths were selected in an attempt to break down the extremely rigid cell walls of *G. gracilis* and hopefully bring out to the extracellular space some of these biomolecules matching the characteristic spectral peaks of R-phycoerythrin and R-phycocyanin. Therefore, a technology employing glass beads of a certain caliber (generally small enough to fit in the tiny tubes compatible with the cell disrupter machine) was implemented for the wild seaweed samples containing a lower content of moisture. As for the fresh cultivated material which presents higher levels of moisture, a procedure encompassing essentially a portable tissue homogenizer was utilized.

A modified version of the method developed by Lawrenz *et al*. (2011) to extract water soluble PBPs from microalgae (namely *Rhodella* sp.) was applied for the wild oven-dried *G. gracilis*. Concisely, 0.1 M phosphate buffer (pH 6.8) was added to the biomass in a ratio of 1 g of biomass to 20 mL of phosphate buffer and stored at -20 °C in 2 mL screw cap micro tubes until frozen (≈ 2 h). Samples were then thawed, a small scoop of glass beads was added, and finally they were subjected to glass bead homogenization (bead beating) in a cell disrupter (FastPrep™ FP120, Thermo Savant, France) for 30 seconds at maximum velocity, being subsequently re-frozen. The process was repeated 5 times and following the final freeze-thaw cycle samples were centrifuged at 12.000 rpm, 4 °C, for 10 min (Centrifuge 5417R, Eppendorf, Germany) to remove cellular debris. Two different caliber of glass beads were tested: smaller pearls with a diameter between 212-300 µm and bigger ones with a diameter of 2 mm. For each of these sizes, additional pre-treatments to the dried biomass were performed before starting the extraction itself, including the following four options summarized in Table III.I: maceration (with mortar and pestle) plus sifting with vases with a mesh of 250 µm; just maceration with no vases; grinding (with a commercial blender) plus sifting with vases; just grinding with no vases. Three sequential extractions, always with addition of new buffer, were executed on the biomass of the "Bigger Glass Beads – Maceration plus sifting with vases" trial by using the sludge obtained immediately in the previous step. All the supernatants were pooled together and the final volume of the extract was 9.5 mL. Throughout all the procedure, aluminum foil was wrapped around the screw cap tubes in order to avoid excessive light exposure. Duplicates were elaborated for every case in Table III.I.

Table III.I: Different pre-treatments applied to the oven-dried wild biomass of *G. gracilis* before the addition of the glass beads (small and big caliber) in the mechanical path of extracting PBPs via bead beating (cell disrupter).

Smaller Glass Beads (212-300 µm)	**Bigger Glass Beads (2 mm)**
- Maceration (mortar and pestle) + sifting with vases	
- Maceration (mortar and pestle) only (without sifting with vases)	
- Grinded (with commercial blender) + sifting with vases	
- Grinded (with commercial blender) only (without sifting with vases)	

Regarding the cultivated samples (Table III.II), so to increase the disruption of cell walls, the extraction of phycobiliproteins followed an adaptation of several works in which authors tried to access phycobilisomes by using the so called traditional (or classic) methods based, in this specific case, on the mechanical action of probes or homogenizers to disrupt seaweed cell walls [24,58,60,70]. It started with the addition of cold distilled water (refrigerated at 4°C), pH 7.1, to approximately 150 mg of cultured *G. gracilis* from each of the assays in a ratio of 1 g of biomass to 20 mL of distilled water. The junction of these components occurred in a commercial blender where the algal material was grinded for 1 minute until complete mashing of its filaments. The resultant mixture was then transferred to Falcon® tubes, standing out evidently two distinct phases: the supernatant, slightly pinkish; and the pellet, composed by algal fragments still very entire. Afterwards, the probe attached to a tissue homogenizer (T18 digital ULTRA TURRAX®, IKA®, Germany) was inserted inside these tubes in a way that its moving and disruptive end could reach and stay in contact with the pellet. Its action over the pellet took about 1 min at the approximate velocity of 17.500 rpm, having as goal a complete homogenization and a more accentuated breakage of cell walls. Lastly, the tubes content was centrifuged for 5 minutes, 4 ºC, at 10.000 rpm (Centrifuge 5810 R, Eppendorf, Germany) to separate the pellet from the supernatant. Another centrifugation cycle may have to be done if pellet leftovers remain buoying among the clean phase. While maneuvering the probe of the tissue homogenizer, the exterior face of the Falcon® tubes should be cooled in ice at the same time due to excessive heat release from the mechanical movement of the probe. Once more, aluminum foil was used to avoid excessive light exposure of our samples. Triplicates for each cultivation condition (Table III.II) were carried out.

As for the quantification methodology, the PBP content, determined equally for samples subjected either to the cell disrupter or to the tissue homogenizer, was estimated from the final supernatant by measuring the absorbance of extracted PBPs at 455, 564, 592, 618 and 645 nm in a spectrophotometer UV/VIS. Phycoerythrin (PE) and phycocyanin (PC) concentrations (mg/mL) were determined spectrophotometrically in every case according to the Beer & Eshel (1985) equations:

[PE] (mg/mL): $[(A_{564}\text{ nm} - A_{592}\text{ nm}) - (A_{455}\text{ nm} - A_{592}\text{ nm}) \times 0.2] \times 0.12$

[PC] (mg/mL): $[(A_{618}\text{ nm} - A_{645}\text{ nm}) - (A_{592}\text{ nm} - A_{645}\text{ nm}) \times 0.51] \times 0.15$

An absorption spectrum was determined by scanning the sample coming from the Independent Trial – "Cool White Fluorescent" - in a range of 200-750 nm wavelengths on an UV-Visible Spectrophotometer (Evolution™ 201, Thermo Scientific™, USA).

3.2.1.3. Microscopic Analysis

Microscopic analyses of pellets were conducted in order to visualize the extent of extraction (essentially degree of cell disruption and release of some cellular content) occurring from the algal material subjected to the mechanical extraction with the cell disrupter. All pellets coming from all situations of pre-treatment of the oven dried wild biomass present in Table III.I were microscopically analyzed along with the pellet resultant from the particular situation of the three sequential extractions (total of four extractions). After centrifugation (as in section 3.2.1.2), small portions of the post-extraction algal filaments (from the pellet) were transferred to a 96-well plate without staining and visualized under light microscope at 200X magnification (Axio Vert.A1, Zeiss®, Germany).

3.2.2. By Enzymatic Methods

3.2.2.1. Sample Preparation

In this case, the biomass used for the enzymatic trials was the same used for the mechanical methodology with the cell disrupter. Ergo, *G. gracilis* came from Lagoa de Óbidos and the sample preparation stage didn´t go through any modifications, being thus dried as depicted in section 3.2.1.1 Sample Preparation.

3.2.2.2. Extraction and Quantification

The extraction process of PBPs through this alternative route summed up to an enzymatic hydrolysis of the previously dried biomass of *G. gracilis* under specific conditions as in studies accomplished by some authors such as Dumay *et al*. (2013) and Nguyen *et al.* (2017). Some changes were made to their standard protocols especially in what regards to the inclusion of a mechanical pre-treatment of the seaweed biomass before the enzyme adding, so to optimize R-PE extraction. These were:

1) grinding of dried biomass; 2) maceration of dried biomass;
3) bead beating of grinded only biomass; 4) bead beating of macerated only biomass

Primarily, around 2 g of pre-treated dry algae was homogenized with 100 mL of acetate buffer 50 mM, pH 5, in a 250 mL Erlenmeyer. Sequentially, a predetermined quantity of the enzyme cellulase C9748 (purified from *Trichoderma longibrachiatum*, Sigma-Aldrich, France) (ratio enzyme/substrate of 20 mg/g dry weight) was added to the mixture and stirred continuously at 175 rpm in a temperature controlled orbital shaker (Stuart SI500, Staffordshire, UK) during approximately 6 hours. During the whole period of the enzymatic hydrolysis, flasks stood and remain at 35 °C always in darkness (covered with aluminum foil) to prevent R-PE degradation. After hydrolysis, the several hydrolysates were centrifuged at 14.000 rpm for 20 min at 4 °C (Centrifuge 5417R, Eppendorf, Germany) to separate undigested residues and solubilized compounds. Supernatants were filtered through 0.2 µm syringe filters and then recovered to be analyzed. In pre-treatment 3) and 4), five bead beating cycles (with 2 mm glass beads) were done without the coupling of any freeze-thaw cycle (as performed in section 3.2.1.2 Extraction and Quantification). Duplicate digestions were carried out, and a control (incubation without any enzymes) was performed simultaneously.

The spectroscopic quantification of PBPs by the Beer & Eshel (1985) equations was carried out from the final supernatant as stated in section 3.2.1.2 Extraction and Quantification without any alterations.

3.3. Selection, Cut and Isolation of Healthy Tips

Several different methods are used for the cultivation of *Gracilaria*. However, with regard to the culture initiation stage, the options available are limited to tip or spore isolation. In this study, the selection, isolation and cleaning/disinfection process of healthy seaweed tips was performed according to Yarish *et al.* (2014), being this stage the most critical in the whole procedure of culturing seaweeds and, therefore, in the obtainment of axenic cultures. Thus, from the previously acclimated *G. gracilis* stock, only the fronds that exhibited a deep dark-red coloration and fleshy thalli, indicative traits of healthy individuals, were chosen. *Gracilaria* fronds were rinsed in a sequence of four beakers, three of them containing clean sterilized seawater, and the last one comprising clean and sterile distilled water. The seaweed was passed through this sequence starting on seawater and finishing on the fresh one. The purpose of the seawater containers was to remove the vast majority of the most evident contaminants visible at bare sight, whereas the final and quick rinse of no longer than 60 seconds (maximum immersion time for not harming the seaweed) in distilled water had as goal the induction of osmotic shock, casting away any adhering organisms harder to detect (e.g. diatoms). The desired cultivating tips (section corresponding to the apical tissue where new and active growth happens) were carefully cut off (approximately 1-2 cm in length, with a scalpel) from the main thicker thalli for each cleaned and rinsed parent frond. Each tip was individually and meticulously wiped down with sterilized cotton-tipped swabs on a stereomicroscope (Stemi™ DV4, Zeiss™, Germany) to better differentiate the eventual contamination, and, subsequently, dragged through an agar gel previously prepared in sterile Petri dishes (1.0 % bacteriological agar, VWR, Radnor, PA USA, in 1:1 distilled water/seawater ratio) to withdraw any remaining microscopic contaminants not removed by the first cleaning with the cotton swabs. Once again, highlighting the extreme importance of obtaining axenic cultures, the process of cleaning the tips with cotton swabs should be, on one hand, careful not to chop off the apical tissue of the tip, and, at the same time, deep and complete to ensure the removal of most epiphytes, adhering organisms and specially diatoms, but, on the other hand, it should also carry a certain delicacy and sensitivity not to damage the outer tissue itself and the tip surface (Fig. 3.1). The drag through agar was performed three times for each tip, and, most importantly, always through unused portions of the agar plate. Forceps, seawater, distilled water and the rest of the tools used in the cleaning process were previously sterilized by autoclave (121 ºC, 20 minutes).

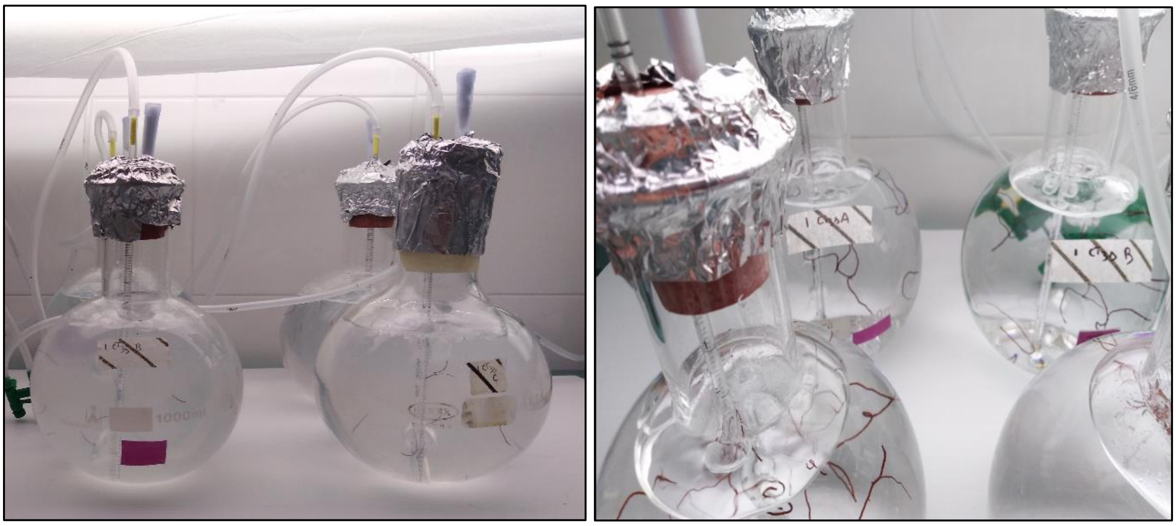


Figure 3.1: Comparison of the aspect of the tips of *G. gracilis*, for the same test conditions and after 5 days of the beginning of cultivation, in two distinct situations: on the left, tips in much less quantity and hardly visible (unhealthy growth) – as result of a rough and excessive cleaning with the cotton swabs; on the right, fleshy and vivacious tips in larger quantity (healthy growth) – as result of a more delicate and attentive cleaning.

3.4. Growth Assays

3.4.1. Media Enrichment & Growth Induction - Establishment of Culture Conditions

Briefly, healthy, clean and weighed seaweed tips were aseptically deposited, recurring to sterile tweezers and a Bunsen burner, into 1000 mL flat bottom flasks (15/30 tips per flask). Cultures were kept in a temperature controlled room (22 ± 1ºC) under constant filtered aeration, with photoperiod set at 12:12 L:D (Light:Dark) and provided either by Plant Growth Fluorescent (Gro-lux) lamps (base value of 39-45 µmol photons $m^{-2}s^{-1}$) or Cool White Fluorescent lamps (base value of 40 µmol photons $m^{-2}s^{-1}$), according to Hayashi *et al*. (2007b) and Yarish *et al*. (2014). Cultures were provided with a liter of sterilized (by autoclave, 121 ºC, 20 minutes) seawater (35 ‰, pH 7) previously enriched with Modified Von Stosch Enrichment (VSE) medium for red algae, prepared according to Redmond *et al*. (2014). The components comprising the VSE media are the macronutrients ammonium (NH_4^+) and phosphate (PO_4^{3-}), the micronutrients iron and manganese, EDTA and the vitamins B_{12}, thiamine and biotin (Appendix A, Table A.I). Nutrient tests were carried out by doubling one, the other, or both concentrations of both macronutrients used. Light intensity tests were performed by variating not only the type of lamp used, but also by oscillating the amount/(quantity) of photons reaching the culture flasks per surface and per time unit. The types of lamps used were the Plant Growth Fluorescent (Gro-lux) lamp (experimenting from 39-45 µmol photons $m^{-2}s^{-1}$ up to 55-85 µmol photons $m^{-2}s^{-1}$) and the Cool White Fluorescent lamp (going from 40 (the flask sections farthest from the lamp) to 110 (the sections closest to the lamp) µmol photons $m^{-2}s^{-1}$). These light intervals were achieved whether by the installation/removal (respectively, decrease or increase of light intensity values) of a paper towel covering the lamp surface or by placing the culture flasks on the top of upside down plastic trays in the hope of increasing the amount of photons converging to them by getting them nearer the light source (Fig. 3.2).

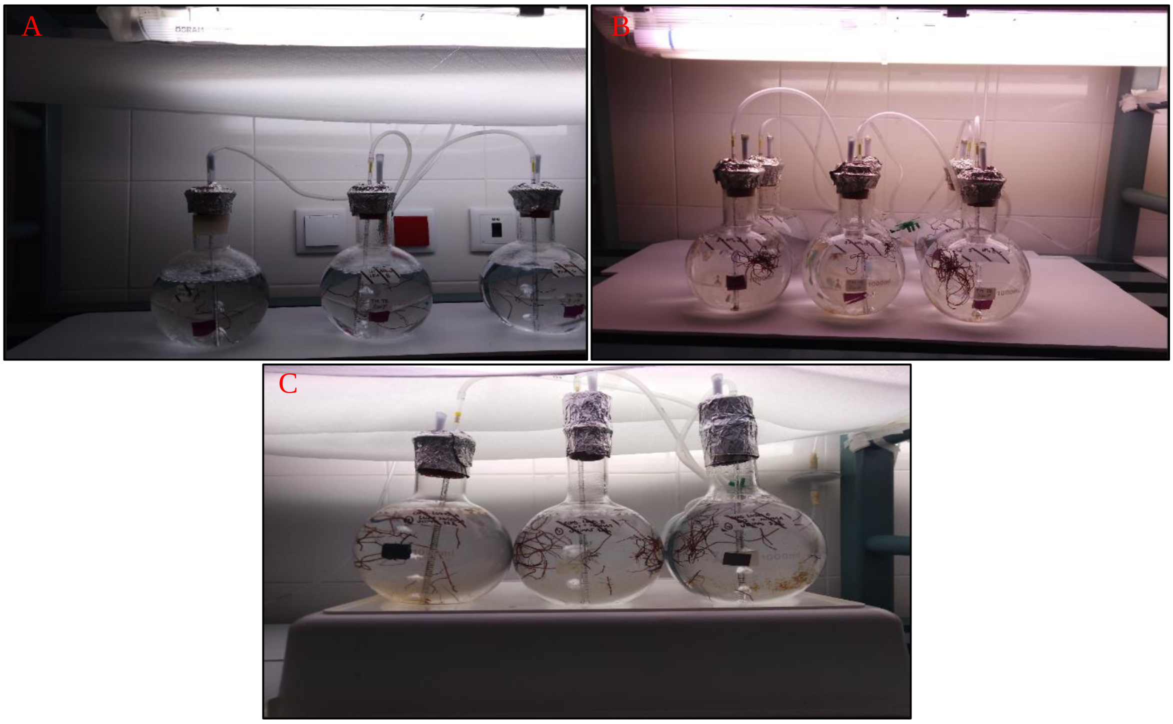


Figure 3.2: Setup of the different strategies adopted laboratorily to vary the intensity of light emited by the Gro-lux lamp that reached the culture flasks: A - installation of a paper towel (39-45 µmol photons $m^{-2}s^{-1}$); B - removal of the paper towel (45-75 µmol photons $m^{-2}s^{-1}$); C - placement of upside down plastic trays along with lamp coverage with a paper towel (55-85 µmol photons $m^{-2}s^{-1}$).

Light intensities were measured with an illuminance meter (T-10A, Konica Minolta, Tokyo, Japan) (given unit is lux), and converted to PPFD (Photosynthetic Photon Flux Density) (given unit is µmol photons $m^{-2}s^{-1}$) according to Thimijan & Heins (1983) and Langhans & Tibbitts (1997). Germanium dioxide (GeO_2, 10 mg/L) was an extra ingredient added to the medium to minimize and prevent the growth and contamination by diatoms. Whenever possible, medium was changed and reestablished fortnightly (without any medium components restrictions) throughout the duration of the experiment. The different range of light intensities used in the several assays, as well as the cultivation time, the variation of nutrients concentration and other conditions, are better depicted in Table III.II. The big difference between the 2nd and the 3rd Set of Independent Trials, in terms of the light effect, consisted fundamentally in varying not only the intensity of the light source, but also the amount of time that cultivating flasks were exposed to those variations - one week or merely 72 hours, respectively. The assay with the cool white fluorescent lamp underwent for more than a month due to an attempt to perform a scale-up of the cultivation tips. It´s important to note that, in order to rise the amount of cultivated biomass available for trials, 30 seaweed tips (instead of 15) were initially introduced solely for the 3rd Set of Trials. Triplicates and controls were performed for all the assays and clean stocks were kept, whenever possible, as backup during the experiment. Assays exhibiting tips where loss of pigmentation soon became evident, visually observed by almost total tip discoloration, were considered to be under stress (e.g. diatom contamination) and without any possible recuperation, being thus promptly terminated.

Table III.II (Part I): Depiction of the different culture conditions (in terms of nutrient concentration, type of lamp, light intensity and cultivation time) adopted in the several trials conducted with tips of *G. gracilis* in a temperature controlled room (22 ± 1ºC) under constant filtered aeration, with photoperiod set at 12:12 L:D (Light:Dark).

	Conditions	**Assay**	**Culture Time** (days)	
Independent Trial	• Cool white fluorescent lamp with paper coverage • Light Intensity: 40-110 µmol photons m^{-2}s^{-1} • VSE medium	Cool White Fluorescent	16 +13 +7	= 36
1st Set of Independent Trials	• Plant Growth Fluorescent (Gro-lux) lamp with paper coverage • Light Intensity: 39-45 µmol photons m^{-2}s^{-1} • VSE medium	Control	14 +14	= 28
	• Same light conditions of the above • VSE medium + 2 x $[NH_4^+]$	Ammonium Increase		
2nd Set of Independent Trials	• Plant Growth Fluorescent (Gro-lux) lamp • Light Intensity: 39-45 µmol photons m^{-2}s^{-1} (paper coverage) - 1st, 3rd and 4th week • Light Intensity: 45-75 µmol photons m^{-2}s^{-1} (no paper coverage) – 2nd week • VSE medium	Light Increase		
	• Same light conditions of the above • VSE medium + 2 x $[NH_4^+]$	Ammonium & Light Increase		
	• Plant Growth Fluorescent (Gro-lux) lamp • Light Intensity: 39-45 µmol photons m^{-2}s^{-1} (paper coverage) for 4 weeks • VSE medium	Control		

+ sign represents media replacement.

Note: different sets of independent trials were established since, during the whole investigation, different harvestings of *G. gracilis* from the Lagoa de Óbidos were conducted. In a season when the lagoon collection spots of *G. gracilis* were suffering from intensive human intervention (besides the oscillation of temperature values as we approached the summer season), compromising the fitness, quality and biochemical composition of the seaweed, the cultivation of *G. gracilis* tips coming from different fronds (aggravating the genetic component) and even from different crops (having in mind this anthropogenic action and natural seasonality) would possibly have an influence on the results obtained for the several study parameters. (continues on the next page…)

Table III.II (Part II): Depiction of the different culture conditions (in terms of nutrient concentration, type of lamp, light intensity and cultivation time) adopted in the several trials conducted with tips of *G. gracilis* in a temperature controlled room (22 ± 1ºC) under constant filtered aeration, with photoperiod set at 12:12 L:D (Light:Dark).

	Conditions	**Assay**	**Culture Time** (days)
3rd Set of Independent Trials	Plant Growth Fluorescent (Gro-lux) lamp with paper coverage Light Intensity: 39-45 µmol photons $m^{-2}s^{-1}$ VSE medium + 2 x $[NH_4^+]$ + 2 x $[PO_4^{3-}]$	Ammonium & Phosphate Increase	14
	Plant Growth Fluorescent (Gro-lux) lamp Light Intensity: 39-45 µmol photons $m^{-2}s^{-1}$ (paper coverage) for 11 days Light Intensity: 55-85 µmol photons $m^{-2}s^{-1}$ (plastic trays & paper cov.) for the last 72 h VSE medium + 2 x $[NH_4^+]$ + 2 x $[PO_4^{3-}]$	Ammonium, Phosphate & Light Increase	
	Plant Growth Fluorescent (Gro-lux) lamp with paper coverage Light Intensity: 39-45 µmol photons $m^{-2}s^{-1}$ VSE medium + 2 x $[PO_4^{3-}]$	Phosphate Increase	
	Plant Growth Fluorescent (Gro-lux) lamp Light Intensity: 39-45 µmol photons $m^{-2}s^{-1}$ (paper coverage) for 11 days Light Intensity: 55-85 µmol photons $m^{-2}s^{-1}$ (plastic trays & paper cov.) for the last 72 h VSE medium	Light Increase	
	Plant Growth Fluorescent (Gro-lux) lamp with paper coverage Light Intensity: 39-45 µmol photons $m^{-2}s^{-1}$ VSE medium	Control	

+ sign represents media replacement.

Note: (…) So, even though it would have been interesting to compare, from a statistical point of view, some of the cultivation conditions between assays from different sets, one opted to limit such analysis within the same sets. Among different sets only visual and numeral analysis were carried out. Yet, due to the extreme importance of performing a comparison between the two types of lamps, statistical analyses were always carried out between the "Independent Trial" (CWF light) and the other sets of independent trials. Ideally, along with the 1st, 2nd and 3rd Independent Sets in Gro-lux light, an assay under CWF light should have also been performed simultaneously for any of these situations (such as the "Control") so that we wouldn't have this variation factor.

3.4.2. Growth Measurements

Growth was solely registered as changes in tip weight along the whole duration of the cultivation trial. Initial measurements were taken at the beginning of each assay whereas final measurements took place after the pre-stablished period of cultivation for each trial/test condition; for trials that underwent for 15 days, i.e. without media renewal, the final weight was recorded only after this period; for more than 15 days, weight measurements were registered each time the media was renewed as well as at the end of the growth experiment. Before measuring tips weight, these were briefly dried in absorbent paper in order to absorb as much water as possible. Daily growth rates were calculated based on the equation below, according to Mtolera *et al.* (1995), Gerang & 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 more accurately recommended to be used as standard for seaweed growth rate determination [74].

$$\text{Daily Growth Rate (\% day}^{-1}\text{)} = \left[\left(\frac{W_t}{W_0}\right)^{\frac{1}{t}} - 1\right] \text{x } 100$$

Where W_0 is the initial wet weight, W_t is the final wet weight, and t is the days of culture.

3.5. Sampling of Cultivated Biomass and Storage

So that the biomass in culture could be used properly in subsequent analyses, namely in the extraction and spectrophotometric quantification of phycobiliproteins (R-phycoerythrin and R-phycocyanin) and determination of the Total Soluble Protein (TSP) content, the new freshly grown tips, that grew into authentic filaments of *G. gracilis*, underwent a small post-culture manipulation. Handling this, in all similar to the cleaning/disinfection methodology with the cotton swabs adopted in section 3.4. of the pre-culture tips, carried out to remove as much as possible of the eventual diatom or other organisms' contamination occurred during the growth of seaweed tips in appropriate culture medium (Fig. 3.3). Thus, with the introduction of this additional step, not only interferences in the absorption peaks of phycobiliproteins would be minimized by the time of spectra plotting, as it could also be possible to reduce exponentially the contamination of the sample itself. Except for two distinct cultivation situations - "Light Increase" and "Control" of the 3rd Set of Independent Trials (referred in section 3.2.1.1 Sample Preparation) - in which fresh biomass was used directly from the culture flasks to estimate the content in phycobiliproteins, the remaining biological material was cautiously sealed in plastic tubes and stored at -20 °C until further analysis.

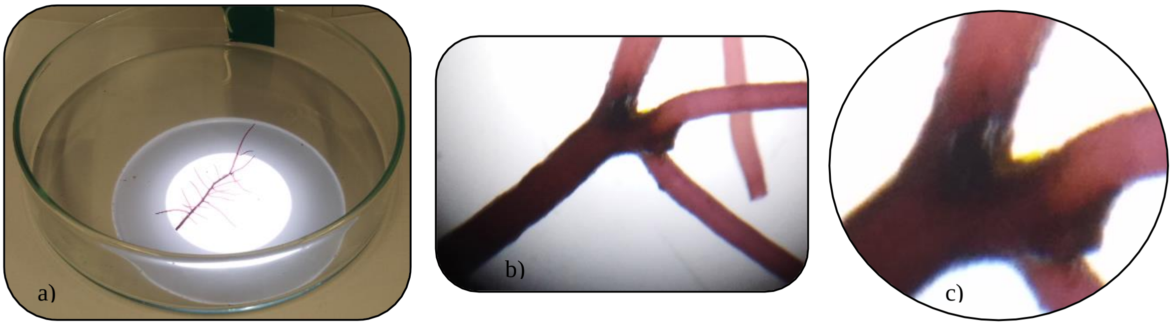


Figure 3.3: Cleaning/disinfection process of grown (post-culture) *G. gracilis* tips. a) – methodology conducted at the binocular microscope; b) tips ramification from a main thicker and darker red thallus (the original cultivation tip); c) amplification of the ramification spot, highlighting the diatom contamination (dark yellow-greenish smudge on the concave area between new tips elongation).

3.6. Estimation of Total Soluble Protein (TSP) Content

3.6.1. Sample Preparation

In order to compare and estimate which portion of the total protein content is normally composed by our proteins of interest - phycobiliproteins -, and from there draw important conclusions about cultivation, it becomes totally imperative to conduct this trial and determine the total protein present in the tissues of this seaweed. The TSP content was estimated solely for the cultivating samples of *Gracilaria*. About 40 mg of lab-grown filaments of each one of the different culture conditions (Table III.II) was manually crushed and fragmented into tiny pieces (thus, increasing the available surface area for the acid in the next step to act) and inserted in 15 mL Falcon® tubes. Afterwards, 2 mL of sulfuric acid (H_2SO_4, 0.1 M) was added to the tubes in a ratio of 0.5 mL of acid to 10 mg of seaweed biomass, being these vortexed for about 30 seconds each. An overnight (≈ 15 h) incubation in a water bath at 37 °C, to let the acidic digestion to occur, followed this agitation preceding step. After this period, samples were submitted to a centrifugation cycle of 10 min, 4 °C, at 13.000 rpm (Centrifuge 5810 R, Eppendorf, Germany) and the obtained supernatant was transferred to new tubes. Triplicate digestions were carried out for each one of the replicas of the different culture assays.

3.6.2. Protein Assay Kit

The estimation of the TSP content was made recurring to a specific kit – the Pierce™ BCA Protein Assay kit (Thermo Scientific™, Rockford, USA) which is a two-component, high-precision, detergent-compatible assay reagent set to measure colorimetrically (A_{562nm}) total protein concentration in a sample compared to a protein standard. The principle of this method is that proteins can reduce Cu^{+2} to Cu^{+1} in an alkaline solution (the Biuret reaction), resulting in a purple color formation by the bicinchoninic acid [75].

The quantification of total protein in our *G. gracilis* culture samples was carried out according to the kit instructions. Briefly, 1 mL of BSA stock (2 mg mL^{-1}, dissolved in H_2O) was prepared and 5 to 8 serial dilutions with a range of 25-2000 µg mL^{-1} were made to generate bovine serum albumin (BSA) standards. The 1 mL of BSA stock solution is present in the kit as albumin standard ampules of 1 mL each. The next step involved the preparation of a BCA (bicinchoninic acid) working reagent (WR) by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B) – the mixture appears to be clear and with a green tonality. The total volume of WR needed is calculated based on the number of BCA standards performed, on the number of samples to analyze, its replicas and volume of WR used per sample. As regards to microplate measurement, 25 µl of each standard or protein sample replicate was pipetted into a microplate well. Right after, 200 µl of WR reagent was added to each well, ensuring an effective and proper mixing of both these components by placing the microplate on a plate shaker for 30 seconds. This step had to be completed in the least time possible because, since the moment the WR is added to the standards or protein samples, the reaction is already taking place before the achievement of the ideal conditions of light and temperature. Nextly, microplates were incubated in the dark at 37 °C for 30 min, allowed to cool at room temperature (RT) for 10 min and the absorbance was read at 562 nm on a plate reader (Synergy H1 Hybrid Reader, BioTek® Instruments, Vermont, USA). Triplicate readings were made for each standard, protein sample replicate and for the blank situations. Water and protein sample preparation buffer were used as blank solutions for standard curve and protein samples, respectively. The average 562 nm absorbance measurement of the Blank standard replicates was subtracted from the 562 nm measurements of all other individual standard and unknown sample replicates. A standard curve was prepared by plotting the average Blank-corrected 562 nm measurement for each BSA standard against its concentration in µg/mL. This curve was used to determine the protein concentration (in µg/mL) of each unknown sample.

3.7. Statistical Analysis

In order to study the effect of the assay (“Cool White Fluorescent”; “Control” and “Ammonium Increase” - 1st Set; “Control”, “Light Increase” and “Ammonium & Light Increase” - 2nd Set; “Control”, “Light Increase”, “Phosphate Increase”, “Ammonium & Phosphate Increase” and “Ammonium, Phosphate & Light Increase” - 3rd Set) on the parameters “Growth Rate”, “Phycoerythrin (PE) content”, “Phycocyanin (PC) content” and “Total Soluble Protein (TSP) content”, a one-way analysis of variance (ANOVA) was performed. All requirements inherent to the analysis (namely data normality and homogeneity of variances) have been validated. In cases where these were not fulfilled, the nonparametric Kruskal-Wallis test was performed [76]. Whenever the results showed statistically significant differences, multiple comparison tests were performed (namely the Dunnett test for control comparisons and the Least Significant Difference (LSD) test for the remaining cases). In addition, to evaluate the statistically significant differences between the PBPs (phycoerythrin and phycocyanin) content of non-frozen and frozen (-20 °C) samples, when comparing the trials “Light Increase” and “Control” (3rd Set of Independent Trials), the *t*-Student test was performed. All differences were considered significant at a level of significance of 5 % (*p-value* < 0.05). All data is expressed as means ± standard deviation (SD). The software IBM SPSS Statistics 25 was used to perform all statistical analyses.

Results and Discussion

4. Results and Discussion

4.1. Determination and Optimization of the Best Methodology for PBPs Extraction

In the light of the objectives of the present study, besides the mechanical methodology of the bead beating cycles associated with freeze-thaw cycles and an approach involving an enzymatic hydrolysis with an enzyme capable of degrading a constituent of the *G. gracilis* cell wall - the cellulose -, it was also intended to experiment another mechanical method to effectively extract phycobiliproteins from *G. gracilis* - a cellular disruption with a tissue homogenizer. Since the quantities of biomass coming from the culture assays were very limited, wild (non-cultivated) biomass of *G. gracilis* harvested from the Lagoa de Óbidos, available in much more abundance, was directly used to perform the trials with the first two referred methodologies. Such choice had as basis the experimentation of different techniques with the goal of determining the best extraction methodology of PBPs before the application itself to the scarce, laboratory-grown, seaweed biomass.

4.1.1. PBPs Extraction of dried *G. gracilis* using the Bead Beading Technology

Before the extraction itself of PBPs by this technology, different pre-treatments were applied to this oven-dried wild biomass of *G. gracilis* (Fig. 4.1 and Table III.I, section 3.2.1.2 Extraction and Quantification of PBPs by Mechanical Methods, in Materials and Methods) with the goal of studying an eventual influenced of these over the extracted content. The main foundation of this technique is that a continuous exposure of biomass to the friction and collision provoked by beads (normally made of glass or steel) leads to cell-wall rupture, resulting in the release of intracellular contents into the solvent medium. By positioning the beads in a cylindrical closed compartment along with the biomass of interest and subjecting them to high-speed agitation, cell walls are disrupted by being physically grinded "against the solid surfaces of beads" in an uninterrupted and violent spinning motion [77,78]. Bead-beating can be applied separately or in combination with chemical techniques or other types of mechanical methods. It's disruption efficiency is mainly dependent on the amount of beads, their size, composition, the degree of contact with cells, the exposure time, the mixing device speed and the characteristics of cell walls [79]. For instance, for microalgae, the optimal beads diameter is 0.5 mm [80].

Figure 4.1 shows the results of this assay. In here, the concentration of PBPs - R-phycoerythrin (R-PE) and R-phycocyanin (R-PC) -, in mg g^{-1} of DW, as function of each one of the different pre-treatment situations, is presented. The type of beads used in the bead beating cycles, after each treatment, is also highlighted on the chart.

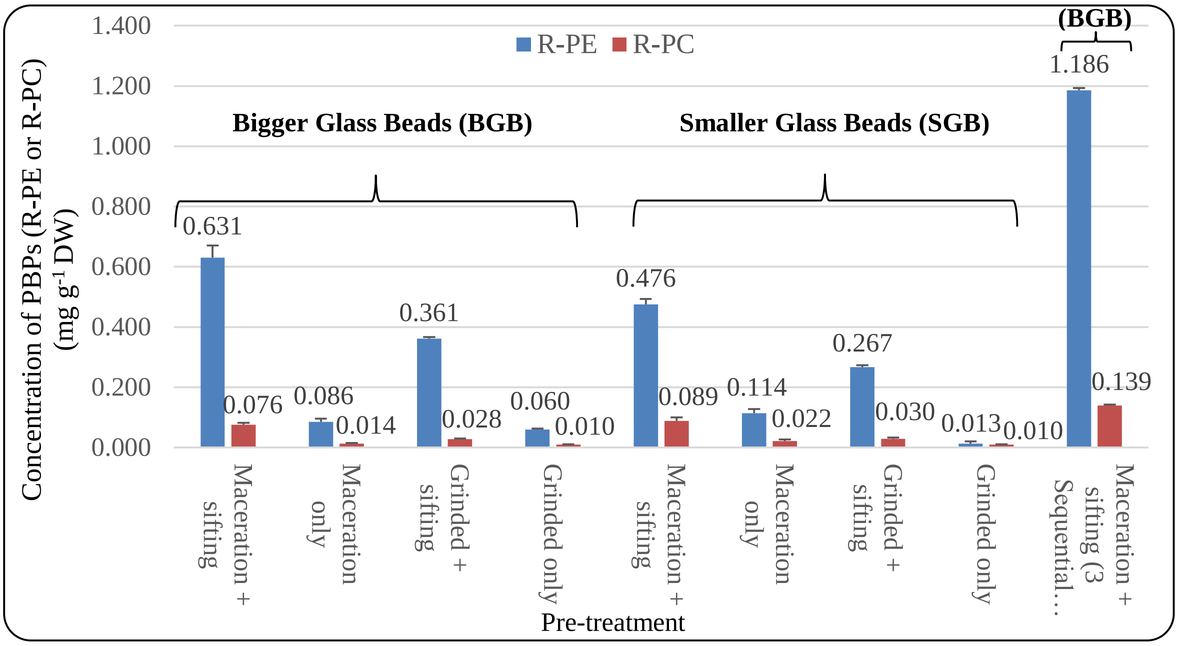


Figure 4.1: Concentration of phycobiliproteins (PBPs) - R-phycoerythrin (R-PE) and R-phycocyanin (R-PC) -, in mg of pigment per g of dry weight (DW) of seaweed, for each one of the different pre-treatment situations of *G. gracilis* biomass. The type of beads used in the extraction process, after each pre-treatment, are highlighted. The color of the bars is not connected to the color of the pigment. Results are expressed as means ± SD (*n* = 2).

In this figure it quickly becomes deducible that higher R-PE and R-PC contents were obtained for the condition where three sequential extractions were performed on the biomass of the "Bigger Glass Beads - Maceration plus sifting with vases" trial/pre-treatment situation, attaining maximum values of 1.186 ± 0.007 mg g^{-1} DW for R-PE and 0.139 ± 0.004 mg g^{-1} DW for R-PC. This trial was chosen, among all the others, to perform the sequential extractions since it was the one which gave better initial results of R-PE concentration. Apart from this situation where better results were already expected - it benefited from the new addition of buffer three more times (higher extraction volume) than the other situations, as well as from a longer lasting and more persistent disruption of *G. gracilis* cell walls -, the highest R-PE concentration was always obtained for a biomass pre-treatment that included maceration (with mortar and pestle) plus sifting with vases: 0.631 ± 0.041 for BGB and 0.476 ± 0.018 mg g^{-1} DW for SGB. The R-PC contents followed the opposite direction, being extracted in slightly greater quantities with the SGB than with the BGB (0.089 ± 0.012 against 0.076 ± 0.007 mg g^{-1} DW). For the rest, exists a concordance between the bead calibre and the remaining assays for both PBPs, in what concerns the highest and lowest concentrations of R-PE and R-PC found: for BGB, "Grinded + sifting" (0.361 ± 0.005 mg R-PE g^{-1} DW / 0.028 ± 0.002 mg R-PC g^{-1} DW) > "Maceration only" (0.086 ± 0.010 mg R-PE g^{-1} DW / 0.014 ± 0.001 mg R-PC g^{-1} DW) > "Grinded only" (0.060 ± 0.003 mg R-PE g^{-1} DW / 0.010 ± 0.001 mg R-PC g^{-1} DW); for SGB, "Grinded + sifting" (0.267 ± 0.007 mg R-PE g^{-1} DW / 0.030 ± 0.004 mg R-PC g^{-1} DW) > "Maceration only" (0.114 ± 0.014 mg R-PE g^{-1} DW / 0.022 ± 0.005 mg R-PC g^{-1} DW) > "Grinded only" (0.013 ± 0.007 mg R-PE g^{-1} DW / 0.010 ± 0.001 mg R-PC g^{-1} DW). Except for the pre-treatment "Maceration only" where the concentration of both R-PE and R-PC extracted with smaller glass beads was superior than that extracted with the bigger ones, in the remaining conditions the extracted

PBP content was always higher when the bigger pearls were employed. Two things have become clear with this study: the best way to extract PBPs seems to be by using the “bigger” glass beads instead of the “smaller” glass beads; as for the best pre-treatment, it seems that decreasing the size of the seaweed fragments on which the beads will act - through the small diameter mesh sifting vases - greatly contributes to raise the concentration of R-PE and R-PC. As for the first observation, it is emphasized that the size of beads is important - the higher the volume ratio of beads to cell suspension (*G. gracilis* in phosphate buffer in this case), the faster the rate of cell disruption [78]. Therefore, “bigger” pearls will damage cell walls more intensively. Contrarily, one must also be aware that the “bigger” ones will cause a less number of collisions than the “smaller” ones. As for the vases, by decreasing the calibre of the algal filaments to values of 250 µm (vase mesh) - close to the calibre of the “smaller” beads (212-300 µm) - we practically obtained an algal powder of similar dimensions and of much more brittle cell walls where beads were capable to act more efficiently and disruptively. By employing the “bigger” beads, it is very likely that the now reduced filaments had been totally crushed by the collision movements, extracting a much larger content in PBPs.

There are some evident drawbacks associated with this extraction methodology. For instance, the tiny portions of biomass able to be introduced at a time in the proper tubes, allied to the energy costs that the disruption process entails as well as the enormity of time that the various freeze-thaw cycles take to be concluded, make an eventual extrapolation of this methodology into the industrial field impracticable. The constant decrease in the algal calibre with the sifting vases (to obtain the best results possible) doesn’t contribute either, at all, for the process profitability. Then, as this is an exclusive methodology for extracting phycobiliproteins from microalgae, there are practically no studies with seaweeds comparable to our results. And yet, if we compare the low R-PE and R-PC values obtained here with the higher ranges that are normally achieved by other authors for the same genus of seaweeds [24,46,81], we can conclude that this may not be the most efficacious path to adopt in the extraction of PBPs. That´s why we decided to perform microscopic analysis of the resultant pellet - to visualize the magnitude of extraction with this methodology -, as well as to test other extraction methods in the hope of obtaining better pigment results.

4.1.1.1. Microscopic Analysis of the resultant pellet

After extracting and spectrophotometrically quantifying the content in PBPs for each of the pre-treatment situations, microscopic observations were conducted, at a magnification of 200x, of the pellets resulting from the various situations in order to visualize and analyze the extent of extraction (essentially the degree of cell disruption and the release of cellular content). In Figure 4.2 stand the microscopic images of *Gracilaria gracilis* macroalgal strands composing the pellet which was obtained after pigment extraction. In a general form, the first assumption to make is that *G. gracilis* cell walls were only weakly damaged by the combined action of bead beating and freeze-thaw cycles, which is justified by an almost non-existent modification in the cell wall morphology (external boundaries) of the algal filaments visible in the images. In fact, in some images more than others, cell walls remain practically intact - for example, B_4 (“Grinded only” trial followed by small calibre glass beads (SCGB)) and A_4 (“Grinded only” trial followed by big calibre glass beads (BCGB))

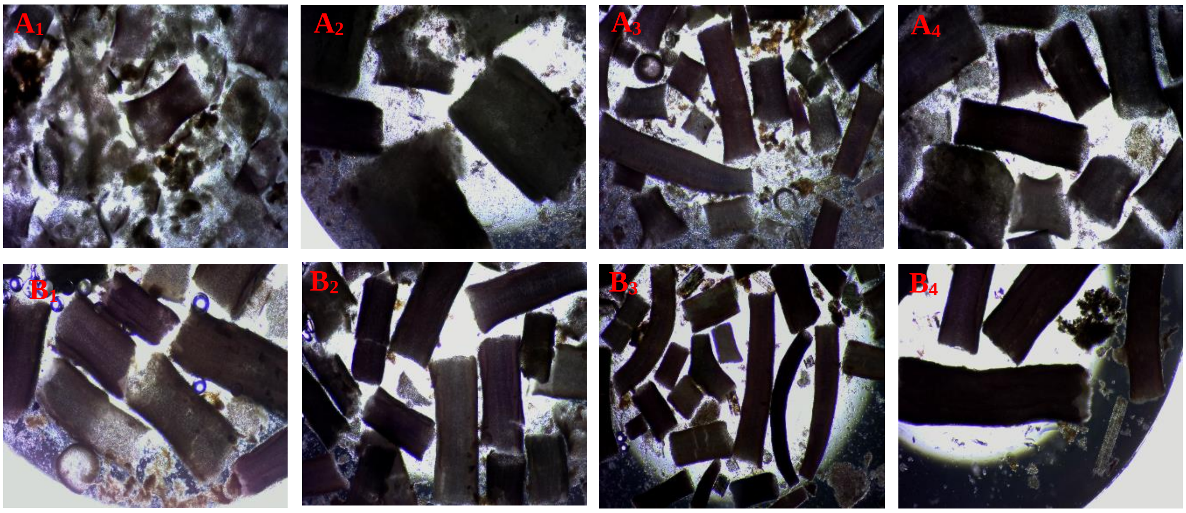


Figure 4.2: Microscopic images, at a magnification of 200x, of *Gracilaria gracilis* macroalgal strands after the mechanic extraction of PBPs via bead beading for each one of the different pre-treatments stated in Table III.I. From the top to the bottom and from the left to right: A) – big caliber glass beads (BCGB): A_1) – macerated and sifted biomass (3 sequential extractions); A_2) – macerated biomass only; A_3) – grinded and sifted biomass; A_4) – grinded only biomass | B) – small caliber glass beads (SCGB): B_1) – macerated and sifted biomass; B_2) – macerated biomass only; B_3) – grinded and sifted biomass; B_4) – grinded only biomass.

corresponding to the biomass pre-treatments from which the smallest amounts of PBPs (R-PE and R-PC) were extracted (see Fig. 4.1). Two other situations where cell walls appear to be relatively more damaged (albeit unclearly) and where there seems to be, in relation to the remaining assays, a more evident rupture with the consequent release of a larger proportion of cellular components, correspond to the images A_3 e B_1. These images resulted, respectively, from the pre-treatments “Grinded + sifting” (followed by BCGB) and “Maceration + sifting” (followed by SCGB) from which some of the highest phycoerythrin values were extracted with this methodology - 0.361 ± 0.005 mg R-PE g^{-1} DW and 0.476 ± 0.018 mg R-PE g^{-1} DW, respectively. The only photograph that clearly stands out from the rest is the A_1, correspondent to the situation where the three sequential extractions were performed and from which maximum contents of R-PE were obtained (1.186 ± 0.007 mg g^{-1} DW) and of R-PC (0.139 ± 0.004 mg g^{-1} DW). In this image, a clearer destruction/disruption of the seaweed cell walls is observed, as well as a greater release of cellular content into the extracellular space. The algal strands in this case appeared much lighter in colour (even though slightly pinkish yet) as result of extracting larger quantities of PBPs from the same amount of added biomass due to its physical maceration. For the remaining conditions, where the extraction process was less accentuated, the pellet still lodged strongly reddish-pinkish hues after the extraction - an indicative sign that considerable amounts of pigment still remained inside the cells.

These findings were similar to the ones found by Mittal *et al.* (2017) when extracting phycobiliproteins from fresh *Gelidium pusillum* through several different methods. Besides reporting that a serial extraction (composed of 5 cycles of maceration using mortar and pestle) with phosphate buffer gave the better results in terms of cell wall disruption, the authors also stated two more things: the employment of freeze-thaw cycles alone resulted in a very weak breakage of cell walls; yet, its combination with a maceration process (with mortar and pestle and facilitated by phosphate buffer) allowed the release of more cellular content and consequent quantification of larger quantities of PBPs. This comparison can only be made at the level of biomass pre-treatment (and freezing & thawing) before the employment of the bead beating cycles since they didn't employ this technology.

4.1.1. PBPs Extraction of fresh *G. gracilis* using the Tissue Homogenizer Technology

As previously mentioned, one of the main basis of this study was to test different methodologies of phycobiliproteins extraction to find the most efficacious one capable of yielding higher amounts of these proteins. As such, another mechanical method employing a tissue homogenizer equipment was applied to fresh cultured biomass of *G. gracilis*. In addition, it was also intended to evaluate the effect that freezing *G. gracilis* culture samples at -20 ºC could eventually have on the R-PE and R-PC contents to be extracted from the cultivation biomass through this methodology. Briefly, in this technique, it's possible to say that the rotor of the homogenizer acts as a centrifugal pump allowing to recirculate the liquid and suspended solids, where shear forces, impact, collision and cavitation are in charge of providing rapid homogenization and consequent tissue disruption. Figure 4.3 compares the PBPs content (mg g^{-1} of WW) of R-PE and R-PC extracted from two distinct groups: the first, consisting of the "Light Increase" and "Control" assays (from the 3rd Set of Trials) wherein the biomass was frozen at -20 °C; the second, composed by the same assays, with the variant that this time the biomass was manipulated and laboured immediately after obtainment from the cultivation. First of all, according to this figure, the concentration of PBPs (mainly R-phycoerythin) extracted with the tissue homogenizer from wet biomass was much higher than those extracted by any of the other methods for dry biomass - maximum values of 4.568 ± 1.773 mg R-PE g^{-1} WW and minimums of 2.384 ± 1.038 R-PE g^{-1} WW, proving that this extracting-with-water mechanical methodology is the most effective of the three in the extraction of phycobiliproteins from *G. gracilis*. It hasn't been reported so far in literature similar approaches involving this specific type of tissue homogenizer and its application in the extraction of PBPs specially from *Gracilaria* sp. However, a study conducted with our species of interest - *G. gracilis* - showed suchlike values of R-PE, even though estimated for dry weight (DW), extracted with a different tissue homogenizer - a Potter homogenizer: 3.5 mg g^{-1} DW [24]. Then**,** what can also be seen from these results (Fig. 4.3) is that, for both R-PE and R-PC contents, there aren't any statistically significant differences between fresh and frozen biomass in what concerns assays of the same type (the only ones that matter to compare) - 4.255 ± 1.043/0.024 ± 0.014 mg g^{-1} WW (R-PE/R-PC) against 4.568 ± 1.773/0.099 ± 0.076 mg g^{-1} WW (R-PE/R-PC) ("Light Increase" (Frozen) against "Light Increase" (Fresh)); 2.384 ± 1.038/0.017 ± 0.017 mg g^{-1} (R-PE/R-PC) against 2.828 ± 0.495/0.036 ± 0.013 mg g^{-1} WW (R-PE/R-PC) ("Control" (Frozen) against "Control" (Fresh)) (*p-value* > 0.05, *t*-student test) (Appendix A, Table A.II and Table A.III). To sum up, the freezing process of cultivation biomass at -20 ºC for periods of time still reasonable (from 1 up to 3 months) doesn't appear to yield significant changes in the R-PE or R-PC content that remains in *G. gracilis* tissues. In other words, freezing at -20 °C does not seem to damage (at least quantitatively) the concentration of R-PE and R-PC present in the biological material as verified by the extraction with the tissue homogenizer. On the other hand, qualitatively, Munier *et al*. (2014) demonstrated that, at -20 °C, the absorption and fluorescence spectra of R-PE had only suffered slightly modifications after 336 h, with absorbance and fluorescence properties being the same as at 0 h. On the contrary, at 4° C, the authors found that R-PE was stable up to 48 h whereas after 2 weeks (336 h) both spectra were completely distorted. As such, not having as basis only these evidences, but also the results of the present study, it appears that conserving seaweed samples at a temperature of -20 °C is not a bad hypothesis at all and considered alternative in the case of impossibility to extract the pigment right after the termination of the cultivation trials.

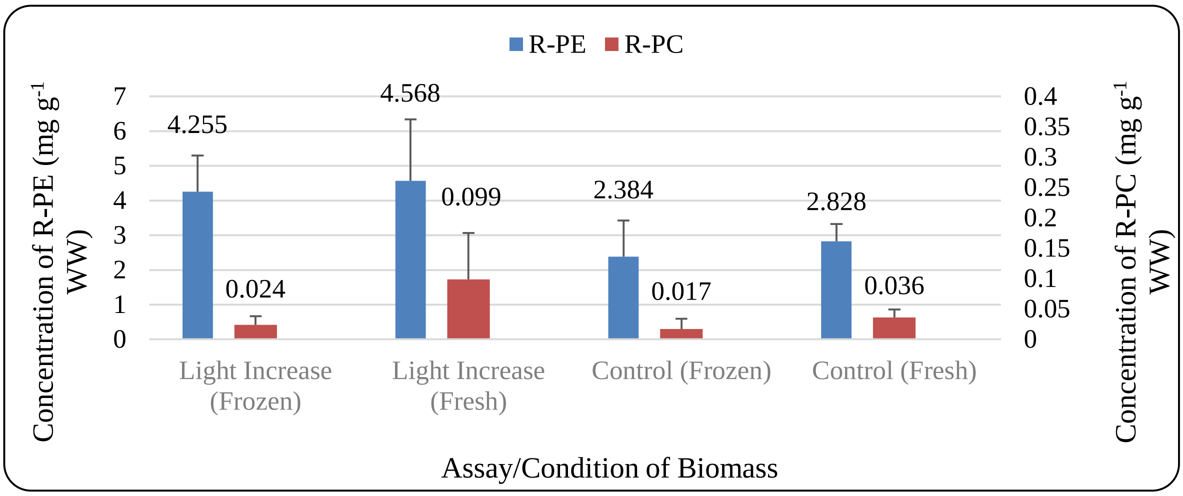


Figure 4.3: Comparison of the phycobiliproteins (PBPs) content - R-phycoerythrin (R-PE) and R-phycocyanin (R-PC) -, in mg of pigment per g of wet weight (WW) of seaweed, between frozen and fresh (non-frozen) biomass of *G. gracilis* from the assays “Light Increase” and “Control” of the 3rd Set of Independent Trials. The color of the bars is not connected to the color of the pigment. Results are expressed as means ± SD (*n* = 3).

4.2.2. PBPs Extraction of dried *G. gracilis* using the Enzyme Cellulase

Besides the application of the mechanical bead beating methodology to extract the phycobiliproteins contents of *G. gracilis*, an approach of different nature was also chosen and conducted for wild biomass harvested from the Lagoa de Óbidos subjected to an oven-dried process in the laboratory - an enzymatic hydrolysis with the enzyme cellulase. Cellulose consists in a polysaccharide composed of a linear chain of several hundred to many thousands of β(1→4) linked D-glucose units and acts as an important structural component of the primary cell wall of, fundamentally, green plants and many forms of algae [13]. It’s one of the key elements that grants cohesion and a spectacularly high rigidity to the cell walls of these groups of organisms. On the other hand, cellulase is one of the enzymes more commonly available to cleave the glycosidic linkage in the cellulose organic polymer, being chiefly produced by protozoans, bacteria and fungi [61,62]. This constitutes thus an alternative route to access algal protein and to increase its extraction yields from macroalgae. The high enzymes cost is the biggest hindrance to the use of this methodology [42,79].

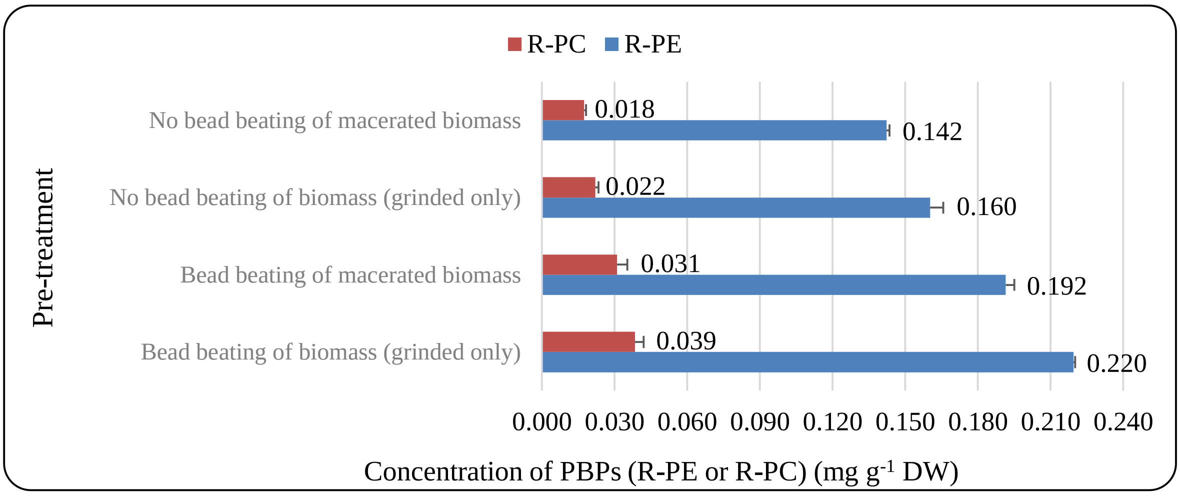


Figure 4.4: Concentration of phycobiliproteins (PBPs) - R-phycoerythrin (R-PE) and R-phycocyanin (R-PC) -, in mg of pigment per g of dry weight (DW) of seaweed, resultant from the enzymatic hydrolysis for each one of the different pre-treatment situations of *G. gracilis* biomass. The color of the bars is not connected to the color of the pigment. Results are expressed as means ± SD (*n* = 2).

Before the enzyme adding, a series of mechanical pre-treatments were conducted on the dried biomass of *G. gracilis*. First of all, the biomass either was previously macerated (with mortar and pestle) or it wasn't macerated (grinded only on a commercial blender). Then, either it suffered a disruption action of its cell walls with glass beads or, on the other hand, it didn't go through this process. The combination of these conditions gave the following four test possibilities: "Bead beating of macerated biomass"; "No bead beating of macerated biomass"; "Bead beating of grinded only biomass"; "No bead beating of grinded only biomass".

Figure 4.4 shows the concentrations of PBPs (R-PE and R-PC), in mg per g of dry weight (DW), obtained for each of the biomass pre-treatment situations after undergoing the enzymatic hydrolysis process. It quickly becomes clear that there isn't a large discrepancy between the various R-PE and R-PC values, which, combined with the absence of a solid statistical analysis, slightly compromises the reliability of the results. Nonetheless, the highest values for both pigments were obtained for the same assay - "Bead beating of grinded only biomass" - with maximum values of 0.220 ± 0.001 mg of R-PE g^{-1} DW and 0.039 ± 0.004 mg of R-PC g^{-1} DW. For the remaining pre-treatments, the concentration of PBPs went by the following order: "Bead beating of macerated biomass" (0.192 ± 0.004 mg of R-PE g^{-1} DW and 0.031 ± 0.004 mg of R-PC g^{-1} DW) > "No bead beating of grinded only biomass" (0.160 ± 0.005 mg of R-PE g^{-1} DW and 0.022 ± 0.002 mg of R-PC g^{-1} DW) > "No bead beating of macerated biomass" (0.142 ± 0.001 mg of R-PE g^{-1} DW and 0.018 ± 0.001 mg of R-PC g^{-1} DW). Based on these results, it was concluded that the introduction of an additional step - the bead beating of biomass in the stage of pre-treatment - is able to slightly increase the content in extracted PBPs.

The great variability of cell wall polysaccharides in marine algae is determined by the specie/taxa, season and habitat, anatomical part of the alga and development and life-cycle stage [82]. Rhodophytas have complex cell walls constituted essentially of cellulose, xylan, or mannan fibrils and sulphated galactans (agars, carrageenans) as the main matrix components [82]. Since *Gracilaria* cell walls are mostly agar in composition [83] (for example, Siddhanta *et al*. (2011) reported only a maximum cellulose content of 5.3 % of dry weight for *Gracilaria* Indian species), temperature increments facilitate their solubility and increase the release of cellular content. However, the ineffectiveness in extracting large quantities of these molecules constitutes the prime limitation in accessing PBPs from this genus. These studies are in agreement with Fleurence *et al*. (1995) who indicate that the degradation of agar of *G. verrucosa* can substantially improve protein extraction. Two things are known: 40 ºC represents the temperature for optimal cellulase activity [62,84]; R-PE exhibits good stability up to 40 ºC [51]. Therefore, an equilibrium temperature between these two conditions of 35 ºC was adopted in this study to conduct the enzymatic hydrolysis, favouring the extraction/dissolution of *G. gracilis* agar and the hydrolysis of cellulose at the same time.

In addition to the excessive time consumed in the enzymatic hydrolysis (≈ 6 h) and energetic expenditures to maintain a constant temperature of 35º C throughout all the hydrolysis, the monetary price of the enzyme itself does not add up any benefits to the overall process. In this case, to extract very low quantities of pigment that hasn't even been purified yet, the use of cellulase may not be a much profitable option from the point of view consumption/production for the extraction of PBPs. Notwithstanding, if the overall enzymatic hydrolysis process is to be optimized (namely incubation time, enzyme/substrate ratio, temperature, pH, etc.), the simultaneous coupling of cellulase with other enzymes such as the κ-carrageenase, the β-agarase or xylanases might be able to extract more considerable amounts of PBPs to such an extent that ends up, eventually, to compensate monetarily the costs of their acquisition [61,62]. For example, Nguyen *et al*. (2017), using response surface methodology (RSM), obtained a much higher R-PE yield - 1.99 mg g^{-1} dw - for *Mastocarpus stellatus* by adding another enzyme to cellulase - the xylanase. Furthermore, the authors found out that neither cellulase nor xylanase alone enhanced R-PE yields. Xylanase is known to exert beneficial effects on cellulose hydrolysis by degrading heterogeneous xylan polymers that shield cellulose fibers in land plants [85,86]. As such, it's important that they act together. Hence, in the future, the junction of xylanase could represent a big plus to the performance of cellulase and consequent extraction of bigger loads of PBPs from *G. gracilis*.

In order to evaluate the protein extraction yields obtained with each of the different methodologies adopted for dry biomass, it may be interesting to compare these results with those obtained with the mechanical intervention of the bead beater (Fig. 4.1). In a first instance, it is revealed that, with the mechanical methodology, much higher values of PBPs can be attained - maximum values of 0.631 ± 0.041 and 0.476 ± 0.018 mg g^{-1} DW of R-PE, and 0.089 ± 0.012 and 0.076 ± 0.007 mg g^{-1} DW of R-PC (except for the sequential extractions situation) against maximums of 0.220 ± 0.001 mg of R-PE g^{-1} DW and 0.039 ± 0.004 mg of R-PC g^{-1} DW for the enzymatic approach. Although quite insignificant due to the proximity of the obtained values and the lack of triplicates, another observation possible to make is that, for the mechanical approach, a maceration of the biomass before the addition of the beads contributed to raise the protein content (e.g. transition of values from 0.013 ± 0.007 mg R-PE and 0.010 ± 0.001 mg R-PC g^{-1} DW for 0.114 ± 0.014 mg R-PE and 0.022 ± 0.005 mg R-PC g^{-1} DW for SGB). In turn, this wasn't the case for the enzymatic approach where a mechanical pre-treatment of the algal strands with a mortar and pestle appears to have contributed little or nothing to the subsequent action of cellulase (Fig. 4.4).

As a final note, we would like to highlight the excellent performance and marvellous extraction yields of R-phycoerythrin (R-PE) obtained with a simple and one-step consisting methodology - the disruption of seaweed cell walls through a tissue homogenizer (T18 digital ULTRA TURRAX®) using solely distilled water as extraction solvent - a cheap, abundant and easily obtainable reagent. Many authors use some of the most complex technologies (e.g. supercritical fluids, ionic liquids or microwave-assisted extraction) [30,59,87] being unable, though, to reach the highest R-PE concentrations obtained for *G. gracilis* in this study - 9.599 ± 1.722 or 6.82 ± 1.066 mg g^{-1} wet weight. In addition, much of the work existing about PBPs extraction refers to the use of extraction solvents of various natures (e.g. phosphate/acetate buffer, mixtures with sodium azide, EDTA or glycerol, Tris-containing buffers, ionic liquids) [24,58,88] which are always, in their whole integrity, more complex, much more polluting, more expensive and difficult to get/prepare than simply water. From an industrial viewpoint, these conditions - advantages of the usage of water and the simplicity of the disruption method - seem more efficient, favorable and profitable in detriment to the use of more complex, laborious and time consuming methods, normally associated with more expensive and elaborated solvents. Perhaps the fact that PBPs are hydrosoluble might have contributed to the magnificent extraction efficacy achieved with water, without the need to recur to other solvents. Still, another aspect to consider is that the extraction of phycobiliproteins from wet biomass is more preferable than from dry biomass since it helps to avoid loss in pigments during drying processes, not requiring additional costs on this process [45,53]. Some authors prefer to remove the humidity from seaweeds, thus presenting the results of pigments as a % of dry weight, while others chose to leave the alga intact, exhibiting, therefore, the results as % of wet weight. Bearing that in mind, it gets a little difficult to compare extraction efficiencies and yield rates of pigment obtainment between, for instance, the tissue homogenizer methodology (which used wet material) and the bead beating technology or even the enzymatic approach with the cellulase (which, in turn, used dry biomass). And the same goes for every other extraction method if the percentage of moisture eliminated in the drying procedure is not known - without knowing the pre-treatment through which the seaweed went by, it´s not correct to attribute the highest pigment values to the best extraction method.

4.2. Cultivation Assays of *G. gracilis* grown in Laboratory

Many of the techniques currently employed to increase growth, PBPs and TSP contents in the algal tissue of different species or groups of seaweed relate themselves with an attempt to manipulate, in cultivation and in the laboratory, the main environmental parameters influencing seaweed metabolism in their natural habitat with the goal of maximizing increments in biomass, the production of desired (bioactive) compounds and manipulating seaweed composition and levels of those specific metabolites [33]. Much of it has already been discussed in section 1.2.1. Main

Environmental Parameters influencing Cultivation (Introduction): the light exposure, temperature, salinity and nutrient uptake are normally the main environmental conditions addressed as determinants for incrementing these parameters' levels.

In this case, for the cultivation of *G. gracilis* in laboratory, the following conditions were stipulated:

- Constant temperature of 22 ± 1 °C, maintained and regulated by a temperature controlled room
- Salinity of 35 ‰
- pH of 7 (neutral at 25 °C) for the seawater medium
- Growth medium: Modified Von Stosch Enrichment (VSE) medium for red algae (Appendix A, Table A.I)
- Water movement, provided by constant filtered aeration through serologic pipettes
- Light: Gro-lux and Cool White Fluorescent lamps (standard intensities of 39-45 µmol photons $m^{-2}s^{-1}$ and 40-110 µmol photons $m^{-2}s^{-1}$, respectively); photoperiod of 12:12 L:D

As for temperature and salinity, we chose to keep these parameters unchanged. On the one hand, for temperature, even though some authors have reported increments in *Gracilaria crassa* R-phycoerythrin and R-phycocyanin contents when increasing the cultivation temperature from 20 to 35 °C (174.41 to 444.72 µg g^{-1} fresh weight (FW) and 241.26 to 389.10 µg g^{-1} FW, respectively), but, on the other side, decreases on DGR values from 5.8 to 3.52 % day^{-1} (corresponding to increases of temperature from 25 to 35 °C at a salinity of 35 ‰) [69], increases in the value of this parameter are generally more propitious to microalgae proliferation and, thus, to contamination [89,90]. Regarding salinity, two studies by different authors reported the same situation for *Gracilaria gracilis*: Özen *et al.* (2018) found that the highest concentrations of phycoerythrin, phycocyanin and total protein were obtained for a salinity close to 35 ‰ - 37 ‰ - when compared with other values of 10, 25 or 48 ‰; Wilson & Critchley (1997) mentioned a maximum DGR of 11.74 % day^{-1} also for 35 ‰ within a range of salinities going from 5 to 50 ‰. Moreover, depending on the balance between freshwater and tidal waters, the Lagoa de Óbidos is subjected to wide shifts in salinity, with values normally ranging from 16 to 36 ‰ [91]. So, even though *G. gracilis* is a euryhaline species capable of supporting a wide range of salinities, a value of 35 ‰ achieved in the central lagoon region, allied to the benefits already mentioned in some studies for this value, seems to be a good candidate to promote the seaweed growth. Based on these evidences, a temperature near 25 °C (22 ± 1ºC) and an optimal salinity of 35 ‰ were chosen for the cultivation of *G. gracilis* from the Lagoa de Óbidos [92].

In addition to these key parameters (allied to others such as pH, growth medium composition, water movement (normally provided through aeration)), the establishment of an axenic culture free from any competing organism able to cause constraints in terms of space, light capture or nutrient intake is highly crucial for the cultivation of algae, whether being seawater or freshwater species. As such, an algal decontamination protocol should be strictly followed taking into account the extent of the contamination, the type, nature and characteristics of the contaminating organism, but also the physiological characteristics and susceptibilities of the species itself being cultivated - for instance, a compound with a potent decontamination action may not be the best option to take if at the same time it damages the tissue structure or other components of our species of interest. The *G. gracilis* macroalgal cleaning/decontamination protocol followed the guidelines of Yarish *et al*. (2014) aiming at four indispensable stages of disinfection: osmotic shock in distilled water; cleaning of tips with sterilized cotton-tipped swabs; dragging though agar plates; and, addition of germanium dioxide (GeO_2) to the growth medium. After several attempts in establishing a pure culture free from any other strains, this goal remained, unfortunately, unachievable. The main contamination in culture was attributed to the survival, proliferation and adherence of diatoms, unicellular microalgae, to seaweed thalli. Either way, it is a known fact that it's practically impossible to establish an axenic culture in its entirety. The disinfection process is never 100 % effective. Arising from the aeration system responsible for promoting gas exchanges with the culture flasks, from the seawater reservoirs supplying part of the culture medium (still without nutrients) or still originated by the persistence in adhering to the biological material coming from the lagoon even after enforcement of the decontamination protocol, the fact is that it was not possible to set in laboratory totally diatom-free *G. gracilis* cultures. GeO_2 is reported to be a specific inhibitor of diatom growth, interfering in some way with the processes of silica frustule formation [93]. Once it is known that germanium potentially replaces silica atoms in compounds, the GeO2 treatment may have been assumed to have no detrimental effects on *G. gracilis* since silica is not a major or essential element for this macroalgae, nor it possesses any known biological function in plant growth [93]. Thus, GeO_2 constitutes a fine example of a compound whose addition to the culture medium would not affect in any way algae growth, while helping at the same time to prevent diatom proliferation.

4.1.1. Influence of Light Intensity and Nutrient Concentration on:

4.1.1.1. Growth Rate

Yong *et al.* (2013) state that growth rate determination is "a basic measurement to determine the growth performance and response of a target culture". As previously mentioned, for the cultivation of *G. gracilis*, two types of fluorescent lamps were used - the Cool White Fluorescent lamp (CWF) and the Plant Growth Fluorescent (Gro-lux) lamp. Both types of fluorescent lamps emit energy mainly in the blue and red, the region's most active in photosynthesis. Unlike CWF lamps, Gro-lux lamps are designed for the sole purpose of promoting plant growth. The cool white lighting has been regarded as the best light source for most seaweed growth systems [34]. Based on this duality, three sets of independent trials were performed along with a special case: a first set of two assays at a constant light intensity provided by the Gro-lux lamp where it was intended to fundamentally test the effect of increasing the ammonium concentration over the parameters DGR, PBP and TSP; a second set of trials (with Gro-lux lamp) composed by three assays in which the separate effect of the light intensity augmentation and of its conjugate effect with the ammonia increase were to be compared towards a control situation; a third and last set of trials where, besides varying the exposure time as the intensity itself of the light source, a new variant was also introduced - the elevation of phosphate concentration; finally, just one assay with the CWF lamp without nutrients increment under which *Gracilaria* was meant to grow at a constant light intensity (Table III.II (Part I & II), Material and Methods - Section 3.4. Growth Assays). Although it is only of interest for this study to conduct comparisons between assays of the same set, it doesn't stop being relevant to compare the CWF light with all the other assays of different sets since we're handling with a different type of lamp. Beyond that, brief comparisons of the culture exposure time to a light intensity increase (example of the last cultivation week in the 2nd Set of Independent Trials and the last 72 h in the 3rd set of Independent Trials) could also be interesting to conduct.

Regarding the cultivation times for each of the trials, *G. gracilis* tips exposed to the CWF light remained in cultivation for 36 days, those from the 1st and 2nd Set of Trials for 28 days and the last ones from the 3rd Set only for two weeks (14 days). In Figure 4.5 it is possible to analyse in more detail the growth rate attained for each of the assays until subsequent culture medium replacement.

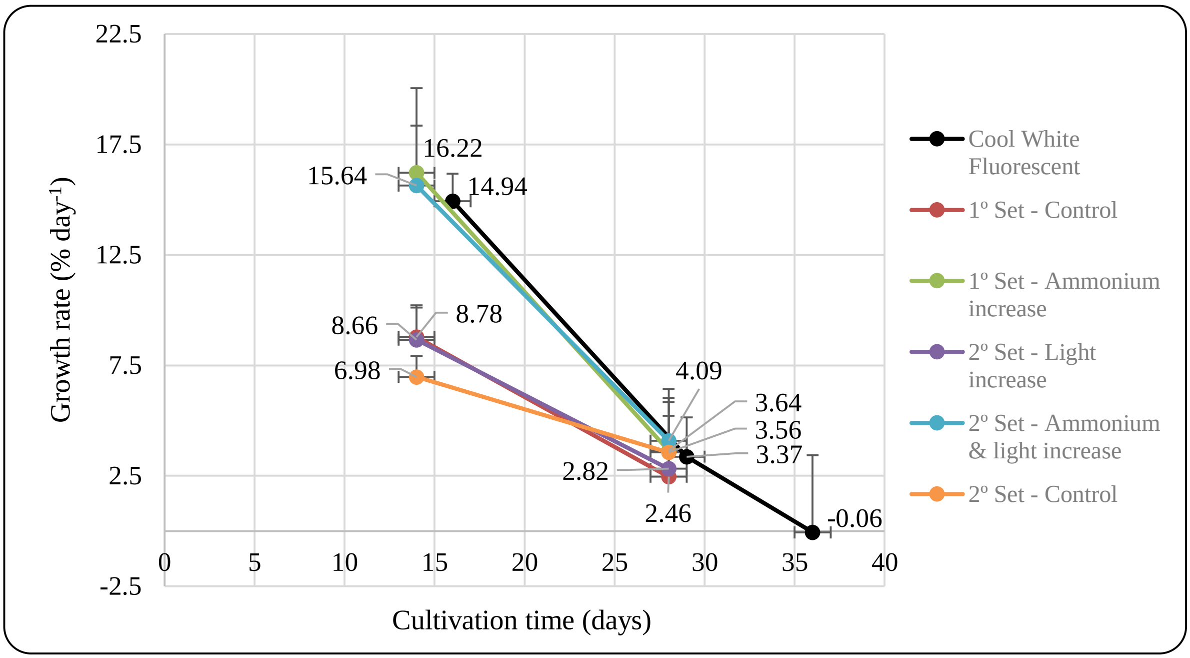


Figure 4.5: Growth rate (% day^{-1}) of *G. gracilis* tips of the assays "Cool White Fluorescent", "Control" and "Ammonium Increase" from the 1st Set and "Control", "Light Increase" and "Ammonium & Light Increase" from the 2nd Set of Independent Trials, estimated after media replacement. Dots represent media replacement. Results are expressed as means ± SD (*n* = 3).

For the control of the 1st Set, the daily growth rate (DGR) (% day^{-1}) reached a value of 8.78 ± 0.88 % day^{-1} in the first 14 days of cultivation. Ended this period, the medium was replenishment, and after more 14 days, the new weighing of the algae tips dictated that, during the second 14 days, the tips had only a weight increase of about 2.46 ± 2.76 % per cultivation day. In other words, a diminishment of more than 6 % between fortnights. The same trend was observed for all trials: in "Ammonium Increase" (1st Set) the DGR decreased from 16.22 ± 22 to 3.64 ± 2.8 % day^{-1}; in "Light Increase", "Ammonium & Light Increase" and "Control (2nd Set), one calculated, respectively, decreases of approximately 8.66 ± 1.56 to 2.82 ± 1.33 % day^{-1}, 15.64 ± 2.71 to 4.09 ± 1.75 % day^{-1} and 6.98 ± 0.95 to 3.56 ± 2.46 % day^{-1}. That is, until the first medium renewal (after 14 days) the DGRs showed some variability between assays, but, once two weeks have passed, the parameter decreases almost exponentially in practically every situation for the same range of values: 4.00-2.00 % day^{-1}. Concerning the assay with the other type of lamp - CWF -, three different DGR values are exhibited since, in an attempt to performed the scale-up of already grown *G. gracilis* tips, one proceeded three distinct times to medium renewal - the first after 16 days, the second after 13 more days and the last one summing 7 additional days in culture (these last ones in 5 L flasks). In this assay, a non-observable phenomenon in any other situation is emphasized - the DGR hit negative values. To clarify, there was a reduction in the growth of the tips itself and not just of its rate. The cultivated tips, in fact, lost some weight until the last day of the trial. Starting in 4.94 ± 1.24 % and falling up to 3.37 ± 1.77 day^{-1} after 29 days, at the 36-day mark it was even observed a value of -0.06 ± 3.5 day^{-1}. Since the same reduction pattern was observed for all assays, an excessive exposure (in terms of time) of the tips to an increment in light or nutrient concentration (for example) cannot be surely held responsible. Alternatively, the contamination by diatoms present in virtually every assays seems to be the source of the occurrence. Diatoms are organisms that adhere to *G. gracilis* thalli, preventing and hampering its growth in a general way mainly through: competition for available nutrients - the same amount of nutrients in the culture medium (initially predestined only for the seaweed) becomes now available for two groups of distinct organisms, decreasing the bioavailability and the nutrient uptake rate for the species to be cultivated. Furthermore, diatoms possess a much faster metabolism and multiplication process in relation to the ones of *G. gracilis*, consuming a large proportion of nutrients in the process [89,90]; by adhering to the *G. gracilis* thalli, they form authentic "carpets" capable of covering completely the main thicker and darker red thalli, leaving only the new growing tips "free" of this "carpet". As such, a shading occurrence by diatoms could arise, leaving the seaweed with very little quantities of the available light required for photosynthesis (very low surface area to collect light photons). If few light reaches the chloroplasts, energetic restrictions will hinder the process by which plants (and algae) obtain energy (in the form of sugars) through light, thereby attenuating their growth. Nonetheless, despite this diatom (or any other contamination), seaweeds in general normally exhibit a characteristic growth pattern differentiated by a rapid initial phase followed by a decreased growth during later stages due to a naturally occurring self-shading phenomenon of their inner regions [74,92]. Thus, in order to determine growth rate more accurately, Yong *et al*. (2013) recommend to adopt a time interval between data as short as a week. Therefore, and since the DGR was diminishing significantly over the duration of the various trials - observation that didn't allow us to predict, with all the security, that the same contamination wouldn't be present in subsequent trials as well -, one opted, for the 3rd Set of Trials, to terminate the cultivation of *G. gracilis* tips after 14 days (2 weeks), not compensating to put them grow much beyond this period. Thus, no decreases in the DGR value (already characteristic for the other sets) would be observed, caused not by variations in the assays conditions, but by other external factors harder to control. Hence, the daily growth rate was compared between the several trials sets for the first two weeks of cultivation only.

The increments in the tips weight after the stipulated cultivation time (originating the daily growth rates possible to be calculated) occurred whether by the lengthening and thickening of the main dark red thallus (the original tip), or by the branching and development of new tips from it through vegetative propagation. Fragmentation and consequent propagation of some of the tips parts contributed as well to weight gains.

The daily growth rates, calculated for seaweed growing under the assays "Cool White Fluorescent" (CWF), "Control" and "Ammonium Increase" of the Independent and 1st Set of Independent Trials, respectively, and determined after 16 cultivation days for CWF and 14 days for the others, are presented in Fig. 4.6 (see Appendix A, Table A.IV).

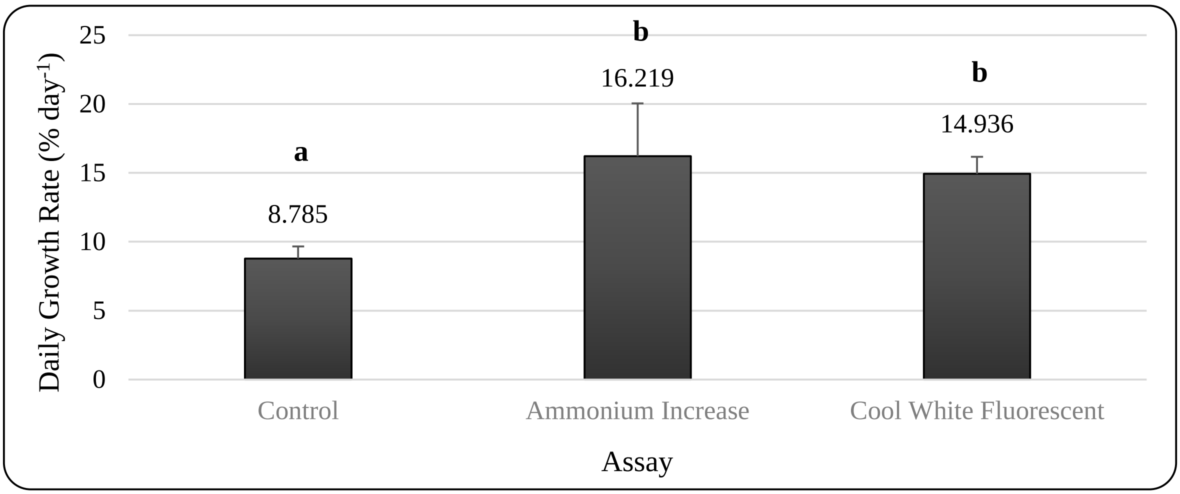


Figure 4.6: Daily Growth Rate (DGR) (% day^{-1}) of *Gracilaria gracilis* cultivated biomass from the assays "Control" (Ct), "Ammonium Increase" (A) and "Cool White Fluorescent" (CWF) determined after 16 days of culture for CWF and after 14 cultivation days for Ct and A. The assays correspond to the "1st Set of Independent Trials" and "Independent Trial", respectively. Results are expressed as means ± SD ($n = 3$). Different letters a-b indicate statistical significant differences (ANOVA followed by LSD and Dunnett's test for control assay; *p-value* < 0.05).

The highest DGR (16.219 ± 3.84 % day^{-1}) was recorded for seaweed tips subjected to an ammonium increase to twice the recommended concentration for the VSE medium (Appendix A, Table A.I)) at a light intensity of 39-45 µmol photons m^{-2}s^{-1}, not demonstrating statistically significant differences in relation to the CWF assay (14.936 ± 1.243 % day^{-1}) (*p-value* > 0.05, LSD test, Appendix A, Table A.V) conducted at 40-110 µmol photons m^{-2}s^{-1}. The "Control" (no nutrients increase, 39-45 µmol photons m^{-2}s^{-1}) presented the lowest DGR (8.785 ± 0.884 % day^{-1}), showing statistically significant differences in relation to both CWF and "Ammonium Increase" assays (*p-value* < 0.05, Dunnett's test). Increases of about 7.5 and 6.2 % day^{-1} of the DGR value from the control situation to the "Ammonium Increase" and CWF assays, respectively, seems to justify not only the importance of adding extra nutrients, but also the necessity of conserving higher light intensities in the achievement of higher growth rates.

Regarding the assays of the 2nd Set of Independent Trials, it is possible to highlight, from a statistical point of view, two groups: the first one consisting of the "Control" assay with a daily growth rate of 6.979 ± 0.954 day^{-1} and of the "Light Increase" assay with a DGR of 8.661 ± 1.559 day^{-1}; a second one composed by the "CWF" and "Ammonium & Light Increase" assays, accounting for, respectively, values of 14.936 ± 1.243 and 15.640 ± 2.711 day^{-1} (Fig. 4.7). Thus, between the "Control" (same conditions as the 1st Set control) and the assay "Light Increase" (no nutrients increase, 39-45 µmol photons $m^{-2}s^{-1}$ for the 1st week/45-75 µmol photons $m^{-2}s^{-1}$ for the 2nd week) there were no statistically significant differences at a significance level of 5 % (*p-value* > 0.05, Dunnett's test). The same occurred between the "CWF" and the "Ammonium & Light Increase" assays (for the latter, same conditions as the "Light Increase" assay but with twice as much the ammonium concentration) (*p-value* > 0.05, LSD test). Yet, both the latter showed statistically significant differences in relation either to the "Control" or to the "Light Increase" test (*p-value* < 0.05, LSD & Dunnett's test). The highest DGR rate was obtained with the increase of both variables (light and nutrients). Notwithstanding, the cool white fluorescent light also triggered a significant and positive impact on growth. In this set of trials, elevating the light intensity from 39-45 to 45-75 µmol photons $m^{-2}s^{-1}$ in the second cultivation week only appears to have caused an increase of nearly 1.7 % day^{-1} whereas doubling the concentration of NH_4^+ increased by 7 % and 8.66 % day^{-1} the DGR relatively to the light augmentation ("Light Increase") and to the control, respectively.

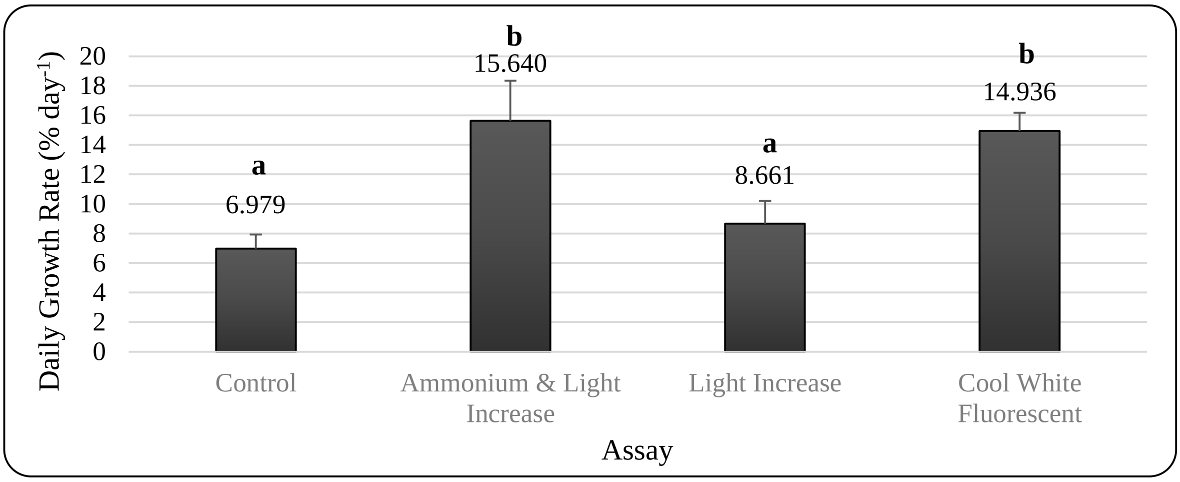


Figure 4.7: Daily Growth Rate (DGR) (% day^{-1}) of *Gracilaria gracilis* cultivated biomass from the assays "Control" (Ct), "Ammonium & Light Increase" (AL), "Light Increase" (L) and "Cool White Fluorescent" (CWF) determined after 16 days of culture for CWF and after 14 cultivation days for Ct, AL and L. The assays correspond to the "2nd Set of Independent Trials" and "Independent Trial", respectively. Results are expressed as means ± SD ($n = 3$). Different letters a-b indicate statistical significant differences (ANOVA followed by LSD and Dunnett's test for control assay; *p-value* < 0.05).

At last, in what concerns the 3rd Set of Independent Trials (Fig 4.8), there were two essential variations in cultivations conditions over the previous set: the concentration of the other macronutrient of the VSE medium - the phosphorus (in the form of phosphate) - was increased; and the light intensity was elevated from 45-75 to 55-85 µmol photons $m^{-2}s^{-1}$, also diminishing the exposure time to this increase from 1 week (168 h) to just 3 days (72 h).

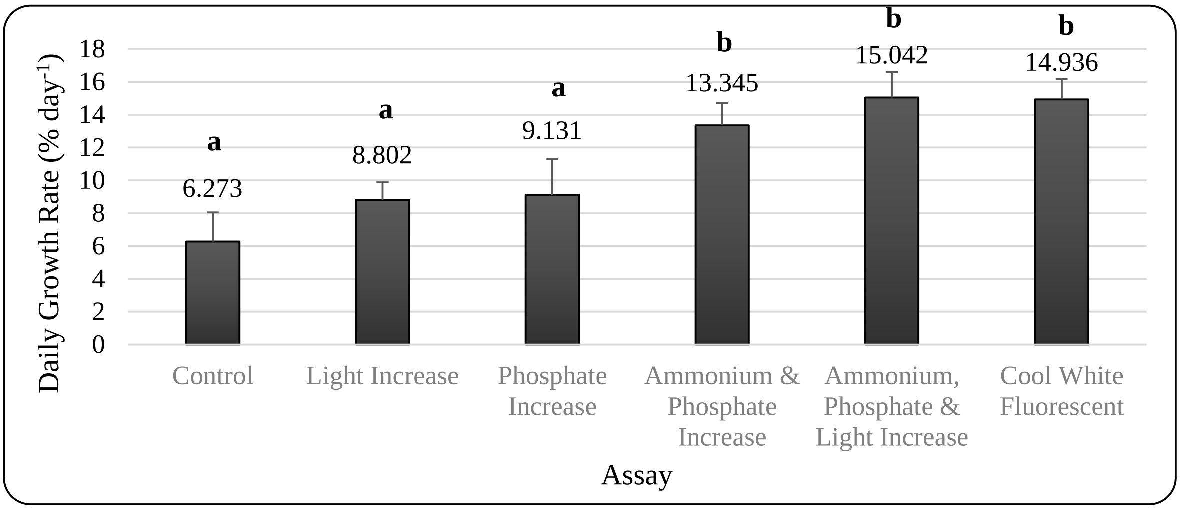


Figure 4.8: Daily Growth Rate (DGR) (% day^{-1}) of *Gracilaria gracilis* cultivated biomass from the assays "Control" (Ct), "Light Increase" (L), "Phosphate Increase" (P), "Ammonium & Phosphate Increase" (AP), "Ammonium, Phosphate & Light Increase" (APL) and "Cool White Fluorescent" (CWF) determined after 16 days of culture for CWF and after 14 cultivation days for Ct, L, P, AP and APL. The assays correspond to the "3rd Set of Independent Trials" and "Independent Trial", respectively. Results are expressed as means ± SD (*n* = 3). Different letters a-b indicate statistical significant differences (ANOVA followed by LSD and Dunnett's test for control assay; *p-value* < 0.05).

The maximum DGR value (15.042 ± 1.552 % day^{-1}) came back to be achieved by the combination of ammonium and light increments, having been added to these, however, twice the phosphate concentration - "Ammonium, Phosphate & Light Increase" assay. Nevertheless, the DGR value of 13.345 ± 1.343 % day^{-1} found for the same assay conditions excepting the light increase (i.e., the assay "Ammonium & Phosphate Increase") demonstrated no statistical significant differences in the daily growth rate of *G. gracilis* between elevating or not elevating the light intensity to 55-85 µmol photons m^{-2}s^{-1} in the last 3 days of culture (*p-value* > 0.05, LSD test). Differences of this nature weren't verified as well between the two assays "Ammonium & Phosphate Increase" and "Ammonium, Phosphate & Light Increase" and the one conducted under cool white fluorescent light. Contrarily, when assessing the effect of supplementing or not supplementing the growth medium with additional ammonium, one found out that, from a statistical point of view, there are significant differences in this duality (decrease of 4.2 % day^{-1} when such supplementation is absent - assay "Phosphate Increase" with DGR of 9.131 ± 2.146 day^{-1} (*p-value* < 0.05, LSD test)). Oppositely, no statistically significant differences (*p-value* > 0.05, LSD & Dunnett's test) were detected among three assays - the "Control", the "Light Increase" and the "Phosphate Increase" (6.273 ± 1.776, 8.802 ± 1.085 and 9.131 ± 2.146 day^{-1}, respectively) -, demonstrating with total certainty the following: extra supplementation of the growth medium with solely phosphate (that is, without ammonium as well) has no influence in the DGR of *G. gracilis*. Phosphate, unlike ammonium, appears to be macronutrient with a lesser role in the seaweed growth rate - something already proved by many investigations [27,43,94]; increasing or not increasing light in the last 72 h of the assay is relatively indifferent in the evaluation of this parameter (this time, a comparison based on the control was made). Obviously, and once again, statistically significant differences were found between the group composed by these three assays and the CWF one (*p-value* < 0.05, LSD & Dunnett's test). An increase of about 8.8 % day^{-1} of the DGR value from the control situation to the "Ammonium, Phosphate & Light Increase" assay was registered.

Nutrient availability possesses a relevant importance on growth. Among nutrients, ammonium plays a very important role in controlling the growth of *Gracilaria* [41]. By proceeding to an extra supplementation of N (and P in some cases), higher concentrations of these nutrients had become available for the seaweed, thus increasing its growth rate. Some studies have revealed similar trends in what concerns the effect of adding determined proportions of nitrogen and phosphorus on the growth of seaweeds. Joniyas *et al*. (2016) demonstrated a correlation between the ratio of the two nutrients and the specific growth rate (SGR) of *Gracilaria manilaensis*. In a range from 0/0 to 300/30 NH_4^+/PO_4^{3-} µM, the SGR increased gradually until it reached the maximum value of 5.72 % day^{-1} for the highest nutrients ratio. They reached these values with an ammonium supplementation 10 times higher than that of phosphate, highlighting, similarly to the present study, the superior importance of ammonium in relation to phosphate in the DGR of two different species of the genus *Gracilaria*. For *G. gracilis*, the evidences of that are: when $[PO_4^{3-}] = 0$ & $[NH_4^+] = 0$ ("Control" of the 1st and 3rd Set), the DGR is equal to 8.785 ± 0.884 and 6.273 ± 1.776 % day^{-1}, respectively; $[PO_4^{3-}] = 0$ & 2x $[NH_4^+]$ ("Ammonium Increase" - 1st Set), DGR = 16.219 ± 3.84 % day^{-1}; 2x $[PO_4^{3-}]$ & $[NH_4^+] = 0$ ("Phosphate Increase" - 3rd Set), DGR = 9.131 ± 2.146 % day^{-1}; 2x $[PO_4^{3-}]$ & 2x $[NH_4^+]$ ("Ammonium & Phosphate Increase" - 3rd Set), DGR = 13.345 ± 1.343 % day^{-1}. Yu & Yang (2008) demonstrated a similar tendency for *G. lemaneiformis* regarding the supplementation of nitrogen and phosphorus, having obtained a maximum SGR of 1.62 % day^{-1} after a cultivation period of 7 days for a N/P ratio of 400/25 µM while a value of circa 0.58 % day^{-1} was obtained for 0/0 N/P µM. For this genus, an investigation concluded that ratios higher than 400/25 µM (N/P) tend to decelerate not only seaweed growth (by disrupting regular metabolism of photosynthesis and protein synthesis), but also to inhibit PBPs production [41]. In what concerns the effect of light over this parameter, Yang *et al.* (2015) state that the *Gracilaria* genus generally requires a high light intensity for normal growth (optimal illumination intensity varies from 35–60 µmol $m^{-2}s^{-1}$). Some other studies report the following: for a temperature of 22 ºC and within an interval of irradiances composed by three values - 70, 100 and 170 µmol photons $m^{-2}s^{-1}$-, the last two managed to obtain the highest SGR values - 6.78 and 7.16 % day^{-1}, respectively - for *G. gracilis* [27]. Even though the authors don't discriminate the type of lamp used, we can equate these results to the DGR obtained for *G. gracilis* grown under the cool white fluorescent lamp (CWF) (light intensities weren´t too distant from 40-110 µmol photons $m^{-2}s^{-1}$) - 14.936 ± 1.243 % day^{-1}; Bunsom & Prathep (2012) also evaluated the influence of light on the growth rate of *Gracilaria tenuistipitata* estimated as the percentage of weight gain (% WG) in the seaweed biomass. Within a range going from 150 to 1000 µmol photons $m^{-2}s^{-1}$, the smallest value (the closest to the CWF intensity) allowed the obtainment of the highest WG value (≈ 116 %) for a sediment-free condition (as in our study) and at a salinity of 31 psu (close to the 35 % adopted for *G. gracilis* cultivation); finally, Xu *et al*. (2009), by studying the effect of light on growth rates of two species of *Gracilaria*, revealed that the best conditions for the highest daily specific growth rates (% increase in wet weight) were determined to be 287.23 µmol $m^{-2}s^{-1}$ for *G. lichenoides* (16.26 % d^{-1}) and 229.07 µmol $m^{-2}s^{-1}$ for *G. tenuistipitata* (14.83 % d^{-1}) within a range of the following intensities:

20, 40, 60, 80, 100, 120, 160, 200, 240 and 300 µmol $m^{-2}s^{-1}$. Similarly to our study, both seaweed species were cultivated under cool-white fluorescent lamps. For *G. gracilis*, we obtained similar growth rate values (specially towards *G. tenuistipitata*) - 14.936 ± 1.243 % day^{-1} - without the need of such high light intensities (maximum of 110 µmol $m^{-2}s^{-1}$).

A rather interesting case seems to be comparing the two types of light bulbs. While for lower light intensities of 39 to 85 µmol photons $m^{-2}s^{-1}$, achieved with Gro-lux, the extra ammonium supplementation exerts a preponderant function in increasing growth rates, higher intensities from 40 to 110 µmol photons $m^{-2}s^{-1}$, achieved with CWF, somehow appear to replace this supplemental nutrient addition - fact corroborated by the lack of statistically significant differences between the situations/assays where more ammonium is present and the CWF assay of any of the different sets (*p-value* > 0.05, LSD test). Helson (1964) compared the effect of Gro-lux and Cool White Fluorescent (CWF) lamps (light intensities of circa 1337 and 2303 footcandles (fc) converted, respectively, to 200 and 765 µmol photons $m^{-2}s^{-1}$) on the growth and development of tomato plants. They concluded about the superiority of the Gro-lux lamp, stating that plants grown under this lamp had 34 % more flowers, 20 % riper fruits and 32 % heavier fruit than those grown under cool white light. Such results appear to be contradictory to those obtained for the seaweed *G. gracilis* in a situation where nutrients increments are not considered - 14.936 ± 1.243 % day^{-1} obtained with CWF (higher light intensities) against 8.785 ± 0.884 % day^{-1} ("Control", 1st Set, for instance) with Gro-lux (lower light intensities). Yet, further and consolidated conclusions are not allowed to make since there are practically no studies comparing the effects of both these types of lamps on seaweed (or even microalgae) growth.

The resemblance between a value of 8.661 ± 1.559 day^{-1} (2nd Set, assay "Light Increase") and of 8.802 ± 1.085 (3rd Set, "Light Increase") might also indicate that, for the low range of intensities defined for the Gro-lux lamp, there are no major differences in increasing the exposure time from 72 to 168 h to the light increments respective for each set. Moreover, based on Fig. 4.8, one more conclusion is crucial to be made: the absence of statistically significant differences (*p-value* > 0.05, LSD test) between the CWF assay and the "Ammonium & Phosphate Increase" and "Ammonium, Phosphate & Light Increase" assays (increases of solely nutrients and of both nutrients and light, respectively) indicates that all these three conditions are considered equally good options for maximizing the growth of *G. gracilis*. Well, between a situation that only requires the placement of *G. gracilis* tips under a specific type of light - the cool white fluorescent light - and two more situations demanding extra monetary costs in terms of nutrients or supplementary energetic expenses to achieve similar growth results, the first one is definitively preferable from an economic point of view for growth augmentation. A finding that is indeed extremely interesting in case of an eventual extensive cultivation of this seaweed.

4.1.1.2. TSP Content

Proteins are essential nutrients required by the human body for growth and maintenance. Composing one of the building blocks of body tissue, proteins constitute polymer chains made of amino acids linked together by peptide bonds [50,95]. Even though the majority of these amino acids can be biosynthesized by the organism, there are nine essential amino acids which adult humans must obtain from their diet in order to prevent protein-energy malnutrition and other complications: phenylalanine, methionine, threonine, lysine, tryptophan, isoleucine, histidine, valine and leucine [95]. Dietary sources of protein normally include products of animal or plant origin: fish, eggs, meat, dairy products, or legumes, nuts, grains, vegetables, etc. Among these, a global interest is increasingly emerging around algae (microalgae and seaweeds) as viable sources of protein [50]. Justified by the high levels found in their tissues often superior than those found in many traditional sources such as soybean, eggs and peas, a grand piece of scientific community focused on this field of research has been referencing abundantly the potential of seaweeds as authentic protein and amino acids providers. Admittedly, protein values can range from 5 to 47 % (this last one found in red seaweeds, particularly *Porphyra tenera*, currently regarded as a synonym of *Pyropia tenera* (Kjellman) N.Kikuchi, M.Miyata, M.S.Hwang & H.G.Choi [25]), according to the species, environmental conditions, maturity degree, habitats and applied methods for protein and amino acid determination [96]. Unfortunately, with the world population increasing exponentially, there is an inherent need to find/create new sources of these biomolecules and to increase the amount that the existing ones already produce in order to supply the nutritional deficiencies that characterize essentially the least developed countries. Therefore, and for the obvious and simple reason that wild seaweed reserves are not inexhaustible, production sustainable systems of seaweeds in cultivation may well be the key to make this essential nutrient reach the places where it’s most needed. Beyond that, the protein benefits (as also are the phycobiliproteins R-PE and R-PC) are not limited to suppressing the nutritional scarcity. Some of their remarkable biological functions such as anti-fungal, anticancer, antioxidant, antiviral and angiotensin-converting enzyme inhibitory activity [95], among many others, further justify the need to produce more and more biomass to obtain considerable amounts of these crucial compounds to human beings.

In this study, after an acidic digestion to extract the TSP content of the cultivated *G. gracilis* samples, one proceeded to its estimation by using a specific kit - the Pierce™ BCA Protein Assay kit - that measures colorimetrically the total protein concentration of a sample compared to a protein standard. Other methods currently employed to extract and quantify the proteinic content in red seaweeds are: the Bradford, the Lowry, the Biuret and the Kjeldahl (the most common one) [95].

Figure 4.9 reveals these contents (in % of dry weight) as a function of the various assays performed for the 1st Set of Independent Trials and for the Independent Trial.

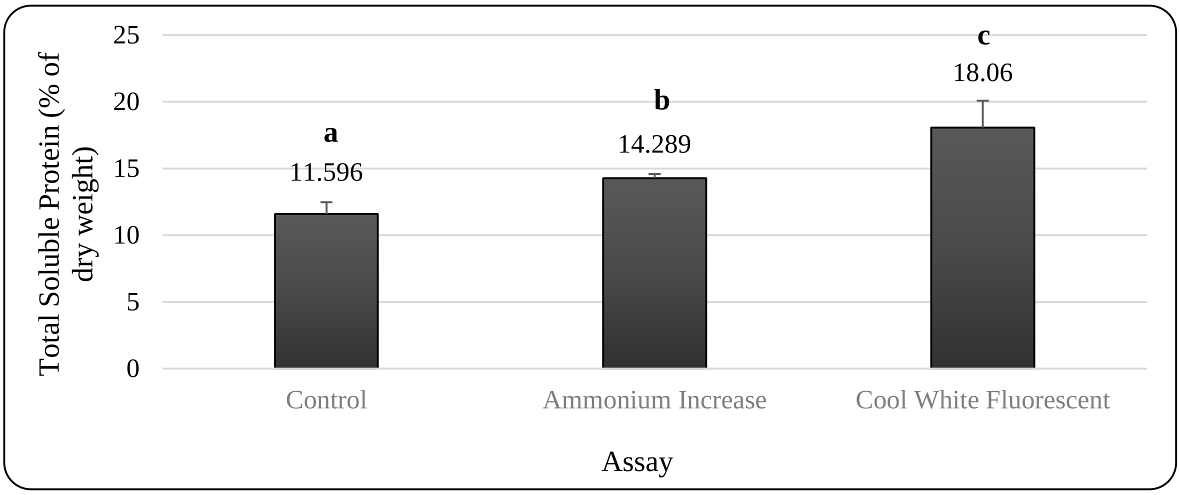


Figure 4.9: Total Soluble Protein (TSP) content (% of dry weight (DW)) of *Gracilaria gracilis* cultivated biomass extracted from the assays “Control” (Ct), “Ammonium Increase” (A) and “Cool White Fluorescent” (CWF). The PBPs content was estimated for a culture time of 36 days for CWF and after 28 cultivation days for Ct and A. The assays correspond to the “1st Set of Independent Trials”and “Independent Trial”, respectively. Results are expressed as means ± SD (*n* = 3). Different letters a-b indicate statistical significant differences (Kruskal-Wallis followed by LSD and Dunnett’s test for control assay; *p-value* < 0.05).

From there, we can realize that the assay where a higher TSP content was registered was the Cool White Fluorescent (CWF) assay (18.06 ± 2.027 % of dry weight) followed by “Ammonium Increase” (14.289 ± 0.309 % of dry weight) and, at last, by the “Control” assay, accounting for the lowest percentage (11.596 ± 0.89 % of dry weight) (Appendix A, Table A.VI). Statistical analysis revealed significant differences between all assays (*p-value* < 0.05, LSD & Dunnett’s test) (Appendix A, Table A.VII). That is, without much more to add, when compared with the control situation (39-45 µmol photons $m^{-2}s^{-1}$), cultivating *G. gracilis* under CWF light (40-110 µmol photons $m^{-2}s^{-1}$) stimulates a larger production of protein by it than by doubling the ammonium concentration under the Gro-lux light regime (39-45 µmol photons $m^{-2}s^{-1}$). The CWF assay registered positive differences of circa 6.5 and 3.8 % of dry weight of the TSP content in comparison to the assays “Control” and “Ammonium Increase”, respectively.

Switching to the 2nd Set of Independent Trials (Fig. 4.10), the main observation made is related to increasing the light intensity of the Gro-lux assays from 39-45 µmol photons $m^{-2}s^{-1}$ to 45-75 µmol photons $m^{-2}s^{-1}$ and is as follows: the referred increase of light to these values in the 2nd week of cultivation alone doesn’t induce higher protein production (proven by the absence of statistically significant differences between the “Control” (12.566 ± 2.503 % of dry weight) and “Light Increase” assays (12.961 ± 2.128 % of dry weight)) (*p-value* > 0.05, Dunnett’s test); on the other hand, the combined effect of increasing ammonium and light intensity is already capable of statistically improving the total soluble protein content in the seaweed (12.961 ± 2.128 % of dry weight of “Light Increase” against 17.692 ± 1.35 % of dry weight of “Ammonium & Light Increase”) (*p-value* < 0.05, LSD test). In this last situation, it was even verified that the combination of both conditions (nutrients and light) was sufficient to almost match the protein values obtained for CWF. Still, we face a lack of statistically significant differences in this situation - 17.692 ± 1.35 % (“Ammonium & Light Increase”) against a TSP content of 18.06 ± 2.027 % of dry weight (“Cool White Fluorescent”) (*p-value* > 0.05, LSD test). The CWF assay registered positive differences of circa 5.5 and 0.37 % of dry weight of the TSP content in comparison to the assays "Control" and "Ammonium & Light Increase", respectively.

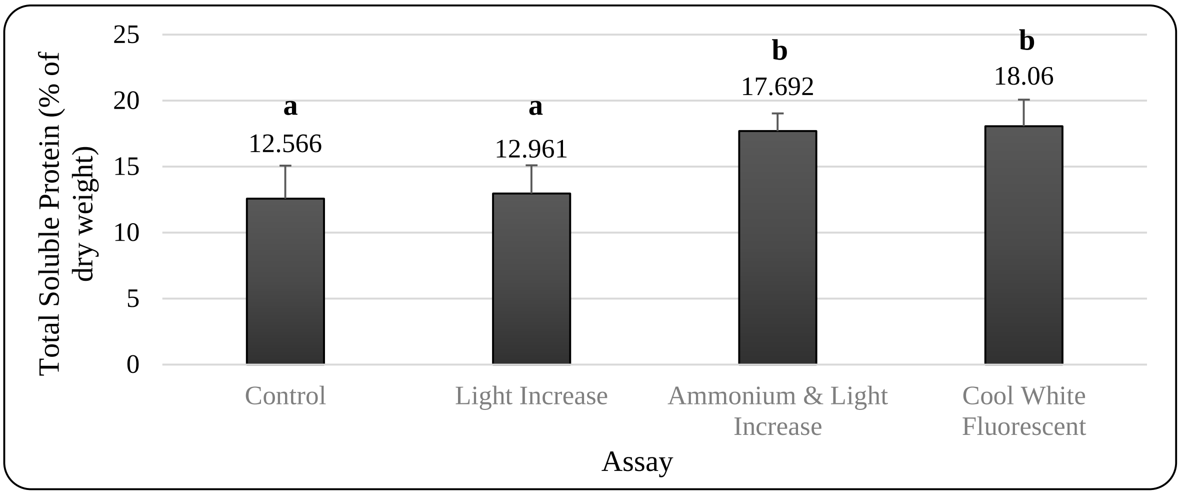


Figure 4.10: Total Soluble Protein (TSP) content (% of dry weight (DW)) of *Gracilaria gracilis* cultivated biomass extracted from the assays "Control" (Ct), "Ammonium & Light Increase" (AL), "Light Increase" (L) and "Cool White Fluorescent" (CWF). The PBPs content was estimated for a culture time of 36 days for CWF and after 28 cultivation days for Ct, AL & L. The assays correspond to the "2nd Set of Independent Trials" and "Independent Trial", respectively. Results are expressed as means ± SD ($n = 3$). Different letters a-b indicate statistical significant differences (Kruskal-Wallis followed by LSD and Dunnett's test for control assay; *p-value* < 0.05).

Finally, relatively to the last set of trials, Figure 4.11 denotes some interesting variations between trials. The first thing to note in this case is that the assay CWF wasn't the one which exhibited the highest TSP content as it has usually been in the other assay sets. This time, the said role was occupied by the "Ammonium, Phosphate & Light Increase" assay which accounted for a value of 18.865 ± 1.083 % of dry weight - that is, in 100 g of dried seaweed, about 18.9 g is protein. In other words, 18.9 % of its dry weight is composed solely by proteins. However, there were still no statistically significant differences between these two assays (*p-value* > 0.05, LSD test), reason why both are considered equally good options for maximizing protein production by *G. gracilis*. None of the other assays - "Control", "Light Increase", Phosphate Increase" or "Ammonium & Phosphate Increase" - was able to equate, from a statistical point of view, the highest values obtained in CWF and "Ammonium, Phosphate & Light Increase" (*p-value* < 0.05, LSD & Dunnett's test).

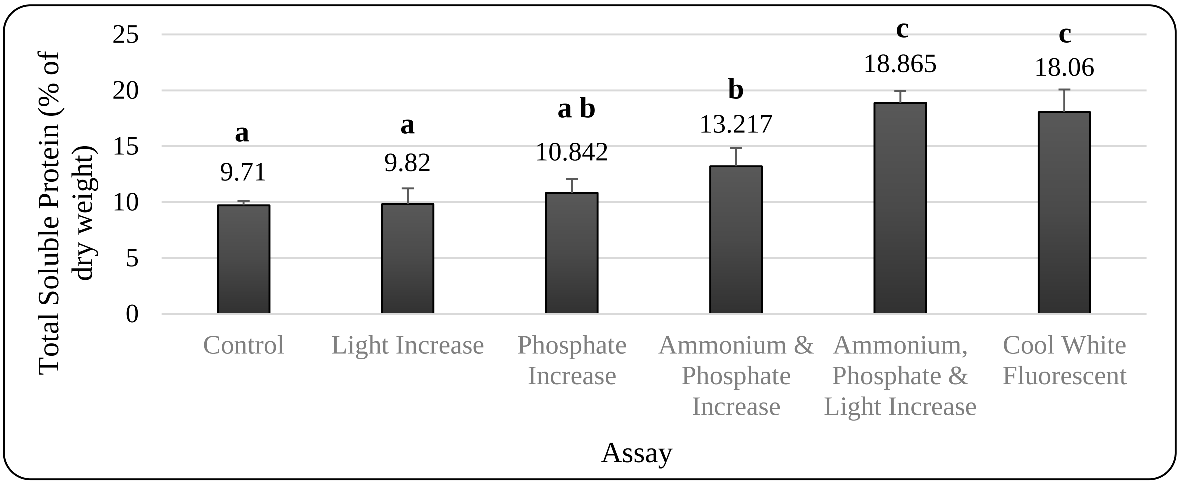


Figure 4.11: Total Soluble Protein (TSP) content (% of dry weight (DW)) of *Gracilaria gracilis* cultivated biomass extracted from the assays “Control” (Ct), “Light Increase” (L), “Phosphate Increase” (P), “Ammonium & Phosphate Increase” (AP), “Ammonium, Phosphate & Light Increase” (APL) and “Cool White Fluorescent” (CWF). The TSP content was estimated for a culture time of 36 days for CWF and after 14 cultivation days for Ct, L, P, AP and APL. The assays correspond to the “3rd Set of Independent Trials” and “Independent Trial”, respectively. Results are expressed as means ± SD (*n* = 3). Different letters a-b indicate statistical significant differences (Kruskal-Wallis followed by LSD and Dunnett’s test for control assay; *p-value* < 0.05).

Three more situations worth highlighting are: increasing only light to intensities of 55-85 µmol photons $m^{-2}s^{-1}$ over the last 72 h of cultivation won’t contribute barely nothing to increase the protein production rate by the alga. The same goes to the extra supplementation with phosphate alone - total inexistence of statistically significant differences between the assays “Light Increase” and “Phosphate Increase” when compared with the control situation (9.82 ± 1.406 and 10.842 ± 1.255 % of dry weight, respectively, against 9.71 ± 0.364 % of dry weight) (*p-value* > 0.05, LSD test); yet, if we compare the assays “Phosphate Increase” and “Ammonium & Phosphate Increase”, we quickly realize that there are no gains, in statistical terms, to the TSP content when, after extra phosphate is added to the growth medium, the concentration of ammonium is also duplicated. However, this doesn’t mean at all that the combined addition of both nutrients won’t have a significantly positive influence over the protein synthesis mechanism - evidence of statistically significant differences between the “Ammonium & Phosphate Increase” (13.217 ± 1.637 % of dry weight) and “Control” (9.71 ± 0.364 % of dry weight) situations (*p-value* < 0.05, Dunnett’s test); at last, between “Ammonium & Phosphate Increase” and “Ammonium, Phosphate & Light Increase”, there are also differences of this nature (13.217 ± 1.637 % against 18.865 ± 1.083 % of dry weight) (*p-value* < 0.05, LSD test), confirming that, allied to the doubling of both nutrients concentration, the referred Gro-lux light increase for this set is capable of triggering a higher protein production by *G. gracilis*. From the “Control” to the CWF and “Ammonium, Phosphate & Light Increase” assays, an increase of circa 8.35 and 9.16 % of dry weight in the TSP content was respectively estimated.

Once again, by comparing the exposure time to the light intensity elevations stipulated between sets, the difference between a value of 12.961 ± 2.128 % of dry weight (2nd Set, assay “Light Increase”) and of 9.82 ± 1.406 (3rd Set, “Light Increase”) may indicate (even though without statistical certainties) that variations in the *G. gracilis* TSP content exist when the exposure time is increased from 72 to 168 h. Estimating a relationship between light intensity and nutrients concentration (speaking only of ammonium) for this parameter, both seem to play an equally important action. On the one hand, we have the high light intensities of the CWF lamp (40 up to 110 µmol photons $m^{-2}s^{-1}$) that somehow seem to abolish the need for extra nutritional supplementation - a fact that is proven by, in practically the totality of times, triggering a larger production of soluble proteins in the algal tissue. On the other hand, we have the assays with the Gro-lux lamp in which, if we don't interfere with the nutrient concentration, the TSP content values obtained for the increase of light only are relatively low and quite inferior, statistically, to those obtained for CWF. As such, only a supplementation of essentially ammonium (beyond the basis formulation already established in Appendix A, Table A.I), coupled with an increment in light over the last 72 or 168 h, seems to stimulate the protein production in the same way the CWF does.

When assessing the influence that the "Nutrients" factor has over the "TSP content" parameter, Yu & Yang (2008) reported maximum values of 3.955 % wet weight for the highest nutrient ratio (N/P) used - 600/37.5 µmol/L - when cultivating *Gracilaria lemaneiformis* for 7 days. Lower values were obtained for shorter and longer cultivation times: 3.433 % WW for 3 days and 2.311 % WW for 15 days. Although there are differences over the exact quantities of nitrogen and phosphorus supplemented to the growth medium, this study, along with ours, demonstrate that increasing the concentration of these nutrients (more nitrogen than phosphorus) will result in an augmentation of the content in total soluble proteins. The divergence between the maximum protein values obtained by Yu & Yang (2008) for *G. lemaneiformis* and the TSP maximum content achieved for *G. gracilis* in the present study - 18.865 ± 1.083 % of DW - is owed precisely to the state/condition of the biomass over which the trials were conducted. Whereas in our study the TSP content was estimated in % of dry weight, where the % of humidity had already been removed (same mass of protein for a smaller total mass of alga), for *G. lemaneiformis* the same content was calculated in % of wet weight where the % of humidity had still been accounted (same mass of protein for a bigger total mass of alga), justifying the inferiority of the obtained values. Regarding the influence of the exposure time to N and P increments over the TSP content, our results cannot be compared with those of this study since the estimation of this parameter was only performed after 14 days (when medium replacement took place) and none other times during the duration of cultivation. As for the effect that the "Light Intensity" factor plays over the same parameter, Ak & Yücesan (2012) also managed to obtain an interval of crude protein contents ranging from 20.17 to 24.19 % DW when using the following light intensities: 25 µmol photons m^{-2} s^{-1} (24.19 %); 50 µmol photons m^{-2} s^{-1} (22.23 %); 75 µmol photons m^{-2} s^{-1} (20.91 %); 100 µmol photons m^{-2} s^{-1} (20.22 %); 150 µmol photons m^{-2} s^{-1} (20.17 %). Although in our study the maximum TSP content (18.865 ± 1.083 % DW) has been obtained when light intensity was increased to values of 55-85 µmol photons m^{-2} s^{-1} in the last 3 days of cultivation after 11 pre-established days under intensities of 39-45 µmol photons m^{-2} s^{-1}, as opposed to the 25 µmol photons m^{-2} s^{-1} of this study, the proximity between the proteinic contents of *G. gracilis* (18.865 ± 1.083 % DW) and of *G. verrucosa* (20.91 % DW), obtained for a light intensity near the 75 µmol photons m^{-2} s^{-1}, should be noted. And yet, if we compare, for instance, the maximum total protein content obtained in our cultures with those obtained for wild material representative of the same species, considerable differences can be clearly noticed - it's the case of the seaweed *G. gracilis* from the Lesina Lagoon (Italy) for which Francavilla *et al*. (2013) obtained maximum values of 45 % dry weight in January. Unless species-specific requirements are known to an extreme, artificial conditions (whether be light, nutrients, temperature, etc.) hardly surpass what optimal natural conditions do best. Nonetheless, the protein levels obtained for *G. gracilis* from the Lagoa de Óbidos cultivated in laboratory are comparable to other venerable protein sources such as: tuna (23 %), cod (21 %), mussels (17 %), chicken eggs (12 %), cottage cheese (13 %), walnuts (15 %) and chickpeas (8 %) [97], making it possible and advantageous to combine this edible vegetable source with others of animal origin and its consequent integration in a healthy diet regime [28].

Photosynthesis generally results in an increase in biomass (and consequent growth, as seen before) since more carbohydrates (sugars/glucose) are produced in algae [98,38]. In turn, the energy provided by these reduced carbon (organic) compounds is used for the synthesis of the diverse macromolecules essential to plants as is the cellulose of cell walls, nucleic acids, starch, pigments, fats and proteins (promoting even more their growth) [38,99]. However, plants cannot synthesize protein from pure carbohydrate since proteins contain nitrogen not produced through photosynthesis [99]. In this case, when we talk about seaweed cultivation systems, the inorganic compounds intaken by seaweeds are simply supplemented/provided through pre-established growth media or other sources containing these compounds - e.g. eutrophic lake waters). This supplementation is of a paramount importance since fixed inorganic nitrogen compounds are required for the biosynthesis of all nitrogen-containing organic compounds such as amino acids and proteins, nucleoside triphosphates and nucleic acids [38,100]. Composing one of the essential structural blocks of body tissue, when favourable conditions are created for increasing protein content in cultivation, the best circumstances for seaweed growth are also being normally defined at the same time since much of its growth is due to the production of new proteins through the protein synthesis mechanisms. Thus, based on the above evidences, the function exerted by nutrients and light in seaweed growth, as well as in protein generation and composition (of which phycobiliproteins, mentioned just below, are also members) is readily understood.

4.1.1.3. Phycoerythrin and Phycocyanin Content

Amongst the four groups of PBPs already described for Rhodophytes, only two had interesting features for this study - the Phycoerythrin (PE) and the Phycocyanin (PC). The fact that their origin, i.e., the biological material from which these proteins were extracted, refers to the red algae phylum Rhodophyta, Florideophyceae, Gracilariales, which encloses the study species - *Gracilaria gracilis*), the pigments present are the R-phycoerythrin (R-PE) and the R-phycocyanin (R-PC). Effects that temperature and salinity may have on this content have already been briefly mentioned. Many of the advantages and utilities of these fluorescent pigments have been described too in section 1.4.2. Importance and Applications (Introduction) as well as some of the most commonly used methods for their extraction (section 1.5. R-Phycoerythrin Conventional Extraction Procedures). Even so, in this study, the content in R-PE and R-PC was mechanically extracted from *G. gracilis* culture samples through a tissue homogenizer. Being already known a huge slice of its benefits and patented the vast majority of its applications [45], many are the studies devoted not only to the development of strategies to increase the content of phycobiliproteins in cultivating algae, but also to the creation and improvement of the already existing extraction and purification methodologies for the purpose of taking full advantage of these pigments potentialities. For being present in a higher proportion in red algae (it's a pinkish-red pigment), the evaluation of R-phycoerythrin levels will be more relevant than those of R-phycocyanin for this study.

So, going straight to the point, it was found that, in the 1st Set of Trials, the highest R-PC value didn't go beyond the 0.156 ± 0.043 mg g^{-1} of wet weight (WW) of seaweed (registered for the CWF assay) while the lowest was attained for "Ammonium Increase" - 0.128 ± 0.09 mg g^{-1} WW (Fig 4.12) (see Appendix A, Table A.IX).

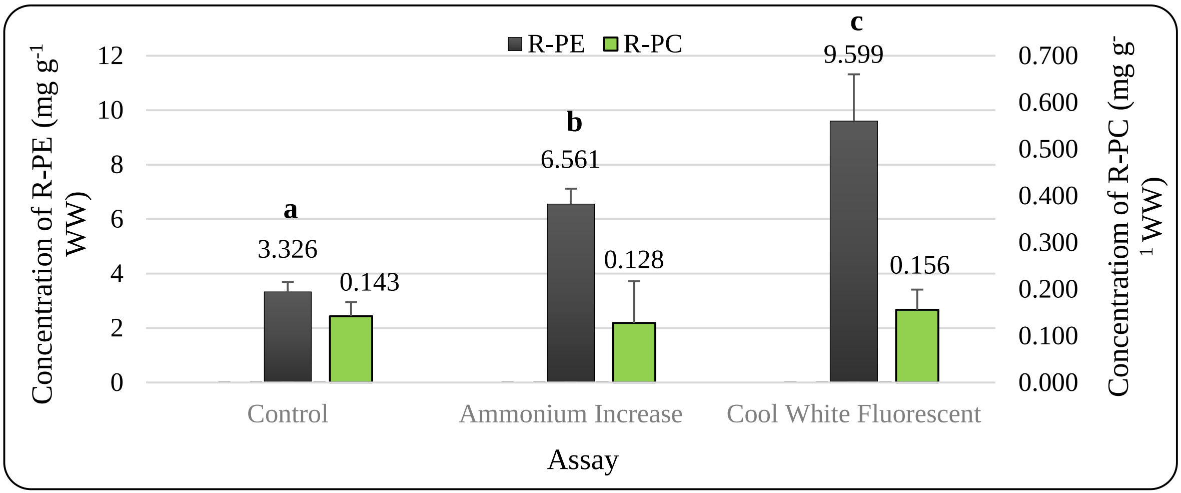


Figure 4.12: Concentration of R-phycoerythrin (R-PE) and R-phycocyanin (R-PC) (mg g^{-1} wet weight (ww)) of *Gracilaria gracilis* cultivated biomass extracted from the assays "Control" (Ct), "Ammonium Increase" (A) and "Cool White Fluorescent" (CWF). The PBPs content was estimated for a culture time of 36 days for CWF and after 28 cultivation days for Ct and A. The assays correspond to the "1st Set of Independent Trials" and "Independent Trial", respectively. Results are expressed as means ± SD ($n = 3$). Different letters a-b indicate statistical significant differences (ANOVA followed by LSD and Dunnett's test for control assay; *p-value* < 0.05).

Yet, there were no statistically significant differences either between these two assays or in relation with the "Control" (*p-value* > 0.05, LSD & Dunnett's test, Appendix A, Table A.XI). That is, neither additional ammonia increments nor higher light intensities (as those provided by CWF) were sufficient to significantly increase the R-PC content in *G. gracilis* vegetative tissues. Regarding the R-PE concentration (also estimated in mg per g of wet weight of seaweed), statistically significant differences were observed between all assays (*p-value* < 0.05, LSD & Dunnett's test, Appendix A, Table A.X): the CWF assay showed the highest value (9.599 ± 1.722 mg of R-PE per g of WW), the "Control" the lowest one (3.326 ± 0.372 mg g^{-1} WW) and the "Ammonium Increase" assay an intermediate value (6.561 ± 0.56 mg g^{-1} WW) (see Fig. 4.12 and Appendix A, Table A.VIII). Apparently, the superior light intensities provided by the CWF lamp (40-110 µmol photons $m^{-2}s^{-1}$) allow to obtain higher contents of R-PE when compared to the Gro-lux lamp. For this latter type of lamp, comparatively to a control in this regime, the extra ammonium supplementation (assay with a light intensity of 39-45 µmol photons $m^{-2}s^{-1}$) wasn't enough to beat the high intensities of CWF. And, on top of that, this last assay didn't even need the same ammonium concentration of that to happen. The CWF assay registered positive differences of circa 6.3 and 3.0 mg per g of wet weight of the R-PE concentration in comparison to the assays "Control" and "Ammonium Increase", respectively.

From a statistical point of view, in the 2^{nd} Set of Trials happens the exact same thing to the R-PC values (Fig. 4.13). Although being relatively smaller than those extracted for the previous set (minimums in the "Control" situation - 0.010 ± 0.005 mg g^{-1} WW - and maximums in "Light Increase" - 0.066 ± 0.048 mg g^{-1} WW -, excepting for CWF), neither the increase in light in the second cultivation week nor the combination of this augmentation with that of nutrients was sufficient to induce statistically significant differences in the R-PC concentration between any of the assays (*p-value* > 0.05, LSD & Dunnett's test).

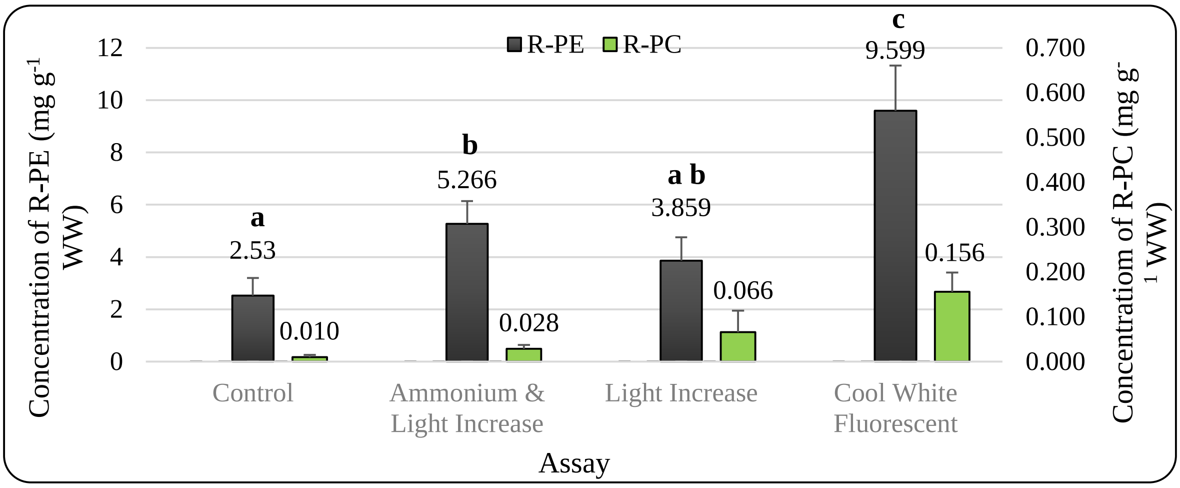


Figure 4.13: Concentration of R-phycoerythrin (R-PE) and R-phycocyanin (R-PC) (mg g^{-1} wet weight (ww)) of *Gracilaria gracilis* cultivated biomass extracted from the assays "Control" (Ct), "Ammonium & Light Increase" (AL), "Light Increase" (L) and "Cool White Fluorescent" (CWF). The PBPs content was estimated for a culture time of 36 days for CWF and after 28 cultivation days for Ct, AL & L. The assays correspond to the "2^{nd} Set of Independent Trials" and "Independent Trial", respectively. Results are expressed as means ± SD (n = 3). Different letters a-b indicate statistical significant differences (ANOVA followed by LSD and Dunnett's test for control assay; *p-value* < 0.05).

Regarding the R-PE pigment, for Gro-lux, three situations stand out: elevating light intensity in the second week to 45-75 µmol photons $m^{-2}s^{-1}$ doesn't seem to have much influence on the production of more R-PE by the alga - 3.859 ± 0.901 mg g^{-1} WW for the "Light Increase" assay against the 2.53 ± 0.668 mg g^{-1} WW of the "Control" (*p-value* < 0.05, Dunnett's test); in what touches the relationship between "Ammonium & Light Increase" and "Light Increase", it can be verified that associating a duplication of the ammonium concentration with the referred light increase won't be statistically beneficial (3.859 ± 0.901 mg g^{-1} WW of the "Light Increase" assay against 5.266 ± 0.872 mg g^{-1} WW of the "Ammonium & Light Increase" test (*p-value* > 0.05, LSD test). In other words, for this type of lamp in a situation where light is only increased in the last week to the values already mentioned, adding or not adding ammonium will not significantly influence the seaweed's R-PE production rate; finally, both conditions (nutrients and light) ("Ammonium & Light Increase" assay), if raised, will already show statistically significant differences in relation to the condition where neither of them is increased - the "Control" (5.266 ± 0.872 mg g^{-1} WW of the "Ammonium & Light Increase" test against 2.53 ± 0.668 mg g^{-1} WW of the "Control" assay) (*p-value* < 0.05, Dunnett's test). Yet, once again, none of these three situations failed to present statistically significant differences towards the highest R-PE values attributed to the CWF trial. (*p-value* < 0.05, LSD & Dunnett's test). The CWF assay registered positive differences of circa 7.1 and 4.3 mg per g of wet weight of the R-PE concentration in comparison to the assays "Control" and "Ammonium & Light Increase", respectively.

Ultimately, nothing new occurs in the 3rd Set regarding the blue pigment (Fig. 4.14): values continue to fluctuate within the same general range of values - 0.017 ± 0.017 ("Control") to 0.114 ± 0.083 mg g^{-1} WW ("Ammonium & Phosphate Increase") - without any statistically significant differences between the assays under the Gro-lux lamp and the assay going under the Cool White Fluorescent lamp (*p-value* > 0.05, LSD & Dunnett's test Appendix A, Table A.XI). That is, considering as goal the increase of R-PC concentration, none alteration in the cultivation conditions of *G. gracilis* stood out from the remaining ones.

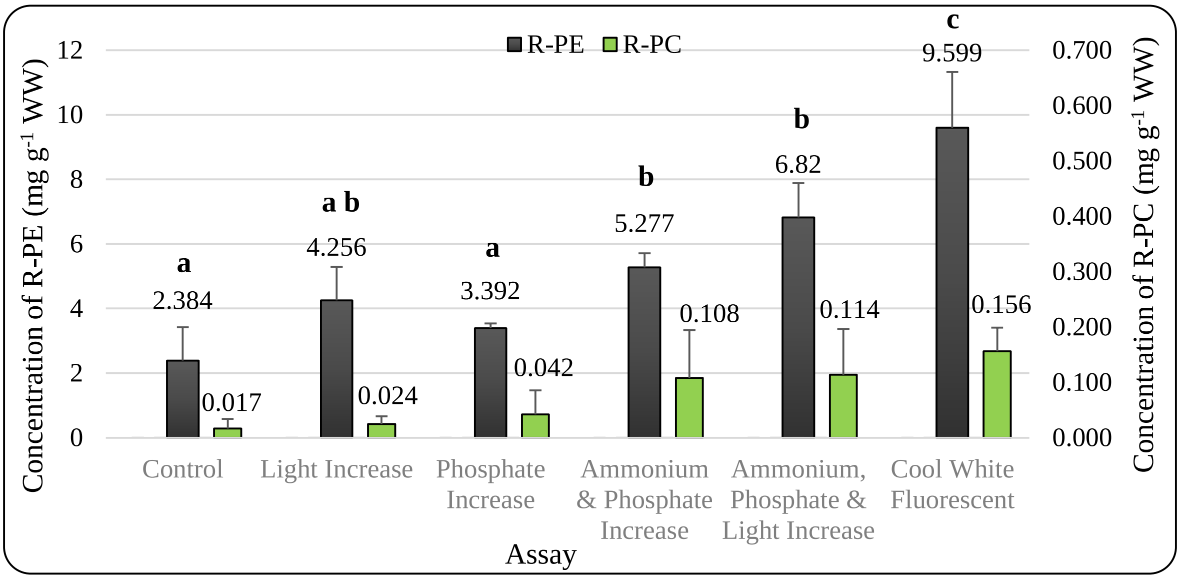


Figure 4.14: Concentration of R-phycoerythrin (R-PE) and R-phycocyanin (R-PC) (mg g^{-1} wet weight (ww)) of *Gracilaria gracilis* cultivated biomass extracted from the assays "Control" (Ct), "Light Increase" (L), "Phosphate Increase" (P), "Ammonium & Phosphate Increase" (AP), "Ammonium, Phosphate & Light Increase" (APL) and "Cool White Fluorescent" (CWF). The PBPs content was estimated for a culture time of 36 days for CWF and after 28 cultivation days for Ct, L, P, AP and APL. The assays correspond to the "3nd Set of Independent Trials" and "Independent Trial", respectively. Results are expressed as means ± SD (n = 3). Different letters a-b indicate statistical significant differences (ANOVA followed by LSD and Dunnett's test for control assay; *p-value* < 0.05).

In what concerns the R-phycoerythrin, results bear some similarities with the pink pigment concentrations recorded for the previous set. First of all, it is important to note that, once more, none of the Gro-lux assays outperformed the one of CWF. Then, Figure 4.14 shows us that neither the supplementation of the growth medium exclusively with additional phosphate nor the increase in light to values of 55-85 µmol photons $m^{-2}s^{-1}$ in the last 72 cultivation hours elevated significantly the concentration of R-PE (as evidenced by the absence of any statistically significant differences between the assays "Control", "Light Increase" and "Phosphate Increase" - R-PE concentrations of 2.384 ± 1.038, 4.256 ± 1.043 and 3.392 ± 0.144 mg g^{-1} WW, respectively (*p-value* > 0.05, LSD & Dunnett's test). Another aspect already expected was that, with ammonium supplementation, the R-PE would have increased significantly. A situation that ended up being true - proven by the existence of statistically significant differences between the assays “Ammonium & Phosphate Increase” and “Phosphate Increase” (or “Control”) (5.277 ± 0.436 against 3.392 ± 0.144 or 2.384 ± 1.038 mg g^{-1} WW, respectively) (*p-value* < 0.05, LSD & Dunnett’s test). However, when comparing the “Ammonium & Phosphate Increase” assay value with the “Light Increase” and “Ammonium, Phosphate & Light Increase” ones, it’s pretty clear that, in this situation, there weren’t significant differences at the statistical level - the three assays presented R-PE values close to each other (5.277 ± 0.436, 4.256 ± 1.043 and 6.82 ± 1.066 mg g^{-1} WW, respectively) (*p-value* > 0.05, LSD test). Basically, these findings suggest that cultivating *G. gracilis* solely with increments of ammonium and phosphate, with stronger light in the last 72 h or with a combination of these two situations is relatively indifferent (statistically) for the production of R-PE. Even so, after extra-supplementing the growth medium with NH_4^+ and PO_4^{3-}, an exposure to a higher light intensity over the last 72 h allowed to increase by nearly 1.6 mg g^{-1} WW the content of R-PE produced by *G. gracilis*. It should be also noted that, as anticipated, the assay “Ammonium, Phosphate & Light Increase” evidenced itself statistically better than the “Control” and “Phosphate Increase” assays in the production of R-PE (*p-value* < 0.05, LSD & Dunnett’s test). The visual aspect of the R-PE and R-PC-containing supernatants resultant from the extraction of PBPs from seaweed biomass corresponding to some of the assays within this 3rd Set, is illustrated in Fig. 4.15.

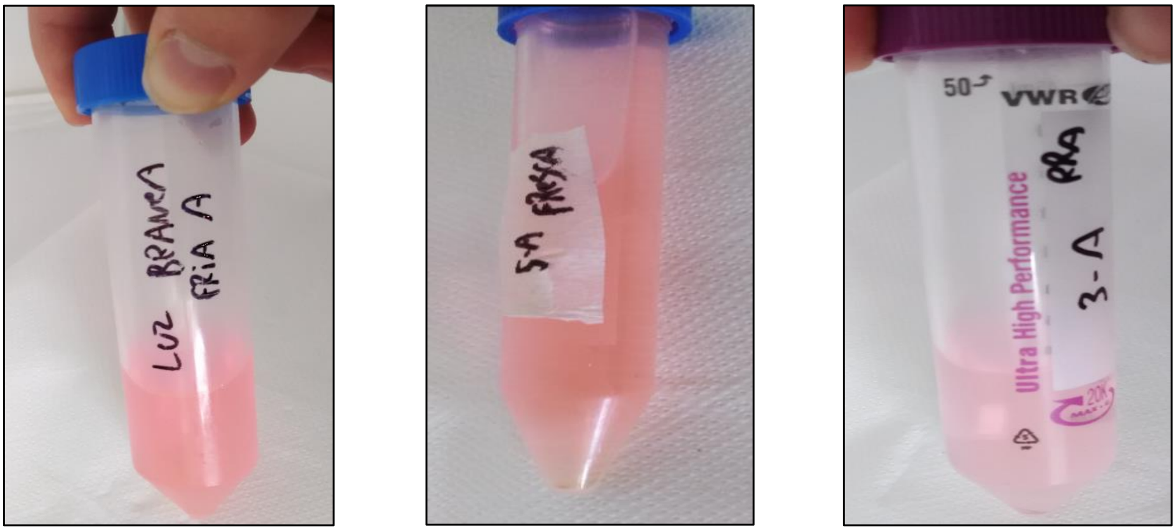


Figure 4.15: Photographs illustrating the visual aspect of the R-PE and R-PC-containing supernatants resultant from the mechanical extraction of PBPs with the tissue homogenizer applied to *G. gracilis* culture samples; from left to right, the assays “Cool White Fluorescent” (CWF), “Ammonium, Phosphate & Light Increase” and “Control”, respectively, from the Independent and 3rd Set of Independent Trials.

Herein, we visualize that the strongest/solidest R-PE color and highest fluorescence are associated with the assays from where higher concentrations of R-PE were withdrawn - the CWF and the “Ammonium, Phosphate & Light Increase” assays. On the opposite side, we have the control situation accounting very low contents of R-PE that match the clearest red/pink tonality of the supernatant. As for R-PC, being present in much lower concentrations, its characteristic blue hue is masked by the heavier/more robust tonality of the R-PE pigment present in the supernatant in much higher concentrations. The CWF assay registered, again, positive differences of circa 7.2 and 2.8 mg per g of wet weight of the R-PE concentration in comparison to the assays “Control” and “Ammonium, Phosphate & Light Increase”, respectively.

For this parameter (R-PE and R-PC concentration), the light intensity seems to play a bigger role than nutrients, evidenced by the constant superiority of the CWF assay values in relation to the Gro-lux values regardless of the situation/assay in question. Statistically speaking, it compensates more, in terms of production and obtainment of pigment, simply cultivating *G. gracilis* under a CWF light with a standard composition of the VSE medium than actually expending resources (additional nutrients) on cultivation under Gro-lux light. Fact corroborated by the existence of statistically significant differences between the situations/assays where more ammonium, phosphate or both are present and the CWF assay in any of the sets (*p-value* < 0.05, LSD test). A similarity between a value of 3.859 ± 0.901 mg R-PE g^{-1} WW (2nd Set, assay “Light Increase”) and of 4.256 ± 1.043 mg R-PE g^{-1} WW (3rd Set, “Light Increase”) might also indicate that for the low range of Gro-lux intensities we worked with, there are no major differences in raising the exposure time from 72 to 168 h (two weeks) to the light increases respective for each set. Even though statistical analysis does not prove great significant differences, the highest R-PC contents were found for CWF in all trial sets.

Phycobiliprotein synthesis depends on the supply of assimilable nitrogen in the environment. On the other side, these proteins are able as well to serve as a nitrogen reserve [45,53]. According to Parjikolaei *et al*. (2013), the alterations in pigment concentration are determined by the interaction between two factors - light intensity and nutrient availability. This interaction determines whether the pigment concentration (essentially chlorophyll *a* and PBPs) should increase or decrease in order to stimulate the photosynthetic capacity or avoid photo-oxidative damage, respectively [101,102]. In the photosynthesis process in red seaweeds, the chlorophyll *a* pigment never stops playing the preponderant role in converting light energy to chemical energy, being phycoerythrin and phycocyanin in charge of collecting additional light from wavelengths of the spectrum not absorbed by chlorophyll *a* or *b* [48]. Phycoerythrin, the most abundant accessory pigment in red algae, is also involved in the adaptation to sudden irradiance and light spectral changes due to its light-harvesting and linker function [47,48]. The main response mechanisms to sunlight (or artificial light) variations occur in the photosynthetic system: on the one hand, high light exposure (as during the summer months or achieved artificially in laboratory conditions) requires seaweeds to acclimatize, avoiding inhibition of photosynthesis and degradation of the photosynthetic apparatus [38,39,99]. When this situation occurs, one usually evidences it by the bleaching of seaweeds - a phenomenon not witnessed by *G. gracilis* (specially with CWF light where irradiance was higher), which justifies that light intensities weren’t high enough to induce this damage. Protection, granted by pigments such as carotenoids and PBPs, against the harmful effects of superexcitation by supersaturating light intensity is a fundamental survival mechanism of seaweeds (e.g. in the intertidal zone) [103,104,105]. On the other hand, during low photon flux conditions, algae have to harvest maximum light, generally increasing the PBPs and clorophyll a concentration. A study regarding the effect of light intensity on the pigment composition of *Gracilaria verrucosa* (Rhodophyta), currently regarded as a synonym of *Gracilariopsis longissima* [25], as concluded that the maximum phycoerythrin (≈ 5.39 mg g^{-1} wet weight (WW)) and phycocyanin (≈ 3.31 mg g^{-1} WW) contents were achieved after a period of 72 and 60 h, respectively, for a light intensity of 25 µmol photons $m^{-2}s^{-1}$ when compared to other studied intensity values – 50, 75, 100 and 150 µmol photons $m^{-2}s^{-1}$ [103]. Generally, the phycocyanin and phycoerythrin contents of *G. verrucosa* decreased with light intensity increments and tended to increase with increments on time exposure from 0 to 72 h to this intensity (at least for 25 µmol photons $m^{-2}s^{-1}$). This study is a fine example of how light intensity and exposure time affects the PE and PC concentrations produced in the algal tissue. If we compare it to our results, some contradictions are found: first of all, the minimum intensities we afforded and tested were of 39 µmol photons $m^{-2}s^{-1}$ (for Gro-lux) - a little higher than the ones responsible for the best results in this study - and were even associated with the lowest R-PC and R-PE contents achieved for *G. gracilis*; this study's experience took place over 72 h, the same period of time that *G. gracilis* tips were exposed to a higher light intensity (55-85 µmol photons $m^{-2}s^{-1}$) - assays "Light Increase" and "Ammonium, Phosphate & Light Increase". Although there is no agreement on the values of light intensity that allowed to obtain the highest tenors of both pigments, the similarity attainable between the two studies is that the increase of the exposure time up to 72 h for certain fixed values of light intensity (55-85 µmol photons $m^{-2}s^{-1}$ for *G. gracilis* / 25 and 50 µmol photons $m^{-2}s^{-1}$, at least, for *G. verrucosa*) leads to an increment of this parameter. Among other factors and target parameters, Bunsom & Prathep (2012) studied the effects of light intensity on pigments of *Gracilaria tenuistipitata*, finding that the highest PE concentration (0.8 mg g^{-1} FW) was attained for an irradiance of 150 µmol photons $m^{-2}s^{-1}$ within a range going from 150 to 1000 µmol photons $m^{-2}s^{-1}$. Albeit these irradiance values are much higher than the ones used in the present study - which prevents the results from being comparable -, one is only interested in pointing out that light intensities well above 150 µmol photons $m^{-2}s^{-1}$ do not contribute to increase the content in PE of one of the many species of the genus *Gracilaria*, even occurring a photoinhibition phenomenon around 1000 µmol photons $m^{-2}s^{-1}$. As for the nutrient source, seaweeds necessarily require them usually in the most common form of nitrate (NO_3^-) or ammonia (NH_4^+) (primary sources), but also phosphate (PO_4^{3-}), under the limitation of occurring a dramatic effect, if absent, on the cellular pigmentation leading to the inhibition of biliproteins synthesis and, therefore, decreasing the quantity of R-PE present in the macroalgae tissue [106,107]. Joniyas *et al*. (2016) demonstrated that specific ratios of ammonium/phosphate (NH_4^+/PO_4^{3-}), besides influencing the DGR as seen before, could also affect the chemical composition (PE and PC) of the seaweed *Gracilaria manilaensis*: in a range from 20/2 NH_4^+/PO_4^{3-} µM (excluding the control situation) to 300/30 NH_4^+/PO_4^{3-} µM, this last ratio of nutrients exhibited the highest PE and PC concentrations (4.32 and 2.20 mg/g DW, respectively). Hence, PE and PC were strongly dependent on the inorganic concentrations of N and P. Meanwhile, Yu & Yang (2008), for a similar range of N and P applied to the growth of *G. lemaneiformis*, obtained a maximum value of 0.268 mg/g WW of PE after 7 days in cultivation for 400/25 µmol/L (N/P). They discovered that increasing N/P concentration would lead to an increase of chlorophyll *a* and PE contents, accelerating, consequently, the photosynthesis rate. Associated with this phenomenon was, obviously, an increase in the growth of *G. lemaneiformis* which the authors justified as being caused by a raise in nutrient supply capable of increasing the substrate concentration (and enhancing its assimilation) of a series of growth-related physiological processes of the seaweed. Although we cannot solidly compare our results with those of these studies since we didn't establish concrete ratios between the two nutrients for *G. gracilis* - we simply doubled or didn´t double their concentrations to the contents already stipulated in the VSE medium -, these two studies only corroborate the conclusions we have reached to: the nitrogen has a greater influence than phosphorus on the PBP content; increasing the concentration of ammonium (the nitrogen form) until certain limits not stipulated in the present study contributes significantly to elevate the PBPs contents (and, in particular, R-phycoerythrin).

The spectral profile is a commonly used approach to indicate the non-degradation of PBPs [58]. Therefore, as being the sample with the highest R-phycoerythrin (and also R-PC) content recorded, an absorption spectrum was determined for the Cool White Fluorescent assay by scanning the supernatant resulting from the extraction on an UV-Visible Spectrophotometer in a range of 200-750 nm wavelengths. The result is portrayed in Fig. 4.16.

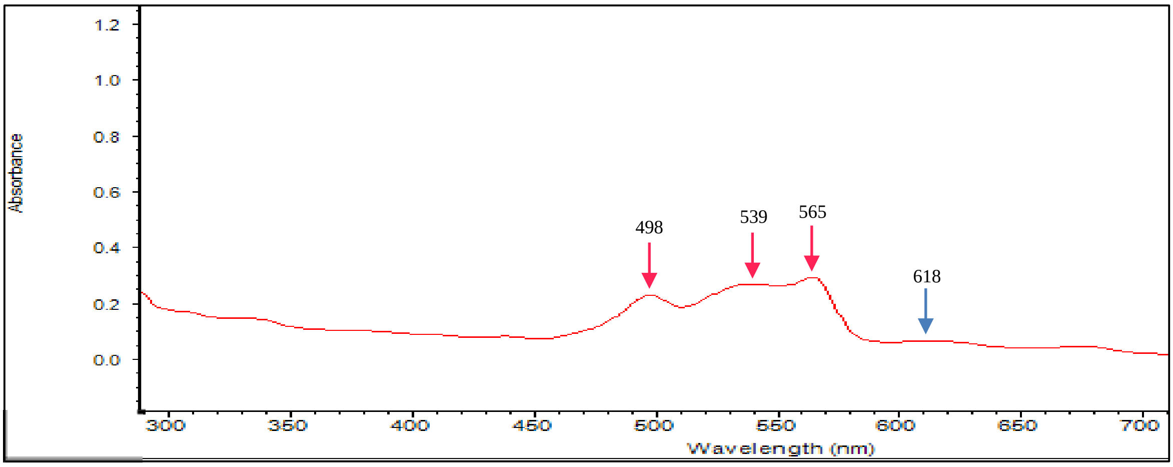


Figure 4.16: Absorption spectrum of the phycobiliproteins (PBPs) R-phycoerythrin and R-phycocyanin extracted through the tissue homogenizer methodology from lab-cultivated *Gracilaria gracilis*. The spectrum corresponds solely to the independent trial with Cool White Fluorescent lamp (light intensity of 40-110 µmol photons $m^{-2}s^{-1}$, VSE medium). The absorption maxima were at 498, 539 and 565 nm (red/pink arrows), also denoting a tenuous plateau at 618 nm (blue arrow).

Two distinct situations can be observed: firstly, it's possible to identify three maximum absorbance peaks (0.228, 0.287, 0.334) at 498, 539 and 565 nm, respectively; then, even though less noticeable and visible, a fourth absorbance peak (0.139) exists at about 618 nm. As seen from the absorption spectrum specific for R-PE (Fig. 1.3, section 1.3 Phycobiliproteins (PBPs) amongst other Algal Proteins, Introduction), the wavelengths at which the pigment has the highest absorbance values are at 498, 539 and 565 nm - the same wavelengths at which the maximum absorbance peaks for *G. gracilis* were detected in this study (Fig. 4.16). Fig. 1.3 (B) still encloses a small portion of the typical absorption spectrum for R-phycocyanin (R-PC) in which it is noticeable that, at approximately 618 nm, the pigment absorbs the maximum radiation. The same finds to be true for our CWF culture sample where, at 618 nm, a peak is observable (albeit in the form of a tenuous plateau due to the low R-PC values extracted) corresponding to this emblematic maximum absorbance ordinarily found for the R-PC pigment [46]. In conclusion, the similarity between the typical R-PE and R-PC peaks normally characteristics of the purified pigments and those obtained for the CWF absorption spectrum allow us to deduce that the sample didn't suffer considerable degradation of PBPs.

Even though the PBP content (specially R-PE) extracted from *G. gracilis* was relatively high when compared to other studies, we have to bear in mind an extremely important thing: none purification step was applied to the enriched extract in PBPs. Confirmed by the absorption spectrum obtained for CWF, our results confirm one thing: it was indeed possible to make the most of the R-PE extraction from the cultivated *G. gracilis* biomass, but if the intention is to employ it in any of the several domains of applicability, this study and this extraction are not enough - it's imperative to purify the pigment. Rossano *et al*. (2003) reported on a new, one step procedure for the extraction and purification of R-PE from the Mediterranean red algae *Corallina elongata* Ellis & Solander. After the extraction of phycobiliproteins, the subsequent purification step involved an adsorption process on a hydroxyapatite (HA) column and the application of the red coloured resultant fractions on a Superdex 75 gel filtration column. In this case, as opposed to ours, the absorption spectrum obtained by the authors for R-PE was the result of this post-extraction purification process and not just extraction. As a consequence, much more salient, isolated and perceptible pigment peaks (than those achieved in our study) were obtained for the wavelengths of 495, 539 and 566 nm [71]. Thus, in this study, we quickly become aware of the importance of purifying the sample through one or more methods of the vast diversity currently available for the effect, as well as of the spectrophotometric consequences entailed to the process.

In a general way, there seems to be an agreement between the PBP and TSP content among the several assays of different sets. That is, the cultivation conditions impelling the seaweed to produce higher quantities of R-PE and R-PC appear to be the same ones that stimulated it to synthesize higher contents of total soluble proteins. Being the phycobiliproteins a specific class of proteins directly involved in light harvesting (photosynthesis process), it would be expected that, to some extent, an augmentation in light intensity would trigger off a greater production of these coloured biomolecules. Furthermore, due to the fact of being included in the group of soluble proteins, an increase in PBPs is necessarily associated with an increase in TSPs as well. When comparing the conditions affecting both parameters, we promptly make the following conclusions: the PBP content is more affected by light than by nutrients, although the latter also enjoys of a strong influence; the TSP content is fundamentally affected by the increase in nutrients whereas light is in charge of considerable raisings in this parameter as well. Evidences can be seen in some figures: for PBP, Fig. 4.12 - an increase of nutrients caused an increment of about 3.2 mg R-PE g^{-1} DW while higher light intensities (cool white fluorescent lamp) raised in circa 6.2 mg g^{-1} DW the concentration of R-PE, always relatively to a control without increments of any kind. Fig. 4.14 - for Gro-lux, an increase of ammonium and phosphate managed to augment, compared to the control, the concentration of R-PE by nearly 2.9 mg g^{-1} DW whereas the increase of light over the last 72 h added, for this assay, more 1.5 mg g^{-1} to the content of this pigment; for TSP, Fig. 4.10 - increasing light in the second week of cultivation elevated from 12.566 ± 2.503 to 12.961 ± 2.128 % of dry weight the TSP content whilst the addition of ammonium to the already existing light increase caused the alga to raise its proteinic content by more 5 % (for Gro-lux); Fig. 4.11 - the duplication of both nutrients caused a TSP increment of approximately 3.5 % and when light was also augmented in the last 72 h of culture, the increase was even bigger - 5.6 % of dry weight (Gro-lux). When compared with other studies, Joniyas *et* al. (2016) also reported that the same conditions - the highest ratio of nutrients (300 µM NH_4^+ for 30 µM PO_4^{3-}) - were the ones that allowed the obtainment of the highest contents of both PBPs and TSPs (4.32 mg of PE and 1.61 mg of PC g^{-1} DW, respectively, and 16.57 mg of TSP g^{-1} DW). In another study, Ak & Yücesan (2012) defined the light conditions of 25 µmol photons $m^{-2}s^{-1}$ as the best ones to extract as much of both PE (5.39 mg g^{-1} WW) and PC (3.31 mg g^{-1} WW) as well as of TSP (24.19 %). However, they don´t specify the type of lamp used.

Another detail worth mentioning in this study is the possibility of establishing a relationship between the heterogeneity of values (quite evident in some cases) obtained among cultivation replicas for the three study parameters - DGR, PBP and TSP content - and the cultivation conditions under which the tips of *G. gracilis* grew. This discrepancy can be observed by looking into the graphs or tables respective for each of the parameters: Fig. 4.6-4.14 and Tables A.II, A.IV, A.VI, A.VIII and A.IX. (Appendix A) - where it becomes evident that, in some trials, the standard deviation values are rather high, i.e., there is a wide dispersion of values around the mean value. Leaving aside any natural heterogeneity possibly existing among replicas in a statistical analysis, the phenomenon can be fundamentally attributed to two factors: firstly, the *G. gracilis* tips placed on each culture flask (each one corresponding to one replica) could have associated different degrees of diatom contamination. Thus, the flasks more contaminated would possibly lead to the achievement of lower values of the various parameters as a consequence of an increased competition which, in turn, would contribute to a deregulation, in cultivation, of growth and compounds production rates in *G. gracilis*; then, despite an absolute and rigorous control over the proportions of added nutrients, the same cannot be said about light intensity. During cultivation, the three flasks corresponding to the three replicates of each assay were positioned at the same height and equal distance from the light source. However, for reasons of spatial distribution and/or lamp positioning, it might occur that the distribution of light photons reaching the algae tips won't be uniform for each flask, contributing to the oscillation of parameter values between replicas.

With the exception of the "Growth Rate" parameter about which Helson (1965) compares the effect that light sources composed of Gro-Lux and Cool-White Fluorescent lamps have on growth and development of tomato plants, rare are the studies conducting comparisons between this two types of lamps. And if we limit the research to their application in the cultivation of seaweeds, results are even scarcer. Notwithstanding, another very important aspect also capable of influencing the growth and biochemistry of seaweeds must be taken into consideration when comparing the two types of light - the light quality - and not only its intensities. Light characteristics (spectral composition, quantity and duration) have a profound influence on algae metabolism and development [108,109].

In a seaweed culture system, excessively high intensities of light tend to lead to photo-inhibition and photo-oxidation phenomena whereas, on the opposite side, extremely low light intensities can constitute a major growth-limiting factor [110]. As such, intensity, duration and light wavelengths should be optimized to determine the critical point from which algal growth in culture starts to become saturated and energetically unsustainable. Besides that, many experiments have demonstrated that different light wavelengths (red, green and blue light) can produce “finely modulated responses” towards a seaweed relative pigment composition [111]. In another words, they enjoy the ability of having a special influence in regulating photosynthetic pigments synthesis. This complementary chromatic adaptation (CCA) - the acclimation to different light qualities - has been demonstrated to occur in many species of phycobiliproteins-containing algae and cyanobacteria [112]. Red algae, as a pivotal group of PBPs producers don’t escape, obviously, to this sphere [113]. Whereas red and orange growth light has shown to stimulate the production of long wavelength-absorbing phycobiliproteins (mainly phycocyanin), green growth light is often associated with the production of PBPs absorbing maximum radiation at short wavelengths [112]. It’s the case of phycoerythrin where is also noticeable that PC content tend to decrease for this wavelength values. Fig. 4.17 and 4.18 illustrate the emission spectra of both types of light used in this study - the cool white fluorescent (CWF) light and the Gro-lux light.

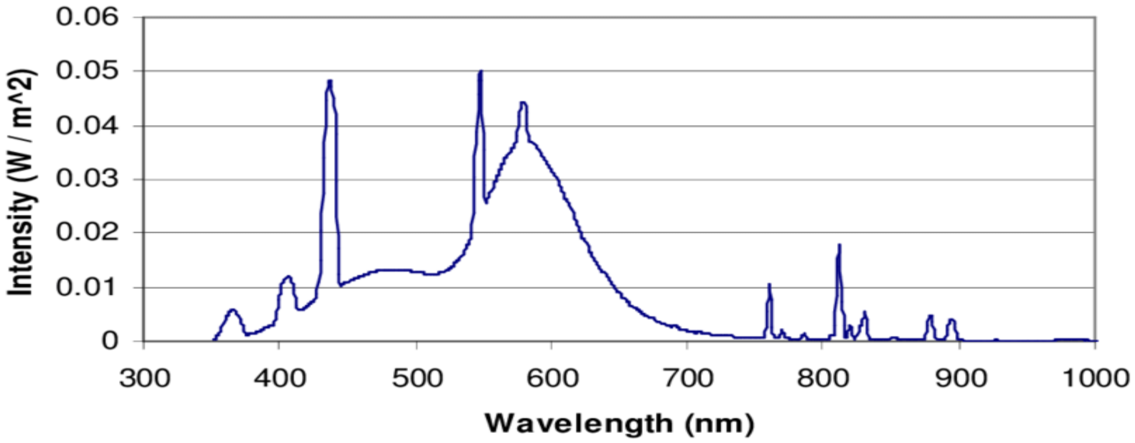


Figure 4.17: Emittance spectrum of Cool White Fluorescent (CWF) lamps. Retrieved from [114].

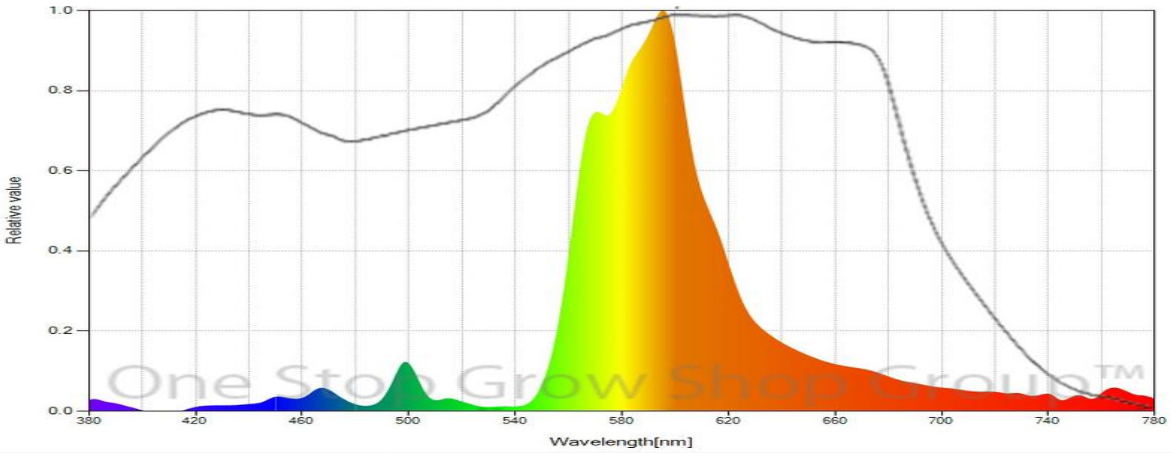


Figure 4.18: Emittance spectrum of Sylvania Gro-lux HPS 600 W 90.000 lumens lamp. Retrieved from [115].

Herein, we can visualize that, for CWF, the maximum of light intensity emitted occurs essentially at three wavelengths bands - close to 430-435 nm, 540-550 nm and 565-580 nm - (in the blue and green spectral zone), while for Gro-Lux light these maxima occur preferably at 560-610 nm (yellow and orange spectral zone). If we conduct a comparison based on the overlapping of these spectra with the absorption ones obtained for R-PC and R-PE in their native state, one can draw some conclusions regarding the content of produced PBPs: having in mind that the wavelengths for which R-PE exhibits the maximum absorbance peaks are located at 498, 539 e 565 (green region), it would be expected that a type of lamp whose maximum light emission occurred near these wavelengths would trigger a greater algal stimulation towards the production of this pigment. Something verified in all assays by the superiority of an amazing value of 9.599 mg g^{-1} WW obtained with the CWF light in relation to the Gro-Lux assays. On the contrary, this latter type of light is characterized by a more concentrated spectral zone of maximum intensity that occurs at slightly longer wavelengths situated more apart from the three maximum absorption peaks for R-PE. Even so, it still encompasses, from the existing three, the maximum absorption peak of R-PE - the one at 565 nm. As such, a lower production of this pigment would be expected when growing *G. gracilis* under Gro-lux light. Something once again verified for all sets of trials and confirmed by the inferiority of R-PE contents obtained under Gro-Lux regime. Finally, out of curiosity, even though R-PC is present in much lower quantities in *G. gracilis* due to the own nature of this alga, if we intend to maximize the production of this pigment, the choice will most likely go through the Gro-Lux light instead of the CWF one. But why? Because the maximum light emission of this lamp occurs very close to the wavelength at which R-PC absorbs maximum radiation - at 618 nm. Jayasankar & Kulandaivelu (2001) studied the influence of different light wavelengths on pigment constituents and absorption spectrum of *Gracilaria corticata*. They discovered, similarly to the present study, that green light (450-610 nm), mimicking the wavelengths of maximum emission that characterize cool white fluorescent light, was the one that stimulated the highest production of R-phycoerythrin by the alga.

In sum, by analysing the emission spectra of each of the types of culture lamps used, the differences in seaweed's yield of produced PBPs when subjected to distinct light qualities quickly become easier to understand. Hence, in an attempt to select the lighting conditions that best maximize the desired parameters in cultivation (whether be the growth/biomass increments or the production of compounds of interest), studies of this nature may attract the research compass into this domain of investigation, as well as open doors to a whole new set of methods employable in macroalgae cultivation systems.

Conclusion

5. Conclusion

Nowadays, the usefulness of marine organisms in the production/synthesis of substances capable of improving human being's life quality is undoubtedly unquestionable. Within the domain of algae, the red alga (rhodophyta) portrayed in this study - *G. gracilis* - possesses a notable importance in several aspects: whether for its interesting biochemical composition, highlighting the high levels of protein, carbohydrates, fatty acids (such as arachidonic acid - PUFA ω-6 and palmitic acid - SFA) and R-phycoerythrin normally found in winter months, or for its remarkable antimicrobial potential (against *B. subtilis* specially), strong antioxidant and radical scavenging activities and high content of total phenols [24,116]. Among these compounds, the phycobiliproteins R-phycoerythrin and R-phycocyanin (associated to rhodophytas) are of a major importance in a panoply of research areas: food, cosmetics, pharmaceutical and biochemical industries, as well as therapeutics and in the field of fluorescence probes. The main obstacle associated with its extraction is related to the solidity of the seaweeds cell wall, being this procedure able to be carried in a considerable number of ways fundamentally associated to three main groups: mechanical (including physical and chemical paths) and enzymatic methodologies or the so called "New Methods" (not detailed in this study) going to the encounter of the forefront of the most efficient extraction methods of PBPs.

One of the specific goals of this study was to determine the best route to extract PBPs (R-PE and R-PC) by comparing different methodologies - mechanicals and an enzymatic one - applied to lab-dried wild biomass or to the fresh culture samples of *G. gracilis*. Then, addressing the specific conditions of light intensity (provided by two different types of lamps) and concentration of essential nutrients (ammonia and phosphate) capable of maximally augmenting either the growth rate, the concentration of PBPs, or even the total soluble protein content of *G. gracilis* grown in laboratory, stood out as a second extremely important objective. It´s important to have in mind that the conditions capable of inducing maximum values of one parameter may not be the same to cause similar effects on any of the other parameters. The cultures initiation stage, which later would lead to the development of authentic algal fragments, began with the cut, cleaning and disinfection of seaweed tips, with their consequent installation and acclimatization in culture flasks supplemented with VSE culture medium and exposed to specific irradiance intensities provided by the lamp bulbs.

From the wild whole specimens collected from the Lagoa de Óbidos used directly in the analysis, to the cultivated samples of *G. gracilis* resultant from tip cultivation, two modifications happened: the condition of the biomass - wet for the cultivating assays and oven-dried for the wild specimens - and the procedure to extract the phycobiliproteins - a mechanical approach with a tissue homogenizer for the wet biomass and two different methodologies consisting on a mechanical (bead beating) and in a enzymatic (with cellulase) approach for the dry biomass. The bead beating methodology coupled with respective freeze-thaw cycles (normally used for microalgae) yielded even considerable amounts of R-PE - 1.186 ± 0.008 mg g^{-1} DW. Much lower quantities were obtained with the use of cellulase - 0.220 ± 0.001 mg R-PE g^{-1} DW - which can be associated with a cell wall of *G. gracilis* poorly constituted by cellulose and mostly consisting of agar which was only partially destroyed by the moderately high temperatures of the enzymatic hydrolysis. In this way, the mechanical process was more efficient than the enzymatic one in the extraction of PBPs. For the wet samples, a comparison between efficiencies it's harder to make since the percentage of humidity wasn't withdrawn. Nonetheless, the first results of R-PE (even though not so much of R-PC) extracted with the tissue homogenizer were pretty satisfactory - maximums of 4.568 ± 1.773/0.099 ± 0.076 mg g^{-1} WW (R-PE/R-PC) and minimums of 2.384 ± 1.038/0.017 ± 0.017 mg g^{-1} (R-PE/R-PC) - proving that this extracting-with-water mechanical methodology is the most effective of the three in the extraction of phycobiliproteins from *G. gracilis*. Consequently, in subsequent analysis, this was the chosen path to extract this type of proteins from the algal tissue. In addition, one more conclusion was able to be made when extracting PBPs through this methodology: freezing *G. gracilis* culture samples at -20 ºC up to 3 months doesn't damage the R-PE nor the R-PC content present in the seaweed tissues. This information may be useful, for instance, if instantaneous extraction of pigment cannot be possible to accomplish right after the end of cultivation trials, or to conserve temporarily our samples.

As for the first cultivation parameter - the daily growth rate (DGR) -, results of the 1st Set of Independent Trials (in Gro-lux light) in comparison with the Cool White Fluorescent (CWF) light demonstrated that nutrients (in particular ammonium) had a greater importance in the DGR relatively to light (even though it wasn´t confirmed statistically). A proof of that was the obtainment of a value of 16.219 ± 3.84 % day^{-1} (for "Ammonium Increase") against a value of 14.936 ± 1.243 % day^{-1} (CWF). In another words, doubling the concentration of ammonium at a constant light intensity of 39-45 µmol photons $m^{-2}s^{-1}$ (base of Gro-lux light) allows to increase the DGR in a greater way that the high light intensities of 40-110 µmol photons $m^{-2}s^{-1}$ (CWF) do. Within the Gro-lux assays, one also concluded that increasing light to values of 45-75 or 55-85 µmol photons $m^{-2}s^{-1}$ in the 2nd week or in the last 72 cultivation hours, respectively, didn't interfere much on the DGR values except when associated with ammonium. Then, the extra-supplementation (2x [PO_4^{3-}]) with phosphate didn´t provoke any significant changes either on the growth of *G. gracilis*.

Regarding the total soluble protein (TSP) content, the highest light intensities provided by the CWF light triggered the production of the major proportions of protein by the seaweed - 18.06 ± 2.027 % of dry weight (a raise of 6.5 % regarding the control) - while doubling ammonia under Gro-lux regime corresponded solely to 14.289 ± 0.309 % of dry weight (a minor raise of 3.8 % regarding the control). Protein values that are, in their essence, comparable to other venerable sources of protein such as tuna, eggs, etc. Afterwards, it's important to note that neither both of the two light increments (2nd week and last 72 h) nor the phosphate nutrient contributed to elevate these proteinaceous indexes. However, when coupled with ammonium, the increments on the TSP contents were statistically significant and, sometimes, even higher than those produced by the CWF lamp. In sum, for Gro-lux assays, the nutrient duplication (specially ammonium) dominated over the effect of light in the production of protein, whereas the higher irradiance values of CWF seem to replace the increment of both conditions.

At last, in what concerns the R-PE and R-PC (phycobiliproteins) content, the main conclusion taken out is that cultivating *G. gracilis* under CWF light allows to obtain the highest values for this parameter - a similar tendency to the TSP parameter. If we look at the 1st Set of Independent Trials (CWF: 9.599 ± 1.722 mg of R-PE g^{-1} WW and 0.156 ± 0.043 mg of R-PC g^{-1} WW; "Ammonium Increase": 6.561 ± 0.56 mg of R-PE g^{-1} WW and 0.128 ± 0.09 mg of R-PC g^{-1} WW), one easily sees that light has a stronger impact on R-PE and R-PC concentrations than nutrients (also playing a significant role specially in the assays with Gro-lux light). As for the increasing of light in the 2nd week of cultivation (2nd Set), it alone didn't bring anything new in relation to the control. Only its association with the nutrient ammonium was able to raise statistically the R-PE concentration. Exactly the same situation was verified for the last 72 h light increasing (3rd Set), one having only ascertained statistically significant differences when associated to the increase of both nutrients. Ergo, to obtain the highest possible PBPs concentration and avoiding at the same time the unnecessary waste of resources, *G. gracilis* should be cultivated under CWF light, always having in mind an eventual relationship between the exposure time and the seaweed photosensibility to these high irradiances. Still regarding this parameter, a spectral profile proved the non-degradation of PBPs during the extraction process, allowing the visualization of the spectral peaks characteristics for R-PE and R-PC. Overall, the PBP and TSP content seem to be more affected by light than nutrients while this latter plays the preponderant role in the DGR.

With slight variations in some cases, this study is in general agreement with most of the investigations in what concerns the role of the nutrients and light intensity on the growth, protein and pigment content of rhodophytas and, specially, *Gracilaria* species. These parameters are dependent and ultimately regulated by phenomena such as photosynthesis and nutrient (N and P, in the form of nitrogenous compounds (e.g. NH_4^+, NO_3^- and NO_2^-) or phosphorus-containing compounds (e.g. PO_4^{3-}, HPO_4^{2-} or $H_2PO_4^-$)) assimilation. On the contrary of several studies in which very high light intensities tend to sometimes stagnate the growth and even the synthesis of certain compounds (such as PBPs and other proteins) due to photo-oxidative damage, such situation doesn't seem to have occurred in this study for the low irradiance values of both lamps and the short exposure times.

In sum, the tenuous optimization of some of the cultivating conditions established in this study along with considerable amounts of extracted R-PE, may certainly justify the deepening of the role of both nutrients employed (and relationship between them), the influence of each one of the specific types of cultivation lamps and still the improvement of the best light intensities and exposure times to these increases in order to maximize the growth and production of compounds of interest in *G. gracilis*. The gathering and a better understanding of these conditions may motivate and contribute for the large-scale cultivation of this macroalgae species from the Lagoa de Óbidos.

Final Remarks and Future Research/Perspectives

6. Final Remarks and Future Research/Perspectives

Much of the findings of this study have already been summarized in section 5. Conclusion. However, some additional considerations should be made in order to consummate the purpose of this research, alienated with the mention of some perspectives eventually very interesting for the future with the view of giving continuity to the cultivation and harnessing the full potential of *G. gracilis* from the Lagoa de Óbidos.

If the aim of the study had been the extraction and quantification of R-PE and total protein from native algae, the results would only reflect the natural conditions in the time of harvest. In that situation, a detailed study regarding the effect of seasonal variation on this contents should be performed in order to discover and estimate the best season of the year (months) to collect seaweed specimens for analysis - preferable, obviously, when contents are the highest possible. Already under manipulable conditions, depending much on the culture situation, *G. gracilis* exhibited interesting growth patterns and appealing productivity yields (of PBPs and TSP). Concerning PBPs, depicted by the end of this section, a subsequent step of purification should be the next stage to follow. As for total protein, once determined its values, an accessory study regarding the content, digestibility, proportion and bioavailability of composing amino acids could be conducted in a near future in order to further assess the seaweed's nutritional profile and the quality of edibility [50]. By finding the exact magnitude of different culture conditions (e.g. temperature, nutrients, pH, salinity, light, sediment) capable of increasing to a maximum a specific algal nutrient, one can make experiments by varying the amplitude of the several conditions to test if the amounts of other constituting nutrients of interest are also increased by the same interval of values. And, if besides the desired nutritional value an optimal growth of biomass is also achieved for the same specific range, we can call it a win-win situation.

Two additions that would have been capable of supplementing/complementing the present study are the following: the increase of the nutrients concentration (initially only of ammonium) to the double in the assay of cool white fluorescent (CWF) light; the study of the influence of given cultivation conditions over another plant pigment - the clorophyll *a* (Chla). In the first case, without this additional supplementation of both these nutrients to the growth medium, this assay practically demonstrated the highest values for all parameters. Who knows if, similarly to Gro-lux assays, doubling the NH_4^+ concentration (and later an attempt with PO_4^{3-}) couldn't increase even further the growth rate and the production of PBPs and total proteins by *G. gracilis*. For the second situation, one is aware that Chla and PE are the key pigments that transform light energy into chemical energy during photosynthesis in red algae [13,48]. Their cellular level is an important physiological index for photosynthesis of algae [38,99]. Therefore, by quantifying Chla contents, it would become possible to evaluate the effect that determined light intensities (of both lamps) would have on the photosynthesis mechanism performed by *G. gracilis*. For instance, if Chla levels were found to be high at a certain moment of cultivation, it probably meant that the seaweed was going through a stage/period of low photon flux conditions which requires the organism to harvest maximum light (stimulating photosynthetic capacity), thus increasing Chla (and PE) concentrations [103,104].

As mentioned before in section 1.1.2. *Gracilaria gracilis* and its Contribution (Introduction), *Gracilaria* algae species are mainly applied in the phycocolloids industry (one of the largest in the realm of harnessing algae products) due to the high presence of these compounds in their composition [117]. Phycocolloids are water-soluble polysaccharides (polymers of sugars units) produced by seaweeds and constitute the main structural components of seaweed cell walls. Whereas two of its main products are extracted from red seaweeds - the agar and carrageenan -, the alginates are specifically extracted from brown seaweeds [117]. Among these three phycocolloids, the agar is the principal product of *Gracilaria* spp [29]. As seen in Table I.I (Section 1.2. Algal Cultivation - Introduction), the world aquaculture production/cultivation of *Gracilaria* seaweeds has been suffering a non-stopping increase (in thousand tons) since 2005 to recent days which can be fundamentally justified by the interest of extracting agar from their tissues. But why agar? Besides its use mainly as a gelling and stabilizing agent and for controlling viscosity (applications in human food industry), agar also possesses a number of biological activities: hepatoprotective, antioxidant and antitumor activity along with some effects as the decrease in the concentration of blood glucose and an antiagregation action on red blood cells [117]. Moreover, agar constitutes the basis of the gel substrate in biological culture media (useful for bacteriological research) and enters in the constitution of laxatives, capsules, tables, suppositories and anticoagulants (important in medical and/or pharmaceutical fields) [117]. Hence, similarly to what has been done with the DGR, the PBP and TSP contents in this study, a promising future application could be the employment and variation of the same culture conditions (nutrients and light intensity) with the view of optimizing the production of this highly valued compound in culture by *G. gracilis*.

As previously seen, nutrients and light are key environmental factors able to determine biomass yield and productivity of an aquaculture system. In tank (or flasks like the present study) culture, it is possible to control all environmental factors, making the knowledge of nutrient eco-physiology of seaweeds of an extremely importance for maximising growth rates and/or the product required [102,118]. Still, understanding this nutrient physiology is a synonym of perceiving some basic concepts such as nutrient uptake rates, nutrient assimilation, nutrient storage and critical tissue nutrient concentrations. When growing a particular species of seaweed in a cultivation system, the knowledge of the C:N:P ratio of the alga is crucial under nutrient saturating conditions [118,119]. Such information can make, thus, the delivery of these nutrients more economical. Nutrient concentrations are expressed as µM for practically all nitrogen compounds [118]. As for the ratio of two specific nutrients (N:P), required for maximal growth of a seaweed, it is called of optimum ratio. In this study, for *G. gracilis,* a nutrient ratio between ammonium and phosphate wasn´t established even though that would have been possible by recurring to the VSE medium formulation. Instead, one proceeded to a different strategy which consisted in duplicating or not duplicating the concentrations of these compounds prescribed by the classical VSE medium. Hence, the concentrations of N and P added were far above the generally recommended units - µM (as seen in

Appendix A - Table A.I). It's a known fact that ammonium may be toxic to some species in the mM range unless the species in question has been studied for NH_4^+ toxicity [106,118]. Besides the concentration, the exposure time to such compounds can also be determinant for its toxicity. However, it didn't seem to be the case for *G. gracilis* - in 14 days of cultivation only, instead of interfering negatively with the various parameters in this study due to concentrations higher than the standardized ones, the adding of ammonium always led to positive and high increments. Furthermore, several studies point out to the exceptional potential of nitrogen uptake by *G. gracilis* without suffering significant damages in its physiology [120]. Regardless of these evidences, to follow the guidelines of similar investigations, in future studies with *G. gracilis* from the Lagoa de Óbidos perhaps a ratio of N:P could be pre-established before the culture initiation in order to determine, at a certain extent, not only the exact proportions of each of these nutrients that are capable of maximizing our parameters of interest, but also to define the limiting ratio from which increases in ammonium and phosphate concentrations no longer contribute to improve/increase the intended goal - possibly even preventing a super-saturation of nitrogen (essentially) in *G. gracilis*, thus avoiding eventual negative impacts on both growth and pigmentation of the seaweed.

During the past decades, compounds separation and purification technologies have significantly contributed to advances in the field of pharmaceutics and biotechnological industries. Phycobiliproteins are no exception to this sphere. The rapid purification and enrichment of the quality of PBPs by the various existent conventional methods - dialysis, precipitation (e.g. with ammonium sulphate), gel permeation, column chromatography, electrophoresis, etc. - play a central role for quality of resultant products [45,50,72,73]. According to the purity index obtained, PBPs may have as destiny different industrial sectors. Also according to the purification techniques employed, PBPs may end up having a higher or a lower index value. In recent times, a great portion of investigators commonly couple an initial purification step - regularly with dialysis membranes and precipitation with $(NH_4)SO_4$ - to a specific type of chromatography - ion exchange chromatography -, and/or to a gel filtration step on specific columns [58]. On top of all that, a SDS-PolyAcrylamide Gel Electrphoresis (SDS-PAGE) is usually conducted afterwards to confirm the purification of extracted PBPs by separating the respective constituting subunits of each pigment based on their molecular weight due to the presence of an electric field [46,49,58]. Therefore, these could be good strategies to purify our samples after extracting the R-PE and R-PC contents.

At a more advanced stage of post-purification research, a detailed study about the bioactivities of *G. gracilis*'s purified PE could also gain notability. Namely, regarding their eventual existing antibacterial, antifungal and antioxidant activities as well as anticancer properties - as demonstrated recently by some investigations to be existent and significant for PE isolated from the cyanobacterium *Microchaete* and from the red alga *Portieria hornemannii* [121,122].

Finally, in what concerns the several existing types of light sources to cultivate seaweeds, we could not fail to briefly address an increasingly emerging technology - the light emitting diodes (LEDs). Conventional artificial lighting for indoor seaweed cultivation usually involves fluorescence lamps which have wide emission spectra, including, very often, wavelengths that stimulate a poor/low photosynthetic activity of the species we intend to cultivate [123]. As such, much of the light energy produced by conventional illumination is not utilized during macroalgae growth. Alternatively, with the goal of eliminating the emission of light by unusable wavelengths and, at the same time, minimizing energy consumption, LEDs can be used as a more efficient, durable, viable and economical light source [124,125]. Capable of providing a more sustainable control of supplemental light during seaweed growth, they also work as a tool to adjust the biochemical composition of the biomass and to promote the production of desired compounds [125,126,127]. LEDs are solid-state, long-lasting, semiconductors which emit light with a very narrow emission peak - their bandwidth generally lies between 10 and 30 nm [124] -, being able to overlap the photosynthetic absorption spectrum. Finally, they exhibit high conversion efficiencies (low heat emission and low power consumption) [125]. Taking as an example the pigment production, by selecting single and specific emission wavelengths at different intensities of the light source, we will be directly directing the light photons to wavelengths used by algae to specifically fabricate these colourful biomolecules. Ergo, in order to maximize even more the PBPs production in *G. gracilis*, its cultivation under the influence of LEDs at pre-selected wavelengths could be a very appealing approach for a near future.

The first steps taken in the present work along with promising perspectives here described linked to the farming of this seaweed, constitute solely tiny fundamentals to the incitement of a larger portion of investigation focused on the cultivation of *G. gracilis* from the Lagoa de Óbidos. A lot more has still to be done. Perhaps in a utopian future propelled by such findings, the adoption of a cultivation system (indoor as this case or outdoor in forthcoming attempts) could be an important piece of the puzzle to stop completely the excessive harvesting of native species, avoiding not only its uncontrolled devastation, but also contributing to maintain natural populations stable and preserved.

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7. Bibliography

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Appendix

Appendix

Appendix A

Table A.I: Composition of the stock solutions required for the Modified Von Stosch Enrichment (VSE) medium preparation, 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 solutions is added. Solutions 3 and 4 were combined immediately prior to this addition to sterilized seawater. GeO_2 was also added at the concentration of 10 mg/L, 2 mL of this stock solution for 1 L of seawater.

Solution Components (*Ingredients****)***	***Quantity (****grams/L of distilled deionized water****)***
Solution 1: Nitrogen	⬇
Ammonium Chloride (NH_4Cl)	26.75
Solution 2: Phosphate	⬇
Sodium Phosphate . Dibasic, 12-hydrate, crystal ($Na_2HPO_4 \cdot 12H_2O$)	0.4
Solution 3: Iron	⬇
Ferrous Sulfate ($FeSO_4 \cdot 7H_2O$)	0.278
Solution 4: EDTA	⬇
Disodium Ethylenediamine Tetra Acetate (Na_2EDTA)	3.72
Solution 5: Manganese	⬇
Manganese Chloride ($MnCl_2 \cdot 4H_2O$)	0.0198
Solution 6: Vitamins	⬇
Thiamine	0.2
Biotin	0.001
Vitamin B_{12}	0.002

Figure A.I: Diagram illustrating the two different main methodologies adopted on the phycobiliproteins (PBPs) extraction (mechanical and enzymatic), the condition of the biomass employed (dried or fresh), its origin (wild or cultivated), and the chosen paths (e.g. bead beating, enzymatic hydrolysis, …) within these to access its content and proceed to their extraction from the algal tissue.

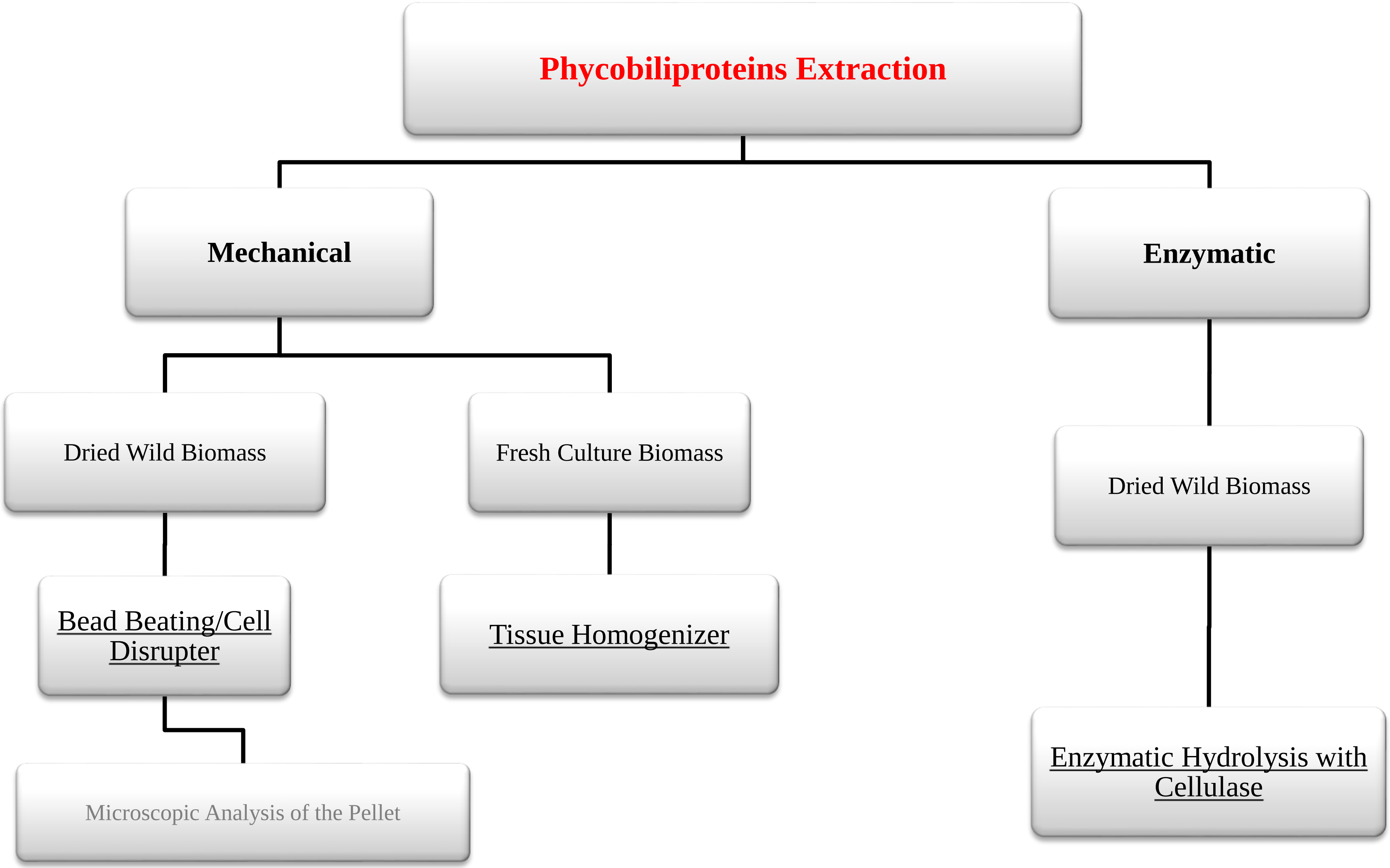
Phycobiliproteins Extraction
Mechanical
Enzymatic
Dried Wild Biomass
Fresh Culture Biomass
Dried Wild Biomass
Bead Beating/Cell Disrupter
Tissue Homogenizer
Enzymatic Hydrolysis with Cellulase
Microscopic Analysis of the Pellet

Table A.II: Phycobiliproteins (PBPs) - R-phycoerythrin (R-PE) and R-phycocyanin (R-PC) – concentration of *G. gracilis* cultivating biomass in two different conditions – frozen and fresh (non-frozen) – from the trials “Light Increase” (L.I.) and “Control” (Ct) of the 3rd Set of Independent Trials. The PBP content was extracted with the tissue homogenizer. Results are expressed in mg of pigment per g of wet weight (WW) of seaweed and were calculated according to the Beer & Eshel (1985) equations. 1, 2 and 3 correspond to the triplicates performed for each assay (*n* = 3).

		L.I. *(Frozen)*	***L.I.*** *(Fresh)*	***Ct*** *(Frozen)*	***Ct*** *(Fresh)*
R-PE	1	4.450	2.521	1.260	2.946
	2	3.129	5.628	2.586	3.253
	3	5.187	5.555	3.307	2.285
	Mean	4.255	4.568	2.384	2.828
	SD	1.043	1.773	1.038	0.495
R-PC	1	0.034	0.142	0.006	0.036
	2	0.031	0.143	0.037	0.049
	3	0.008	0.011	0.008	0.022
	Mean	0.024	0.099	0.017	0.036
	SD	0.014	0.076	0.017	0.014

Table A.III: *t*-student statistical analysis performed according to assay, upon the phycobiliproteins (PBPs) - R-phycoerythrin (R-PE) and R-phycocyanin (R-PC) – concentration of *G. gracilis* cultivating biomass in two different conditions – frozen and fresh (non-frozen) – from the trials “Light Increase” (L.I) and “Control” (Ct) of the 3rd Set of Independent Trials. Greyish values are associated to redundant situations. Combinations of trials few relevant for this analysis are represented by the signal -. Statistical significant differences (*p*-value < 0.05) are shown in bold (*n* = 3).

	Assay	***L.I*** *(Frozen)*	***L.I*** *(Fresh)*	***Ct*** *(Frozen)*	***Ct*** *(Fresh)*
R-PE	**L.I** (Frozen)		0.805	-	-
	L.I (Fresh)	0.805		-	-
	Ct (Frozen)	-	-		0.540
	Ct (Fresh)	-	-	0.540	
R-PC	**L.I** (Frozen)		0.232	-	-
	L.I (Fresh)	0.232		-	-
	Ct (Frozen)	-	-		0.204
	Ct (Fresh)	-	-	0.204	

Table A.IV: Daily Growth Rate (DGR) (% day^{-1}) of *Gracilaria gracilis* cultivated biomass from the several assays conducted in the study, and calculated according to the equation $[(W_t/W_0)^{1/t} - 1] \times 100$ (Mtolera *et al*., 1995; Gerang & Ohno, 1997; Aguirre-Von-Wobeser *et al*., 2011; Bulboa *et al*., 2007; Hayashi *et al*., 2007a; Hayashi *et al*., 2007b; Hung *et al*., 2009; Hayashi *et al*., 2011), based on the difference between the tip weights estimated in the beginning and ending of a 16 or 14-days culture trial. 1, 2 and 3 correspond to the triplicates performed for each assay (n = 3).

	Assay	*1*	*2*	*3*	*Mean*	*SD*
Independent Trial	**Cool White Fluorescent**	14.313	16.37	14.128	14.936	1.243
1st Set of Independent Trials	**Control**	9.773	8.513	8.069	8.785	0.884
	Ammonium Increase	16.278	20.03	12.35	16.219	3.84
2nd Set of Independent Trials	**Light Increase**	10.023	6.961	8.997	8.661	1.559
	Ammonium & Light Increase	12.677	16.248	17.995	15.64	2.711
	Control	7.265	5.915	7.757	6.979	0.954
3rd Set of Independent Trials	**Ammonium & Phosphate Increase**	13.452	11.951	14.632	13.345	1.343
	Ammonium, Phosphate & Light Increase	13.257	15.79	16.08	15.042	1.552
	Phosphate Increase	11.377	8.915	7.101	9.131	2.146
	Light Increase	8.464	7.927	10.016	8.802	1.085
	Control	4.377	6.544	7.897	6.273	1.776

Table A.V: Post Hoc Multiple Comparisons tests - Least Significant Difference (LSD) and Dunnett's test - performed to compare individual means of the parameter "Growth Rate" between *G. gracilis* biomass from the assays depicted in Table III.I and with the control situation, respectively. Greyish values are associated to redundant situations. Combinations of trials few relevant for this analysis are represented by the signal -. Statistical significant differences (p-value < 0.05) are shown in bold (n = 3).

			IT	*1st Set*	*2nd Set*		*3rd Set*			
			CWF	NH_4^+ Increase	Lt Increase	NH_4^+ & Lt Increase	NH_4^+ & PO_4^{3-} Increase	NH_4^+, PO_4^{3-} & Lt Increase	PO_4^{3-} Increase	Lt Increase
LSD	*IT*	CWF		0.470	0.002	0.690	0.373	0.952	0.004	0.003
	1st Set	NH_4^+ Increase	0.470		-	-	-	-	-	-
	2nd Set	Lt Increase	**0.002**	-		**0.001**	-	-	-	-
		NH_4^+ & Lt Increase	0.690	-	0.001		-	-	-	-
	3rd Set	NH_4^+ & PO_4^{3-} Increase	0.373	-	-	-		0.342	**0.027**	**0.019**
		NH_4^+, PO_4^{3-} & Lt Increase	0.952	-	-	-	0.342		**0.004**	**0.002**
		PO_4^{3-} Increase	**0.004**	-	-	-	0.027	0.004		0.852
		Lt Increase	**0.003**	-	-	-	0.019	0.002	0.852	
Dunnett	*1st Set*	Ct	**0.034**	**0.016**	-	-	-	-	-	-
	2nd Set	Ct	**0.001**	-	0.487	**0.002**	-	-	-	-
	3rd Set	Ct	**0.000**	-	-	-	**0.001**	**0.000**	0.155	0.233

CWF - Cool White Fluorescent CWF; NH_4^+ - Ammonium; PO_4^{3-} - Phosphate; Lt - Light; Ct - Control; IT - Independent Trial

Table A.VI: Total Soluble Protein (TSP) content of *Gracilaria gracilis* cultivated biomass from the several assays conducted in the study, and calculated in accordance with the Pierce™ BCA Protein Assay kit, based on a calibration curve of standard BSA [26]. Results are expressed as % of dry weight (DW) of seaweed. 1, 2 and 3 correspond to the triplicates performed for each assay (*n* = 3).

	Assay	***1***	***2***	***3***	***Mean***	***SD***
Independent Trial	**Cool White Fluorescent**	20.396	17.011	16.771	18.06	2.027
1st Set of Independent Trials	**Control**	12.607	10.934	11.246	11.596	0.89
	Ammonium Increase	14.069	14.643	14.157	14.289	0.309
2nd Set of Independent Trials	**Light Increase**	11.339	15.37	12.174	12.961	2.128
	Ammonium & Light Increase	16.298	17.783	18.994	17.692	1.35
	Control	15.407	10.686	11.606	12.566	2.503
3rd Set of Independent Trials	**Ammonium & Phosphate Increase**	12.344	12.203	15.105	13.217	1.637
	Ammonium, Phosphate & Light Increase	19,754	19.183	17.658	18.865	1.083
	Phosphate Increase	10.571	9.746	12.211	10.842	1.255
	Light Increase	9.064	11.442	8.953	9.82	1.406
	Control	9.365	9.674	10.09	9.71	0.364

Table A.VII: Post Hoc Multiple Comparisons tests - Least Significant Difference (LSD) and Dunnett's test - performed to compare individual means of the parameter "Total Soluble Protein (TSP) content" between *G. gracilis* biomass from the assays depicted in Table III.I and with the control situation, respectively. Greyish values are associated to redundant situations. Combinations of trials few relevant for this analysis are represented by the signal -. Statistical significant differences (p-value < 0.05) are shown in bold (n = 3).

			IT	*1st Set*	*2nd Set*		*3rd Set*			
			CWF	NH_4^+ Increase	Lt Increase	NH_4^+ & Lt Increase	NH_4^+ & PO_4^{3-} Increase	NH_4^+, PO_4^{3-} & Lt Increase	PO_4^{3-} Increase	Lt Increase
LSD	*IT*	CWF		0.007	0.001	0.587	0.001	0.572	0.000	0.000
	1st Set	NH_4^+ Increase	**0.007**		-	-	-	-	-	-
	2nd Set	Lt Increase	**0.001**	-		**0.002**	-	-	-	-
		NH_4^+ & Lt Increase	0.587	-	0.002		-	-	-	-
	3rd Set	NH_4^+ & PO_4^{3-} Increase	**0.001**	-	-	-		**0.009**	0.075	**0.014**
		NH_4^+, PO_4^{3-} & Lt Increase	0.572	-	-	-	0.009		**0.003**	**0.001**
		PO_4^{3-} Increase	**0.000**	-	-	-	0.075	0.003		0.430
		Lt Increase	**0.000**	-	-	-	0.014	0.001	0.430	
Dunnett	*1st Set*	Ct	**0.002**	**0.012**	-	-	-	-	-	-
	2nd Set	Ct	**0.000**	-	0.113	**0.000**	-	-	-	-
	3rd Set	Ct	**0.000**	-	-	-	**0.011**	**0.001**	0.955	1.000

Table A.VIII: R-phycoerythrin (R-PE) concentration of *Gracilaria gracilis* cultivated biomass from the several assays conducted in the study, and calculated according to the Beer & Eshel (1985) equations, based on the maximum absorbance of phycoerythrin. Results are expressed in mg of pigment per g of wet weight (WW) of seaweed. 1, 2 and 3 correspond to the triplicates performed for each assay ($n = 3$).

	Assay	***1***	***2***	***3***	***Mean***	***SD***
Independent Trial	**Cool White Fluorescent**	9.461	11.386	7.95	9.599	1.722
1st Set of Independent Trials	**Control**	3.657	3.397	2.923	3.326	0.372
	Ammonium Increase	6.71	5.941	7.031	6.561	0.56
2nd Set of Independent Trials	**Light Increase**	3.485	4.887	3.206	3.859	0.901
	Ammonium & Light Increase	4.314	5.455	6.03	5.266	0.873
	Control	1.918	2.43	3.243	2.53	0.668
3rd Set of Independent Trials	**Ammonium & Phosphate Increase**	5.374	4.801	5.656	5.277	0.436
	Ammonium, Phosphate & Light Increase	6.655	7.96	5.846	6.82	1.066
	Phosphate Increase	3.417	3.237	3.523	3.392	0.144
	Light Increase	4.45	3.13	5.187	4.256	1.043
	Control	1.26	2.586	3.307	2.384	1.038

Table A.IX: R-phycocyanin (R-PC) concentration of *Gracilaria gracilis* cultivated biomass from the several assays conducted in the study, and calculated according to the Beer & Eshel (1985) equations, based on the maximum absorbance of phycocyanin. Results are expressed in mg of pigment per g of wet weight (WW) of seaweed. 1, 2 and 3 correspond to the triplicates performed for each assay (n = 3).

	Assay	***1***	***2***	***3***	***Mean***	***SD***
Independent Trial	**Cool White Fluorescent**	0.195	0.11	0.162	0.156	0.043
1st Set of Independent Trials	**Control**	0.176	0.131	0.120	0.143	0.03
	Ammonium Increase	0.027	0.156	0.2	0.128	0.09
2nd Set of Independent Trials	**Light Increase**	0.014	0.108	0.076	0.066	0.048
	Ammonium & Light Increase	0.038	0.027	0.02	0.028	0.009
	Control	0.014	0.005	0.011	0.010	0.005
3rd Set of Independent Trials	**Ammonium & Phosphate Increase**	0.01	0.139	0.175	0.108	0.087
	Ammonium, Phosphate & Light Increase	0.008	0.170	0.117	0.114	0.083
	Phosphate Increase	0.026	0.091	0.009	0.042	0.043
	Light Increase	0.034	0.031	0.008	0.024	0.014
	Control	0.006	0.037	0.008	0.017	0.017

Table A.X: Post Hoc Multiple Comparisons tests - Least Significant Difference (LSD) and Dunnett's test - performed to compare individual means of the parameter "R-Phycoerythrin (R-PE) content" between *G. gracilis* biomass from the assays depicted in Table III.I and with the control situation, respectively. Greyish values are associated to redundant situations. Combinations of trials few relevant for this analysis are represented by the signal -. Statistical significant differences (*p*-value < 0.05) are shown in bold (*n* = 3).

			IT	*1st Set*	*2nd Set*		*3rd Set*			
			CWF	NH_4^+ Increase	Lt Increase	NH_4^+ & Lt Increase	NH_4^+ & PO_4^{3-} Increase	NH_4^+, PO_4^{3-} & Lt Increase	PO_4^{3-} Increase	Lt Increase
LSD	*IT*	CWF		0.001	0.000	0.000	0.000	0.003	0.000	0.000
	1st Set	NH_4^+ Increase	**0.001**		-	-	-	-	-	-
	2nd Set	Lt Increase	**0.000**	-		0.090	-	-	-	-
		NH_4^+ & Lt Increase	**0.000**	-	0.090		-	-	-	-
	3rd Set	NH_4^+ & PO_4^{3-} Increase	**0.000**	-	-	-		0.065	**0.028**	0.208
		NH_4^+, PO_4^{3-} & Lt Increase	**0.003**	-	-	-	0.065		**0.000**	**0.005**
		PO_4^{3-} Increase	**0.000**	-	-	-	0.028	0.000		0.284
		Lt Increase	**0.000**	-	-	-	0.208	0.005	0.284	
Dunnett	*1st Set*	Ct	**0.001**	**0.018**	-	-	-	-	-	-
	2nd Set	Ct	**0.000**	-	0.159	**0.012**	-	-	-	-
	3rd Set	Ct	**0.000**	-	-	-	**0.020**	**0.001**	0.654	0.163

CWF - Cool White Fluorescent CWF; NH_4^+ - Ammonium; PO_4^{3-} - Phosphate; Lt - Light; Ct - Control; IT - Independent Trial

Table A.XI: Levene's test to evaluate the homogeneity of variances among testing categories, and Kruskal-Wallis non-parametric test applied to the parameter R-phycocyanin (R-PC) content. Situations where *p*-value < 0.05 are shown in bold (*n* = 3).

R-phycocyanin (R-PC)	*Homogeneity*	*Non-parametric test*	
Levene's test based on mean	**0.012**		Null hypothesis retained
Kruskal-Wallis of independent samples		0.195	