

Cultivating seaweed associated with a recirculating fish aquaculture system as a nutrient valorisation tool

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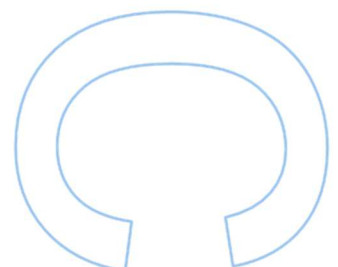
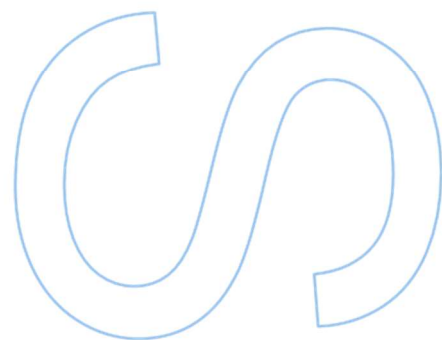
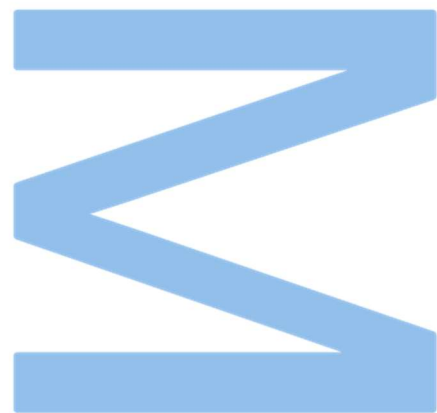
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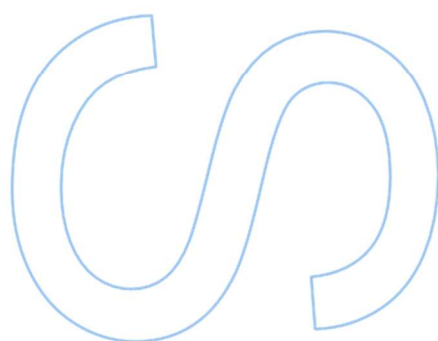
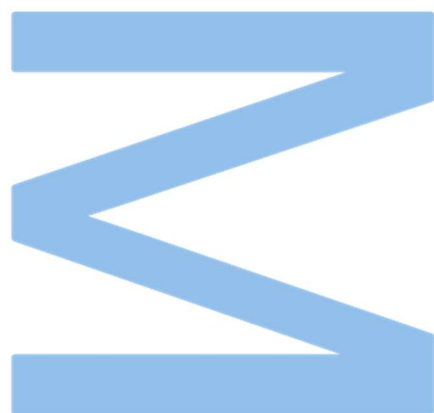
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Resumo

A aquacultura multitrófica integrada tem vindo a ganhar maior relevância científica e industrial, devido ao potencial de biorremediação de nutrientes do efluente das aquaculturas, mas também pelo interesse crescente nas macroalgas como fonte de compostos de interesse biotecnológico. Assim, surge a necessidade de estudar as espécies de macroalgas com maior potencial económico, procurando aprimorar os seus métodos de cultivo, à escala industrial e laboratorial.

Recolheram-se indivíduos de várias espécies de macroalgas presentes na costa portuguesa, com o objetivo de testar o seu desempenho num sistema de cultivo de algas alimentado pelo efluente de uma piscicultura em diferentes condições. Para *Chondrus crispus* realizou-se um ensaio de densidade de cultivo e luminosidade, em *Codium tomentosum* testou-se o método de fragmentação para aumentar o crescimento, para *Gracilaria gracilis* testaram-se as densidades 3Kg/m² e 5Kg/m² e para *Ulva* sp. as condições de baixa e alta luminosidade. Realizaram-se também ensaios de propagação vegetativa e reprodução sexuada de *Chondrus crispus* em laboratório. Para *Gracilaria gracilis* e *Ulva* sp. analisou-se em laboratório o seu conteúdo bioquímico, procurando estudar de que forma as condições de cultivo conduziam a uma biovalorização dos organismos.

As espécies com maiores taxas de crescimento no sistema foram *Ulva* sp. e *Gracilaria gracilis*. No cultivo de *Ulva* sp., o conteúdo de polissacarídeos, compostos fenólicos e clorofila b foi favorecido pela condição com maior crescimento. *Gracilaria gracilis* apresentou crescimento semelhante entre densidades, mas 5Kg/m² favoreceu o conteúdo em conteúdo total de polissacarídeos e fenóis. *Chondrus crispus* não se desenvolveu em contacto com o efluente, no sistema de cultivo, porém apresentou crescimento em laboratório, aquando da propagação vegetativa. Em termos de reprodução sexuada, considerou-se que a condição de 14°C e 20 µmol fotão m⁻² s⁻¹ a foi a mais favorável para o seu desenvolvimento.

Estes ensaios permitiram obter mais informações sobre as condições mais adequadas para o cultivo destas espécies num sistema de aquacultura multitrófica integrada à escala piloto, bem como em contexto laboratorial.

Palavras-chave: *Chondrus crispus*; *Codium tomentosum*; *Ulva* sp.; *Gracilaria gracilis*; Aquacultura multitrófica integrada; bioatividade, biorremediação

Abstract

In the last decade, integrated multitrophic aquaculture (IMTA) has gained scientific and industrial relevance, due to the nutrient bioremediation potential but also to the growing interest in macroalgae as a source of compounds of biotechnological interest. As a result, there is a need for research into the macroalgae species with the greatest economic potential to improve their cultivation methods on an industrial and laboratory scale.

Individuals of different species of macroalgae present along the Portuguese coast were collected to test their performance in an algae cultivation system fed with fish farm effluent under different conditions. For *Chondrus crispus*, a cultivation density and luminosity test was carried out, for *Codium tomentosum* a fragmentation procedure was tested, for *Gracilaria gracilis* a stocking density trial with 3Kg/m² and 5Kg/m² was elaborated and for *Ulva* sp. the conditions of low and high luminosity were tested.

Laboratory tests were also carried out on the vegetative propagation and sexual reproduction of *Chondrus crispus*. The biochemical content of *Gracilaria gracilis* and *Ulva* sp. was analysed in the laboratory to study how the culture conditions affect the biochemical composition of the species.

The species with the highest growth rates in the system were *Ulva* sp. and *Gracilaria gracilis*. In the cultivation of *Ulva* sp. the content of polysaccharides, phenolic compounds and chlorophyll b was favoured by the high light treatment, the condition with the highest growth rate. *Gracilaria gracilis* showed similar growth between densities, but 5Kg/m² favoured the content of total polysaccharides and phenolics. *Chondrus crispus* did not grow in contact with the effluent, in the cultivation system but showed growth in the laboratory when propagated vegetatively. In terms of sexual reproduction, the condition of 14°C and 20 µmol photon m⁻² s⁻¹ was considered the most favourable for development.

These tests provided more information on the most suitable conditions for growing these species in a pilot scale IMTA system and in the laboratory.

Keywords: *Chondrus crispus*; *Codium tomentosum*; *Ulva* sp.; *Gracilaria gracilis*; Integrated multi-trophic aquaculture; bioactivity, bioremediation

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List of Abbreviations

IMTA	INTEGRATED MULTI- TROPHIC AQUACULTURE
RAS	
VSE	VON STOSCH ENRICHMENT
C	CARBON
N	NITROGEN
CN RATIO	CARBON AND NITROGEN RATIO
UV	ULTRAVIOLET
GeO ₂	GERMANIUM DIOXIDE
CO ₂	CARBON DIOXIDE
NH ₄ ⁺	AMMONIA
NO ₂ ⁻	NITRITES
NO ₃	NITRATES
PO ₄ ³⁻	PHOSPHATE
RGR	RELATIVE GROWTH RATE
FW	FINAL FRESH WEIGHT
IW	INITIAL FRESH WEIGHT
%DW	DRY WEIGHT PERCENTAGE
PO ₄ ³⁻	PHOSPHATE
μmol	MICROMOLE

1. Introduction

1.1. Aquaculture current state

In the 21st century, aquaculture as a food production sector has become more prominent and important (Food and Agriculture Organization of the United Nations [FAO], 2022; Naylor et al., 2021; Neori, 2008). In 2020, approximately 51% of aquatic animals consumed by humans came from marine and inland fisheries, while 49% originated from aquaculture (FAO, 2022). However, in the face of the challenges of modernity, concerns about climate change, the collapse of fish stocks and the environmental impact of human food production have become more prominent. In this context, the search for more efficient and environmentally friendly technological solutions applied to fisheries and aquaculture is needed (FAO, 2022; Naylor et al., 2021).

In order to increase the efficiency and environmental sustainability of intensive aquaculture, Recirculating Aquaculture Systems (RAS) were developed. RAS are production facilities based on the reuse of water for cultivation. Processes such as the elimination of excreted metabolites, oxygenation and the maintenance of the parameters of each cultivated species are essential (Dinis & Rocha, 2021). Nowadays these closed systems are on worldwide expansion, though, according to Chen et al. (1993) these methods weren't used in the past mainly because of their high production costs.

Considering all aquaculture production systems used today, RAS allows the possibility to reach a high production while reducing considerably impacts on environment and ecology (Martins et al., 2010). This cultivation technology is applied to freshwater species (ex. *Oreochromis niloticus*) as much as marine species (ex. *Solea senegalensis*), currently developing mainly in Europe (FAO, 2022; Martins et al., 2010).

However, the major difficulties of these systems are the removal of surpluses such as solid particles matter, that mainly comes from fish or residual feed, as well other hazardous substances as ammonia, nitrite, nitrate, and phosphate, antibiotic and antibiotic resistant genes, steroids, heavy metals, etc., thus these pollutants can be removed by specific water treatments. (Li et al., 2023)

There are three types of procedures applied to RAS wastewater treatment, primary, secondary, and tertiary. The first one consists of solid waste removal, methods such as sedimentation, centrifugation, and mechanical filter. These methods are used to remove suspended solids and larger residues. Then, the secondary treatment is destined to

remove the remaining particles from previous treatment as well as some dissolved and colloidal substances. At this point, biological filtration is used, employing nitrifying bacteria per example (Dinis & Rocha, 2021). According to Suprivono et al. (2023) incorporating biofilters and microbubbles yields a higher cultivation efficiency. Furthermore, physical fractionation filtration (production of foam) and chemical adsorption filtration (activated charcoal) are applied. The last wastewater treatment is considered water sterilization by UV radiation, aerification and thermoregulation (Dinis & Rocha, 2021).

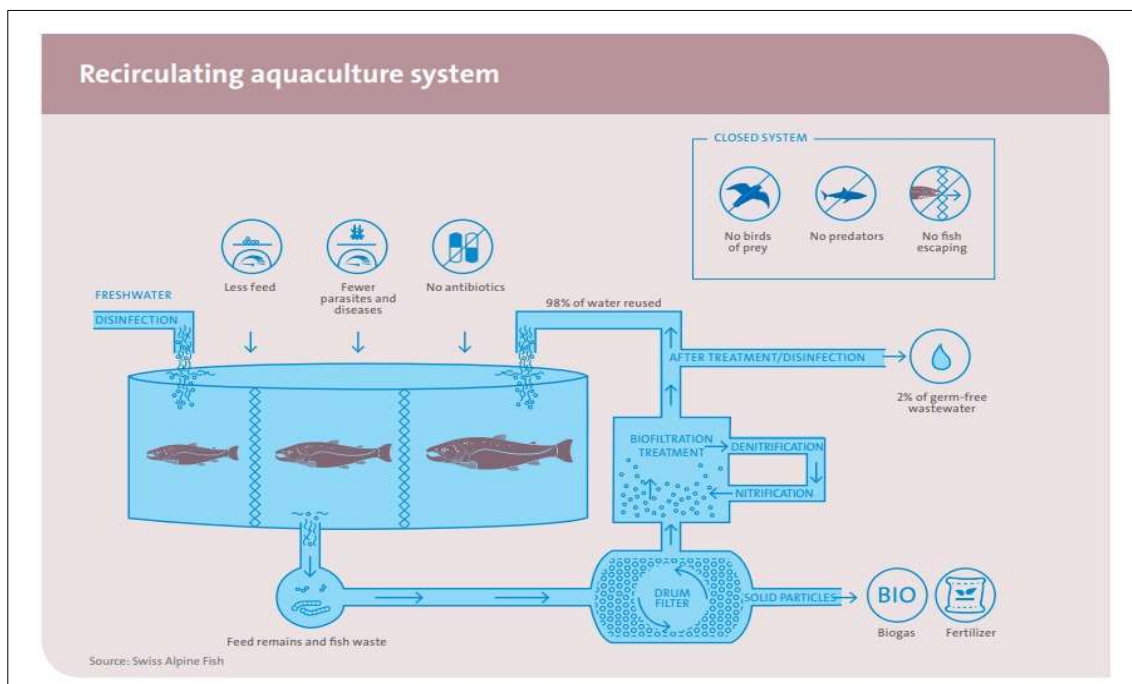


Figure 1 - Functioning of a recirculating aquaculture system (RAS) with a water treatment system, according to Hofmann (2019).

Despite the development of these advanced treatment technologies and the drastic reduction in effluent produced by RAS, there is still a significant amount of nutrients being released into the environment. Seaweed can play an important role in the bioremediation of these nutrient-rich effluents (Chopin, 2013).

The production of algae in 2020 corresponded to around 36 million tonnes of fresh weight, 97% of which came from aquaculture. This branch of aquaculture has experienced very significant growth over the last two decades, with production reaching 12 million tonnes in 2000 and 21 million tonnes in 2010. The world's largest algae producers are in Asia, with China accounting for 59.75% and Indonesia for 27.55% of world production. In 2020, the most produced species were *Laminaria japonica* (12,469

tonnes), species of the genus *Eucheuma* sp. (8,129 tonnes) and *Gracilaria* sp. (5,180 tonnes). At the European level, 68% of seaweed production comes from harvesting, most of which is done manually (85%) and a small part mechanically (15%) (FAO, 2022). The most harvested species are *Laminaria digitata* and *Laminaria hyperborea*, and *Ascophyllum nodosum*. In the case of Portugal, manual harvesting was used to collect *Gelidium* sp. for industrial uses. The remaining 32% comes directly from aquaculture, mainly marine (76%) but also land-based (24%), with *Saccharina latissima*, *Alaria esculenta* and *Ulva* sp. standing out as the most produced (Araújo et al., 2021). Norway is the largest producer of seaweed in Europe, with mechanical harvesting of kelp being prominent (Araújo et al., 2021; FAO, 2022). However, considering the long-term sustainability of marine ecosystems, wild algae stocks are monitored by the authorities and cannot be harvested above a certain pre-established level per season. Thus, as interest in the potential of macroalgae grows, the concept of integrated multi-trophic aquaculture as a more sustainable alternative to traditional intensive aquaculture is gaining international prominence (Araújo et al., 2021; Neori, 2008; FAO, 2022).

Tank macroalgae cultivation has gained increasing importance in aquaculture due to macroalgae applications in food, biofuels, and pharmaceuticals, as well as environmental concerns. According to Kumar et al., (2023), tank cultivation brings significant advantages once compared to traditional algae cultivation. For instance, the ability to adjust optimally to a much wider range of seaweed species. The main strategy for this kind of cultivation is based on water agitation to maintain seaweed circulating in the water column and subjected to optimal light conditions. This, as well as the addition of nutrients and water reusing from other sources such as fish aquaculture allows a more efficient cultivation. (Chopin et al., 2001).

The challenges of land-based cultivation tend to focus more on the location of production, as it is extremely expensive to relocate existing structures. This way, it is important to consider geography, topography, water quality, sun radiation, climate conditions, availability of workers and electricity (Hafting et al, 2012). Factors such as temperature, salinity, pH levels, and nutrient concentration predominantly impact sea-based cultivation. However, these factors can be easily regulated in land-based cultivation (Kumar, et al., 2023).

1.2. Integrated Multi-trophic aquaculture

Intensive aquaculture effluent treatment has been a problem addressed by several scientific studies (Naylor et al., 2021; Troell et al., 1999; Marinho-Soriano et al., 2011),

given the increasing anthropogenic pressure over the shoreline and eutrophication caused by nutrient overload, namely nitrogen (N) compounds, which are present in effluent that reach aquatic ecosystems and cause their deterioration (Naylor et al., 2021).

To alleviate this problem, the concept of Integrated Multi-Trophic Aquaculture (IMTA) has emerged, which takes a perspective of environmentally sustainable, economically, and socially beneficial aquaculture, recognised as a beneficial solution for sustainable aquaculture with monetary potential at local and regional levels (Chopin, 2013; Neori, 2008).

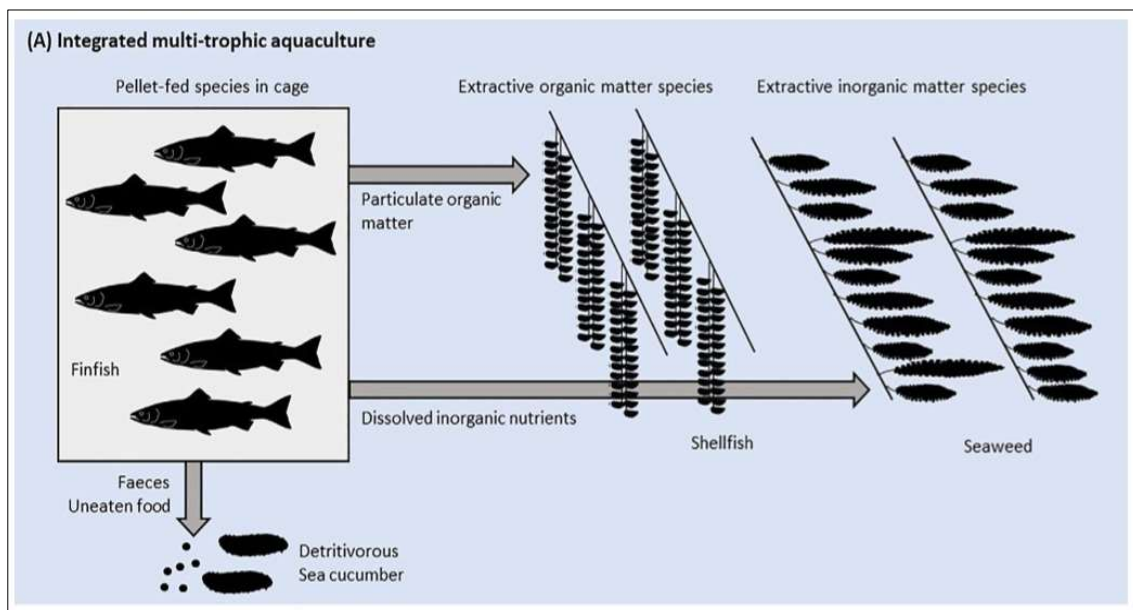


Figure 2 - Schematic illustration of an Integrated multi-trophic aquaculture system, according to Thomas et al. (2021).

Integrated multitrophic aquaculture aims to reduce food waste as far as possible, increasing, and diversifying production by introducing filter-feeding species, such as bivalves, and extractive species, such as macroalgae. In these systems, the function of filtering species corresponds to the removal of particulate organic matter, in this case, originating from the food not consumed by fish. The extractive species remove dissolved inorganic nutrients present in the effluent, originating from the fish metabolism. These two types of organisms are inserted in accessory cultivation modules. By passing aquaculture effluent through these, the final load of nutrients and the level of CO₂ reaching nature is decreased (Chopin, 2013).

Thus, the cultivation of macroalgae as an extractive species, from an "ecological engineering" perspective, can be seen as an alternative for reducing waste and increasing productivity in aquaculture (Troell et al., 1999). Examples of algae that can be

used as extractive species are *Chondrus crispus* and *Codium* sp, *Ulva* sp. and *Gracilaria gracilis*, species widely cultivated in aquaculture (Correia & Pereira, 2015; Redmond et al., 2014). There are several examples of the successful use of these species in IMTA systems. Matos et al. (2006) tested the efficiency of *Chondrus crispus*, *Gracilaria bursa pastoris* and *Palmaria palmata* when integrated into an aquaculture system, with the aim of assessing the most favourable conditions for their cultivation and obtained favourable results. In his review, Chopin (2001) mentions *Ulva lactuca*, *Gracilaria* sp., *Chondrus crispus* and other species that are viable options for use in integrated multi trophic aquaculture due to their potential as biofilters or their economic value and bioactive potential. Neori et al (2000) refer to the use of *Ulva lactuca* and *Gracilaria conferta* as biofilters in an integrated sea bream aquaculture with a gastropod module. In this study, *Ulva lactuca* showed good results as a biofilter, with good growth rate and N removal, and was also used to feed the gastropods produced, creating a more sustainable and profitable system.

1.3. Seaweed

Algae is the generic term for photosynthetic eukaryotic organisms inhabiting aquatic environments. Colloquially, as this is not a taxonomic distinction, they are usually divided into microalgae (unicellular, colonial or cenocytic microscopic organisms) and macroalgae (marine macroscopic organisms). Macroalgae can display a high degree of biological complexity and can reach several metres in length. Their ecological role in ecosystems is also important, providing refuge from predators, food for organisms, carbon sequestration and oxygen production (Correia & Pereira, 2015; Graham & Wilcox, 2000).

At the taxonomic level, red algae belonging to the phylum Rhodophyta can be considered as containing chlorophyll *a*, phycobilins (R-phycocyanin and R-phycoerythrin) and carotenoids (β -carotene, lutein and zeaxanthin), while green algae belonging to the phylum Chlorophyta contain chlorophyll *a* and *b* and carotenoids (β -carotene, lutein, violaxanthin and zeaxanthin). Brown algae belong to the phylum Ochrophyta and have chlorophyll *a* and *c* and carotenoids such as β -carotene, fucoxanthin and violaxanthin (Pereira, 2010; Correia & Pereira, 2015).

In addition to their role in nutrient bioremediation in aquaculture, seaweeds have other applications and commercial value in industry and biotechnology. In Portugal, mixtures of seaweed and marine plants have traditionally been used as fertiliser for agriculture in coastal regions. Today, this practice has fallen into disuse, but there are still regions in

the north of the country that use "sargaço" as a fertiliser for agricultural fields. "Sargaço" is a mixture of seaweeds collected from the rocky coast, including *Fucus* sp., *Laminaria* sp., *Chondrus* sp., *Codium* sp., *Saccorhiza* sp., *Gelidium* sp. and *Palmaria* sp.. Another seaweed mixture that was once used as a fertiliser was "moliço", from Ria de Aveiro, made up of *Ulva* sp., *Gracilaria* sp., *Rhizoclonium* sp. and marine angiosperms such as *Zoostera* sp. and *Rupia* sp. (Pereira, 2010; Correia & Pereira, 2015; Gaspar et al., 2019). Another application of seaweed is the extraction of phycocolloids, such as agar and alginates, organic compounds with biotechnological application in food, pharmaceutical and cosmetic industries (Graham & Wilcox, 2000; Correia & Pereira, 2015). In Portugal industrial extraction of agar began in the mid-20th century with the harvesting of *Gelidium corneum* from the coast (Gaspar et al., 2019). However, this practice declined due to the over-exploitation of wild populations, which made it unsustainable and less profitable (Correia & Pereira, 2015; Gaspar et al., 2019). Other agarophyte species exploited in Portugal were *Pterocladia capillacea* and *Gelidium microdon*, found on the Azorean coast. Carrageenophytes such as *Chondrus crispus* and *Mastocarpus stellatus* were also collected by hand in Portugal for export. Apart from the Azores, human consumption of seaweed is not traditional in Portugal, unlike in Asian countries, but it has become more important over the years (Gaspar et al., 2019).

1.3.1. *Chondrus crispus*

Chondrus crispus is a red algae species, belonging to the Gigartinaceae family, phylum Rhodophyta. It is distributed along the rocky coastline, in the lower mid-littoral and infralittoral areas of the northern Atlantic infralittoral, encompassing the North American and European coasts.



Figure 3 - *Chondrus crispus* distribution map according to "the marine life information network (www.marlin.ac.uk/species/detail/1444).



Figure 4 - *Chondrus crispus* individual (from www.seaweed.ie/descriptions/Chondrus_crispus.php).

It is a perennial species, whose growth is more prominent during the summer, and whose longevity is generally greater than 2 years (Redmond et al., 2014). Morphologically it can present itself in divergent forms, with colours ranging from dark red to purplish with dichotomously divided fronds, with variable thickness. It adheres to the substratum of a discoid holdfast from which several stipes emerge and gradually expand to form the fronds (Fredericq et al., 1992; Redmond et al., 2014).

This species can be produced in aquaculture and there are records of its use in integrated multi-trophic systems (Redmond et al., 2014; Correia & Pereira, 2015). Recommended stocking densities range from 4 to 6 kg/m² at low densities to 8 to 12 kg/m² at high densities (Bidwell et al., 1985; Redmond et al., 2014). The latter require efficient aeration systems that prevent the accumulation of biomass in 'dead zones' of the tanks to prevent their degradation and the emergence of epiphytes, which tend to be more problematic in the spring and summer months. Some of the epiphyte species that may appear are the genera *Ulva* sp. and *Ectocarpus* sp. (Bidwell et al., 1985).

The life cycle of *Chondrus crispus* is divided into 3 generations: a tetrasporophyte (diploid) generation, which reproduces asexually by releasing tetraspores; a gametophyte (haploid) generation, which reproduces sexually (male and female gametophytes); and a microscopic generation called carposporophyte, which develops on the fronds of female individuals after fertilisation by the male gamete (Chen & McLachlan, 2011; Redmond et al., 2014). Although they are isomorphic in the vegetative phase, it is possible to distinguish the two generations during their reproductive stages because of the differences in their structures (Redmond et al., 2014; Fredericq et al., 1992; Chen & McLachlan, 2011). In the case of the female gametophyte, the cystocarp develops in the frond after fertilisation. From the cystocarp, the carpospores (carposporophyte generation) are released, which, after attachment to a stable surface, develop and give rise to the tetrasporophyte individual (Redmond et al., 2014). In nature it is uncommon to find male gametophyte specimens, as female specimens appear to be more common, especially in summer months (Redmond et al., 2014; Fernandez & Menendez, 1991).

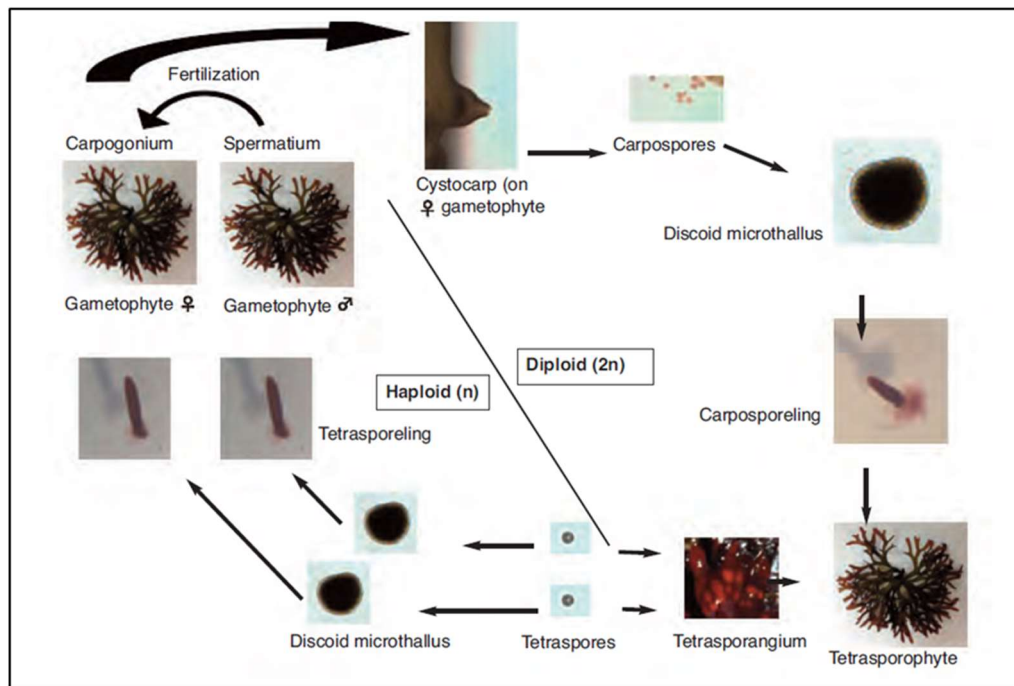


Figure 5 - *Chondrus crispus* life cycle, according to Redmond et al. (2014).

Compounds of biotechnological interest are extracted from *Chondrus crispus* and other algae of the order Gigartinales, namely carrageenans, which are a component of their cell walls. In the food industry, they act as food stabilisers and gelling agents (Correia & Pereira, 2015). It also has a high protein content, vitamin A, minerals, and polyunsaturated fatty acids. and is widely used in biotechnology (Pereira, 2010). In addition, it is recognised that some red algae can be used as fertiliser for plants, stimulating their growth in the early stages of development and promoting greater development in adult plants (Hamouda, 2017).

1.3.2. *Codium tomentosum*

Codium tomentosum is a green alga species, belonging to the family Codiaceae, order Bryopsidales, phylum Chlorophyta. It is distributed along the rocky coastline of the European Atlantic coast, in the infralittoral zone, also inhabiting tidal pools.



Figure 6 - Distribution map of *Codium tomentosum* in Europe, according to “the marine life information network” (www.marlin.ac.uk/species/detail/1474).



Figure 7 - *Codium tomentosum* individual (from pt.informaciongastronomica.com/alga-codium-variante-con-sabor-gallego/).

Morphologically it is characterised by a thallus with a soft and spongy consistency, dark green in colour and with dichotomous division. It usually appears as a dense aggregate of dark green filaments (Correia & Pereira, 2015; Rey et al. 2020).

In nature, it is impossible to distinguish *Codium tomentosum* from another exotic species also found in European rocky shores, *Codium fragile*. Their morphological differences are not obvious at first sight (Trowbridge, 2014), but can be observed under the microscope (Silva, 1955). *Codium tomentosum* differs from *Codium fragile* in its morphology, more precisely in the utricles, elongated vesicular structures related to sexual reproduction, that develop from the stalk. Gametangia develop from the base of some utricles (Borden et al., 1969). *Codium fragile* has long, broad utricles with mucronate apices (acute apex), whereas the utricles of *Codium tomentosum* are long but lack mucronate apices (Trowbridge, 2014).

It is often covered by epiphytes, namely *Ectocarpus* sp., *Ceramium* sp., *Ulva* sp. and *Chaetomorpha* sp. (González et al., 2004), which tends to be more problematic in open sea culture, but more manageable in land-based aquaculture conditions (Hafting et al., 2012). Growth in both winter and summer tends to be positive (Gonçalves, 2018) and it can also be applied to integrated multi-trophic aquaculture systems (Marques et al., 2021). According to Gonçalves (2018), cultivation densities of between 5g/L and 15g/L are suitable for growing *Codium tomentosum*.

This seaweed has bioactive properties that makes it biotechnologically relevant, such as the presence of antioxidant compounds and high lipid content, which are of interest for the food and cosmetics industry. *Codium tomentosum* is becoming increasingly popular as a delicacy and is much sought after in gastronomy. (Correia & Pereira, 2015; Rey et al. 2020).

1.3.3. *Ulva* sp.

The genus *Ulva* is a group of green algae belonging to the phylum Chlorophyta, order Ulvales. Widespread along the Portuguese coast, they inhabit the mid-littoral zone and are usually attached to rocks by means of a small basal disc. The species of the genus *Ulva* are light to dark green in colour and may have tubular or lamellar stems. Its holotype is *Ulva lactuca*, which is widely used in aquaculture (Correia & Pereira, 2015; Pereira, 2010).



Figure 8 - Distribution map of *Ulva lactuca* in Europe, according to "the marine life information network (<https://www.marlin.ac.uk/species/detail/1467>).



Figure 9 - *Ulva lactuca* individual (from <https://www.biolib.cz/en/taxon/id138096b/>).

According to Neori et al. (1991), the best culture density for *Ulva lactuca* cultivation is between 1 kg/m² and 2 kg/m², with the growth rate decreasing as the density increases. Al-Hafedh et al. (2015) reached the same conclusion, stating that lower densities and high flow rates are the most favourable conditions for growing this seaweed.

The recurrence of sporulation episodes tends to hinder the production of *Ulva* sp. cultures, rendering them unviable or with reduced productivity. The dynamics of sporulation follows cyclical patterns and is influenced by the ratio of young to mature biomass in the culture. The presence of a higher percentage of young biomass determines later sporulation events and consequently higher growth rates. However, in the long term, a mixture of mature and young biomass is more advisable to mitigate these effects (Gao et al., 2010; Obolski et al., 2022).

Ulva lactuca, can be used for direct human consumption (Correia & Ferreira, 2015), as well as for phenol extraction (Connan, 2015), polysaccharide extraction for use in biorefineries and animal feed (Bikker et al., 2016), pharmaceutical uses (Yu-Quin et al. 2016) and bioremediation of aquaculture wastewater (Metaxa et al., 2006; Al-Hafedh et al., 2014).

1.3.4. *Gracilaria gracilis*

Gracilaria gracilis is a red alga belonging to the phylum Rhodophyta, order Gracilariales. It inhabits semi-exposed or sheltered areas in the lower mid-littoral and infralittoral zones.

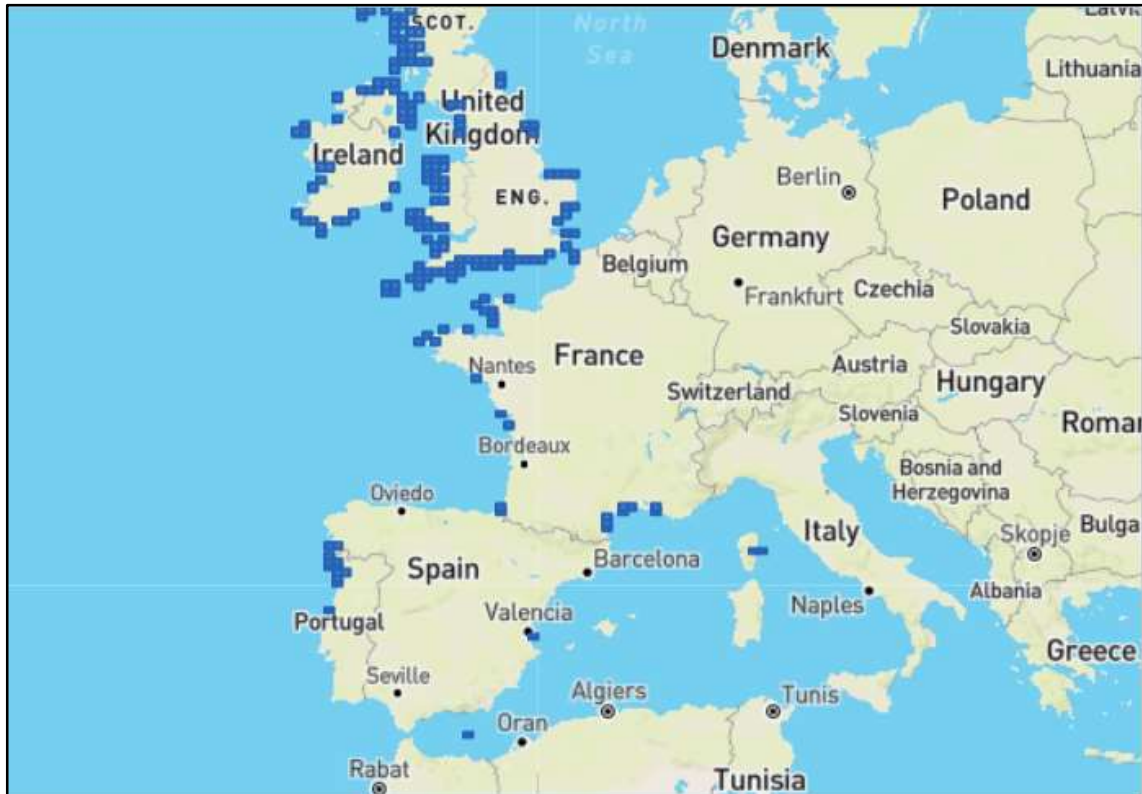


Figure 10 - Distribution map of *Gracilaria gracilis* in Europe, according to "the marine life information network (www.marlin.ac.uk/species/detail/2349).



Figure 11 - *Gracilaria gracilis* (from www.aphotomarine.com/red_seaweed_gracilaria_gracilis.html).

It is resistant to salinity oscillations, being present in estuarine waters as well as in marine environments, in the presence of sediments. It can reach 50 to 60 cm in length and has

a small basal appendage with which it attaches itself to the sand. It is red in colour, has cylindrical stems and is cartilaginous in consistency, presenting cystocarps when it is reproductive (Correia & Pereira, 2015; Redmond et al. 2014).

This seaweed genus is cultivated in aquaculture and harvested from nature in several countries (Abreu et al., 2011; Francavilla et al., 2013; Correia & Pereira, 2015). The simplest and most common method of cultivation is tip isolation to obtain epiphyte-free and genetically identical cultures. Associated with tip isolation is tumbled tank culture, which gives favourable results. The *Gracilaria* genus is better cultivated with stocking densities between 2 g/L and 4 g/L (Redmond et al. 2014).

One of the main purposes for *Gracilaria* sp. cultivation and harvesting is to extract agar, a phycocolloid with industrial applications in microbiology as culture media for bacteria, biotechnology, pharmacology, cosmetics, and the food industry as an emulsifying, stabilizing, and suspending agent. *Gracilaria gracilis* is not currently harvested to extract agar in Portugal, but it is harvested in countries such as Namibia and South Africa (Gaspar et al., 2019; Correia & Pereira, 2015).

1.4. Objectives and thesis structure

The goal of this dissertation was to evaluate the performance of several macroalgae, when cultivated in a fish farm effluent, and to study the different forms of reproduction of *Chondrus crispus*, in the laboratory. The choice of species used in this dissertation was based on criteria such as local abundance and seasonal availability, as well as their economic interest (Correia & Pereira, 2015).

To accomplish this goal, the following objectives were set:

- a) Evaluate the influence of different production factors on the growth of *Ulva* sp. (light intensity), *Gracilaria gracilis* (stocking density), *Codium tomentosum* (fragmentation) and *Chondrus crispus* (stocking density and light intensity) in a fish farm effluent.
- b) Study the nursery stage of *Chondrus crispus*, by vegetative and sexual reproduction.
- c) Analyse the biochemical composition of the best performing species in the integrated multitrophic aquaculture system.

This dissertation is organised in 6 chapters. Chapter 1 provides a general introduction to the topics and species covered in the work. This is followed by a general characterisation of the seaweed cultivation system used in the pilot tests (Chapter 2). Each subsequent chapter corresponds to one of the species studied and presents the corresponding trials, including methodology, results, and discussion.

Chapter 3 describes the trials carried out with *Chondrus crispus*, in the laboratory (sexual reproduction and vegetative propagation) and on a pilot scale. Chapter 4 refers to the *Codium tomentosum* fragmentation test. Chapter 5 covers the *Gracilaria gracilis* stocking density trial and the *Ulva* sp. light intensity trial, as well as the chemical analyses carried out on the seaweed to determine its potential for biovalorization. The final chapter - Chapter 6 - contains general considerations and conclusions of all trials.

2. System characterization

2.1 Cultivation system and study site

The pilot scale work of this thesis was carried out at the Aquacria S.A. aquaculture farm, part of the "Sea8" group, located in Torreira (40° 45' 38" N 8° 42' 20" W), in the municipality of Aveiro. The company carries out intensive brackish water aquaculture in a recirculating water system (RAS). It operates as a *Solea senegalensis* cultivation unit with a production capacity of 250 tonnes per year.

The seaweed cultivation system installed at the Aquacria S.A. facilities consisted of a set of 24 tanks (volume = 60 L), organised into four rows of 6 tanks, each with a manually adjustable water supply tap. The tanks were fed by a water supply system, which provided effluent from the RAS system water treatment plant before it passed through the biological filter. Before reaching the tanks, the effluent passed through a sand filter installed to retain the larger particles, to avoid clogging the pipework and the accumulation of debris in the tanks. The outlet pipe was covered with a removable mesh to prevent algae from passing into the drainage system. To prevent the accumulation of debris, the system was purged every 2 weeks. Throughout the experiments, data loggers were installed in tanks to record temperature and light data.



Figure 12 - Seaweed cultivation system installed at the Aquacria S.A. facilities.

2.2. Effluent composition

The effluent used in the seaweed cultivation system came from the fish farm and entered the seaweed cultivation system before passing through the biological filter. The effluent reaching the seaweed cultivation system was characterised during the first pilot scale experiment, in December 2022. Nutrient levels were $43,7 \pm 11,6 \mu\text{mol/L}$ (n=6) for ammonia (NH_4^+), $12,6 \pm 4,4 \mu\text{mol/L}$ (n=6) for nitrites (NO_2^-), which was the least abundant, $1186,2 \pm 73,9 \mu\text{mol/L}$ (n=6) for nitrate (NO_3^-) which was the most abundant, and $31,8 \pm 13,5 \mu\text{mol/L}$ (n=6) for phosphate (PO_4^{3-}).

3. *Chondrus crispus*

Trials with *Chondrus crispus* were conducted in the laboratory at CIIMAR and at Aquacria S.A. (at pilot scale). In the laboratory, the nursery stage of *Chondrus crispus* was studied, specifically cultivation by vegetative propagation and sexual reproduction. In the fish farm, trials were conducted to study the influence of light intensity and stocking density on the growth of *Chondrus crispus*.

3.1. Vegetative propagation of *Chondrus crispus*: Effect of temperature and culture medium on vegetative growth

3.1.1. Objectives

This laboratory test aimed to assess how the aquaculture effluent and temperature would influence the vegetative propagation of *Chondrus crispus*, to assess how this species would perform when cultivated at a pilot scale in the fish farm. As a control, Von Stosch enriched (VSE) seawater was utilized. Growth performance was evaluated at two distinct temperatures - 16 and 18°C.

3.1.2. Methodology

3.1.2.1. Experimental procedure

The biological material used in this trial was collected at Praia da Granja (41° 02' 11" N, 8° 38' 54" O) on the 12th of October, packed in a thermal box and transported to the laboratory. In the laboratory, epiphytes and sediments were removed with filtered seawater, and the macroalgae were kept in CIIMAR facilities until use, in a 100L tank of filtered seawater at 80 $\mu\text{mol photons m}^{-2} \cdot \text{s}^{-1}$.

Apices from healthy and epiphyte-free *Chondrus crispus* specimens were selected for the experiment using a scalpel (approximately 30g). The excised apices were placed in a 5 L autoclaved flask containing 5 L of VSE nutrient medium, at 16°C and 60 $\mu\text{mol photons m}^{-2} \cdot \text{s}^{-1}$ and a 12h/12h light/dark cycle for 7 days with continuous aeration, as an acclimation to reduce variability between individuals.

The experiment was conducted using 0,5 L autoclaved flasks, 6 of which were filled with 0.5 L of effluent from the aquaculture and another 6 with seawater supplemented with VSE.

Sets of 2 g of *Chondrus crispus* apices from the 5L flask were weighed and placed in the previously prepared flasks (4g/L). The flasks were labelled according to the experimental treatments (Effluent at 16°C and 18°C; VSE seawater at 16°C and 18°C). Three replicates were used for each treatment. Thus, 3 flasks containing effluent and 3 others

containing VSE nutrient medium, were incubated at 16°C and the other 6, at 18°C for 4 weeks at approximately 60 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at a 12h/12h light/dark cycle.

The biomass in each flask was weighed weekly to assess growth. After weighing, density was restored, and the culture medium was changed in accordance with the defined treatments.

3.1.2.2. Calculations and Statistical analyses

Relative growth rate (RGR) was calculated per day using the following formula (Abreu et al., 2011):

$$RGR (\% \text{ day}^{-1}) = \frac{\ln(FW) - \ln(IW)}{T} \times 100$$

Where FW represented “final fresh weight”, IW, “initial fresh weight”, and T, “days in culture”. A mean growth rate was calculated from replicates of each treatment, corresponding to the weekly average RGR ($\% \text{ day}^{-1}$) per treatment. Additionally, an overall mean RGR ($\% \text{ day}^{-1}$) calculation for each treatment was presented, using the mean growth rate obtained for each week.

A two-way ANOVA was performed to assess the effect of temperature (2 levels: 16, 18; fixed) and culture medium (2 levels: VSE, Effluent; fixed) and their interactive effects on the dependent variable RGR.

When a significant difference between sample means or interaction of factors was revealed by the test, a pairwise comparison among levels of factors was performed and means were considered significantly different at $p < 0.05$. These pairwise comparison tests applied were Tukey’s multiple comparisons. All statistical analysis were performed using GraphPad Prism (v 8.0.1).

3.1.3. Results

Chondrus crispus not only grew in seawater supplemented with VSE but also in the fish farm effluent, although this species had higher growth rates in VSE than in effluent.

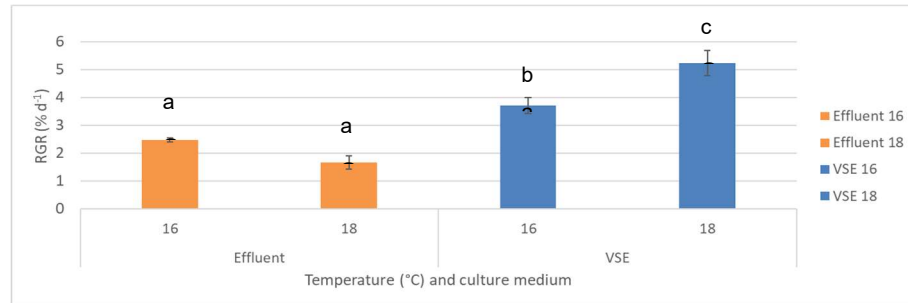


Figure 13 - Mean growth rate (RGR) of *Chondrus crispus* for the different temperature and nutrient medium treatments. Error bar represents standard deviation. Different letters indicate significant difference according to the Tukey's multiple comparison test (p-value = 0,05)

The ANOVA results showed that the effect of nutrient medium on the vegetative growth of *Chondrus crispus* were significant (Table 1, p-value < 0,001), with higher RGR obtained with the VSE nutrient medium. On the other hand, globally, temperature had a non-significant effect on growth (Table 1, p-value > 0,05). There was a significant interaction between temperature and nutrient medium (Table 1, p-value = 0,0014).

Table 1 - ANOVA results for testing the effects of temperature and nutrient medium on the vegetative growth of *Chondrus crispus*. Values in bold represent significant differences.

Source	Df	MS	F	P.
Medium =M	1	51,93	52,91	<0,0001
Temperature=T	1	1,116	1,138	0,2941
M * T	1	12,08	12,31	0,0014

The highest RGR recorded was $5,23 \pm 0,48$ % d⁻¹, for 18°C VSE. The lowest growth rate recorded was for 18°C effluent with $1,67 \pm 0,23$ %/d.

It was observed that for the effluent treatments the temperature with the highest growth rate was 16°C, while for the VSE medium it was 18°C, showing an opposite tendency.

Table 2 - Tukey's multiple comparisons test for the effects of temperature and nutrient medium on the vegetative growth of *Chondrus crispus*. Values in bold represent significant differences.

Compared groups	P- values
EF:T16 vs. EF:T18	0,3269
EF:T16 vs. VSE:T16	0,0555
EF:T16 vs. VSE:T18	<0,0001
EF:T18 vs. VSE:T16	0,0006
EF:T18 vs. VSE:T18	<0,0001
VSE:T16 vs. VSE:T18	0,0142

Results from the Tukey's multiple comparisons showed that RGR was significantly lower at 16°C than 18°C with VSE, (Table 2, p-value = 0,014). However, RGR was similar for cultivation at 16°C and 18°C with effluent (Table 2, p-value = 0,327).

3.1.4. Discussion

The performance of *Chondrus crispus* in terms of growth rate (RGR %day⁻¹) was evaluated in this trial. We hypothesised that the performance of *Chondrus crispus* in contact with the effluent would be similar to that in VSE nutrient medium, but this was not the case.

The individuals grown in VSE – a well-accepted media to grow red seaweed (Redmond et al 2014) exhibited higher growth rates than the ones being cultivated in the aquaculture effluent. Since VSE is an optimised nutrient media, containing high nutrient levels, micronutrients and vitamins, high growth rates were expected. Since the effluent also presents high nutrient levels, namely nitrates, the lower performance may be due to an unbalanced nutrient ratio or lower content in micronutrients and vitamins comparatively to VSE medium. Comparing the effluent composition to VSE medium, in g/L, the former contains a higher nitrate content (76,4 g/L) than what is administered when preparing VSE (42,5g of NaNO₃), however, the VSE medium contains Vitamin B12, Thiamine and Biotin and other vitamins, depending on the specificity of culture (Okauchi & Kawamura, 1997; Redmond et., 2014) which are important for seaweed development, but are not present in the fish farm effluent. Nutrient ratio in the nutrient media is also carefully adjusted to the necessities of seaweed, containing a much wider variety of organic compounds to ensure optimal growth (Redmond et al., 2014). Compounds resulting from fish metabolism or aquaculture feed waste may also have adversely affected growth.

According to Redmond et al. (2014), the ideal temperature for the growth of *Chondrus crispus* is 20°C, with survival reported at a wide range of temperatures from -1,9°C to 26°C. This was observed in cultivation with VSE, since the temperature closest to 20°C (18°C VSE) showed a higher growth rate than 16°C.

It can therefore be assumed that temperature, as an isolated parameter, is not the most preponderant factor for a higher growth rate of *Chondrus crispus*. However, depending on the culture medium used, this may change. It can be assumed that the composition of the effluent and the level of nutrients available compared to VSE were the main determinants of the differences in growth between the treatments with different culture media.

It is recognised that an increase in temperature can affect the photosynthetic rate of algae, biochemical composition, as well as their growth rate, by reducing it. However, this occurs at temperature ranges outside what is considered ideal for each species (Kumar et al., 2020).

3.2. Sexual reproduction of *Chondrus crispus*: Germination under different light and temperature conditions

3.2.1. Objectives

The present trial aimed to determine the best conditions for the sexual reproduction of *Chondrus crispus* at the laboratory, to support the development of the nursery stage of this species. To accomplish this goal, fixation, survival and growth of *Chondrus crispus* carpospores (sexual reproduction) was assessed at two different temperatures and three light intensities. For this purpose, we performed a bifactorial assay evaluating the following light conditions: 20, 40 and 80 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and two temperatures: 14 and 18°C.

3.2.2. Methodology

3.2.2.1. Experimental procedure

Sampling was carried out at Praia Norte, Viana do Castelo (41° 41' 49.034" N, 8° 51' 2.941" W) to obtain reproductive individuals of *Chondrus crispus* in good condition. The seaweeds were packed in a thermo box and taken back to the laboratory. In the laboratory, sampled algae were washed with filtered seawater to remove sediment and invertebrates. Seaweeds were maintained in 2L of seawater supplemented with VSE at 16°C, 100 $\mu\text{mol photons m}^2/\text{s}$, and a 12h/12h light/dark cycle for 1 week.

The procedure for spore release was based on the methods described in Redmond et al. (2014), Tasende & Fraga (1997) and Chen et al. (1972).

Cystocarps were excised from the algae with a scalpel and then washed with autoclaved seawater. The surface of the cystocarp was cleaned with sterile cotton wool to remove any epiphytes present on the surface. Cystocarps (n=50) were placed in an autoclaved beaker containing 300 mL of autoclaved seawater at room temperature (~20°C) and light to release the carpospores. The beaker was placed on an orbital shaker (VWR 3500) for 24 hours. The next day, the presence of carpospores was observed under an inverted microscope (Nikon TE300).

After spore release, the suspension was transferred to 6 well culture plates, 10 mL per well. Temperature was set to 14 and 18°C and light intensity to 20, 40, and 80 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ e a 12h/12h light/dark cycle. Five replicates were used.

After one week, three images were taken of each well of the culture plates under an inverted microscope to estimate the total number of spores released (T0). To monitor the germination process, three images were taken of each well of each culture plate each week and the culture medium was renewed. Seawater was replaced by 10 ml of VSE medium and the plates were returned to the pre-established conditions. Germanium dioxide (GeO₂) was added in proportion to the medium to control the growth of epiphytes in the wells, each week. This procedure was followed for 8 weeks.

3.2.2.2. Calculations and Statistical analysis

As mentioned above, each treatment had 5 replicates and for each replicate 3 images were taken at 4x magnification under an inverted microscope (Nikon TE300)

The fixation rate (%) was measured at T1, and the survival rate (%), was measured at T8. The quantity of spores present in each well each week was estimated based on the number of spores in each visual field, its respective area, and the area of the well, considering a uniform distribution of the carpospores throughout the culture well. The number of spores estimated was compared with the number of spores present at T0 (start of the experiment), translating the results into percentages.

$$\text{Fixation rate (\%)} = ((n \text{ of carpospores in T1}) \div (n \text{ of carpospores in T0})) \times 100$$

$$\text{Survival rate (\%)} = ((n \text{ of carpospores in T8}) \div (n \text{ of carpospores in T0})) \times 100$$

To obtain the diameter of the carpospores over the course of the experiment, the scale shown in the images was used, translated into pixels, and the diameter of each spore present was estimated using image analysis, followed by an average and standard deviation for the diameter of the carpospores per treatment at each time point (T0; T1; T2; T4; T6; T8).

For statistical calculations, the Shapiro-Wilk test was applied to verify the normality of the data. Homogeneity of the variance was assessed by Levene's test. A two-way ANOVA was performed to assess the effect of temperature (2 levels: 14, 18; fixed) and light (3 levels: 20, 40, 80; fixed) and their interactive effects on the dependent variables. All statistical analysis were performed using GraphPad Prism (v 8.0.1).

3.2.3. Results

Regarding the fixation rate (calculated one week after the introduction of the spores), the highest overall percentage was estimated to be found at 18°C and 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and the lowest at 14°C and 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

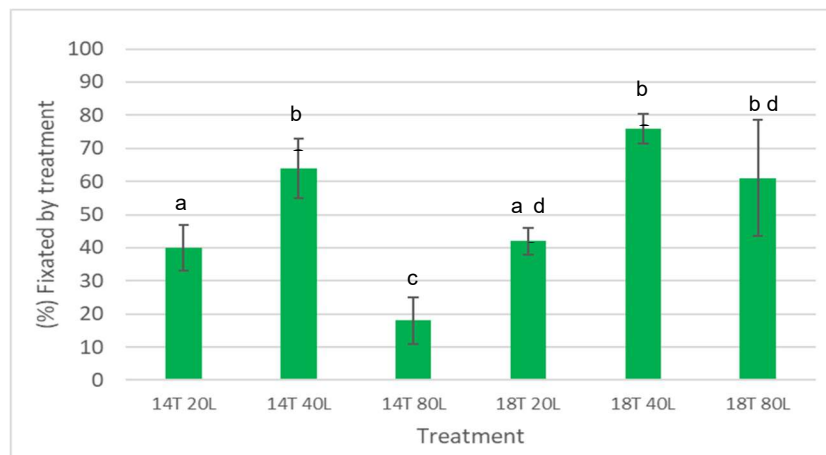


Figure 14 - The effects of temperature and luminosity on *Chondrus crispus* Fixation rate (%). Abbreviations refer to treatment conditions; 14T20L: 14°C 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; 14T40L: 14°C 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; 14T80L: 14°C 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; 18T20L: 18°C 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; 18T40L: 18°C 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; 18T80L: 18°C 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Error bar represents standard deviation. Different letters indicate significant difference according to the Tukey's multiple comparison test (p-value = 0,05).

At 14°C, the luminosity estimated to ensure the greatest fixation was 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (14T40L), with $64 \pm 9\%$, and the one with the lowest fixation rate was 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (14T80L), with $18 \pm 7\%$, corresponding to the lowest fixation rate value among all the treatments.

As aforementioned, for 18°C, the highest fixation rate was found for the light intensity of 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (18T40L) with $76 \pm 5\%$ and the lowest for 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (18T20L) with $42 \pm 4\%$.

There were significant differences regarding the influence of luminosity (Table 3; p-value<0,0001) and temperature (Table 3; p-value<0,0001) on fixation rate. Interaction between factors was also registered (Table 3; p-value=0,0003).

Table 3 - ANOVA results for the effects of temperature and luminosity on *Chondrus crispus* carpospores fixation rate (%). Values in bold represent significant differences.

ANOVA	Df	MS	F	P
Luminosity=L	2	2627	31,44	<0,0001
Temperature=T	1	2407	28,80	<0,0001
L * T	2	1016	12,15	0,0003

Table 4 - Tukey's multiple comparisons test for the effects of temperature and luminosity on *Chondrus crispus* carpospores fixation rate (%). Values in bold represent significant differences.

Compared groups	P- values
20:14°C vs. 20:18°C	0,9994
20:14°C vs. 40:14°C	0,0090
20:14°C vs. 40:18°C	<0,0001
20:14°C vs. 80:14°C	0,0115
20:14°C vs. 80:18°C	0,0267
20:18°C vs. 40:14°C	0,0279
20:18°C vs. 40:18°C	0,0002
20:18°C vs. 80:14°C	0,0090
20:18°C vs. 80:18°C	0,0735
40:14°C vs. 40:18°C	0,3980
40:14°C vs. 80:14°C	<0,0001
40:14°C vs. 80:18°C	0,9969
40:18°C vs. 80:14°C	<0,0001
40:18°C vs. 80:18°C	0,1857
80:14°C vs. 80:18°C	<0,0001

According to Tukey's multiple comparisons (Table 4), even though the fixation rate was higher for the 18°C 40 $\mu\text{mol photons m}^2 \text{ s}^{-1}$ condition, there were not significant differences between this condition and 18°C80 $\mu\text{mol photons m}^2 \text{ s}^{-1}$.

In terms of carpospore survival, the highest survival rate of all treatments was also ensured by the 18°C and 40 $\mu\text{mol photons m}^2 \text{ s}^{-1}$ (18T40L) treatment. The lowest survival was observed for 14°C and 80 $\mu\text{mol photons m}^2 \text{ s}^{-1}$ (14T80/L).

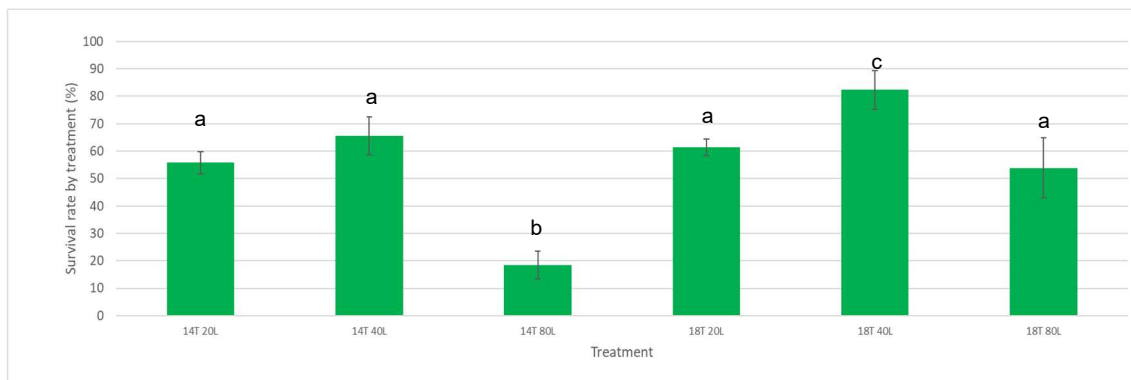


Figure 15 - Survival rate (%) of carpospores at the end of eight weeks by treatment - Sexual reproduction of *Chondrus crispus* trial. 14T20L: 14°C 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; 14T40L: 14°C 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; 14T80L: 14°C 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; 18T20L: 18°C 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; 18T40L: 18°C 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; 18T80L: 18°C 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. Error bar represents standard deviation. Different letters indicate significant difference according to the Tukey's multiple comparison test ($p\text{-value} = 0,05$).

At 14°C, the light estimated to ensure the highest spore survival rate was 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, with $66 \pm 7\%$ (14T40L). The lowest survival was at 14°C 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with $19 \pm 5\%$ (14T80L), which corresponded to the lowest survival value for both treatments.

For the 18°C treatments, as aforementioned, the highest survival rate was found at 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with $82 \pm 7\%$ (18T40L) and the lowest at 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with $54 \pm 11\%$ (18T80L).

There were significant differences regarding the influence of luminosity (Table 5, $p\text{-value} < 0,0001$) and temperature (Table 5, $p\text{-value} < 0,0001$) on fixation rate. Interaction between factors was also registered (Table 5, $p\text{-value} = 0,0002$).

Table 5 - ANOVA results for the effects of temperature and luminosity on *Chondrus crispus* carpospores survival rate (%). Values in bold represent significant differences

ANOVA	df	MS	F	P
Luminosity=L	2	3551	79,20	<0,0001
Temperature=T	1	2613	58,29	<0,0001
L * T	2	575,8	12,84	0,0002

Table 6 - Tukey's multiple comparisons test for the effects of temperature and luminosity on *Chondrus crispus* carpospores survival rate (%). Values in bold represent significant differences.

Compared groups	P- values
20:14°C vs. 20:18°C	0,8415
20:14°C vs. 40:14°C	0,2095
20:14°C vs. 40:18°C	<0,0001
20:14°C vs. 80:14°C	<0,0001
20:14°C vs. 80:18°C	0,9967
20:18°C vs. 40:14°C	0,8415
20:18°C vs. 40:18°C	0,0006
20:18°C vs. 80:14°C	<0,0001
20:18°C vs. 80:18°C	0,5737
40:14°C vs. 40:18°C	0,0105
40:14°C vs. 80:14°C	<0,0001
40:14°C vs. 80:18°C	0,0859
40:18°C vs. 80:14°C	<0,0001
40:18°C vs. 80:18°C	<0,0001
80:14°C vs. 80:18°C	<0,0001

According to Tukey's multiple comparisons (Table 6), even though there was interaction between the factors, the 18°C 40 $\mu\text{mol photons m}^2 \text{ s}^{-1}$ condition, was still significantly different from all other conditions evaluated.

Regarding spore diameter, growth was observed in all conditions tested. However, at the end of the trial, the condition 14°C 20 $\mu\text{mol photons m}^2 \text{ s}^{-1}$ showed the highest degree of carpospore development, followed closely by the 18°C 20 $\mu\text{mol photons m}^2 \text{ s}^{-1}$ condition (Figure 16, Figure 17). The 14°C 20 $\mu\text{mol photons m}^2 \text{ s}^{-1}$ was the only condition to develop apices (Figure 18). The lowest development occurred at 18°C 40 $\mu\text{mol photons m}^2 \text{ s}^{-1}$ (Figure 19).

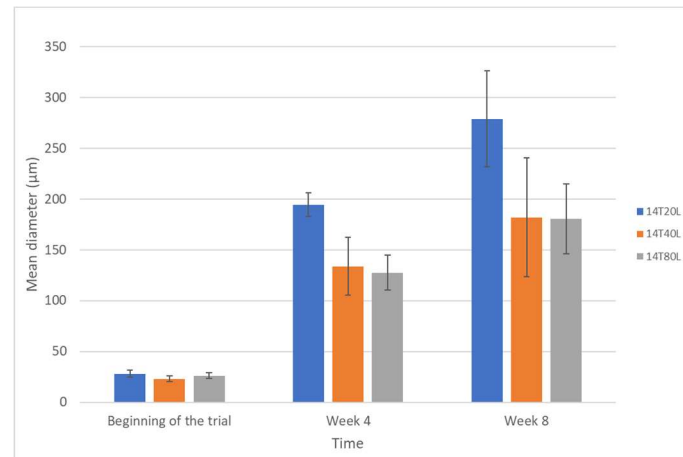


Figure 16 - Mean diameter of *Chondrus crispus* carpospores during the sexual reproduction trial (14°C). Error bar represents standard deviation

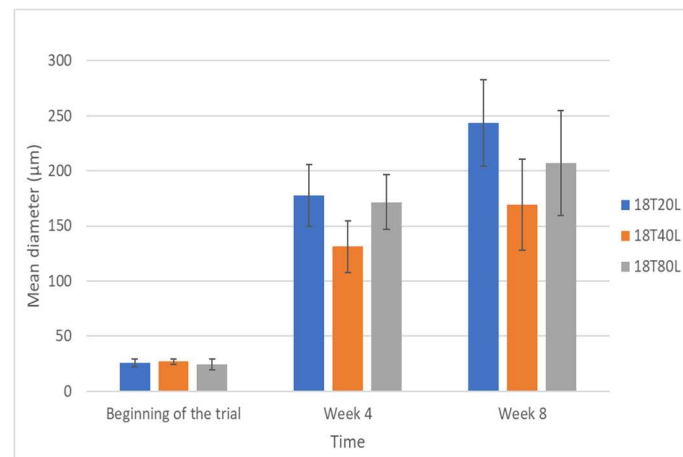


Figure 17 - Mean diameter of *Chondrus crispus* carpospores during the sexual reproduction trial (18°C). Error bar represents standard deviation

At the beginning of the assay (T0) the size of the spores after release varied between approximately 23 and 28 µm, rounded to the nearest micrometre.

At the end of the experiment, after eight weeks, T8, there were significant differences in spore size between treatments. As in T4, the largest spores were observed at 14°C 20 µmol photons m² s⁻¹ (14T20L). The effect of luminosity was significant (Table 7, p-value < 0,0001), but the effect of temperature was not (Table 7, p-value=0,5427). There was no interaction between factors (Table 7, p-value=0,1013).

Table 7 - ANOVA results for the effect of temperature and luminosity on *Chondrus crispus* carpospore diameter, end of the trial (T8). Values in bold represent significant differences.

ANOVA	df	MS	F	P
Luminosity=L	2	40707	19,66	<0,0001
Temperature=T	1	777,2	0,3753	0,5427
L * T	2	4949	2,390	0,1013

The evolution of carpospores growth in the treatment with the highest (14T20L) and lowest (18T40L) organism growth is shown below (Figure 18 Figure 19).

Although the evolution of growth is not shown for all treatments, only the 14°C 20 μmol photons m² s⁻¹ (14T20L) treatment had apical growth (Figure 18 at T6 and T8). However, it was not possible to determine its exact length due to the position of the image.

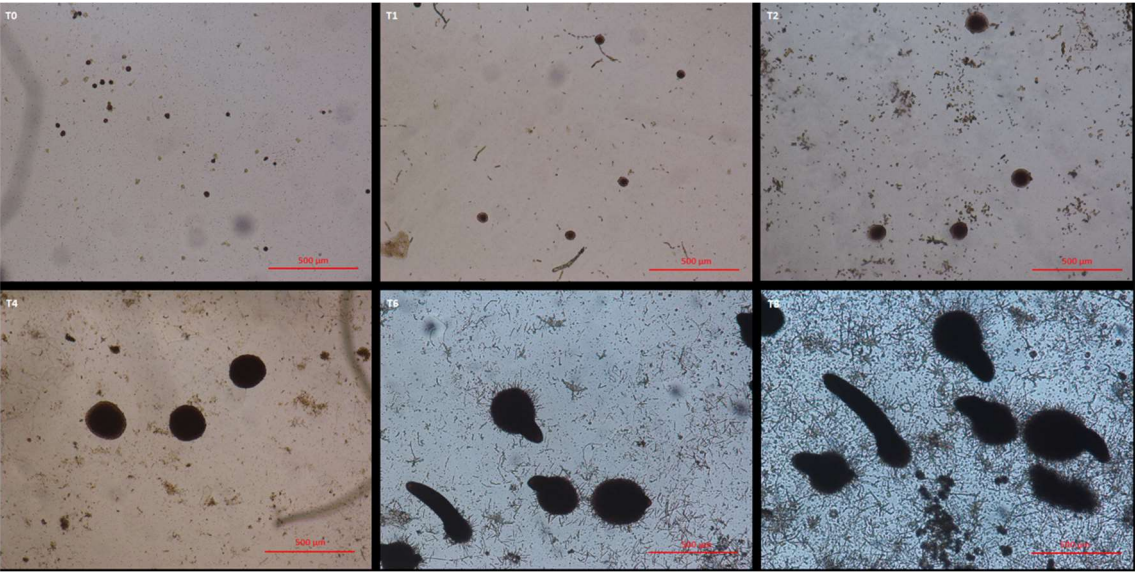


Figure 18 - Carpospore’s development throughout the Sexual reproduction trial for the 14°C 20 μmol photons m² s⁻¹ (14T20L) treatment. Images were taken at a 4x magnification, with a 500μm scale for comparison. Abbreviations refer to different time points; T0: beginning of the trial; T1: One week later; T2: two weeks later; T4: Four weeks later; T6: Six weeks later; T8: Eight weeks later, end of the trial.

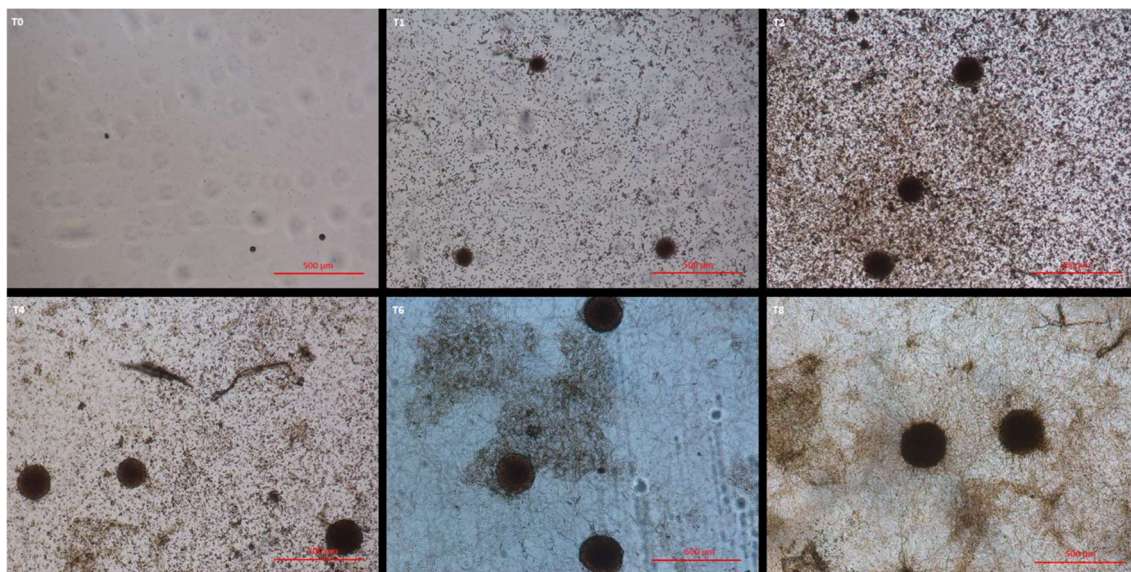


Figure 19 - Carpospore's development throughout the Sexual reproduction trial for the 18°C 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (18T40L) treatment. Images were taken at a 4x magnification with a 500 μm scale for comparison. Abbreviations refer to different time points; T0: beginning of the trial; T1: One week later; T2: two weeks later; T4: Four weeks later; T6: Six weeks later; T8: Eight weeks later, end of the trial.

3.2.4. Discussion

Different results were obtained regarding the best conditions for carpospore fixation, survival and grow. While the best condition for carpospore fixation and survival was 18°C and 40 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (fixation rate: $76 \pm 5\%$; survival rate: $82 \pm 7\%$), this was the one that ensured the least growth of carpospores out of the 6 conditions evaluated. The condition that ensured the best growth of the carpospores was 14°C and 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, as this was the only one in which the apexes developed. The 18°C and 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ closely followed, however in this condition there was no apical growth. According to Chen & McLachlan (1992) *Chondrus crispus* is capable of germinating in conditions from 5 to 20°C and 100 to 1000 ft-c, which translates to 2 to 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. However, according to Redmond et al. (2014), the best germination conditions are found between 10 to 15°C and 10 to 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, which gives a wider range of light intensities viable to this process but narrows the optimal temperature conditions. Chen & McLachlan (1992) also point out that germination can only be detected in about 20% of the carpospores, which is a much lower value than the one obtained in our best condition (18T40L: $82 \pm 7\%$) and that germination at more than 20°C is not viable. The temperature conditions of this trial were also in the range tested by Burns & Mathieson (1972) who concluded that *Chondrus crispus* is capable of germinating at temperatures between 11 and 19°C, and at salinities between 15 and 45%, with the best conditions at 11°C and 35% salinity. These temperature conditions

also coincide with those evaluated in this trial and, similarly to what the authors state, the best result for carpospores development in our trial was at 14°C, the lowest temperature evaluated. Similarly, to Redmond et al. (2014), Burns & McLachlan (1972) also affirm that the best condition for adult *Chondrus crispus* to develop is at around 20°C. However, as mentioned above, lower temperatures are preferable for germination of carpospores.

The higher light conditions (above 20 $\mu\text{mol photons m}^2 \text{s}^{-1}$) also increased the potential for contamination to develop in the culture wells, which did not help the carpospores to grow. Although germanium dioxide (GeO_2) was used in all conditions evaluated to mitigate this problem, it persisted throughout the test (Figure 18 and Figure 19). Germanium dioxide is a compound that inhibits the growth of diatoms by interfering with silicon metabolism, but it has been reported to have no effect on *Chondrus crispus* at any stage of its life cycle (Markham et al. 1982), making it suitable for carpospore cultures.

It is, however, recognised that a longer test without contamination would be required to ensure more consistent results. According to the results obtained, we can consider that, after the release of the *Chondrus crispus* carpospores by a procedure consistent with that mentioned by Redmond et al. (2014), Tasende & Fraga (1997) and Chen et al. (1972), they should spend the first week of their development in conditions of 18°C and 40 $\mu\text{mol photons m}^2 \text{s}^{-1}$, to ensure greater chances of fixation. After the fixation period, it is best to place the carpospores in conditions of 14°C and 20 $\mu\text{mol photons m}^2 \text{s}^{-1}$ to ensure their maximum growth in the following weeks. Even though the 14°C and 20 $\mu\text{mol photons m}^2 \text{s}^{-1}$ were not the conditions to reportedly ensure the highest survival rate (56 $\pm$ 4%) amongst the evaluated conditions, these were the best conditions for carpospore growth, which is prioritizing compared to sheer survival capacity.

3.3. *Chondrus crispus* IMTA system trials: Stocking density and luminosity trial (March) and stocking density trial (April)

3.3.1. Objectives

The aim of this pilot-scale study was to determine the performance of *Chondrus crispus* when cultivated in a fish farm effluent. The factors of light intensity and stocking density were evaluated in order to determine the most ideal conditions for its cultivation.

3.3.2. Methodology

3.3.2.1. Experimental procedure (March)

Chondrus crispus sampling was carried out on the rocky shores of Granja (22/1/2023), Vila Chã (24/1/2023), Viana do Castelo (25/1/2023) and Angeiras (20/2/2023) with the objective of collecting non-reproductive individuals in good condition. The seaweeds were packed in a thermo box and taken back to the laboratory. Seaweeds were washed with filtered seawater to remove sediments and epiphytes and kept in a 100L tank with filtered seawater at 16°C and 100 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$.

After a month of growth in the CIIMAR facilities, the algae were transferred to the aquaculture, where they were acclimated for a week in contact with the company's effluent. The algae were then distributed to the tanks (volume = 60 L) according to the 6 pre-defined treatments based on density and light intensity (cultivation densities of 1Kg/m², 2Kg/m² and 4Kg/m² and normal light conditions and covered with netting). Three replicates were used for each condition and a tank was also installed to accommodate the excess biomass from the trial. Cultivation proceeded under ambient conditions, with a continuous effluent flow between 277 and 360 L/h for the duration of the trial.

Weekly visits to the aquaculture were made to monitor the evolution of algal growth. A multi-parameter probe was used to measure pH, temperature (°C), salinity (‰) and dissolved oxygen (mg/L). The algae were removed from the tanks and placed in draining boxes, the tanks were cleaned and after a drying period of 90 minutes each replicate was weighed. The predefined densities for each tank were re-established and the flow rate of each tank was manually adjusted.

3.3.2.2. Experimental procedure (April)

After the early termination of the *Chondrus crispus* stocking density and luminosity trial, the remaining algae in the best condition were collected and cultivated in high density tanks to grow biomass for the next trial.

The seaweeds remained growing for about 6 weeks, from 23 March to 28 April. At this time, the algae were distributed according to the biomass available between the tanks intended for the test, according to the 2 pre-defined treatments based on density (stocking densities of 2Kg/m² and 3Kg/m²). 3 replicates were used for each condition and a tank was also installed to accommodate the excess biomass from the trial. Cultivation proceeded under ambient conditions, with a continuous effluent flow between 277 and 360 L/h for the duration of the trial.

Weekly visits to the aquaculture were made to monitor the development of seaweed growth in contact with the effluent using a multi-parameter probe to measure pH, temperature (°C), salinity (%) and dissolved oxygen (mg/L). The algae were removed from the tanks and placed in draining boxes, the tanks were cleaned and after a drying period of 90 minutes each replicate was weighed.

3.3.3. Results

Chondrus crispus stocking density and luminosity trial had an expected duration of 12 weeks, starting at 2 March, however the trial was interrupted 3 weeks later, on 23 March. The biomass of *Chondrus* was found to be excessively epiphytic, with whitening of the fronds and apparent signs of degradation. At the final date of the trial, there was an outgrowth registered, however the development of *Ulva intestinalis* on the fronds, made it impossible to evaluate the true growth of the culture (Figure 20). Therefore, it was not considered appropriate to present the values for the RGR (% d⁻¹) growth rate as well as the physical-chemical parameters. The algae in the worst condition were discarded and the ones in better condition were kept on a tank to conduct another trial a few weeks later, this time evaluating only the stocking density, given the amount of biomass available.

In April, another attempt was made to grow *Chondrus crispus* on a pilot scale. The densities used in the April trial (2kg/m² and 3kg/m²) were lower than those used in the March trial due to the lack of biomass available in the wild and the reduced amount of time available to carry out the trials as part of the thesis.

Chondrus crispus stocking density trial was initiated on 28th April, however on 10th May, it was interrupted, once again, due to excessive epiphytisation by *Ulva* sp. and general biomass degradation (Figure 21). Attempts were made to remove the epiphytes from the algal fronds, but with little success as they were severely affected. At the end of the test, the amount of epiphytes removed from *Chondrus crispus* and the effective mass of the algae studied were weighed. It was then estimated that the total mass of *Ulva* sp. was at least 41% of the total culture, as mentioned in the pie chart (Figure 22). The presentation of graphs calculating the daily growth rate (RGR (% d⁻¹)) or statistical analyses was therefore not considered adequate.



Figure 20 - *Chondrus crispus* epiphytes at the end of the Stocking density and luminosity trial (March).

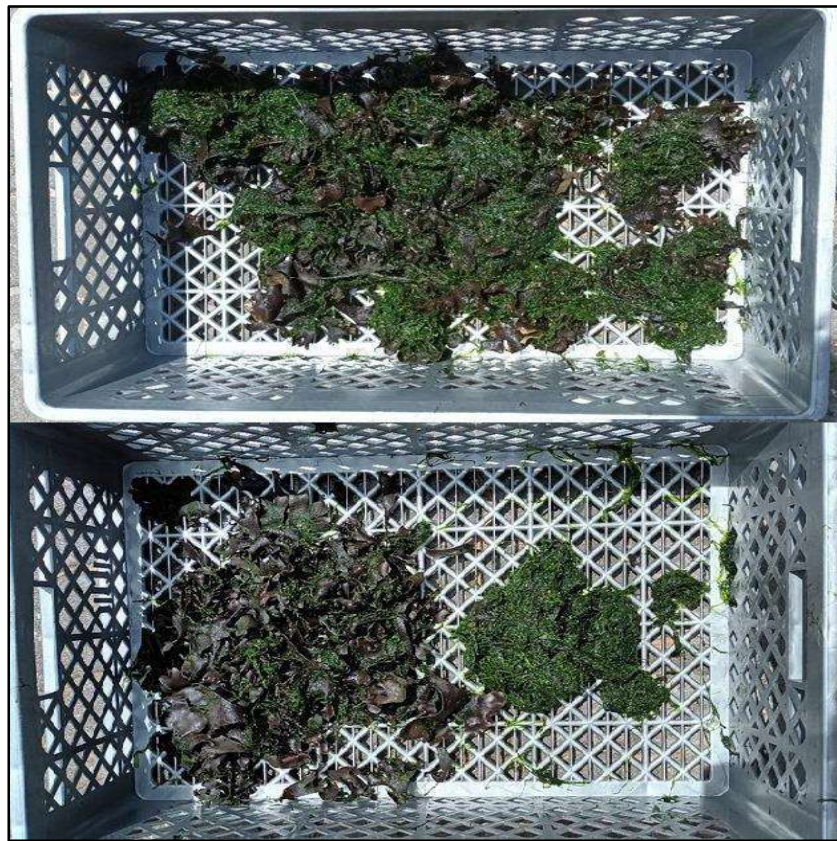


Figure 21 - *Chondrus crispus* epiphytes at the end of the Stocking density trial (April). In the lower photograph of the assembly, *Chondrus crispus* (left side) is observed after manual removal of the epiphytes (right side).

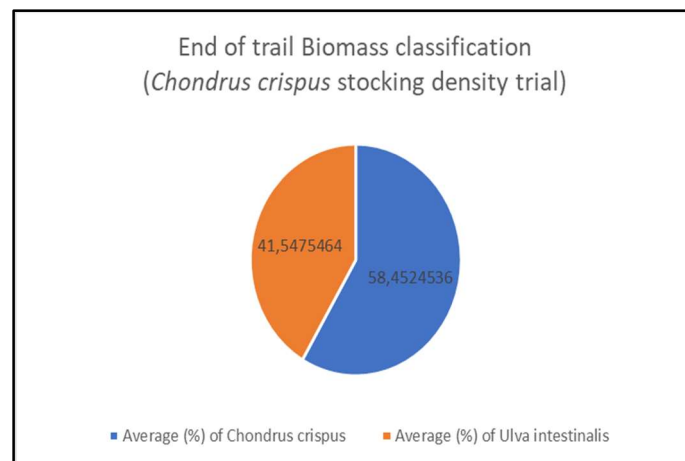


Figure 22 - Proportion of *Chondrus crispus* and epiphyte weight at the end of the Stocking density trial (April).

3.3.4. Discussion

Both attempts to cultivate *Chondrus crispus* on our IMTA system showed unsatisfactory and inconclusive results. The epiphytes gave the impression that the culture was growing, but this was not the case as the epiphytes got worse each week and the culture degraded. In both trials we registered high levels of epiphytisation and degradation of the algae. At the end of the test, photographs were taken to show the condition of the macroalgae, and a pie chart was elaborated to illustrate the degree of epiphytisation as a percentage of the total weight (%). According to the pie chart (Figure 22), the epiphytic algae accounted for an average of 41% of the fresh weight of *Chondrus crispus*. Therefore, no data were collected on the growth rate of *Chondrus crispus* in this experiment.

The densities used in both the March (1Kg/m², 2Kg/m² and 4Kg/m²) and April (2Kg/m² and 3Kg/m²) trials were in or below the lower range (4Kg/m² to 6Kg/m²) recommended by other authors (Bidwell et al., 1985; Redmond et al., 2014). This fact may have favoured the development of epiphytes. According to Bidwell et al. (1985), growing *Chondrus crispus* at low densities and without any mitigating measures, such as the use of selective herbicides, may favour the development of epiphytes, which may impair their development and jeopardise good results.

Another possibility, applicable to small-scale cultivation, is the use of herbivorous arthropods for selective grazing of epiphytes to control the development of this problem in macroalgae cultivation. A species of amphipod, *Gammarus lawrencianus*, has been tested in *Chondrus crispus* cultivation and is capable of consuming epiphytes without affecting the growth of the main algae (Schacklock & Doyle, 1983). However, the use of these organisms would involve additional effort and cost, and the practicality of this procedure on a large scale is questionable.

Future trials in this cultivation system should therefore study higher densities of *Chondrus crispus* to prevent the occurrence of epiphytes

4. *Codium tomentosum* IMTA system Trial: Effect of fragmentation on growth

4.1. Objectives

The present trial aimed to assess whether *Codium tomentosum* was able to grow in this RAS effluent at a pilot scale and test if fragmentation of the biomass to smaller pieces influenced growth.

4.2. Methodology

4.2.1. Experimental procedure

Codium tomentosum individuals were collected at Praia da Granja (41° 02' 11" N, 8° 38' 54" W) on October 12th, packed in a thermal box and transported to the laboratory. Seaweeds were washed with filtered seawater to remove sediments and epiphytes and kept in a 100L tank with filtered seawater at 16°C and 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 3 weeks.

Seven individuals of the collected seaweeds were observed under the inverted microscope (Nikon TE300) to identify the *Codium* species. All individuals were identified as *C. tomentosum* because their utricles were long and lacking mucronate apices, contrarily to *C. fragile* (Trowbridge, 2014).

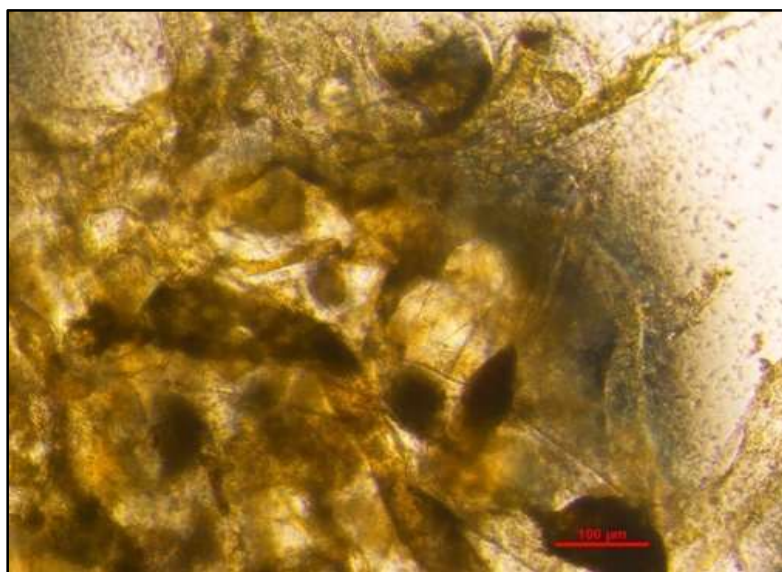


Figure 23 - *Codium tomentosum* utricles (10x magnification).

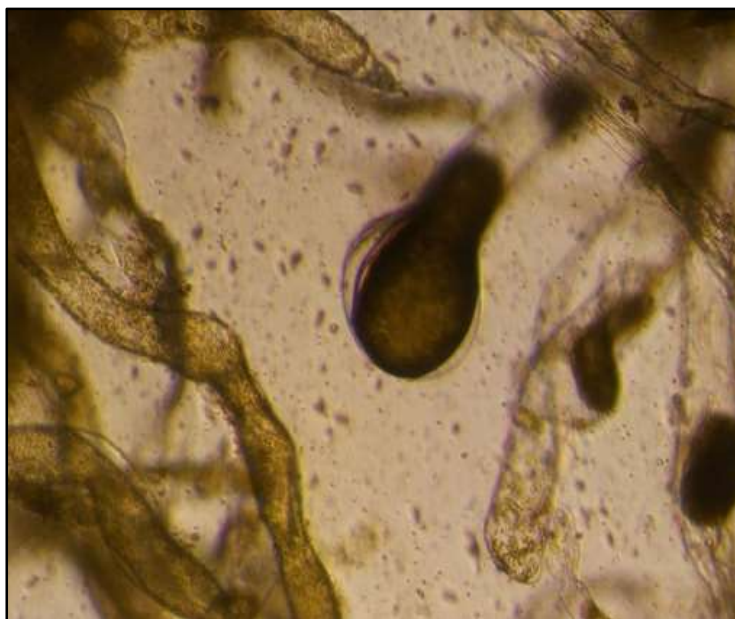


Figure 24 - *Codium tomentosum* utricles (40X magnification).

Seaweeds were weighted and separated in two groups of equal weight. In one of the groups, individuals were cut into fragments of 5 cm (fragmented *Codium*) and the other one was left whole (whole *Codium*). These two groups were maintained under 16°C, 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and a 12h/12h light/dark cycle for one week.

The biomass was transferred to the seaweed cultivation system at the fish-farm and acclimated for two weeks. The macroalgae were distributed in 4 tanks (volume = 60 L), 2 per treatment, at 5g/L stocking density. Cultivation proceeded under ambient conditions, with a continuous effluent flow between 90 and 180 L/h for 8 weeks.

Seaweed growth and physicochemical parameters were monitored weekly. A multi-parameter probe (Hanna HI98194 multiparameter) was used to measure salinity (%), pH, temperature (°C), and dissolved oxygen (%) in the water before weight assessment. Seaweeds were removed from each tank, poured into draining boxes, and left outdoors for 30 minutes, during which time the tanks were cleaned to remove epiphytes and accumulated debris. At the end of the draining period, the macroalgae were weighed on a digital scale (Becken BKS-2389) for growth measurement. The biomass was then returned to its respective tanks, resetting the stocking density to its target value. The water flow rate was adjusted manually to reach the desired flow, between 90 and 180 L/h.

4.2.2. Calculations and Statistical analyses

Relative growth rate (RGR, % day⁻¹) was calculated according to the following formula (Abreu et al., 2011):

$$RGR (\% \text{ day}^{-1}) = \frac{\ln(FW) - \ln(IW)}{T} \times 100$$

Where FW represented “final fresh weight”, IW, “initial fresh weight”, and T, “days in culture”. Both growth rates and environmental conditions were calculated by averaging replicates from all timepoints for each treatment.

The Shapiro-Wilk test was used to evaluate the distribution of the data. Homogeneity of the variance was assessed by Levene’s test. If the data were normally distributed, a two-sample parametric t-test was used to evaluate significant differences between cultivation methods for RGR and all environmental conditions. However, if the data were not normally distributed, the Mann-Whitney test was used to evaluate significant differences between cultivation methods for RGR and all environmental conditions (non-parametric t-test). All statistical analysis were performed using GraphPad Prism (v 8.0.1).

4.3. Results

4.3.1. Environmental conditions

Water temperature varied from 16,4 to 19,0 °C with an average of 17,9 °C. The salinity in the effluent averaged 22,6%, showing at the beginning of the trial a maximum of 25,2%. The dissolved oxygen levels in the water varied between 93% and 114%, with an average of 101% (table 8). Temperature, salinity, and dissolved oxygen were similar for both cultivation methods, whereas the “whole seaweeds” cultivation method showed a pH of 7,6, which was significantly higher than the pH registered in the effluent with fragmented seaweeds (Table 8, p-value < 0.05)

Table 8 - Environmental conditions assessed in the effluent for each *C. tomentosum* treatment. S.D represents standard deviation.

Environmental parameters	Fragmented			Whole		
	n	mean $\pm$ S. D	Range	N	mean $\pm$ S. D	Range
Temperature (°C)	6	17,9 $\pm$ 0,9	16,4-18,9	6	18,0 $\pm$ 0,9	16,9 – 19,0
pH	6	7,4 $\pm$ 0,1	7,3 – 7,8	6	7,6 $\pm$ 0,1	7,5 – 7,8
Salinity	6	22,8 $\pm$ 1,1	21,9 – 25,2	6	22,8 $\pm$ 1,2	21,9 – 25,3
Dissolved O2 (%)	6	101,5 $\pm$ 4,1	95,8 – 112,5	6	101,1 $\pm$ 1,5	95,0 – 113,9

4.3.2. Growth rate

Codium tomentosum grew in the fish-farm effluent, with RGR ranging from 0,14 to 2.69% a day. No significant differences were found between cultivation methods (Figure 25, p-value > 0,05). However, this species showed a slightly higher growth rate when cultivated intact (1,70% $\pm$ 0,59% d⁻¹) than when using the fragmentation method (1,25% $\pm$ 0,82% d⁻¹).

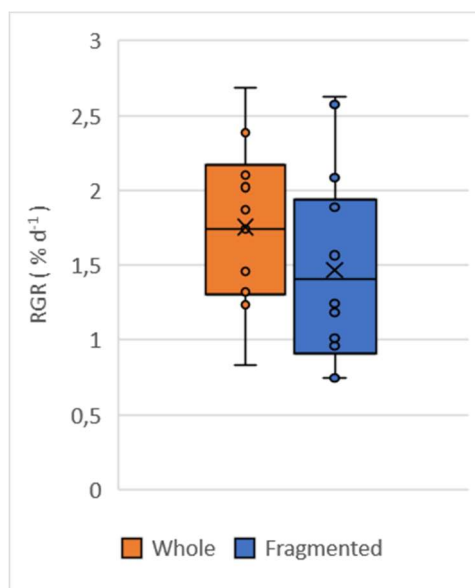


Figure 25 - Boxplot with RGR for each treatment on *Codium tomentosum* fragmentation trial. Error bar represents standard deviation.

4.4. Discussion

The aim of this trial was to find out whether the method of fragmentation of *Codium tomentosum* would be advantageous for its cultivation in aquaculture. The seaweed managed to grow in contact with the fish farm effluent, however, statistics revealed there was no significant difference between cultivating *Codium tomentosum* whole or fragmented. Nevertheless, the “Whole Codium” treatment presented slightly higher growth rates than “fragmented Codium”.

The higher pH obtained for the “whole Codium” treatment may be related to a higher photosynthetic rate due to the higher growth rates observed for this treatment (Graham & Wilcox, 2000). Several previous studies have tested the cultivation of *Codium tomentosum* in an IMTA context, testing light conditions and verifying biochemical changes in the composition of the algae (Marques et al., 2021; Marques et al., 2022).

In one of their trials, Marques et al. (2021), took *Codium tomentosum* from an IMTA system aquaculture and subjected it to a photoperiod experiment in laboratorial conditions (2 L flask with 1 L of VSE saltwater and between 0 and 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) making it possible to compare our results to those with an ideal culture media and controlled light conditions. According to the author, under conditions of short photoperiod (8 hours of light and 16 hours of darkness) growth rate was $\text{RGR}=2.9\pm0.3\%/ \text{day}$. These photoperiod conditions can be equivalent to the autumn and winter seasons, the time of year during which the fragmentation test was carried out. Compared to the results obtained in this thesis, the average RGR values (%/d) for fragmented Codium ($\text{RGR} = 1.25 \pm 0.82\%/ \text{d}$) and for “whole Codium” ($\text{RGR} = 1.70 \pm 0.59\%/ \text{d}$) were lower than those documented by the author. However, the aforementioned test was carried out under controlled laboratory conditions and with an optimised nutrient medium (VSE medium), whereas our test was carried out on an outdoor IMTA system and using the aquaculture effluent.

Some variability in growth rate was observed along the experiment in the RGR, which is probably related to unfavorable weather conditions, namely during the weeks of 14/12/2022 and 22/12/2022 (data not shown), which saw periods of cloudiness and heavy rainfall (Instituto português do mar e da atmosfera [IPMA], 2022). As the pilot scale cultivation system is outdoors, this external factor may have had an impact on the growth of the algae.

As we did not collect light intensity data during this test, and as light intensity under natural conditions varies throughout the day and with climatic conditions, we cannot draw a complete parallel with the article by Marques et al. (2021).

Given that there are not many studies reporting the cultivation of *Codium tomentosum* in IMTA, this study adds to the knowledge of cultivating the species. Ultimately, the method of fragmenting *Codium tomentosum* to increase its growth rate has proved to be counterproductive. Thus, according to our results, it is therefore preferable to cultivate the seaweed whole, under aquaculture conditions.

5. *Gracilaria gracilis* and *Ulva* sp.: IMTA system trials

To assess the performance of *Gracilaria gracilis* and *Ulva* sp. when cultivated in a fish farm effluent, the following trials were conducted at a pilot scale, at the fish farm. A trial was conducted to examine the effect of stocking density on *Gracilaria gracilis* cultivation and another to examine the effect of light intensity on *Ulva* sp. cultivation.

5.1. *Gracilaria gracilis* stocking density trial

5.1.1. Objectives

This stocking density trial was carried out to determine the most favourable stocking density for *Gracilaria gracilis*. The growth rate of the algae at densities of 3 kg/m² and 5 kg/m² was evaluated over a period of 5 weeks. Samples were taken to determine the biomass biochemical composition (total polysaccharide content, phenols, total pigments and quantification of phycobiliproteins) throughout the experiment.

5.1.2. Methodology

5.1.2.1. Experimental procedure

Gracilaria gracilis sampling was carried out on 30 March 2023 in the Ria de Aveiro, near Torreira (40° 45' 38" N 8° 42' 20" W), under favourable tidal conditions.

The algae were placed in sampling bags, packed in a thermobox and taken to the laboratory. At the laboratory the seaweeds were washed with filtered seawater to remove sediments and epiphytes and kept in a 100L tank with filtered seawater at 16°C and 100 µmol photons m²/s 12h/12h in a dark/light cycle, for one week.

The algae were then transferred to the aquaculture facilities on 6 April, where they were acclimated for two weeks in contact with the company's effluent. This period was used as a growth phase to ensure sufficient biomass for the experiment.

On the 28th of April, the algae were distributed among the tanks (volume = 60 L) intended for the test according to the 2 pre-defined treatments based on density (stocking densities of 3Kg/m² and 5Kg/m²). Three replicates were used for each condition and an additional tank was installed to accommodate the excess biomass from the trial for

reposition in case of biomass loss in the trail tanks. Cultivation proceeded under ambient conditions, with a continuous effluent flow between 90 and 180 L/h for the duration of the trial.

Weekly visits to the aquaculture were made to monitor the development of algal growth in contact with the effluent, environmental parameters were measured using a multi-parameter probe (Hanna HI98194 multiparameter) to measure pH, temperature (°C), salinity (%) and dissolved oxygen (%). The algae were removed from the tanks and placed in draining boxes, the tanks were cleaned and after a drying period of 90 minutes each replicate was weighed using a digital scale (Becken BKS2389).

After weighing, algae samples were taken for future biochemical analysis and placed in separate plastic bags in a thermobox. The predefined densities for each tank were re-established and the flow rate of each tank was manually adjusted.

Samples for biochemical analysis were taken at the beginning of the experiment, corresponding to the end of the acclimatisation period (28/04), at mid-term (16/05) and at the end of the experiment (08/06), from each tank to monitor the evolution of the biochemical composition of the seaweed.

5.1.2.2. Biochemical analysis procedure

5.1.2.2.1. Samples pre-processing

In the laboratory, the sampled algae were weighed, and dried in an oven at 60 °C for 24 hours, weighted again and then homogenised into powder using an electric grinder. The homogenised samples were stored in labelled Eppendorf tubes and packed in the desiccator for further analysis.

5.1.2.2.2. Elemental analysis (carbon (C), nitrogen (N) and CN ratio

The total carbon and nitrogen content of the macroalgae samples was analysed using a Flash 2000 elemental analyser (Thermo Fisher Scientific/USA-Germany).

5.1.2.2.3. Total polysaccharide content determination

The polysaccharide content was determined according to Hentati et al. (2018). The weight of the dry extract was recorded the following day. The results were expressed as a percentage of the dry weight of the algae (%DW).

5.1.2.2.4. Total pigments

The content of total pigments (chlorophylls *a* and *b* and total carotenoids) was calculated using the methods described by Ritchie et al. (2015) and Lichtenthaler et al. (2001). The contents were calculated using formulae developed for green and red algae. The results were expressed as a percentage of the dry weight of the algae (%DW).

5.1.2.2.5. Phenolic compounds

The total soluble phenolic compounds of the macroalgae extracts were determined in triplicate by the Folin-Ciocalteu method, modified in microplate according to Magalhães et al. (2010). The results were calculated from the gallic acid calibration curve and expressed as a percentage equivalent to the weight of the extract (%E).

5.1.2.2.6. Quantification of total phycobiliproteins by spectrophotometry

The total quantification of phycobiliproteins was determined from the absorption spectra of photosynthetic pigments according to the method described by Bennet & Bogorad (1973) and adapted for red algae by Kursar et al. (1983). This protocol was only applied to *Gracilaria gracilis* samples.

5.1.2.3. Calculations and Statistical analyses

Relative growth rate (RGR, % day⁻¹) was calculated according to the following formula (Abreu et al., 2011):

$$RGR (\% \text{ day}^{-1}) = \frac{\ln(FW) - \ln(IW)}{T} \times 100$$

Where FW represented “final fresh weight”, IW, “initial fresh weight”, and T, “days in culture”. Both growth rates and environmental conditions were calculated by averaging replicates from all timepoints for each treatment.

The Shapiro-Wilk test was used to evaluate the distribution of the data. Homogeneity of the variance was assessed by Levene’s test. If the data were normally distributed, a two-sample parametric t-test was used to evaluate significant differences between cultivation methods for RGR and all environmental conditions. However, if the data were not normally distributed, the Mann-Whitney test was used to evaluate significant differences

between cultivation methods for RGR and all environmental conditions (non-parametric t-test).

For the biochemical analyses, the Shapiro-Wilk test was also applied, and 2-way ANOVA with Tukey's multiple comparisons was used to assess significant differences between treatments and between sample times. All statistical analysis were performed using GraphPad Prism (v 8.0.1). The protein content was indirectly calculated using the nitrogen-to-protein conversion factor of 6,25, as mentioned in Angell et al. (2016). Nitrogen removal was determined indirectly according to Kambey et al. (2020).

5.1.3. Results

5.1.3.1. Environmental conditions

Temperature, salinity, dissolved oxygen, and pH were similar for both cultivation methods (Table 9), there were no significant differences between treatments. Water temperature varied from 19,6 to 21,7 °C with an average of 20,7 °C. The pH ranged between 7,4 and 8,3 with an average of 7,8. The salinity in the effluent averaged 22,3%, varying between 21,9 and 22,6%. The dissolved oxygen levels in the water varied between 87,5 % and 101,7 %, with an average of 95,0 %.

Table 9 - Environmental conditions assessed in the effluent for each *G. gracilis* treatment. S.D represents standard deviation.

Environmental parameters	3 Kg/m ²			5 Kg/m ²		
	N	mean ± S.D	Range	N	mean ± S.D	Range
Temperature (°C)	13	20,7 ± 0,7	19,8-21,7	13	20,6 ± 0,5	19,6 - 21,6
pH ()	13	7,7 ± 0,2	7,2 - 8,2	13	7,8 ± 0,2	7,4 - 8,3
Salinity (%)	13	22,2 ± 0,3	21,9 - 22,6	13	22,2 ± 0,3	21,9 - 22,6
Dissolved O ₂ (%)	13	95,0 ± 4,4	87,5 - 100,6	13	95,0 ± 4,2	88,8 – 101,7

5.1.3.2. Growth rate

The growth of *Gracilaria gracilis* in the aquaculture effluent ranged from 0,68 to 7,35% per day. No significant differences were found between the different cultivation methods (Figure 26, p value > 0.05). However, this species showed a slightly higher growth rate when cultivated at 3 kg/m² ($3,8 \pm 2,5$ % d⁻¹) than when cultivated at 5 kg/m² ($3,0 \pm 1,5$ % d⁻¹).

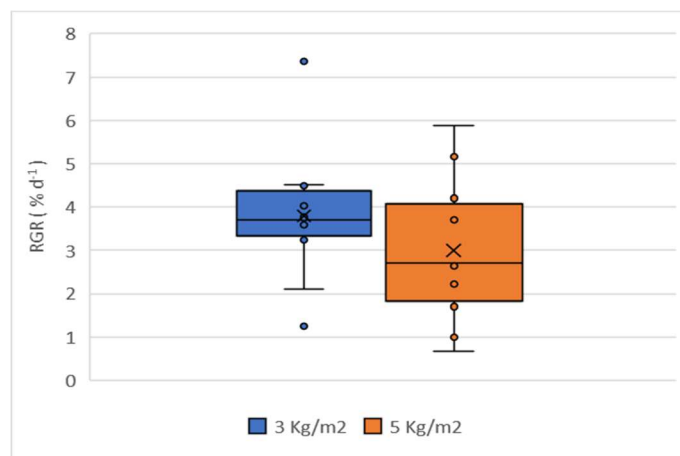


Figure 26 - Boxplot with RGR per treatment for *Gracilaria gracilis* stocking density trial. Error bar represents standard deviation

5.1.3.3. Biochemical characterization of *Gracilaria gracilis*:

Elemental analysis (carbon (C), nitrogen (N) and CN Ratio of *Gracilaria gracilis*

The nitrogen removal for the 3Kg/m² density varied between $2,5 \pm 0,7$ and $14,5 \pm 7,7$ g/m²/week. For the 5Kg/m² it varied between $6,4 \pm 1,2$ and $11,5 \pm 4,3$ g/m²/week.

Table 10 - Elemental analysis results for *Gracilaria gracilis* stocking density trial.

Day	Treatment	Element		
		Carbon (C)	Nitrogen (N)	CN Ratio
28/04	3Kg/m ²	30,4±0,9 % (n=2)	5,0±0,2 % (n=2)	7,2±0,2 % (n=2)
	5Kg/m ²	30,5±1,2 % (n=2)	4,7±0,1 % (n=2)	7,7±0,1 % (n=2)
16/05	3Kg/m ²	32,5±1,4 % (n=3)	5,3±0,3 % (n=3)	7,3±0,3 % (n=3)
	5Kg/m ²	31,1±0,9 % (n=3)	5,3±0,3 % (n=3)	7,0±0,4 % (n=3)
08/06	3Kg/m ²	29,5±1,1 % (n=3)	5,4±0,3 % (n=3)	6,5±0,3 % (n=3)
	5Kg/m ²	31,0±0,6 % (n=3)	5,1±0,2 % (n=3)	7,1±0,2 % (n=3)

There was no statistically significant difference between cultivation densities (Table 11, p-value = 0,916) or between sampling times (Table 11, p-value = 0,0613) for carbon content (%) on *Gracilaria gracilis*. There was no interaction between cultivation density and sampling time (Table 11, p-value=0,0952). The mean Carbon (C) content varied between $29,5 \pm 1,1$ and $32,5 \pm 1,4$ %.

Table 11 - ANOVA results for the effect of sampling time and stocking density on *Gracilaria gracilis* Carbon (C) content during the stocking density trial.

ANOVA	Df	MS	F	P
Sampling time = S	2	4,027	3,740	0,0613
Stocking density=D	1	0,01246	0,01157	0,9165
S * D	2	3,233	3,002	0,0952

For the Nitrogen (N) content, there was no significant difference between cultivation densities (Table 12, p-value = 0,203), but there were significant differences between sampling times (Table 12, p-value= 0,028). There was no interaction between cultivation density and sampling time (Table 12, p-value=0,4944). The highest value recorded was $5,4 \pm 0,3\%$ (08/06) for the 3 kg/m² cultivation density. The lowest value was $4,7 \pm 0,1\%$ (28/04) for the 5 kg/m² cultivation density. For both treatments, the lowest values were recorded on 28/04. Even though there was no significant variation between cultivation densities, when comparing the values for 28/04 and 08/06, the Nitrogen percentage (%) increased for both densities, which corresponds to the variation over sampling time.

Table 12 - ANOVA results for the effect of sampling time and stocking density on *Gracilaria gracilis* Nitrogen (N) content during the stocking density trial. Values in bold represent significant differences.

ANOVA	df	MS	F	P
Sampling time = S	2	0,3125	5,200	0,0283
Stocking density=D	1	0,1112	1,850	0,2036
S * D	2	0,04546	0,7565	0,4944

Regarding the Carbon and Nitrogen (CN) Ratio there was no significant difference between cultivation densities (Table 13, p-value = 0,133) but there was variation over the sampling time (Table 13, p-value= 0,024). There was interaction between cultivation density and sampling time (Table 13, p-value=0,0438). The highest value recorded was $7,7 \pm 0,1\%$ (28/04) for the 5 Kg/m² density and the lowest was $6,5 \pm 0,3\%$ (08/06) for 3

Kg/m² density. Even though there was no significant difference between cultivation densities, comparing the values for 28/04 and 08/06, the CN ratio decreased for both densities, which corresponds to the significant variation over sampling time.

Table 13 - ANOVA results for the effect of sampling time and stocking density on *Gracilaria gracilis* Carbon and Nitrogen (CN) Ratio during the stocking density trial. Values in bold represent significant differences.

ANOVA	df	MS	F	P
Sampling time = S	2	0,4633	5,567	0,0237
Stocking density=D	1	0,2226	2,675	0,1330
S * D	2	0,3618	4,347	0,0438

Table 14 - Tukey's multiple comparisons test for the effects of sampling time and stocking density on *Gracilaria gracilis* Carbon and Nitrogen (CN) Ratio during the stocking density trial.

Compared groups	P- values
28/04:3kg/m ² vs. 28/04: 5 Kg/m ²	0,6368
28/04:3kg/m ² vs. 16/05:3kg/m ²	0,9979
28/04:3kg/m ² vs. 16/05:5 Kg/m ²	0,9532
28/04:3kg/m ² vs. 08/06:3kg/m ²	0,1818
28/04:3kg/m ² vs. 08/06:5 Kg/m ²	0,9993
28/04:5Kg/m ² vs. 16/05: 3kg/m ²	0,7812
28/04:5Kg/m ² vs. 16/05: 5 Kg/m ²	0,1970
28/04:5Kg/m ² vs. 08/06: 3kg/m ²	0,0140
28/04:5Kg/m ² vs. 08/06:5 Kg/m ²	0,3850
16/05:3kg/m ² vs.16/05: 5 Kg/m ²	0,7261
16/05:3kg/m ² vs. 08/06: 3kg/m ²	0,0577
16/05:3kg/m ² vs. 08/06: 5 Kg/m ²	0,9543
16/05:5Kg/m ² vs.08/06: 3kg/m ²	0,4148
16/05:5Kg/m ² vs. 08/06: 5 Kg/m ²	0,9910
08/06:3kg/m ² vs. 08/06:5 Kg/m ²	0,1978

Protein content

The Protein content showed no significant difference between the luminosities (Table 15, p-value = 0,706) or over sampling time (Table 15, p-value = 0,346). There was no interaction between cultivation density and sampling time (Table 15, p-value = 0,616). Neither the sampling time nor the cultivation density influenced the protein content in the algae. The mean protein content varied between $29,4 \pm 1,1$ and $33,9 \pm 2,8$ %.

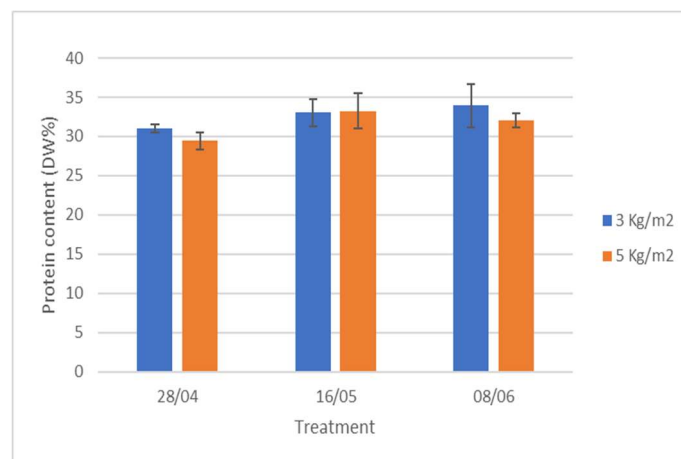


Figure 27 - The effect of sampling time and stocking density on Protein content over the course of *Gracilaria gracilis* stocking density trial. Error bar represents standard deviation.

Table 15 - ANOVA results for the effect of sampling time and stocking density on *Gracilaria gracilis* Protein content during the stocking density trial.

ANOVA	df	MS	F	P
Sampling time = S	2	12,21	3,495	0,0706
Stocking density=D	1	4,343	1,244	0,2909
S * D	2	1,776	0,5085	0,6162

Total polysaccharide content

The Total Polysaccharide content showed a significant difference between cultivation densities (Table 16, p-value=0,0002), but not between sampling times (Table 16, p-value=0,1679). There was no interaction between cultivation density and sampling time (Table 16, p-value=0,2759). The cultivation density influenced the Total polysaccharide content of *Gracilaria gracilis* with the stocking density of 5 kg/m² showing the highest concentrations.

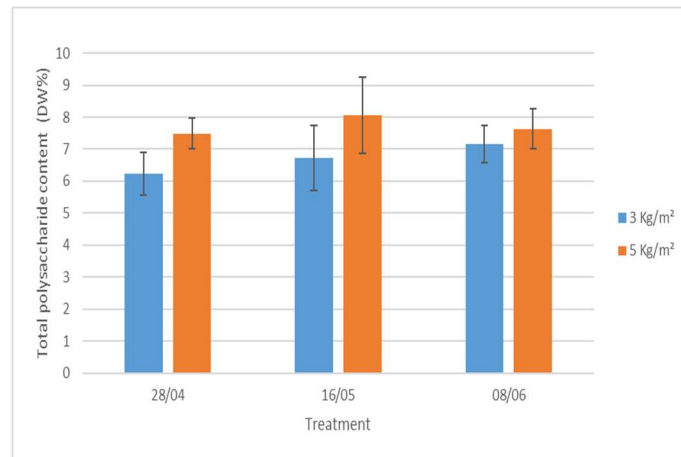


Figure 28 - The effect of sampling time and stocking density on Total polysaccharide content over the course of *Gracilaria gracilis* stocking density trial. Error bar represents standard deviation.

For the 3 kg/m² stocking density, total polysaccharide content ranged between 6,2±0,7% and 7,2±0,6%. A slight increase over time was observed, although not statistically significant. For the stocking density of 5 kg/m² total polysaccharide content ranged between 7,5±0,5% and 8,1±1,2%.

Table 16 - ANOVA results for the effect of sampling time and stocking density on *Gracilaria gracilis* total polysaccharide content during the stocking density trial. Values in bold represent significant differences.

ANOVA	Df	MS	F	P
Sampling time = S	2	1,287	1,864	0,1679
Stocking density=D	1	11,69	16,94	0,0002
S * D	2	0,9173	1,329	0,2759

Total pigments

The concentration of “Chlorophyll *a*” exhibited a significant difference between sampling times (Table 17, p -value=0,04), but not between cultivation densities (Table 17, p -value=0,84). There was no interaction between cultivation density and sampling time (Table 17, p -value=0,11).

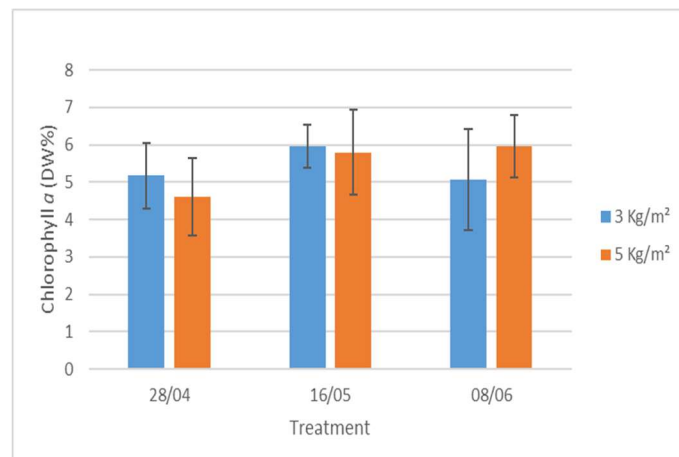


Figure 29 - The effect of sampling time and stocking density on Chlorophyll *a* content over the course of *Gracilaria gracilis* stocking density trial. Error bar represents standard deviation.

The highest value recorded was $6,0 \pm 0,8\%$ (08/06) on the 5 kg/m^2 cultivation density and the lowest was $4,6 \pm 1,0\%$ (28/04) on the 3 kg/m^2 cultivation density. It was therefore concluded that the treatment did not significantly affect the “Chlorophyll *a*” concentration of *Gracilaria gracilis*, even though some variations occurred during sampling time. The concentration of the pigment increased over sampling time in the 5 Kg/m^2 stocking density treatment.

Table 17 - ANOVA results for the effect of sampling time and stocking density on *Gracilaria gracilis* chlorophyll *a* content during the stocking density trial. Values in bold represent significant differences.

ANOVA	df	MS	F	P
Sampling time = S	2	0,0003504	3,469	P=0,0403
Stocking density=D	1	4,155e-006	0,04113	P=0,8403
S * D	2	0,0002280	2,257	P=0,1172

The concentration of chlorophyll *b* showed a statistically significant difference over sampling time (Table 18, p -value=0.003), but not between cultivation densities (Table 18, p -value=0,79). There was no interaction between cultivation density and sampling time (Table 18, p -value=0,9435)

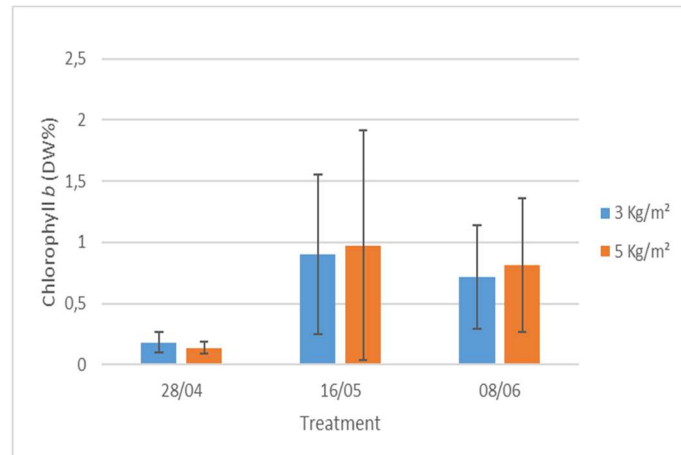


Figure 30 - Chlorophyll b content over the course of *Gracilaria gracilis* stocking density trial. Error bar represents standard deviation.

The highest value recorded was $0.98 \pm 0.9\%$ (08/06) and the lowest was $0.1 \pm 0.5\%$ (28/04), both in the 5 kg/m² density. It was therefore concluded that the cultivation density did not significantly affect the chlorophyll *b* concentration of *Gracilaria gracilis* but over sampling time its concentration was positively affected in both treatments.

Table 18 - ANOVA results for the effect of sampling time and stocking density on *Gracilaria gracilis* chlorophyll b content during the stocking density trial. Values in bold represent significant differences.

ANOVA	Df	MS	F	P
Sampling time = S	2	0,0002291	6,708	0,0030
Stocking density=D	1	2,221e-006	0,06504	0,7999
S * D	2	1,990e-006	0,05827	0,9435

The concentration of carotenoids showed a significant difference over time, (Table 19, p-value=0,0289), but not between treatments (Table 19, p-value=0,37). There was no interaction between cultivation density and sampling time (Table 19, p-value=0,2160).

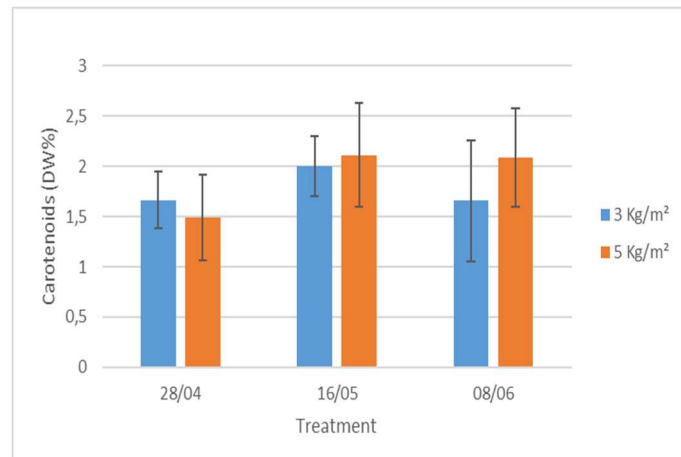


Figure 31- Carotenoid content over the course of *Gracilaria gracilis* stocking density trial. Error bar represents standard deviation

The lowest value recorded was $1.5 \pm 0.4\%$ (28/04) and the highest value was $2.1 \pm 0.5\%$ (16/05) both registered in the 5 Kg/m^2 cultivation density. The density of 5 Kg/m^2 showed the most apparent and stable increase in concentration. It was therefore considered that the cultivation densities did not significantly affect the carotenoid concentration of *Gracilaria gracilis*, although there were some changes over time.

Table 19 - ANOVA results for the effect of sampling time and stocking density on *Gracilaria gracilis* carotenoid content during the stocking density trial. Values in bold represent significant differences.

ANOVA	Df	MS	F	P
Sampling time = S	2	8,303e-005	3,859	0,0289
Stocking density=D	1	1,733e-005	0,8054	0,3746
S * D	2	3,421e-005	1,590	0,2160

Total phycobiliproteins content

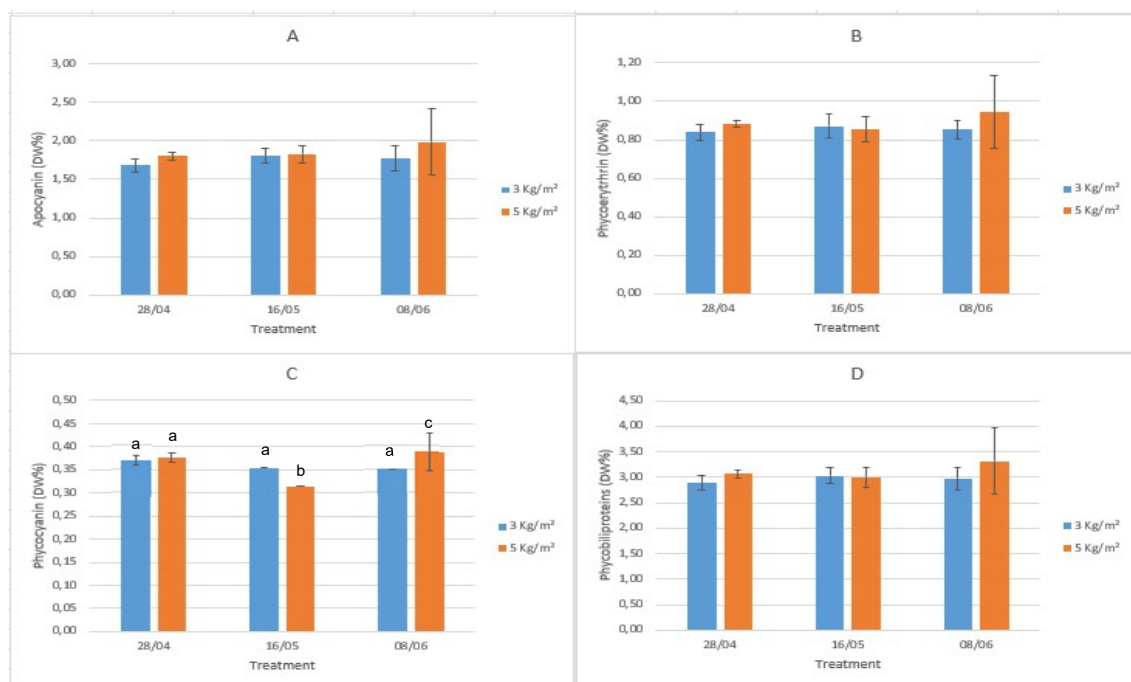


Figure 32 - Phycobiliprotein content over the course of the *Gracilaria gracilis* stocking density trial. Graphic A corresponds to Apocyanin content; B to Phycoerythrin; C to Phycocyanin and D to Total phycobiliproteins. Error bar represents standard deviation. Different letters indicate significant difference according to the Tukey's multiple comparison test (p -value = 0,05).

The Apocyanin content did not show statistically significant variation between cultivation densities (Table 20, p -value=0,0627) nor between sampling times (Table 20, p -value=0,235). The lowest Apocyanin concentration was $1,7 \pm 0,1\%$ (28/04) for the 3 Kg/m² stocking density and the highest was $2,0 \pm 0,5\%$ (08/06) on the 5 Kg/m² stocking density. Overall, neither the cultivation density nor the sampling time affected the total Apocyanin content of *Gracilaria gracilis*.

Table 20 - ANOVA results for the effect of sampling time and stocking density on *Gracilaria gracilis* Apocyanin content during the stocking density trial

ANOVA	Df	MS	F	P
Sampling time = S	2	2,716e-005	1,496	0,2357
Stocking density=D	1	6,636e-005	3,655	0,0627
S * D	2	1,695e-005	0,9339	0,4010

The Phycoerythrin content did not show statistically significant variation on any of the cultivation densities (Table 21, p -value=0,17) nor throughout the sampling time (Table 21, p -value=0,43). There was no interaction between factors (Table 21, p -value=0,24).

Neither the cultivation densities nor the sampling time affected the total Phycoerythrin content of *Gracilaria gracilis*.

The lowest Phycoerythrin concentration was $0,8 \pm 0,1\%$ for both densities (on 28/04 for the 3Kg/m^2 and on 16/05 for the 5 Kg/m^2) and the highest value was $0,9 \pm 0,2\%$ (08/06) for the 5 Kg/m^2 .

Table 21 - ANOVA results for the effect of sampling time and stocking density on *Gracilaria gracilis* Phycoerythrin content during the stocking density trial.

ANOVA	Df	MS	F	P
Sampling time = S	2	3,135e-006	0,8560	0,4321
Stocking density=D	1	7,143e-006	1,951	0,1699
S * D	2	5,406e-006	1,476	0,2401

The phycocyanin content did not show no statistically significant difference between treatments (Table 22, p-value = 0,654). However, there was variation over sampling time (Table 22, p-value < 0,001) and interaction between factors (Table 22, p-value < 0,001). The lowest value was $0,3 \pm 0,001\%$ (16/05) and the highest value was $0,4 \pm 0,04\%$ (08/06) both for the 5 kg/m^2 stocking density.

Table 22 - ANOVA results for the effect of sampling time and stocking density on *Gracilaria gracilis* Phycocyanin content during the stocking density trial. Values in bold represent significant differences.

ANOVA	Df	MS	F	P
Sampling time = S	2	2,945e-006	20,20	<0,0001
Stocking density=D	1	2,971e-008	0,2037	0,6540
S * D	2	2,745e-006	18,83	<0,0001

Table 23 - Tukey's multiple comparisons test for the effect of sampling time and stocking density on *Gracilaria gracilis* Phycocyanin content during the stocking density trial. Values in bold represent significant differences.

Compared groups	P- values
28/04:3kg/m ² vs. 28/04: 5 Kg/m ²	0,9629
28/04:3kg/m ² vs. 16/05:3kg/m ²	0,7576
28/04:3kg/m ² vs. 16/05:5 Kg/m ²	<0,0001
28/04:3kg/m ² vs. 08/06:3kg/m ²	0,5272
28/04:3kg/m ² vs. 08/06:5 Kg/m ²	0,3263
28/04:5Kg/m ² vs. 16/05: 3kg/m ²	0,2413
28/04:5Kg/m ² vs. 16/05: 5 Kg/m ²	<0,0001
28/04:5Kg/m ² vs. 08/06: 3kg/m ²	0,1163
28/04:5Kg/m ² vs. 08/06:5 Kg/m ²	0,8494
16/05:3kg/m ² vs.16/05: 5 Kg/m ²	0,0010
16/05:3kg/m ² vs. 08/06: 3kg/m ²	0,9983
16/05:3kg/m ² vs. 08/06: 5 Kg/m ²	0,0056
16/05:5Kg/m ² vs.08/06: 3kg/m ²	0,0036
16/05:5Kg/m ² vs. 08/06: 5 Kg/m ²	<0,0001
08/06:3kg/m ² vs. 08/06:5 Kg/m ²	0,0016

The total phycobiliprotein content showed no statistically significant difference over sampling time (Table 24, p-value=0,304) or between cultivation densities (Table 24, p-value=0,099). There was no interaction between factors (Table 24, p-value=0,22). Neither the cultivation densities nor the sampling time affected the total phycobiliprotein content of *Gracilaria gracilis*. The phycobiliprotein content varied between 2,9±0,1% (28/04 on the 3 kg/m² stocking density) and 3,3±0,7% (08/06 on the 5 kg/m² stocking density).

Table 24 - ANOVA results for the effect of sampling time and stocking density on *Gracilaria gracilis* Phycobiliproteins content during the stocking density trial.

ANOVA	Df	MS	F	P
Sampling time = S	2	5,177e-005	1,223	0,3045
Stocking density=D	1	0,0001208	2,854	0,0986
S * D	2	6,549e-005	1,547	0,2247

Phenolic content

The phenolic content showed a significant difference between sampling times, (Table 25, p -value=0,0081), and between cultivation densities, (Table 25, p -value=0,0246) with interaction between the factors (Table 25, p -value = 0,006).

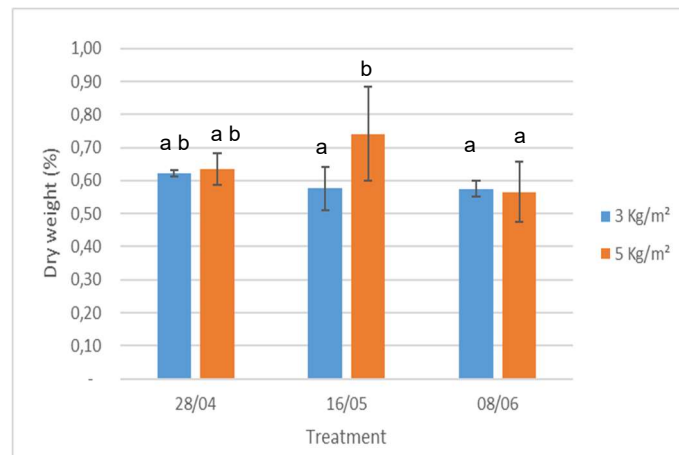


Figure 33 - Total Phenolic content over the course of *Gracilaria gracilis* stocking density trial. Error bar represents standard deviation. Different letters indicate significant difference according to the Tukey's multiple comparison test (p -value = 0,05).

The lowest values for both treatments were recorded on 08/06. For the 3 kg/m² stocking density, the lowest phenolic content was 0,57±0,02% (08/06) and the highest was 0,62±0,01% (28/04). For the stocking density of 5 kg/m² the lowest value was 0,57±0,02% (08/06) and the highest value was 0,74±0,14% (16/05), which corresponded to the highest value registered overall. It was therefore concluded that cultivation densities and sampling time influenced the content of phenolic compounds in *Gracilaria gracilis*.

Table 25 - ANOVA results for the effect of sampling time and stocking density on *Gracilaria gracilis* Phenolic content during the stocking density trial. Values in bold represent significant differences.

ANOVA	Df	MS	F	P
Sampling time = S	2	0,1436	5,416	0,0081
Stocking density=D	1	0,1441	5,433	0,0246
S * D	2	0,1538	5,801	0,0060

Table 26 - Tukey's multiple comparisons test for the effect of sampling time and stocking density on *Gracilaria gracilis* Phenolic content during the stocking density trial. Values in bold represent significant differences.

Compared groups	P- values
28/04:3kg/m ² vs. 28/04: 5 Kg/m ²	0,9998
28/04:3kg/m ² vs. 16/05:3kg/m ²	0,8969
28/04:3kg/m ² vs. 16/05:5 Kg/m ²	0,0826
28/04:3kg/m ² vs. 08/06:3kg/m ²	0,8804
28/04:3kg/m ² vs. 08/06:5 Kg/m ²	0,7818
28/04:5Kg/m ² vs. 16/05: 3kg/m ²	0,7628
28/04:5Kg/m ² vs. 16/05: 5 Kg/m ²	0,1516
28/04:5Kg/m ² vs. 08/06: 3kg/m ²	0,7384
28/04:5Kg/m ² vs. 08/06:5 Kg/m ²	0,6111
16/05:3kg/m ² vs.16/05: 5 Kg/m ²	0,0014
16/05:3kg/m ² vs. 08/06: 3kg/m ²	>0,9999
16/05:3kg/m ² vs. 08/06: 5 Kg/m ²	0,9998
16/05:5Kg/m ² vs.08/06: 3kg/m ²	0,0012
16/05:5Kg/m ² vs. 08/06: 5 Kg/m ²	0,0006
08/06:3kg/m ² vs. 08/06:5 Kg/m ²	>0,9999

5.1.4. Discussion

According to statistics, there was no significant difference between cultivating *Gracilaria gracilis* with 3 kg/m² or 5 kg/m² stocking density, although the 3 kg/m² density presented a higher growth rate than the 5 kg/m². Even though it is not corroborated by statistic, we can find this to be consistent with what was reported by Abreu et al. (2011), where the species was cultivated in an integrated system with a fish farm for a period of 9 months at stocking densities of 3 kg/m², 5 kg/m² and 7 kg/m². The density with the highest growth rate was 3 kg/m². The 7 kg/m² density had the poorest performance, losing biomass and having a negative RGR at one time of the year (autumn). The author affirms that growth rate tends to diminish as stocking density gets higher, which is confirmed by our trial.

According to Redmond et al. (2014), the best density for the cultivation of macroalgae of the genus *Gracilaria* is between 2 and 4 g/L. In the case of our cultivation, in 60 L tanks, this would correspond to between 120 and 240 g/L, but the densities used in our experiment, converted to g/L, were 9.5 g/L (a total of 570 g, corresponding to 3 kg/m²) and 15.8 g/L (a total of 950 g, corresponding to 5 kg/m²) - much higher than those given by the author. As lower stocking densities were not tested, we cannot draw a parallel

with the author. However, *Gracilaria gracilis* managed to grow at both densities in contact with the aquaculture effluent.

Regarding environmental conditions, there were no significant variations for any of the parameters evaluated. Therefore, it can be considered that the physical-chemical parameters were not associated with differences in growth rate (RGR) between one stocking density and another.

Regarding elemental analysis (C, N and CN ratios), there were no significant differences between treatments, meaning that none of the densities studied had a higher content of these elements than the other. Only the nitrogen (N) content showed a significant difference over time, with an increase in both densities during cultivation which signifies that *Gracilaria* was able to accumulate nitrogen in its tissues.

The increase in nitrogen content in *Gracilaria gracilis* accompanied by little variation in carbon content explains the decrease in the CN ratio over the course of the test, with a higher value at the beginning of the test than at the end.

The hydrodynamics of the cultivation site influence the removal of nitrogen from the water, as the rate of removal increases with the level of agitation of the water (Smit, 2002). The author also mentions *Gracilaria gracilis* as an organism with good affinity for NO_3 removal.

In terms of biochemical composition, the only significant difference between treatments was in total polysaccharide and phenolic content. The 5 kg/m² stocking density had higher values than the 3 kg/m² stocking density. Even though statistics did not recognize one stocking density as more efficient than the other, the lower stocking density had a larger growth rate than the higher density. It could be hypothesised that if the primary objective of algae cultivation is not to maximize the biomass increase but to modify their biochemical composition, cultivation at a stocking density of 5 kg/m² could be carried out in contact with an effluent with a composition similar to the aquaculture in which the tests were carried out (section 2.2 Effluent composition). According to Rasyid et al. (2019), not only polysaccharides, but carbohydrates in general, account for approximately 63% of the dry weight composition (DW%) of *Gracilaria gracilis* in the wild. However, the total polysaccharide content obtained in this study was approximately between 6% and 8% of DW. As the remaining carbohydrates were not analysed, a full parallel cannot be drawn. The carbohydrate content of algae varies between species and depends on the physico-chemical parameters during cultivation (temperature, salinity, nutrients) (Said et al., 2018). Regarding phenolic compounds, Francavilla et al. (2013) recognise

that its content is highly variable and seasonal, even in conditions with little variation. In this study, the highest levels of phenolics were found in the summer, but as our study only took place in the spring, we can't draw a parallel.

Considering total pigments, no differences between stocking densities were observed, only variations over time. The content of the three pigments fluctuated over the sampling weeks, with an increase (from 28/4 to 16/5) and a decrease (from 16/5 to 08/06) in pigment content for both stocking densities. This translates into an unsatisfactory result in terms of biovalorization of macroalgae, given that the cultivation conditions appeared to harm the concentration of photosynthetic pigments. Silva-Brito et al. (2021) evaluated the biochemical composition of *Gracilaria gracilis* in IMTA system cultivation with various light spectre conditions and even through no significant differences were registered the chlorophyll a content decreased in all conditions.

Regarding the total quantification of phycobiliproteins, overall neither of the stocking densities promoted a better concentration than the other. Phycocyanin, on the other hand, presented variations over time, but with a decrease in concentration over time. This was also described in a IMTA system trial by Silva-Brito et al. (2021), where all light conditions evaluated conducted to a Phycocyanin content decrease. According to Francavilla et al. (2013), among the phycobiliproteins present in *Gracilaria gracilis*, the least abundant is phycocyanin, which is in line with the results obtained. However, he also states that the most abundant is R-phycoerythrin, which was not the case in our test, since the phycobiliprotein with the highest % relative to the dry weight of the seaweed was apocyanin.

5.2. *Ulva* sp. light intensity trial

5.2.1. Objectives

This light intensity trial was carried out to determine the most favourable luminosity conditions to maximize *Ulva* sp. growth. The objective of this trial was to evaluate whether the reduction in light intensity could inhibit the growth of epiphytes, episodes of sporulation and avoid photoinhibition, if any of these phenomena occurred. The growth rate was evaluated at “low light” and “high light” conditions over a period of five weeks. The biochemical composition (total polysaccharide content, phenols, total pigments) of the produced biomass was determined throughout the experiment.

5.2.2. Methodology

5.2.2.1. Experimental procedure

Ulva sp. sampling was carried out on 30 March 2023 in the Ria de Aveiro, near Torreira (40° 45' 38" N 8° 42' 20" W).

Seaweeds were placed in sampling bags, packed in a thermobox and taken to the laboratory. At the laboratory, seaweeds were washed with filtered seawater to remove sediments and epiphytes and kept in a 100L tank with filtered seawater at 16°C and 100 $\mu\text{mol photons m}^2/\text{s}$, with a 12h/12 h light/dark cycle for one week.

Seaweeds were transferred to the cultivation system located in Aquacria A.S. and acclimated for two weeks in the effluent conditions. The experiment started on April 28th. Seaweeds were distributed in 60L tanks according to the 2 pre-defined treatments based on light intensity (high light and low light), using a stocking density of 5Kg/m². For the low light treatment, tanks had their walls covered with black stretch film, resulting in 53% light attenuation. The high light tanks were left uncovered. Three replicates were used for each condition. Cultivation proceeded under ambient conditions, with a continuous effluent flow between 90 and 180 L/h for the duration of the trial.

Weekly visits to the aquaculture were made to monitor seaweed growth performance. Environmental conditions such as pH, temperature, salinity, and dissolved oxygen were measured using a multi-parameter probe. Seaweeds were removed from the tanks and placed in draining boxes, the tanks were cleaned and after a drying period of 90 minutes each replicate was weighed.

The predefined densities for each tank were re-established and the flow rate of each tank was manually adjusted. The biochemical analysis procedures were the same as those described in section 5.1.2, except for phycobiliprotein quantification. Samples were taken from all tanks at the beginning of the experiment (28/04), at mid-term (16/05) and at the end of the experiment (08/06) to monitor the evolution of the biochemical composition of the seaweed.

5.2.2.2. Calculations and Statistical analyses

Relative growth rate (RGR % day⁻¹) was calculated according to the following formula (Abreu et al., 2011):

$$RGR (\% \text{ day}^{-1}) = \frac{\ln(FW) - \ln(IW)}{T} \times 100$$

Where FW represented “final fresh weight”, IW, “initial fresh weight”, and T, “days in culture”.

Both growth rates and environmental conditions were calculated by averaging replicates from all timepoints for each treatment. Graphic representations were performed using Microsoft Excel (Microsoft 365 v. 2310). Luminosity data extracted from loggers was treated using HOBOWare (Version 3.7.26) and averaged per day, by treatment. Only data from 7h to 20h was considered, nighttime was omitted.

The Shapiro-Wilk test was used to evaluate the distribution of the data. Homogeneity of the variance was assessed by Levene’s test. If the data were normally distributed, a two-sample parametric t-test was used to evaluate significant differences between cultivation methods for RGR and all environmental conditions. However, if the data were not normally distributed, the Mann-Whitney test was used to evaluate significant differences between cultivation methods for RGR and all environmental conditions (non-parametric t-test). For the biochemical analyses, the Shapiro-Wilk test was also applied and 2-way ANOVA with Tukey’s multiple comparisons was used to assess significant differences between treatments and between sampling times. All statistical analysis were performed using GraphPad Prism (v 8.0.1). The protein content was indirectly calculated using the nitrogen-to-protein conversion factor of 6,25, as mentioned in Angell et al. (2016). Nitrogen removal was determined indirectly according to Kambey et al. (2020).

5.2.3. Results

5.2.3.1. Environmental conditions

Temperature, salinity, and pH were similar for both cultivation methods, only dissolved oxygen demonstrated significant differences, with the “high light” treatment averaging a higher content than “low light” treatment (Table 27; p-value < 0.05).

Water temperature varied from 19,8 to 21,9 °C with an average of 20,9 °C. The salinity in the effluent averaged 22,2‰ and varied between 21,9 and 22,6‰. The pH varied between 7,4 and 7,9, averaging 7,6 between treatments. The dissolved oxygen levels in the water varied between 75,6 % and 106,7 %.

Table 27 - Elemental analysis results for *Ulva* sp. light intensity trial. S.D represents standard deviation.

Environmental parameters	High light			Low light		
	N	mean ± S.D	Range	N	mean ± S.D	Range
Temperature (°C)	15	20,7 ± 0,6	19,8-21,8	15	20,9 ± 0,6	19,8 - 21,9
pH	15	7,6 ± 0,04	7,4 - 7,7	15	7,6 ± 0,1	7,4 - 7,9
Salinity (‰)	15	22,2 ± 0,2	22,0 - 22,6	15	22,1 ± 0,2	21,9 - 22,6
Dissolved O ₂ (%)	15	94,5 ± 6,6	84,0 - 106,7	15	86,9 ± 4,4	75,6 - 93,4

Figure 34 shows the variation in luminosity between 17/05 and 08/06, covering the light intensity test on *Ulva* sp. The luminosity data for the 'high light' treatment was higher than that for the 'low light' treatment until around 23/05. Between 23/05 and 29/05 the average values for low light were higher, but high light returned to its initial trend from 30/05 until the end of the experiment.

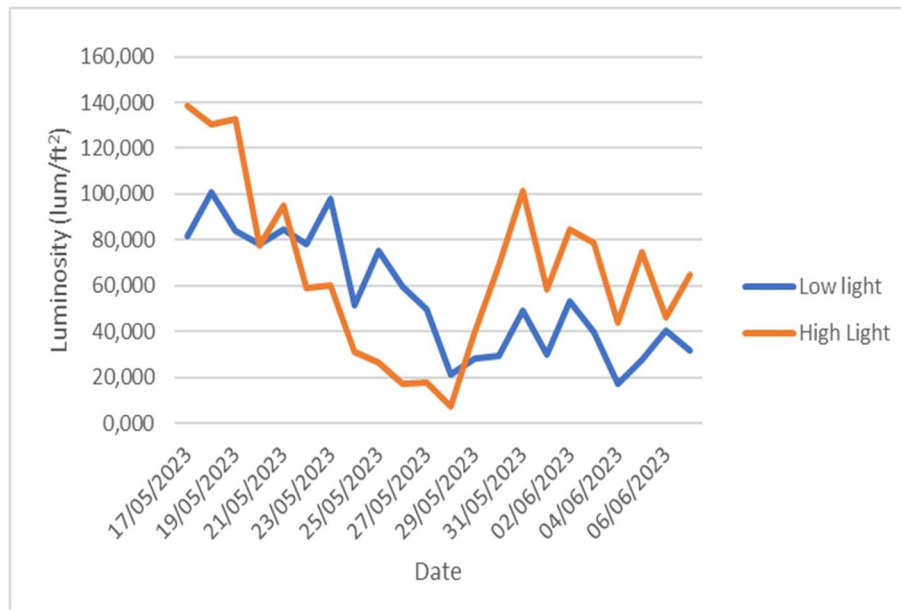


Figure 34 - Mean luminosity per treatment. Values presented per day, calculated using the values for the interval between 7 am and 8 pm, excluding the night hours.

5.2.3.2. Growth rate

Ulva sp. high light treatment performed well in the aquaculture effluent, however the low light treatment showed suboptimal results, averaging negative growth rate (biomass loss) in 4 of the 5 sampling periods (Figure 37). RGR ranged from - 0,7 to 8,5% a day (mean RGR = $5,4 \pm 2,3$ %/d) for the “high light” treatment and from - 2,74 to 2,49% a day (mean RGR = $-0,4 \pm 1,7$ %/d) for the low light treatment. There were significant differences between cultivation methods (Figure 37, $p\text{-value} > 0.05$).

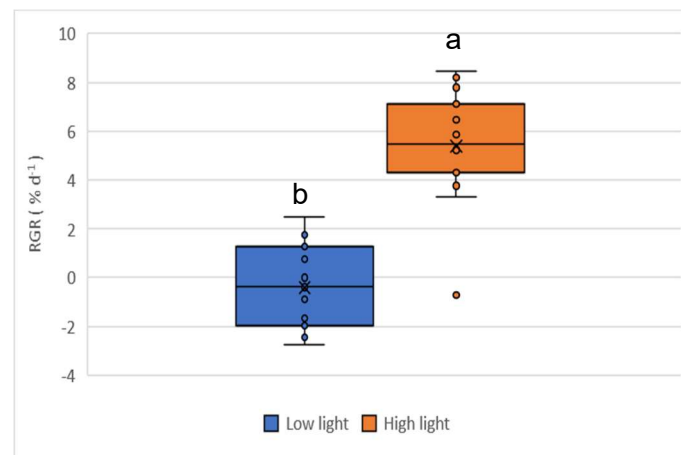


Figure 35 - Boxplot with RGR per treatment for *Ulva* sp. light intensity trial. Error bar represents standard deviation.

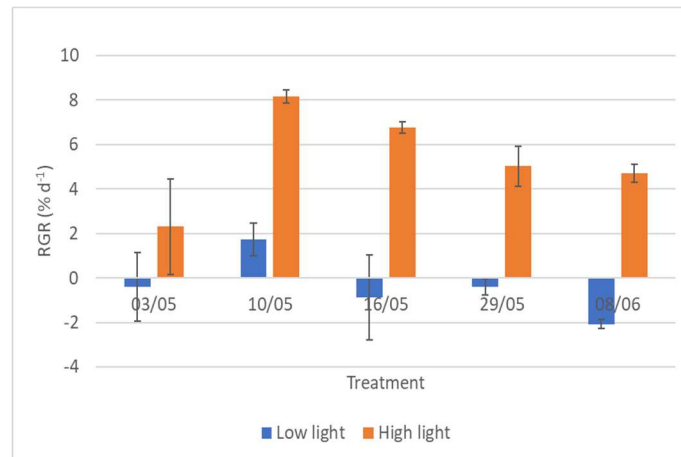


Figure 36 - Mean RGR per week by treatment for *Ulva* sp. light intensity trial. Error bar represents standard deviation

5.2.3.3. Biochemical characterization of *Ulva* sp.: Elemental analysis (carbon (C), nitrogen (N), CN Ratio and Nitrogen removal of *Ulva* sp.

The nitrogen removal for the “high light” treatment varied between $13,15 \pm 2,50$ and $18,28 \pm 4,06$ g/m²/week. For the Low light treatment as there was negative growth rate on several weeks, nitrogen removal values were not considered.

Table 28 - The effects of sampling time and light intensity on *Ulva* sp. elemental content during the light intensity trial.

Day	Treatment	Element		
		Carbon (C)	Nitrogen (N)	CN Ratio
28/04	Low light	34,2±0,8 % (n=3)	4,7±0,1 % (n=3) (b)	8,5±0,1 % (n=3)
	High light	35,0±1,8 % (n=3)	4,8±0,2 % (n=3) (b)	8,8±0,3 % (n=3)
16/05	Low light	35,3±0,5 % (n=3)	5,4±0,2 % (n=3) (a)	7,7±0,2 % (n=3)
	High light	33,4±0,7 % (n=3)	4,6±0,2 % (n=3) (b)	8,6±0,3 % (n=3)
08/06	Low light	35,5±0,6 % (n=3)	5,3±0,0 % (n=3) (a)	8,0±0,2 % (n=3)
	High light	33,8±0,9 % (n=3)	4,7±0,2 % (n=3) (b)	8,4±0,2 % (n=3)

The carbon (C) content showed no significant difference between the luminosities (Table 29, P-value = 0.07) or over sampling time (Table 29, P-value = 0.8). The mean Carbon (C) content varied between $33,4 \pm 0,7$ and $35,5 \pm 0,6$ %. The sampling time and the luminosity didn't influence the percentage of carbon in the algae.

Table 29 - ANOVA results for the effect of sampling time and light intensity on *Ulva* sp. Carbon (C) content during the light intensity trial.

ANOVA	Df	MS	F	P
Sampling time = S	2	0,2166	0,2273	0,8001
Luminosity=L	1	3,764	3,950	0,0702
S * L	2	3,510	3,683	0,0566

The Nitrogen (N) content showed a significant difference between luminosities (Table 30, p-value<0.0001) and over sampling time (Table 30, p-value=0,0262). There was interaction between treatments (Table 30, p-value=0,0006). The "low light" treatment favoured an increase in the nitrogen content in *Ulva* sp., showing a higher content in 2 of the sampling times.

Table 30 - ANOVA results for the effect of sampling time and light intensity on *Ulva* sp. Nitrogen (N) content during the light intensity trial. Values in bold represent significant differences.

ANOVA	Df	MS	F	P
Sampling time = S	2	0,1115	5,011	0,0262
Luminosity=L	1	0,8191	36,81	<0,0001
S * L	2	0,3271	14,70	0,0006

The highest value recorded for the high light treatment was $4.8 \pm 0,2\%$ (08/06) and the lowest was $4,6 \pm 0,2\%$ (16/5). For the low light treatment, the highest value recorded was $5,4 \pm 0,2\%$ (16/05) and the lowest was $4,7 \pm 0,1\%$ (28/04).

Table 31 - Tukey's multiple comparisons test for the effect of sampling time and light intensity on *Ulva* sp. on Nitrogen (N) content during the light intensity trial. Values in bold represent significant differences.

Compared groups	P- values
28/04: Low light vs.28/04: High light	0,9709
28/04: Low light vs. 16/05: Low light	0,0012
28/04: Low light vs. 16/05: High light	0,8878
28/04: Low light vs. 08/06:Low light	0,0060
28/04: Low light vs. 08/06:High light	>0,9999
28/04: High light vs. 16/05:Low light	0,0039
28/04: High light vs. 16/05:High light	0,4939
28/04: High light vs. 08/06:Low light	0,0210
28/04: High light vs. 08/06:High light	0,9792
16/05:Low light vs. 16/05:High light	0,0002
16/05:Low light vs. 08/06:Low light	0,9070
16/05:Low light vs. 08/06:High light	0,0013
16/05:High light vs. 08/06:Low light	0,0011
16/05:High light vs. 08/06:High light	0,8651
08/06:Low light vs. 08/06:High light	0,0066

Carbon and Nitrogen Ratio exhibited significant difference between luminosities (Table 32, P-value = 0,0014) and over sampling time (Table 32, p-value= 0,0075). There was no interaction between factors (Table 32, p-value=0,2084). The “high light” treatment showed higher CN ratio values than “low light” treatment, however the ratio decreased in both treatments, from 28/04 to 08/06.

Table 32 - ANOVA results for the effect of sampling time and light intensity on *Ulva* sp. Carbon and Nitrogen (CN) Ratio during the light intensity trial. Values in bold represent significant differences.

ANOVA	Df	MS	F	P
Sampling time = S	2	0,4733	7,872	0,0075
Light intensity=L	1	1,082	18,00	0,0014
S * L	2	0,1091	1,815	0,2084

For the High light treatment, the highest value recorded was $8,8 \pm 0,3\%$ (28/04) and the lowest was $8,4 \pm 0,2\%$ (08/06). For the Low light treatment, the highest value recorded

was $8.5 \pm 0.1\%$ (28/04) and the lowest was $7.7 \pm 0.2\%$ (16/05). For both treatments, the highest CN ratio values occurred on 28/04.

Protein content

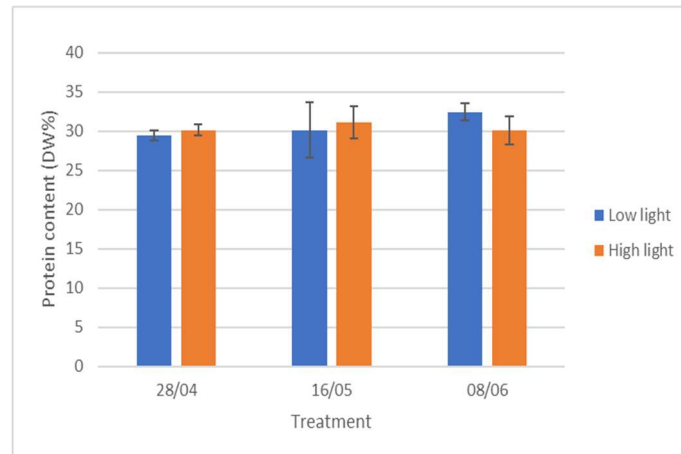


Figure 37 - Protein content over the course of *U/va* sp. light intensity trial. Error bar represents standard deviation.

The Protein content showed no significant difference between the light intensities (Table 33, p-value = 0.77) or over sampling time (Table 33, p-value = 0.346). There was no interaction between factors (Table 33, p-value=0,2236). Neither the sampling time nor the light intensity influenced the protein content in the algae. The mean protein content varied between 29.4 ± 0.7 and 32.5 ± 1.1 %.

Table 33 - ANOVA results for the effect of sampling time and light intensity on *U/va* sp. Protein content during the light intensity trial.

ANOVA	Df	MS	F	P
Sampling time = S	2	3,377	1,168	0,3466
Luminosity=L	1	0,2513	0,08695	0,7736
S * L	2	4,975	1,722	0,2236

Total Polysaccharide content

The total polysaccharide content showed a significant difference between luminosities (Table 34, p-value=0.0123) but not over sampling time (Table 34, p-value=0.8895). There was no interaction between factors (Table 34, p-value=0,604).

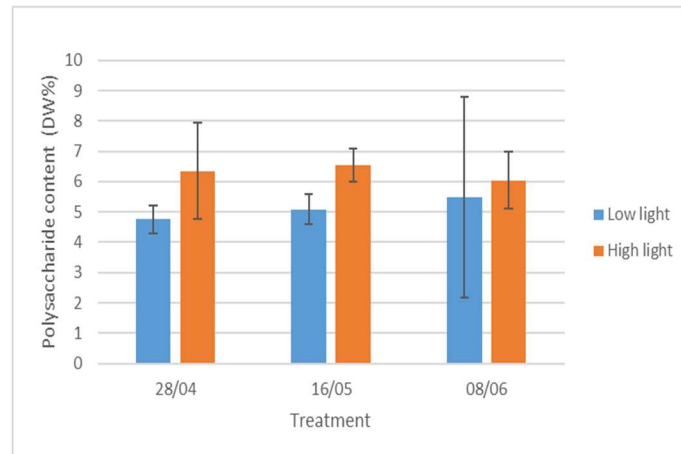


Figure 38 - Total polysaccharide content over the course of the *Ulva* sp. light intensity trial. Error bar represents standard deviation.

The high light treatment exhibited the highest values; however, the content didn't present much change over sampling time for neither of the treatments. The highest value recorded was $6,54 \pm 0,55\%$ (16/05) for "High light" and the lowest was $4,8 \pm 0,5\%$ (28/04) for "low light" treatment.

Table 34 - ANOVA results for the effect of sampling time and light intensity on *Ulva* sp. Total polysaccharide content during the light intensity trial. Values in bold represent significant differences.

ANOVA	Df	MS	F	P
Sampling time = S	2	0,3127	0,1174	0,8895
Light intensity=L	1	18,15	6,812	0,0123
S * L	2	1,356	0,5089	0,6046

Total pigments

The chlorophyll *a* content exhibited significant difference between the luminosities (Table 35, *P* value= 0.004), but not over sampling time (Table 35, *p*-value = 0,8293). There was no interaction between factors (Table 35, *p*-value=0,2573).

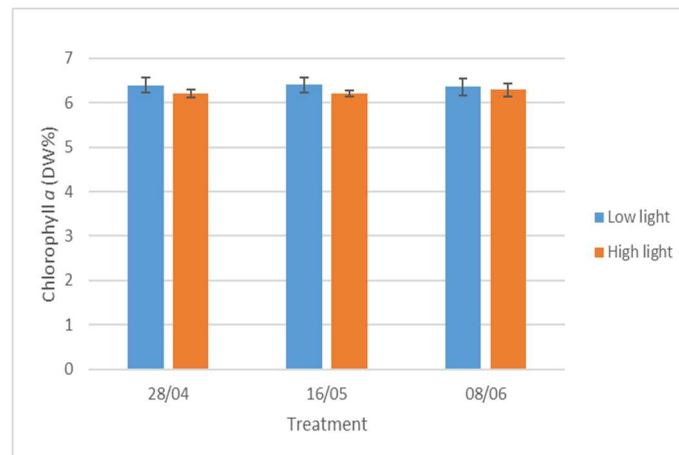


Figure 39 - Chlorophyll *a* content over the course of the *Ulva* sp. light intensity trial. Error bar represents standard deviation.

The “low light” treatment favoured the concentration of chlorophyll *a* in *Ulva* sp. even though there were no significant changes over the course of the experiment. The highest value recorded was $6,4 \pm 0,2\%$ (16/05) for the “low light” treatment and the lowest was $6,2 \pm 0,1\%$ (16/05) for the “high light” treatment.

Table 35 - ANOVA results for the effect of sampling time and light intensity on *Ulva* sp. Chlorophyll *a* content during the light intensity trial. Values in bold represent significant differences.

ANOVA	Df	MS	F	<i>P</i>
Sampling time = S	2	3,776e-007	0,1879	0,8293
Luminosity=L	1	2,902e-005	14,44	0,0004
S * L	2	2,812e-006	1,399	0,2573

The chlorophyll *b* content exhibited a significant difference over sampling time (Table 36, *P*-value < 0,0001) and between luminosities (Table 36, *P*-value <0.0001). There was no interaction between factors (Table 36, *p*-value=0,2120).

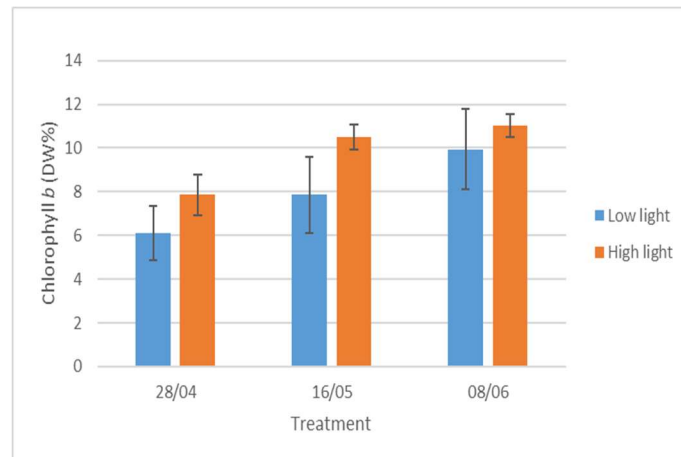


Figure 40 - Chlorophyll b content over the course of the *Ulva* sp. light intensity trial. Error bar represents standard deviation.

There was an increasing trend throughout the trial for both luminosities, but the high light treatment was the one that favoured the most increase over sampling time. The highest value observed was $11,0 \pm 0,5\%$ (08/06) in “high light” treatment and the lowest $6,1 \pm 1,2\%$ (28/04) in the “low light” treatment.

Table 36 - ANOVA results for the effect of sampling time and light intensity on *Ulva* sp. Chlorophyll b content during the light intensity trial. Values in bold represent significant differences.

ANOVA	Df	MS	F	P
Sampling time = S	2	0,005656	38,52	<0,0001
Luminosity=L	1	0,004108	27,97	<0,0001
S * L	2	0,0002358	1,606	0,2120

The carotenoid content exhibited a significant difference over time and between treatments (Table 37, p-value <0,0001). There was no interaction between factors (Table 37, p-value=0,2686).

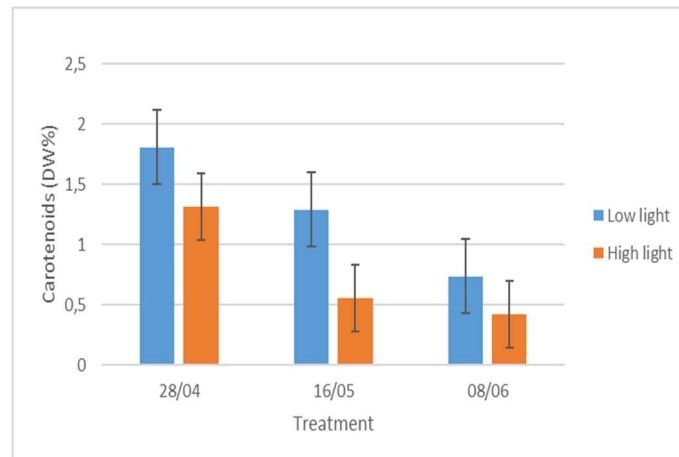


Figure 41 - Carotenoid content over the course of the *Ulva* sp. light intensity trial. Error bar represents standard deviation.

There was a decreasing trend throughout the experiment. For both treatments, the carotenoid content was higher on 28/04 than on 8/06, the end of the experiment. For the “high light” treatment, the highest value corresponded to $1,3 \pm 0,3\%$ (28/04) and the lowest to $0,4 \pm 0,2\%$ (08/06). The highest value for the “low light” treatment corresponded to $1,8 \pm 0,3\%$ (08/06) and the lowest value to $0,8 \pm 0,3\%$ (28/04).

However, the low light treatment showed higher values than the high light treatment at all sampling times, as the decrease was less prominent in this condition. The sampling time had a negative effect on the concentration of carotenoids in *Ulva* sp., since it reduced concentration for both luminosities.

Table 37 - ANOVA results for the effect of sampling time and light intensity on *Ulva* sp. Carotenoid content during the light intensity trial. Values in bold represent significant differences.

ANOVA	Df	MS	F	P
Sampling time = S	2	0,0004456	34,67	<0,0001
Luminosity=L	1	0,0003302	25,70	<0,0001
S * L	2	1,740e-005	1,354	0,2686

Phenolic content

The phenolic content exhibited a significant difference over time (Table 38, p-value < 0,0001) and between treatments (Table 38, p-value = 0,0004). There was no interaction between factors (Table 38, p-value=0,2973).

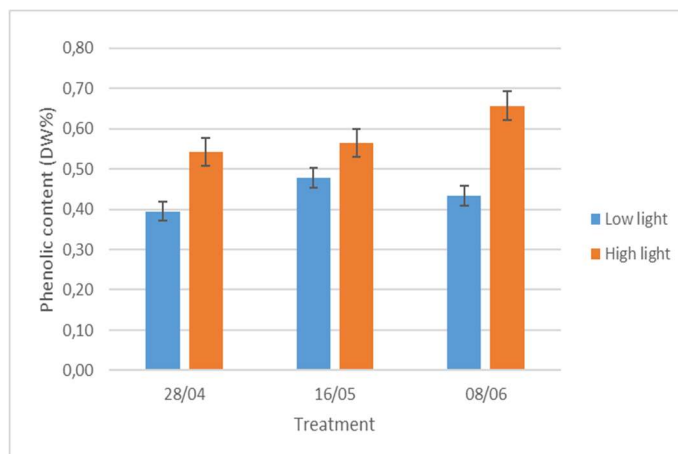


Figure 42 - Total Phenolic content over the course of the *Ulva* sp. light intensity trial. Error bar represents standard deviation.

There was an increasing trend on phenolic content throughout the experiment. For both treatments, the phenolic content was higher on 08/06 than on 28/04. The high light treatment had higher concentrations of phenolic compounds than the low light treatment in all samples. It was concluded that both luminosities and the sampling time positively influenced the phenolic content in *Ulva* sp.

Table 38 - ANOVA results for the effect of sampling time and light intensity on *Ulva* sp. Phenolic content during the light intensity trial. Values in bold represent significant differences.

ANOVA	Df	MS	F	P
Sampling time = S	2	4,800e-005	15,20	<0,0001
Luminosity=L	1	4,635e-005	14,68	0,0004
S * L	2	3,934e-006	1,246	0,2973

5.2.4. Discussion

One of the objectives of this experiment was to test whether reducing light intensity would inhibit epiphyte growth, whether *Ulva* sp. would be photoinhibited by natural light conditions (high light treatment) and whether low light conditions would allow sporulation

(if it occurred). However, no epiphyte growth and no sporulation events were observed in any of the treatments. *Ulva* sp. was not photoinhibited in the high light treatment as the average RGR values were much higher than in the low light treatment. The "low light" treatment proved to be counterproductive in the cultivation of *Ulva* sp., as the mean growth rate obtained was negative. There were losses in biomass (negative growth rates) in 4 of the 5 weeks evaluated. Even though it is recognized that it is most optimal to cultivate *Ulva lactuca* (*Ulva* genus holotype) with 1kg/m² stocking density (Neori et al. 1991), the 5kg/m² in "high light" conditions still managed to present satisfactory growth rates. Given the conditions of our cultivation system, namely the volume of the tanks, 60 L, the density of 5 kg/m² was considered high. This may have determined the loss of biomass in the "low light" treatment, as it exacerbated the effect of shading.

Regarding the environmental conditions, dissolved oxygen presented significant variations between treatments. A higher amount of dissolved oxygen (%) may have been recorded in the "high light" treatment due to the higher growth rate compared to the low light treatment, as this is related to a higher photosynthetic rate and oxygen production (Graham & Wilcox, 2000).

Regarding elemental analysis (C, N and CN ratios), there was no significant difference in content between light intensities nor throughout the sampling time for carbon content. This means that neither the time of exposure to the effluent nor the treatments had any difference in the carbon content of the seaweed. However, for Nitrogen content (%) the "low light" treatment demonstrated a higher nitrogen content (%) than the high light treatment at the end of the trial. This could mean that the "low light" conditions promoted a higher nitrogen concentration on the seaweed tissue than the high light treatment, however the nitrogen (N) removal calculations indicate otherwise. Since there was loss of biomass in 4 of the 5 weeks, the nitrogen removal calculation was mostly negative and was not showcased as an interval in the results. For the "high light" treatment, however, nitrogen removal varied between $13,15 \pm 2,50$ and $18,28 \pm 4,06$ g/m²/week. Shahr & Gutmann, (2021) obtained a higher nitrogen removal using *Ulva fasciata* as part of a biofilter with periphyton to treat an aquaculture effluent. Values obtained by the author were $25,5 \pm 0,1$ g/N/week, however the author did not use tank culture, but plastic nets. The density used was approximately 0,5kg/m² as 1Kg of seaweed was distributed by 2,0 m² of net. As aforementioned, this corresponds to the recommended stocking density for *Ulva* sp. cultivation which is approximately 1Kg/m² (Neori et al. 1991) This method helps with nitrogen removal as seaweed surface is more in contact with

streaming water, given that the process occurs by membrane diffusion (Smitt, 2002; Shahar & Gutmann, 2021).

The hydrodynamics of the cultivation site influence the removal of nitrogen from the water, as the rate of removal increases with the level of agitation of the water (Smit, 2002). A higher level of nitrogen removal could have been achieved by using higher flow rates. However, given the characteristics of the system, the use of very high flow rates could adversely affect the culture as the high water pressure could cause organisms to inadvertently escape through the top of the tank. One solution to this problem would be to place mesh netting over the tanks when high flow rates are used to prevent accidental losses. However, the application of this netting should not cause significant shading in order to maintain good light levels in the tanks.

The increase in nitrogen content in *Ulva* sp. accompanied by little variation in carbon content explains the decrease in the CN ratio over the course of the test, with a higher value at the beginning of the test than at the end.

In terms of biochemical composition, all the compounds analysed showed significant differences between the treatments applied.

The content of polysaccharides, phenolic compounds and chlorophyll *b* was favoured by the high light treatment, while the remaining total pigments (carotenoids and chlorophyll *a*) were favoured by the low light treatment. In the case of phenolic compounds, there were significant differences between treatments and over time, with an upward trend for both treatments. Thus, the cultivation conditions favoured the increase in phenolic content, regardless of the treatment applied. The presence of phenolic compounds, as well as carotenoids, is associated with antioxidant activity to protect against lipid peroxidation in algal membranes. These compounds may be of biotechnological value for extraction (Abd El-Baky et al., 2008). In the case of the total content of polysaccharides, the significant differences were only verified between treatments and not on a time scale. *Ulva* sp. is mainly composed of carbohydrates, such as starch and a characteristic polysaccharide, Ulvan (accounting for approximately 30% of dry weight), which is part of the cell wall composition (Dominguez & Erwann, 2019). The results obtained in this dissertation for polysaccharide content were, therefore, much lower than what is reported.

For chlorophyll *b* there was an upward trend for both treatments, but high light always showed higher values than low light. The carotenoid content of *Ulva* sp. showed a downward trend (statistically proven time-scale variation) in both treatments, but the "low

light" treatment always showed higher values than the "high light" treatment. For chlorophyll *a*, the "low light" treatment always showed higher values than "high light". The higher pigment content (chlorophyll *a* and carotenoids) in the low light treatment is linked to a physiological adaptation of the macroalgae that promotes its increase in the presence of lower light intensity in order to ensure photosynthesis and survival (Zervoudakis & Salachas, 2012).

The protein content of *Ulva* sp. did not vary during our trial and was within the range found in *Ulva* sp. collected from the wild, around 30 % of the dry weight content (Dominguez & Erwann, 2019).

It could be hypothesised that if the primary objective of algae cultivation was not to increase their biomass but to modify their biochemical composition, cultivation with low light conditions could be carried out to increase the content of chlorophyll *a* and carotenoids on seaweed. However, this procedure would be associated with biomass losses, as seen in the trial where negative growth rates were observed in 3 of the 5 weeks studied, which would be counterproductive on an industrial scale.

The light concealment rate is also influenced by the stocking density of algae being cultivated. For lower densities, shading could work better, but it was considered that *Ulva* sp. did not require shading at a stocking density of 5kg, since it grew well with natural light intensity. There was a period during the trial when the 'low light' treatment had a higher average light level than the 'high light' treatment. This may have been due to seaweed growth over the course of the week. As the seaweed grew, there was a gradual reduction in light intensity due to the increase in density, and as the light intensity measurements were made using a logger at the bottom of the tanks, this phenomenon may be exacerbated. The density of 5 kg/m² was considered high for the size of the tanks used in the test. If two loggers had been used, one on the surface and one on the bottom, or just one in the middle of the tank, greater differences in light intensity between the two treatments would have been obtained.

Overall, it can be concluded that growing *Ulva* sp. under "high light" conditions is more favourable than under low light, not only in terms of growth rate, but also in terms of improving the biochemical composition of *Ulva* sp. compared to low light. The characteristics of the effluent favoured the development of this species (see section 2.2 Effluent composition).

6. Conclusion

6.1. General considerations

In the pilot scale tests in this thesis, the exact flow rates are not shown, but rather a range of values, as this was difficult to adjust manually and showed fluctuations over time. As the cultivation system was installed outdoors, the RGR growth rates (% d⁻¹) may also have been influenced by weather conditions.

6.2. Final considerations

Four species of macroalgae were evaluated in contact with the aquaculture effluent and growth was observed in three of the species, *Codium tomentosum*, *Gracilaria gracilis* and *Ulva* sp. The species that performed the best in the pilot scale were *Gracilaria gracilis* and *Ulva* sp. "high light" treatment. The worst performing seaweed was *Chondrus crispus*, as the two attempts to grow it in the seaweed cultivation system were unsuccessful. In addition to *Chondrus crispus*, the "low light" treatment of *Ulva* sp. also proved to be counterproductive.

Chondrus crispus gave unsatisfactory results due to epiphyte development, making the cultures unviable. In a future trial, we recommend using higher densities than those tested in the trials of this thesis to reduce the possibility of epiphytes developing and shading the tanks, in this case using lower cultivation densities to avoid excessive shading of the algae in the lower layers of the tank. If successful, biochemical analyses of *Chondrus crispus* could also be carried out to assess its potential for biovalorisation. It was also observed that even though some growth occurred, the aquaculture effluent was not as favourable for vegetative propagation as the culture medium. It is therefore preferable to carry out the first phase of the hatchery in VSE medium, as recommended in the literature (Redmond et al. 2014). Regarding sexual reproduction in the laboratory, it was observed that the best conditions to achieve good development of the carpospores of *Chondrus crispus* were 14°C e 20 µmol photons m⁻² s⁻¹, since only in this condition was apical growth observed.

As it was observed that the fragmentation method did not favour the growth of *Codium tomentosum*, it is advisable to carry out a pilot scale trial in the future comparing different cultivation densities to obtain more growth data in this area. However, given the weather conditions, it is advisable to carry out this trial in spring or summer, although it will depend on the seasonality of the species to obtain biomass.

Regarding *Gracilaria gracilis*, it can be concluded that, although none of the cultivation densities was considered statistically superior to the other, the algae showed satisfactory growth in the aquaculture effluent. From a biovalorisation point of view, the culture conditions were not the most suitable for improving the overall biochemical composition of the algae. We recommend that an attempt be made to cultivate *Gracilaria gracilis* in contact with the same effluent, a light intensity trial should be executed at a stocking density of less than 3 Kg/m², to see the influence on growth rate, biochemical composition, and appearance of epiphytes.

Ulva sp. was the seaweed with the best performance and highest growth rate in contact with the aquaculture effluent at a density of 5 kg/m². However, in its light intensity test, its shading ("low light" treatment) was found to be very counterproductive in terms of growth rate and it is recommended that it not be used in industrial culture conditions. The content of polysaccharides, phenolic compounds and chlorophyll *b* was favoured by the high light treatment. In the future, a comparison between a low and a much higher density is recommended to see which density gives the highest growth rates and to compare this with the literature which recommends lower densities (Neori et al., 1991). Biochemical analyses could also be carried out on algae grown under both conditions to observe possible differences in the biochemical profile.

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Attachments

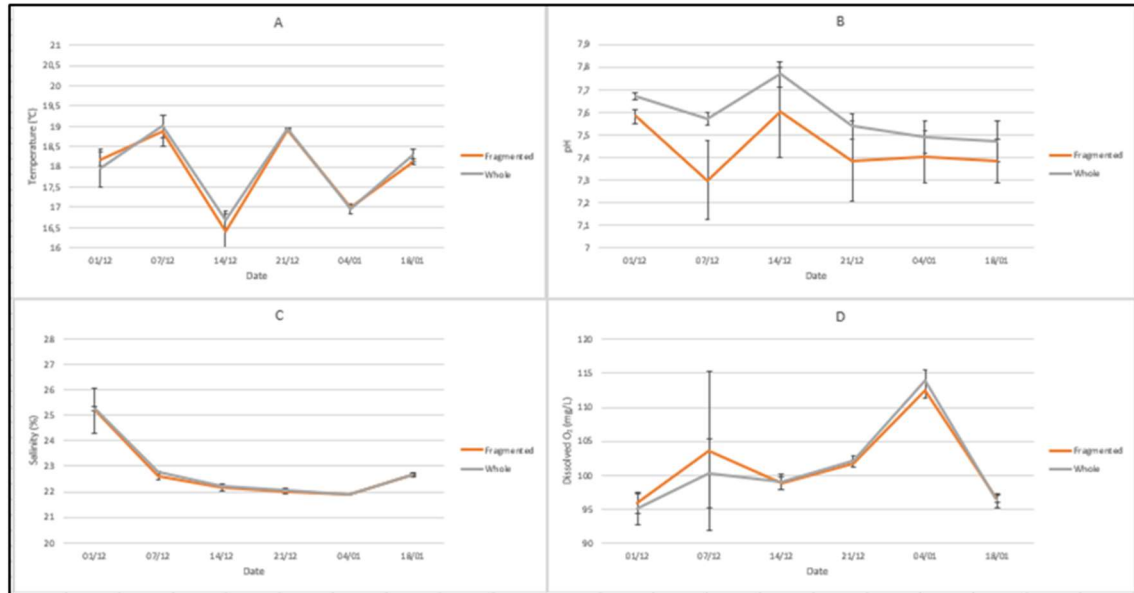


Figure 43 - Environmental conditions *Codium tomentosum* fragmentation trial

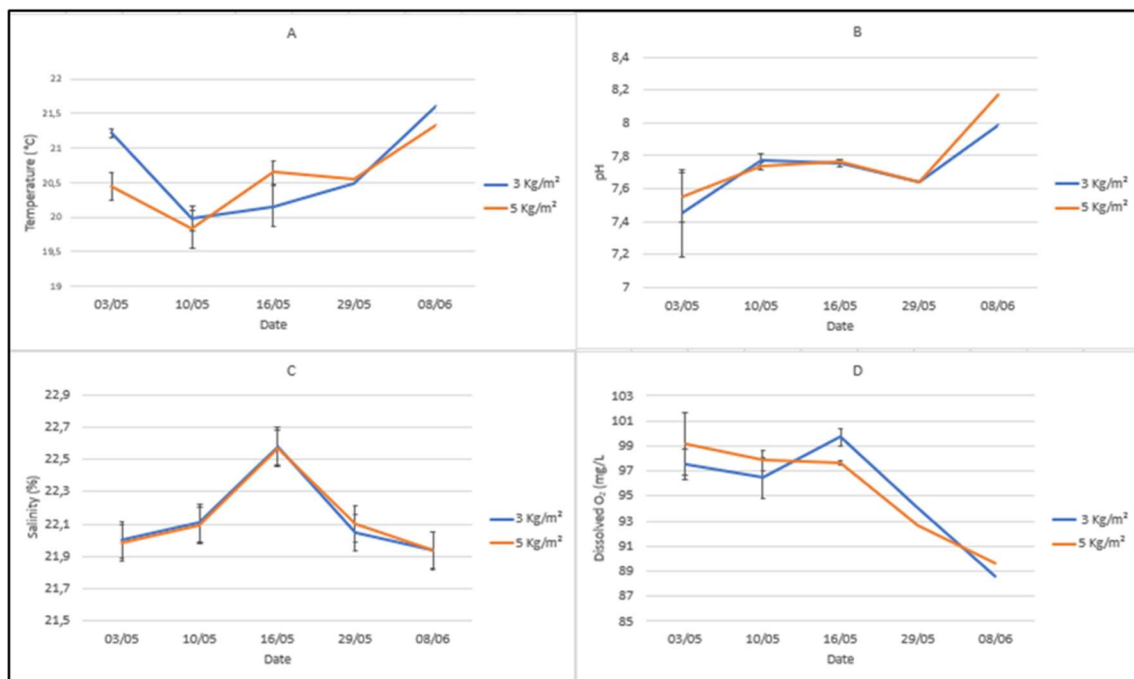


Figure 44 - Environmental conditions *Gracilaria gracilis* stocking density trial (A: temperature; B: pH; C: salinity; D: dissolved O₂).

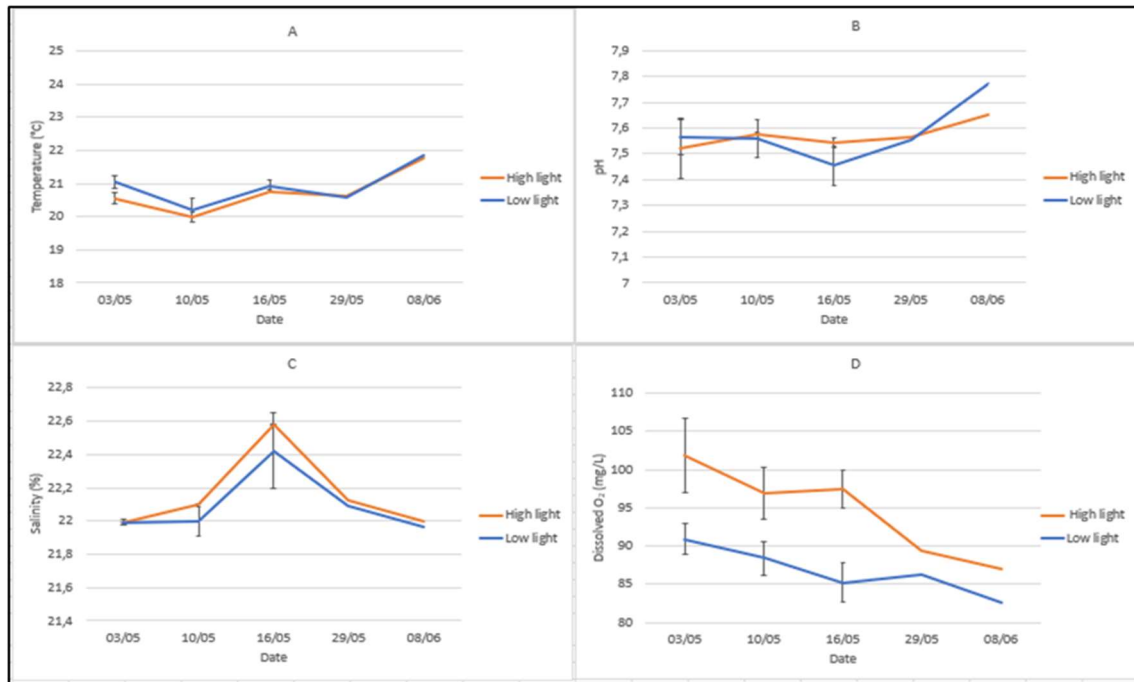


Figure 45 - Environmental conditions *Ulva* sp. light intensity trial (a: temperature b: pH c: salinity d: dissolved O₂).