POLITÉCNICO DE LEIRIA | ESCOLA SUPERIOR DE TURISMO E TECNOLOGIA DO MAR

Initial trials on *Plocamium cartilagineum* (Linnaeus) and *Sphaerococcus coronopifolius* (Stackhouse) cultivation and bioremediation potentials

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Dissertação para obtenção do Grau de Mestre em Aquacultura

Dissertação de mestrado realizada na EPPO-IPMA sob a orientação da Doutora Raquel Quintã (IPMA) e coorientação da Professora Especialista Teresa Baptista (IPL)

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Título: Initial trials on *Plocamium cartilagineum* (Linnaeus) and *Sphaerococcus coronopifolius* (Stackhouse) cultivation and bioremediation potentials.

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Inês Fernandes Freitas

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Vista aérea da Estação Piloto de Piscicultura de Olhão (EPPO) do IPMA onde foi realizado este trabalho.

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“A viagem não acaba nunca. Só os viajantes acabam. E mesmo estes podem prolongar-se em memória, em lembrança, em narrativa. Quando o visitante sentou na areia da praia e disse: “Não há mais que ver”, sabia que não era assim. O fim de uma viagem é apenas o começo de outra (...). É preciso recomeçar a viagem. Sempre.” José Saramago.

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Resumo

O uso de tecnologia para o desenvolvimento de uma aquacultura mais sustentável é fundamental para tornar o consumo mais responsável e sustentável e, por sua vez, uma economia melhor. Neste contexto, os sistemas de aquacultura multitrófica integrada (IMTA) que incluem algas no seu cultivo estão a ser projetados para mitigar os problemas ambientais provocados pelas diversas formas de aquacultura em que se fornece alimento. Várias espécies, nomeadamente algas vermelhas, têm-se mostrado eficientes para o crescimento neste tipo de sistemas. *Plocamium cartilagineum* e *Sphaerococcus coronopifolius* estão presentes em Portugal e prosperam na nossa costa, nomeadamente no Algarve. Estas duas espécies têm despertado a atenção de diversas indústrias devido às suas propriedades. Um sistema experimental de cultivo de algas usando tanques foi instalado numa aquacultura produtora de linguado, usando o efluente dos peixes no primeiro ensaio, e para o segundo e terceiro ensaio, o sistema de cultivo foi mudado de local e um "efluente artificial" foi criado e adicionado ao sistema, substituindo o efluente vindo dos peixes, para avaliar o potencial destas duas espécies como a componente biofiltradora num sistema IMTA. A influência da densidade de cultivo (1 a 9 g L^{-1} para o ensaio 1, e 2, 4, 6 e 8 g L^{-1} para os ensaios 2 e 3) foi testada no cultivo de *P. cartilagineum* e *S. coronopifolius*, adicionalmente o cultivo de *P. cartilagineum* foi testado no inverno e primavera/verão. A produtividade e potencial de remoção de nutrientes, bem como o conteúdo de proteínas, lípidos, cinzas e o conteúdo de compostos fenólicos totais e atividade antioxidante foram avaliados.

Os valores baixos de produtividade obtidos indicam que as condições de cultivo, tais como a temperatura, luz e salinidade, necessitam de estudos mais aprofundados para estas duas espécies. Nos tanques com 4, 6 g L^{-1} (ensaios 1 e 2) e 8 g L^{-1} (ensaio 3) a produtividade média do *P. cartilagineum* foi de 31.30 ± 33.249 g DW m^{-2} wk^{-1} e 23.21 ± 22.103 g DW m^{-2} wk^{-1} e essa biomassa removeu 8.73 x 10^{-3} g DW m^{-2} e 4.77 x 10^{-3} ± 2.572 x 10^{-4} g DW m^{-2} de azoto em 9 (ensaio 1) e 6 (ensaio 2) semanas; e a produtividade de *S. coronopifolius* foi de 19.74 ± 30.513 g DW m^{-2} wk^{-1} e removeu 3.29 x 10^{-3} ± 1.603 x 10^{-3} g DW m^{-2} de azoto em 6 semanas. Também a composição nutricional, o conteúdo de compostos fenólicos e a atividade antioxidante das duas espécies foi analisada; o teor de proteína apresentou resultados próximos aos valores obtidos em vegetais ricos em proteínas, como a soja; o teor de cinzas foi também elevado (28.0 – 29.5 %) apresentando um elevado teor de minerais; o teor de lípidos foi baixo (< 6 %), tal como esperado para as algas; e os extratos mostraram a presença de compostos fenólicos e atividade antioxidante. *P. cartilagineum* e *S. coronopifolius* não obtiveram elevado crescimento e por isso o *N-yield* foi também baixo,

mas se as condições ótimas de cultivo forem encontradas, existe um potencial de remoção de azoto num sistema de IMTA para estas duas espécies, uma vez que apresentaram um conteúdo de azoto elevado no final de cada ensaio (6.82 ± 0.044 %, 6.34 ± 0.204 %, 5.41 ± 0.097 % para os ensaios 1, 2 e 3, respetivamente), por esse motivo são necessários mais estudos para aumentar a produtividade, trazendo benefícios ambientais e potencial económico para a piscicultura.

Palavras-chave: Algas, IMTA, Cultivo, Biorremediação, Azoto.

Abstract

The use of technology to develop more sustainable aquaculture is crucial in order to make a more responsible and sustainable consumption and so a better economy. In this context, Integrated Multitrophic Aquaculture (IMTA) systems that include seaweed in cultivation are being designed to mitigate the environmental problems caused by fed aquaculture. Several species, namely red seaweed, have been shown to be efficient to growth in this type of system. *Plocamium cartilagineum* and *Sphaerococcus coronopifolius* are present in Portugal and thrive on our coasts, namely in Algarve. These two species have been considered for many industries due to their properties. A seaweed experimental cultivation system with tanks was installed at sole land-based aquaculture facility, for the first trial, and for the second and third trials the cultivation system was changed, and an "artificial wastewater" was created and added to the system, replacing the effluent from the fish, to evaluate the potential of these species as the biofilter component of an IMTA system. The influence of stocking density (1 to 9 g L^{-1} for trial 1, and 2, 4, 6 and 8 g L^{-1} for trials 2 and 3) was tested on *P. cartilagineum* and *S. coronopifolius* cultivation, additionally *P. cartilagineum* cultivation was tested in winter and spring/summer. Productivity and nutrient removal potential, as well as their protein, lipid, and ashes content and total phenolics content and antioxidant activity were assessed.

The poor seaweed production obtained indicate that the culture conditions, such as optimal temperature, light, and salinity, require more in-depth studies for these two species. In the tanks with 4, 6 g L^{-1} (trials 1 and 2) and 8 g L^{-1} (trial 3) the production of *P. cartilagineum* was only 31.30 ± 33.249 g DW m^{-2} wk^{-1} and 23.21 ± 22.103 g DW m^{-2} wk^{-1} and this biomass removed 8.73 x 10^{-3} g DW m^{-2} and 4.77 x 10^{-3} ± 2.572 x 10^{-4} g DW m^{-2} of nitrogen in 9 (trial 1) and 6 (trial 1) weeks. The productivity of *S. coronopifolius* was 19.74 ± 30.513 g DW m^{-2} wk^{-1} and removed 3.29 x 10^{-3} ± 1.603 x 10^{-3} g DW m^{-2} of nitrogen in 6 weeks. Also, the nutritional composition, phenolic content, and antioxidant activity of the two species was analyzed; the protein content had results close to values obtained in high-protein vegetables, like soybeans; the ash content was also high (28.0 - 29.5 %) showing a high mineral content; the lipid content was low (< 6 %), as expected for seaweeds, and the extracts showed the presence of phenolic compounds and antioxidant activity. *P. cartilagineum* and *S. coronopifolius* did not have a high growth and therefore the N-yield was also low, but if the optimal cultivation conditions are achieved, there is a potential in these seaweeds to remove nitrogen in a land-based IMTA systems, since they have shown to have a high nitrogen content at the end of the trials (6.82 ± 0.044 %, 6.34 ± 0.204 %, 5.41 ± 0.097 % for trials 1, 2 and 3 respectively), and so more studies are needed to

increase their productivity, to bring environmental and potential economic benefits for the fish farm.

Keywords: Seaweed, IMTA, Cultivation, Bioremediation, Nitrogen.

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ºC	degrees Celsius
%	Percentage
µL	Microliters
µmol	Micromole
AA	Ascorbic acid
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
Al	Aluminium
AOAC	Association of Official Analytical Chemists
Ar	Argon
C	Carbon
Ca	Calcium
CO_2	Carbon dioxide
$CoCl_2.6H_2O$	Cobalt chloride hexahydrate
Cu	Copper
$CuSO_4.5H_2O$	Copper sulphate pentahydrate
d	Day
dgrm	Direção-Geral de Recursos Naturais, Segurança e Serviços Marítimos
DIN	Dissolved inorganic nutrients
DPPH	2,3-diphenyl-1-picrylhydrazyl
DW	Dry weight
EA	Elemental Analyzer
EDTA	Ethylenediaminetetraacetic acid
EPA	The United States Environmental Protection Agency
EPPO	Estação Piloto de Piscicultura de Olhão
EU	European Union
F	Feces
FAO	Food and Agriculture Organization of the United Nations
FCR	Folin-Ciocalteu Reagent
Fe	Iron
$FeCl_3.H_2O$	Ferric Chloride decahydrate
$FeCl_3.6H_2O$	Ferric chloride hexahydrate

FW	Fresh weight
FRAP	Ferric Reducing Antioxidant Power
g	Gram
GAE	Gallic acid equivalents
I	Iodine
ISE	Iron Sulphate equivalents
IMTA	Integrated Multitrophic Aquaculture
IPMA	Instituto Português do Mar e Atmosfera
IRMS	Isotopic Ratio Mass Spectrometer
K	Potassium
L	Liters
m	Meter
M	Molar mass
MAAs	Mycosporine like amino acids
Mg	Magnesium
mL	Milliliters
mM	Millimolar
Mn	Manganese
$MnCl_2.4H_2O$	Manganese chloride tetrahydrate
N	Nitrogen
Na	Sodium
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
Na_2EDTA	Disodium ethylenediaminetetraacetate dihydrate
NaH_2PO_4	Sodium dihydrogen phosphate
$Na_2MoO_4.2H_2O$	Sodium molybdate dihydrate
$NaNO_3$	Sodium nitrate
NH_3^+ NH_4^+	Ammonia
NH_4Cl	Ammonium chloride
nm	Nanometer
NO_3^-	Nitrate
PF	Pseudo-feces
PO_4^{3-}	Phosphate

POM	Particulate Organic Matter
ppm	Parts per million
PUFAs	Polyunsaturated fatty acids
RAS	Recirculating aquaculture system
RGR	Relative Growth Rates
ROS	Reactive oxygen species
rpm	Rotations per minute
s	Second
SFA	Segments Flow Autoanalyzer
TAN	Total ammonia nitrogen
TPTZ	2,4,6-Tris(2-pyridyl)-s-triazine
USA	United States of America
wk	Week
Zn	Zinc
$ZnSO_4.7H_2O$	Zinc sulphate heptahydrate

1. Introduction

The ocean shows potential to provide many resources nonetheless, it needs to be explored wisely, integrating new technology to avoid wearing out this natural resource. For food production, technologies such as Integrated Multitrophic Aquaculture (IMTA), where the production of species from different trophic levels is integrated to reduce waste and maximize profit, are already possible to put into practice and using scientific knowledge we can take a step forward into making a more responsible and sustainable consumption. In parallel, seaweed cultivation is attractive as it shows ecosystem services potential, while the biomass can be used in a wide range of industries. The aim of this work is to contribute to the development of sustainable aquaculture, the blue, and circular economy by investigating the cultivation of novel seaweed species in IMTA.

1.1. Aquaculture trends

Due to world population growth and the fact that world fish consumption per capita has increased from an average of 9,0 kg in 1961 to 20,5 kg in 2018 (FAO, 2020), it has become necessary to develop new ways to obtain fish protein. Fishing can't meet demand, as overfishing has led to a decrease in wild fish stocks, and the maximum exploitation of this resource has been reached (FAO, 2020). Economically viable and environmentally sound alternatives are needed, in fact, aquaculture production has been an area of strong development in recent years. Currently, this activity represents one of the fastest growing food production sectors and is destined to be one of the main ways to guarantee fish at the consumer's table (FAO, 2020), in 2018 world aquaculture fish production reached 82.1 million tons, followed by 32.4 million tons of aquatic algae (FAO, 2020). However, the European Union (EU) doesn't seem to participate in the growth of this sector yet, having had a gradual decrease from the late 80's, but having a slightly recovering, representing 17 % of the global fish production, in 2018 (FAO, 2020). The state of aquaculture in Europe has been studied and several reasons for the degree of development of this sector have been pointed out, such as the difficulty of competing with third countries that have less severe regulatory standards and less licensing which leads to lower costs and so lower prices, limited access to space and water, and difficulties in accessing finance and investment (OECD, 2010; STECF, 2013; Bostock et al., 2016; FAO, 2020). Despite current data, the European Commission (2012), in its EU's Blue Growth Strategy has identified aquaculture as the sector with a job-creation potential, as well as the potential for research

development to deliver technology improvements and innovation that will lead to a blue economy. The EU aquaculture sector can be divided into three main sectors: marine finfish, freshwater finfish, and shellfish, and even though aquatic plants, that include seaweed, are already part of the EU aquaculture sector, they have, thus far, a low impact. The EU's largest producers are Spain, the United Kingdom, France, Italy, and Greece (European Environment Agency, 2018). In 2018, and according to data from the National Institute of Statistics (Instituto Nacional de Estatística, 2019), the total production in Portugal, of all species produced was 13 992 tons; being most of this production relied on mollusks (67.2 %), followed by marine fish (27.6 %); the algae production, that includes macro and microalgae, in 2018 was 35 tons and exports in Portugal were 32 tons, representing a turnover of 243 000 € from international sales. In line with the EU approach, there is a strategic plan for the Portuguese aquaculture (*dgrm*, 2013), that includes the increase and diversification of production and supply of new products, including the installation of new units or modernization of the existing ones, invest to have high food safety standards and research on offshore aquaculture, identifying areas, species and production systems that are better qualified for Portugal.

Traditional aquaculture in Portugal is land-based, characterized mainly by marine extensive and semi-intensive flow-through pond systems located in lagoons, estuaries, and intertidal areas along the coast. Intensive systems are less usual but include the whole of freshwater production (Ramalho & Dinis, 2011). These land-based marine systems rely on water renewal according to the tides, since they are located in estuarine areas, but other systems like raceways and recirculation systems (RAS) are also being put into practice (Ramalho & Dinis, 2011). The raceways and pond systems, or directional flow systems, are systems where water flows through an aquaculture structure with a renewal rate that allows maintaining the desired level of water quality, this requires a large amount of water and the effluent of this type of aquaculture has an apparently clean presentation, which is due to its great dilution (Mirzoyan et al., 2010) and usually, there is no treatment of those effluents (Gross, 2001). RAS, on the other hand, are intensive systems that can achieve high rates of water reuse due to the use of different mechanical, biological, and chemical techniques in water treatment (Yeo et al., 2004). In summary, the RAS reduces water consumption, water discharges effluents, and production costs (Ebeling et al., 2006), for instance in Aveiro, a company uses this type of system for sole production (Site 1).

Considering the traditional aquaculture systems in Portugal, one reason that explains the stagnation of aquaculture is the fact that most coastal farms are in natural reserves and wetlands protected by environmental legislation and only low-tech improvements are

allowed (Ramalho & Dinis, 2011). Therefore, moving cultivation offshore may be advantageous, in parallel with RAS, that was previously described. The geographic location of Portugal's mainland, in the transition from two important oceanographic sub-provinces of the North Atlantic, allows for a great diversity of habitats, and it is considered one of the richest areas in biological terms, namely for its water quality and for the diversity of species (*dgrm*, 2013). However, the geomorphological conditions of the Portuguese coast and the islands' Atlantic waters and, above all, ocean conditions during the winter months, are not the most advantageous for the installation of offshore units, forcing the use of technological solutions adapted to environmental conditions (*dgrm*, 2013). Despite this constraint, several strategic places, including the Algarve coast, have conditions for the practice of aquaculture.

Worldwide, the economic expansion of the aquaculture sector has contributed to obtain clear benefits in several domains, such as social (employment), environmental (bioremediation, habitat structure), and product quality (freshness and security) (Claret et al., 2014; Paul and Vogl, 2011; Slater et al., 2013; Smith et al., 2010; Ottinger et al., 2015). However, despite the need for aquaculture development and its benefits, there are also risks associated to this industry (Utne et al., 2018; Ottinger et al., 2015). Different types of aquaculture generate very different pressures on the environment, and the precise level of impact will, therefore, vary according to the production system, cultured species, production scale, and management technique, as well as local and regional hydrodynamics and chemical characteristics.

One of the environmental negative impacts of fed aquaculture, i.e. culture of species that require added artificial feed, is the nutrient waste. Different species and cultivation systems release different quality and quantity of waste, but generally, waste is released as solid particles in the form of fish faeces and uneaten feed, while dissolved nutrients, including nitrogen and phosphorus, are released through fish gills and in urine, as well as by the solid waste when it breaks down in the water (Gross, 2001; Goldburg et al., 2001; Granada et al., 2016). Nutrient waste released from fish farms can lead to eutrophication, and consequently to alterations on fish stocks, and oxygen depletion, deteriorating the quality of the surrounding water (Rosa et al., 2020), these nutrients are also detrimental for the fish culture itself, when the ammonia concentration is too high, it will decrease the survival, inhibit growth and cause a variety of physiological dysfunctions on the fish (Tomasso, 1994), a problem particularly important in RAS systems. The accumulation of nutrients in the water stimulates anaerobic organic oxidation affecting the ecosystems, including the benthic macrofaunal and microbial communities (Choi et al., 2020). Beneath open water fish farms,

the sediment biogeochemical cycling has also shown changes (Valdemarsen et al., 2012; Bannister et al., 2014).

On the other hand, extractive species can remove particulate or dissolved nutrients, including those wasted from fed species, lowering the nutrient load in the water while building their biomass. Integrated multitrophic aquaculture (IMTA) refers to the co-culture of different species belonging to two or more trophic levels and offers a sustainable approach to aquaculture development (Figure 1.1) (Troell et al., 1997; Rosa et al., 2020). In IMTA the organic extractive aquaculture species such as shellfish can take advantage of the enrichment in small particulate organic matter (POM) in the water coming from the excretion products of fed aquaculture species (e.g., finfish). Also, deposit organic extractive aquaculture species (e.g., echinoids, holothuroids, and polychaetes), can take advantage of the enrichment in large particulate organic matter (POM), and feces and pseudo-feces (F and PF) from finfish and suspension-feeding organisms. While inorganic extractive aquaculture species (e.g., algae and halophytes) take advantage of the enrichment in dissolved inorganic nutrients (DIN). Halophytes have the ability to grow in habitats with a high concentration of salt which makes them capable to grow and recycle the nutrients generated by the fish from saline aquaculture (Buhmann & Papenbrock, 2013). Also, microalgae are used as filters in outdoor tank/pond systems or indoor tubular photobioreactors, they can also be grown in the same pond as fish with an integrated culture of shellfish (Troell et al., 2003). Seaweeds can also lower dissolved nutrients concentration, the nitrogen released from the fed species (NH_3), and dissolved release of phosphorus in

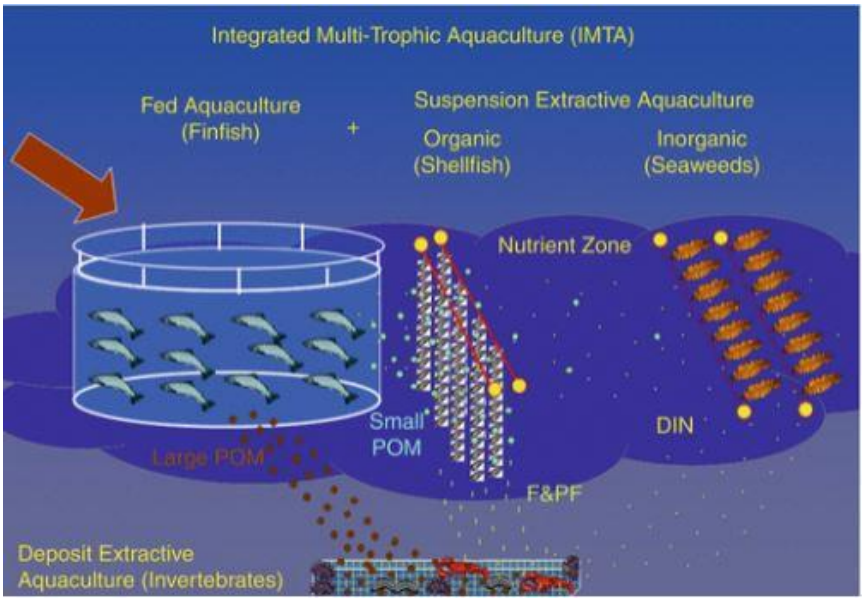

Figure 1.1: Conceptual diagram of an Integrated Multitrophic Aquaculture (IMTA), an open sea cultivation example adapted from Chopin (2013).

the form of phosphate (PO_4^{3-}), which are often the preferred nutrients sources for seaweeds (Troell et al., 2003).

Future aquaculture technologies could increase sustainability by integrating waste generating (fed) and cleaning (extractive) organisms in farms, i.e. IMTA systems (Chopin et al., 2001). This approach to farming and the use of seaweeds is being considered and is attracting attention as a possible future route for sustainable aquaculture, and it is on this aspect that this work will focus.

1.2. Seaweed

Macroalgae can be found in a wide variety of habitats and conditions, like in freshwater, marine water, and on the surfaces of moist soil or rocks (Wang et al., 2015; El Gamal, 2010; McLachlan, 1992). The term seaweed lacks a formal definition, and while it is sometimes used more broadly, in this work it refers solely to marine macroalgae. Seaweed are macroscopic easily observed with the naked eye and are multicellular photoautotrophic organisms, they contain in its constitution chlorophyll and other pigments for photosynthesis, which use light energy from the sun to convert into chemical energy, storing that energy in the form of starch and carbohydrate (McLachlan, 1992; Rout & Kumar, 2015). Seaweed constitute a highly diversified group, which has no common ancestor (Oliveira, 2002). For this reason, they do not constitute a defined taxonomic category, their classification is based on various properties such as pigmentation, chemical nature of photosynthetic reserve products, organization of photosynthetic membranes and other morphological criteria (Carlsson et al., 2007). They are divided mainly into three groups: Chlorophyta (green seaweeds), Rhodophyta (red seaweeds), and Phaeophyceae (brown seaweeds) (Chapman & Chapman, 1980; Pereira, 2015).

As a result of thriving in a variety of environments, they can produce compounds that are not usually found in terrestrial plants, like unique bioactive, complex, exotic acyl lipids and fatty acids (Chapman & Chapman, 1980). Seaweed are a rich source of valuable components including hydrocolloids, that are derived from phycocolloids, such as agar, carrageenan or alginate, other polysaccharides, polyphenols, carotenoids, flavonoids, dietary fibers, vitamins including A, B1, B2, B6, C, and E and some minerals, such as calcium, potassium, magnesium, iron, and iodine. Seaweed contain a high amount of carbohydrates, proteins, fats, and essential minerals (McHugh, 2003; Kiuru et al., 2014; Chapman & Chapman, 1980; Rout & Kumar, 2015; Kumar, 2019; Matanjun et al., 2009).

Environmental conditions such as light (irradiation, light quality, photoperiod) temperature, carbon dioxide, desiccation, salinity, and nutrient supply affect growth and reproduction as well as macronutrients accumulation in its tissues (Singh & Singh, 2015; Figueroa et al., 2003; Zou & Gao, 2010; Zou & Gao, 2013; Han et al., 2013; Yu & Yang, 2008; Liu et al., 2012; Flores-Molina et al., 2014). Ideal conditions for survival, growth, and reproduction vary among species, for example, algae growth rates increase with increasing temperature up to a certain limit because it influences the cellular chemical composition, uptake of nutrients, and carbon dioxide (CO_2) (Singh & Singh, 2015), it also increases up to certain wavelength and irradiance level. Different light intensities induce changes in the growth rates but also affect pigment composition, chlorophyll fluorescence parameters, and antioxidant activity (Wu et al., 2015). Due to the photoinhibition, algal growth decreases after a certain light intensity, this phenomenon is a decrease in photosynthesis rate, when plants are exposed to high irradiance, it basically reduces the photosynthesis capacity (Han, 2002; Mata et al., 2006).

1.2.1. Uses of seaweed

While seaweed have been used for centuries, in recent years there has been an increase related to the applications of seaweed as a result of the identification of compounds synthesized by them, and their resulting properties. Seaweed show great potential for the food, feed, and pharmaceutical industries but also for other industries such as biofuels, biofertilizers, cosmetics, and in the treatment of soil or effluents (Cardozo et al., 2007). As

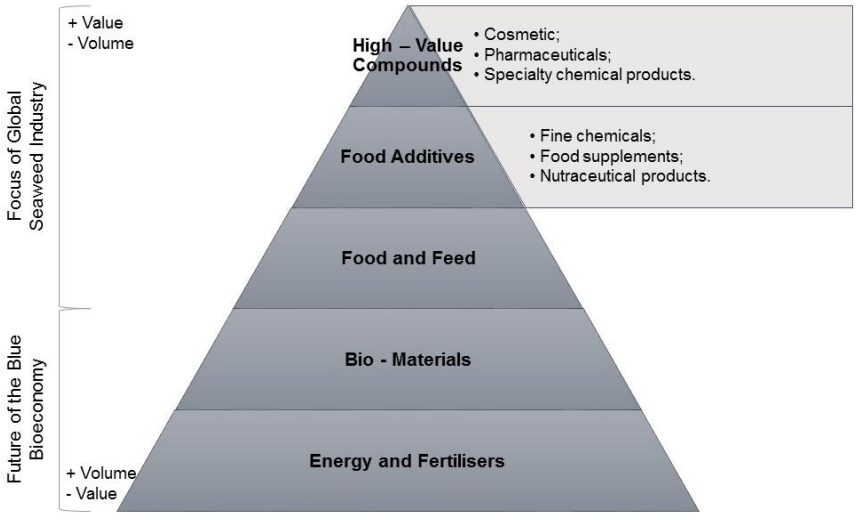

Figure 1.2: Seaweed biomass uses versus value in the global industry. Adapted from Thomas (2018).

shown in Figure 1.2, different industry sectors focus on different products. Algae biomass with specific bioactive compounds increases its value but requires a small volume of production. As we go down on the pyramid, the lower the price gets since it is not necessarily a product with specific compounds, but higher volumes are needed.

Seaweeds were used in traditional medicine from 3000 B.C. due to the presence of therapeutic properties (Kumar, 2019). They are known to have a wide diversity of biological properties, such as antibacterial, anti-inflammatory, antiproliferative, antiviral activity (Cabrita et al., 2010; Xu et al., 2004), antihypertensive, antihyperlipidemic, antioxidant, anticancer (Shalaby, 2011), anticoagulant, antifungal (Mohamed et al., 2012), and antineoplastic (Xu et al., 2004) among other. Seaweed can make a beneficial contribution to treat infections as many of them have antibacterial properties, thus replacing the overuse of antibiotics (Palma, 2011).

The use of seaweed as food has been known to start in Japan and China, in the fourth and sixth centuries, respectively (McHugh, 2003). Many seaweeds such as Viva, Enteromorpha, Caulerpa, Codium and Monostroma (green algae); Sargassum, Hydroclalhrus, Laminaria, Vndaria, Macrocystis (brown algae); Porphyra, Gracilaria, Eucheuma, Laurencia, and Acanthophora (red algae) are used for human consumption (Cardoso et al., 2014; Kumar, 2019). Algal lipids are of immense commercial value as alternative sources of nutritionally important n-3 polyunsaturated fatty acids (PUFAs) and because of that are widely employed as ingredients in functional food formulations (Kumari et al., 2013). In Portugal there is a company that produces seaweeds on a large scale, ALGAplus, it is located in Ílhavo (Aveiro) and cultivates seaweeds for food under the IMTA concept at a fish farm of seabass and seabream. They commercialize *Ulva spp*. and *Porphyra spp*., *Chondrus crispus*, *Gracilaria sp*., *Palmaria palmata*, *Porphyra dioica*, *Codium tomentosum,* and *Fucus spp*. (Site 2).

Seaweed can be used in animal feed as an additive too, production was pioneered in Norway in the 1960s and it was made from brown seaweeds (McHugh, 2003). One of the seaweed used as a feed additive is *Ulva spp*., a green seaweed with a good vitamin and mineral profile and it is rich in glutamic and ascorbic acid, alanine, iron, and dietary fibers (Briand & Morand, 1997; García-Casal et al., 2007). It has been observed that when *Ulva spp*. is added to the diet (10%), fish grow, weight gain, and feed conversion ratio improved (Merrifield, 2009; El-Tawil, 2010). A more recent use of seaweed in animal feed is with *Asparagopsis taxiformis*, a red seaweed, that when used in the diet of lactating dairy cows decreased methane emissions by 80 percent and had no effect on feed intake or milk yield when fed at up to 0.5 percent of feed dry-matter intake (Penn State, 2019).

There are a particular group of molecules present in the seaweeds that are widely used in the food industry as well as in cosmetics since they are hydrophilic molecules that have a high molecular weight and dissolve or disperse in water to give thickening or viscosity building effect, the hydrocolloids (O'Sullivan & O'Mahony, 2016). But the focus of the use of seaweed in the cosmetic industry is on bioactive ingredients, to reduce the usage of toxic chemicals (Wang et al., 2015; Ariede et al., 2017), for instance, a seaweed carotenoid called astaxanthin is being used in anti-aging products (Terao, 1989). In Portugal, Figueira da Foz, there is a brand called Sealgae, which is a registered trademark of natural cosmetics that combines bioactive extracts of Iberian algae (Site 3).

Seaweeds are also used as fertilizers dating back at least to the nineteenth century. The high fiber content of seaweeds acts as a soil conditioner and assists moisture retention, while the mineral content is a useful fertilizer and source of trace elements. Nowadays, with the rising popularity of organic farming, there has been some increasing interest of this industry (McHugh, 2003). In recent years, seaweed extracts are being used as agriculture biostimulants, enhancing plant growth, health, and productivity (Battacharyya et al., 2015; Arioli et al., 2015).

Over the last twenty years, there have been some large projects that investigated the possible use of seaweed as an indirect source of biofuel. Polysaccharides from seaweed can be hydrolyzed for biofuel production (Wei et al., 2013). Since brown algae contain a high amount of carbohydrates compared to green and red algae, they are a suitable resource for bioethanol production (Jung et al., 2013). Seaweed is a readily available and interesting resource for biofuel production and its appropriate fractionation is the main challenge for the development of a marine biorefinery, but currently, seaweeds are not commercially profitable biofuel feedstocks, or in some instances, not even eco-friendly, when the whole production system is taken into account (del Río et al., 2020; Kazemi Shariat Panahi et al., 2019).

From an environmental services perspective seaweed show potential for climate change mitigation, biodiversity increase, and waste nutrients removal (Froehlich et al., 2019; Sondak et al., 2017; Kyo Chung et al., 2013; Dijkstra et al., 2017; Cabrita et al., 2010; Bracken & Williams, 2013; Zhou et al., 2006; Marinho et al., 2015; Neveux et al., 2018). Systems using seaweed for treating effluents from enclosed land-based mariculture systems were initiated in the mid-1970s (Haines, 1975; Ryther et al., 1975; Langton et al., 1977; Harlin et al., 1978), in the 1990s there was a renewed and increased research into the development of macroalgae-based integrated techniques (Vandermeulen & Gordin,

1990; Cohen & Neori, 1991; Neori et al., 1991; Neori et al., 1996; Haglund & Pedersén, 1993; Alejandro Buschmann et al., 1994; Buschmann et al., 1996; Buschmann, et al., 2001; Jiménez del Río et al., 1996; Krom et al., 1995; Neori, 1996; Troell et al., 1997; Neori and Shpigel, 1999; Chopin et al., 1999a; Chopin et al., 1999b) and these studies showed that wastewater from intensive and semi-intensive mariculture is a suitable nutrient source for the intensive production of seaweeds, thereby reducing the discharge of dissolved nutrients to the environment.

1.2.2. Seaweed production

After the Second World War, the industrial uses of seaweed extracts expanded rapidly but were limited by the availability in the wild habitat, ever since the research into life cycles has led to the development of cultivation industries that now supply a high proportion of the raw material for some hydrocolloids (McHugh, 2003).

In total, 35 countries commercialize seaweed, from all the water range temperatures (McHugh, 2003). China is the largest producer of edible seaweeds, harvesting 18 505.7 thousand wet tones (FAO, 2020), the greater part of this is for *kombu*, *Laminaria japonica,* a brown algae. China is followed by Indonesia, Republic of Korea, Philippines, Democratic People's Republic of Korea, and Japan (FAO, 2020). The Japanese production relies on *nori*, a species of *Porphyra,* a red algae (McHugh, 2003). Indonesia has a major influence in the farming development of tropical seaweeds species, such as *Kappaphycus alvarezii* and *Eucheuma spp*., for raw material for carrageenan extraction, in recent years (FAO, 2020). In Europe, the seaweed production is a resource little explored, possibly due to the low commercial value that these algae still have, because of the heavy competition from Asian countries, and because its consumption is not that common in European countries, also the data is limited since in Europe the production of seaweed is done in small-scale farms (FAO, 2020), according to FAO, this industry had an estimated total value of US$ 11 844 163 in 2017. With the seaweed industry relying mainly on food products for human consumption, followed by substances that are extracted from seaweeds, like hydrocolloids, and only a small part is from fertilizers and animal feed additives (McHugh, 2003).

There is a wide range of cultivation methods, including the fixed, off-bottom line method, the floating raft method, and basket method (Ask & Azanza, 2002; Pereira & Yarish, 2008; Hayashi et al., 2010) open water rope cultivation, nearshore bottom cultivation, pond culture and tank cultures (Oliveira et al., 2000; Sahoo & Yarish, 2005; Pereira & Yarish, 2008). It is

necessary to choose which method is more appropriated to the species, but it is also necessary to consider site selection, farm maintenance, and harvesting and drying to have an optimal production. Many species are cultivated in shallow bays, estuaries as well as on intertidal mudflats and propagate by thalli fragmentation, the traditional bottom method carries inherent disadvantages, mainly related to biomass losses and variability in seasonal production (Buschmann et al., 1995; Buschmann et al., 2001; Santelices & Ugarte, 1987). Some cultures are mostly based on the bottom-aeration method (Lapointe & Tenore, 1981; Neori et al., 1991). Aeration stimulates growth by breaking down diffusive boundary layers at the surface of the thalli which would otherwise hinder the uptake of nutrients and inorganic carbon (DeBusk & Ryther, 1984).

In the 1960s, the cultivation of *Gracilaria* started in Taiwan Province of China as a source of raw material for its agar industry. It started with ropes in ditches containing fish pond effluents, but in 1967 they moved the algae to the fish ponds bringing benefits for the fish, that eat the epiphytes, and for the seaweed that used fish waste material as fertilizer (McHugh, 2003). Various strategies have been studied, like seaweed cultivation around the outside of fish cages that has led to significantly better growth seaweed but only partly successful in removing a large number of nutrients coming from the fish cages (Troell et al., 1997). This concept has been utilized in many situations where the effluent from the aquaculture of one species can be utilized by another, reducing the pollution that could come from this production.

As mentioned in the previous sections, in IMTA, seaweeds assimilate the fish-excreted nitrogen, phosphate, and CO_2, converting them into potentially valuable biomass. Its application in land-based intensive fish farms can also be a tool to increase recirculation practices and establish full RAS bringing environment and economic benefits. The cultivation of seaweeds in IMTA promotes higher productivity levels and with less variability than natural seaweed beds due to higher and constant nutrients availability (Abreu et al., 2009; Abreu et al., 2011; Troell et al., 2009; Holdt & Edwards, 2014). Good examples of significant revenues for fish aquaculture when adopting the IMTA concept already exist, for instance for *Porphyra, Gracilaria* (red seaweeds), and *Ulva* (green seaweed). *Porphyra* species are the source of human food, *nori*, and so their use in integrated aquaculture is an attractive economic alternative, particularly because they are very efficient in taking up nutrients. Trials were run on the east coast of Canada and the United States of America to combine *Porphyra* with salmon farming showing positive results (Chopin et al., 1999). Several studies using *Gracilaria* have successfully used this species as biofilters, both in tanks (Buschmann et al., 1996; Matos et al., 2006; Abreu et al., 2011), offshore systems

(Abreu et al., 2009; Buschmann, et al., 2008) and to a lesser extent in ponds (Marinho-Soriano et al., 2002; Haglund & Pedersén, 1993). The majority of these studies achieved good results on productivity and agar content, the main destiny for the biomass. The IMTA-produced *Ulva spp.* represents multiple advantages for the aquaculture sector by considerably reducing nitrogen loads in the effluent, saving water treatment costs, and turning into an additional valuable crop (Bunting & Shpigel, 2009; Holdt & Edwards, 2014).

As seen above, red seaweeds are a good example to use in IMTA systems, both because of their commercial interest and nutrient removal capacity, and in addition, these seaweeds are present on the Algarve coast (Pereira, 2018; Gaspar et al., 2019), which makes their production in aquaculture viable, since they are adapted to the environmental conditions, and will be discussed in greater detail in the next chapter.

1.3. Red seaweed

Red seaweed, Rhodophyceae, includes more than 4 000 species distributed around the world (Sabry et al., 2017). They are found in cold waters such as Nova Scotia (Canada) and southern Chile, in temperate waters, such as the coasts of Morocco and Portugal, and in tropical waters, such as Indonesia and Philippines (McHugh, 2003). They are generally ranging from a few centimetres to about a meter in length, and their colour range is from red to purple and brownish red (McHugh, 2003). Red seaweed get their colour from the pigments phycoerythrin and phycocyanin that mask the other present pigments, chlorophyll *a*, beta-carotene, and a number of unique xanthophylls (Wu et al., 2015). As a result of their predominant growth in temperate and tropical locations, red seaweeds are among the most frequently investigated sources for marine natural products.

As for cultivation methods, as well as the specific ideal environment conditions required for each species growth, some species may be cultivated vegetative or by going through a separate reproductive cycle, involving alternation of generations. Cultivation involving a reproductive cycle, with alteration between a sporophyte and a gametophyte, is required for many red seaweeds. This type of cultivation comes with some difficulties such as management of the transitions from spore to gametophyte to embryonic sporophyte, also since these phases are cultivated in a land-based facility with control of temperature, nutrients, and light, it leads to high costs (Mathieson, 1975; McHugh, 2003), but it also has benefits, such as genetical diversity since they are not all clonal seaweeds, bringing more diversity to the farming.

Typically, in vegetative cultivation, small pieces of seaweed are taken and placed in an environment that will sustain their growth, and when they reach a certain size they are harvested, either by removing the entire plant or by removing most of it, but leaving a small piece that will grow again (McHugh, 2003; FAO, 2018). Seaweed can be attached to long ropes, where pieces of seaweed are tied and then suspended in the water; or placed in a pond or tank and not fixed. Pond cultivation is less labour intensive than ropes farming and has been quite successful, but pond size limit cultivation. In tank culture, it is possible to manage nutrient availability by changing the water flow, which may hold promise for the processing of nutrient-enriched waters from fed aquaculture systems, and depending on the goal of culture, the nutrient supply can be increased in order to produce more biomass or reduced to increase nutrient removal efficiency. Ideally, a system can be optimized to take advantage of both biomass production and nutrient removal, as seen in tank cultivation, still there is some major problems for successfully use this type of cultivation like site selection, tanks design, knowledge about the species life cycle, selection of the best strains, knowledge and control of the ideal environmental conditions, agitation or water movement, nutritional requirements and density in the tank (Buschmann et al., 2001; Craigie & Shacklock, 1995; FAO, 2014, 2018; McHugh, 2003; Pereira & Yarish, 2008; Santelices & Doty, 1989).

There are several examples of red seaweeds already used in IMTA systems. For instance, seaweed from the genus *Gracilaria*, have been shown to be efficient biofilters and are widely investigated in different works showing that these species can be used in an IMTA system. According to Abreu and co-workers (2011), the biomass of *G. vermiculophylla* produced using the effluents of a land-based fish commercial intensive aquaculture farm, revealed to be of excellent quality for applications such as good grade agar, fish feed ingredients and possibly as a source for MAAs (mycosporine like amino-acids) since it exhibited good growth and nutrient removal performance year-round; it also showed that the production of this seaweed in IMTA can also be beneficial for the health of cultured fish. Another promising candidate species for the seaweed cultivation in Europe is *Palmaria palmata*. *P. palmata* has been proven to be a good candidate for biofiltration in IMTA systems (Lüning, 2001; Corey et al., 2013; Corey et al., 2014; Grote, 2016), it was also showed that the integration of this seaweed with fish culture in an IMTA system would result in an increased protein content of the red seaweed. Another example is *Asparagopsis armata*, that has showed a greater potential for rapid uptake of nutrients compared to others. The tetrasporophyte of this species (*Falkenbergia rufolanosa*) was successfully tank-cultivated as a continuous biofilter for the effluent of a commercial fish farm in southern Portugal

(Schuenhoff et al., 2006), showing that *A. armata* is the seaweed biofilter with the highest reported TAN (total ammonia nitrogen) removal in integrated aquaculture.

A review by Troell and co-workers (2003), listed several aspects that will help IMTA future, including species diversification. Although there is already a large number of species used in all sorts of industries, *Plocamium cartilagineum* and *Sphaerococcus coronopifolius* are among the potential interesting new species to use in cosmetic, pharmaceutical and medical industries, because of its properties. Besides the commercial interest, both species are abundantly present in the Algarve coast (Araújo et al., 2009; Pereira, 2008) and therefore were chosen for this study.

1.3.1. *Plocamium cartilagineum*

The genus *Plocamium* (family Plocamiaceae, order Gigartinales), marine red algae, includes approximately 40 species (Wynne, 2002), traditional taxonomy distinguishes between *Plocamium* species largely based on the number of ramuli in alternating series, the morphology of the lower ramulus, the length, width, color and consistency of the thallus, and the morphology and arrangement of tetrasporangial stichidia and cystocarps (Simons, 1964; South & Adams, 1979; Gabrielson & Scagel, 1989). Besides all of those criteria, biogeographical needs to be considered as well (Cremades et al., 2011).

P*locamium* species found in Algarve are *P. cartilagineum* (Figure 1.3) and *P. maggsiae* (Afonso et al., 2000; Pereira, 2018; Alves et al., 2018; Díaz-tapia & Bárbara, 2014). *P. cartilagineum* covers extensive areas and are bigger in size than *P. maggsiae.*

Figure 1.3: *Plocamium cartilagineum* (Linnaeus). Recorded in Portugal (June 6th, 2020).

Plocamium cartilagineum is found on coasts of strong to moderate wave action and are found in depths of 2-26 m (Womersley, 1994) and it is present in the Mediterranean Sea, English Channel, Belgium, France, Ireland, Netherlands, Sweden, Madagascar, North Atlantic Ocean and Portugal. It is a bright scarlet seaweed up to 30 cm in length with branching fronds, the branching occurs alternately along the fronds and becomes more frequent towards the tips, that are incurving and ultimate branching occurs only to one side (Guiry, 2020). The physical structure of the algal thallus and metabolites found in *P. cartilagineum* makes it a perfect host to several amphipod species, where they can get protection from fish predation (Zamzow et al., 2010), this relationship occurs as a mutualistic one, where this species also eat endo and epiphytic algae from the surface of *P. cartilagineum* (Aumack et al., 2011). Plocamium is characterized by its interesting secondary metabolites, being a rich source of diverse polyhalogenated monoterpenes, with a high degree of halogen incorporation, they vary for the given species depending on collection, location and season (Abreu and Galindro, 1996; Kladi et al., 2004; Young et al., 2013; Sabry et al., 2017). According to San-Martin and Rovirosa (1986), *P. cartilagineum* collected from different places presented a similar composition with only some differences in the percentage of their components, but some compounds were not present in all samples. The isolated metabolites are interesting because of their diverse biological properties ranging from ichthyotoxicity to antifeedant, antimicrobial and antifungal activity (Crews et al., 1984). Many of the halogenated monoterpenes isolated from *Plocamium spp.* have been proven to be active in pharmaceutical screening possessing mutagenic, cytotoxic activities and inhibiting oxidative phosphorylation as well as in agrochemical testing (König et al., 1990; König et al., 1999; Williard and Grab, 1984).

Even though there are previous studies about the properties of this species and commercial interest due to those properties, there is still little knowledge on its cultivation potential. *P. cartilagineum* life cycle (Figure 1.4) has three stages, with alternating haploid and two diploid phases (Hommersand & Fredericq, 1990). In the haploid phase, the macroscopic algae can be female or male (dioecious). The female haploids produce carpogonia (eggs) that are fertilized by the spermatia (sperm) produced in the male haploids. After fertilization the diploid zygote divides to form the diploid carposporophyte inside female gametophyte tissue, presenting as circular macroscopic structures known as cystocarps, where carpospores are developed. Carpospores are eventually released and grow into nonsexual diploids, tetrasporophyte, those then eventually grow stichidia that release spores that grow into male or female haploids (Cremades et al., 2011). The gametophyte and the tetrasporophyte are then similarly looking macroscopic phases. According to Kain (1987),

when *Plocamium* is cultivated in tanks exposed to reduce mid-summer daylight they grew more slowly than uprights receiving less irradiance, which means that this species is a "shade-loving".

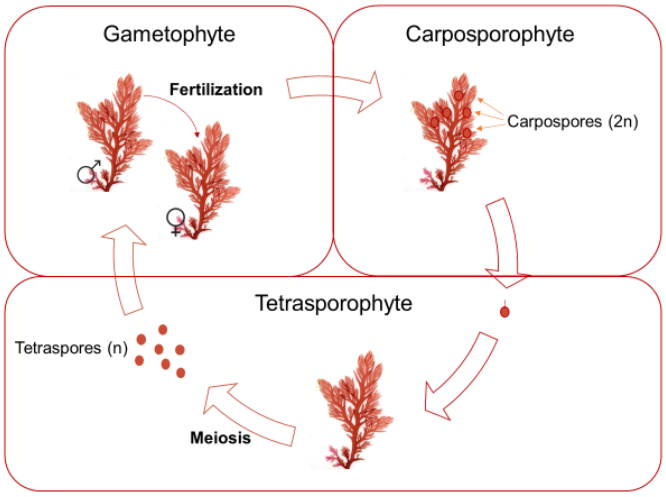

Figure 1.4: Schematic representation of *P. cartilagineum* life cycle. The boxes separate the different phases (Gametophyte, Carposporophyte and Tetrasporophyte).

1.3.2. *Sphaerococcus coronopifolius*

The bright-red seaweed *Sphaerococcus coronopifolius* (Figure 1.5), Stackhouse 1797 (family Sphaerococcaceae, order Gigartinales), generally grows on rocks in shallow areas up to 20 meters. It distinguishes from *P. cartilagineum* from its narrow, compressed, two-edged, cartilaginous, scarlet fonds, main axes dark brownish-red; it has abundant branching

Figure 1.5: *Sphaerococcus coronopifolius* (Stackhouse). Recorded in Portugal (August 7th, 2020).

with short marginal proliferations. It is common on the Atlantic and Mediterranean coasts, including the Algarve coast, and has been known since the 1980s (Caccamese et al., 1981).

This species is an unusually prolific source of diterpenes (Etahiri et al., 2001; Smyrniotopoulos et al., 2009; Smyrniotopoulos et al., 2010a; Smyrniotopoulos et al, 2010b), however, only a few reports have been published in the isolation and characterization of bioactive compounds from this species, and even more restricted information is available on their biological activity. There has been an interest about this seaweed because of its insecticidal, antibacterial, antifungal, antitumoral, anti-inflammatory, and antiviral activities (El Sayed et al., 1997; Caccamese et al., 1980; König & Wright, 1997; Sakemi et al., 1986; Smyrniotopoulos et al., 2008; Salhi et al., 2018; Alves et al., 2020) and despite these properties, this seaweed has not yet been exploited commercially or cultivated on a large scale.

S. coronopifolius, like *P. cartilagineum*, has three stages, with alternating haploid and two diploid phases, but unlike the previous species, where the three stages look similar, this one has a heteromorphic life cycle with an alteration of a monoecious, erect and bushy gametophyte (*S. coronopifolius*), and a crustose tetrasporophyte (*Haematocelis fissurata)* rarer to found in nature on both the Atlantic and Mediterranean coasts (Abdelahad et al., 2016). However Abdelahad and co-workers (2016) observed that carpospores grown in culture gave rise directly to erect gametophytes, demonstrating that this species can reproduce without fertilization through a direct-type life history, as Maggs (1988) also showed. According to Abdelahad and co-workers (2016), the gametophytes are found throughout the year along the coast of Lazio, with cystocarps being more numerous in the winter months.

1.4. Objectives

This work aims to investigate the cultivation of *Plocamium cartilagineum* and *Sphaerococcus coronopifolius* due to the potential environmental and commercial applications of these species. Specifically, the work sets out to investigate *P. cartilagineum* and *S. coronopifolius* biomass production, nitrogen biofiltration potential, and characterizations, under different cultivation densities in IMTA. Results are expected to contribute to the conversion of current aquaculture production systems into more sustainable ones, following the guidelines for blue growth, minimizing de impacts of the effluents of aquaculture.

2. Material and Methods

2.1. Seaweed collection and processing

Seaweed collection took place on 3 occasions, September 30th, June 5th, and July 22nd, at the Arrifes' beach in Albufeira (37° 4' 33.72" N; 8° 16' 38.73" W). For the different trials *Plocamium cartilagineum* (trial 1 and 2) or *Sphaerococcus coronopifolius* (trial 3), were removed from the substrate as gently as possible, in order to avoid damage to the seaweed itself and the environment. The biological material was collected into net bags and moved to buckets with seawater for quick transport to the laboratory (~1h). Upon arrival to the laboratory, seaweed was kept on the experimental system without added nutrients, for four to ten days while it was manually cleaned to remove attached organisms or soil using forceps and new filtered and UV-ed seawater.

After cleaning and before each trial, an initial seaweed sample was frozen (-20 °C) and three replicate samples were weighed before and after oven drying at 60 ºC and kept in airtight bags at room temperature in the dark until further analysis.

2.2. Seaweed cultivation

Cultivation trial systems were established at an aquaculture research centre (EPPO, IPMA) in Southern Portugal, Olhão. White polyethylene buckets were adapted for seaweed tank cultivation and set to receive independent flows of water, each with a 20 L, and a surface area of 0.08 m^2 (Figure 2.1 a). The water flow was checked daily during weekdays and adjusted manually for each tank when necessary, to maintain at 68 L h^{-1} (4 volumes h^{-1}) for the duration of the trials. The cultivation system received chilled water but being an outdoor system water temperature fluctuation is still expected as a result of air temperature strong changes, both reflecting seasonal changes, weather conditions, and daily cycles. Seaweed were kept in constant movement by air supplied via tubes placed in the bottom of the tanks and pierced at regular intervals (Figure 2.1 b), to keep the seaweed in movement and exposed to light and nutrients in the water. The effluent form fish cultivation (trial 1) or artificial wastewater (trials 2 and 3) passed a reservoir tank (Figure 2.1 c) before entering the seaweed cultivation system and was filtrated through glass wool to remove solid waste (Figure *2.1* d). The outflow from the seaweed tanks passed through a net (0.5 mm mesh size) (Figure 2.1 d) to ensure there was no loss of biomass and water was directed and disposed of into the environment.

Water temperature was monitored through the study, 3 sensors (iBCod, Portugal) were placed in 3 tanks randomly every week to continuously register the average half-hourly temperature. Salinity was measured every week, with a multi-parametric sensor (Hanna instruments, Portugal), in each tank. The pH water levels were monitored using a pH sensor (Fisher Scientific, Porto Salvo), twice a day on weekdays, morning and afternoon in Trial 1; and once a day on weekdays, morning, for Trials 2 and 3. Irradiance was measured using a SP-510-SS Upward-Looking Thermopile Pyranometer (Apogee instruments, Inc., USA) which recorded values every ten minutes.

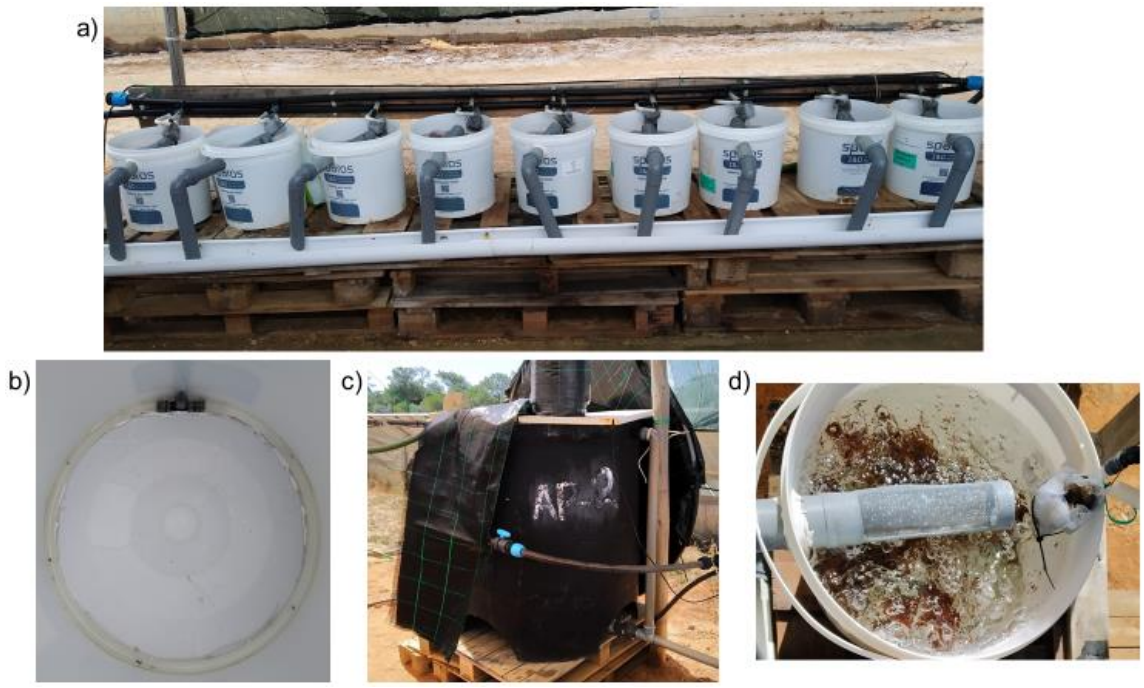

Figure 2.1: System for seaweed cultivation: a) 9 white polyethylene tanks (20 L), continuously supplied with the effluent (Trial 1); b) Seaweed tank in detail, where it is possible to see the tubes placed in the bottom of the tanks and pierced at regular intervals; c) Reservoir tank; d) Seaweed tank in detail, where it is possible to see the inflow water, glass wool filter, and outflow apparatus.

Water samples were collected at the inflows of the tanks (n = 2) once a week, filtered and frozen (-20 ºC) for nitrate (NO_3^-), total ammonia nitrogen (TAN: NH_3+NH_4^+), and phosphate (PO_4^{3-}) analyses. The levels of NO_3^-, TAN and PO_4^{3-} were determined in the Skalar Sanplus Colorimetric Segmented Flow Autoanalyzer (SFA) using, respectively, the Skalar methods: M461-318 (EPA 353.2), M155-008R (EPA 350.1), and M503-555R (Standard Method 450-P I), adding in some samples known concentration of the analysed nutrients; these analyses were carried out at Marinnova facilities in September 2020.

2.2.1. Winter trial – *Plocamium cartilagineum* density (Trial 1)

Nine *P. cartilagineum* stocking densities, from 1 to 9 g FW L^{-1} were tested. The experiment ran for a period of 9 weeks (October 10th to December 12th of 2019). In this trial, the effluent used was from *Solea senegalensis* cultivation tanks (EPPO, IPMA).

Every 7 days, the biomass was totally removed from each tank and weighed. Before weighing, the excess superficial water was removed using a salad spinner, then biomass was weighted on a digital balance, and the process repeated until no differences in weight existed (fresh weight). Each week, the excess *P. cartilagineum* biomass from each unit was removed and individually oven-dried at 60 ºC until a stable dry weight was measured. At each sampling point, when the seaweed was removed for weighing, the tanks were emptied and cleaned with bleach and freshwater. To minimize any effect of the position, every week after sampling the treatment´s positions were randomised again, and the tanks restocked accordingly.

2.2.2. Spring/summer trial – *Plocamium cartilagineum* density (Trial 2) and *Sphaerococcus coronopifolius* density (Trial 3)

Based on preliminary data analysis from trial 1, the experiment design was changed to include four *P. cartilagineum* (Trial 2) and four *Sphaerococcus coronopifolius* (Trial 3) stocking densities, 2, 4, 6, and 8 g FW L^{-1} tested in duplicate. The experiments ran in a period of 6 weeks each (Trial 2: June 9th to July 21st of 2020; Trial 3: July 27th to September 7th of 2020) in EPPO, IPMA. In these trials the effluent used was different, since the fish wastewater was no longer available, instead, an "artificial wastewater" was created, adding to the reservoir tank nutrients (Figure 2.1 c). Target nutrient levels in wastewater were decided based on preliminary analysis of the TAN, NO_3^- and PO_4^{3-} levels present on the effluent from fish culture. Nutrient stock solutions of approximately 4 M NH_4Cl, 2 M $NaNO_3$, and 2 M NaH_2PO_4 were prepared throughout the experiment, and used within 6 days to 2 weeks to maintain the quality of the solutions, to obtain the targeted concentrations in the nutrient's reservoir and consequently in the artificial wastewater. Based on f/2 medium (Guillard & Ryther, 1962), two stock solutions of vitamins and trace metals were also prepared and added to the deposit (approximately 32.7 mL and 62.3 mL, respectively). The nutrients were added to a deposit with sterile saltwater, which was sterilized using bleach (13 %), which was then neutralized in a proportion of 2:1 with sodium thiosulphate (150 g L^{-1}). The nutrient supply was done with a continuous flow (0.52 L h^{-1}), that was checked and

adjusted every day, to obtain a targeted final concentration on the effluent as presented in Table 2.1.

Table 2.1: Target concentration (µM) of Nitrogen and Phosphorus in the deposit of 30 L.

	Target concentration (µM)
NH_4Cl	80
$NaNO_3$	8
NaH_2PO_4	6

Like in the first trial, the biomass was totally removed from each tank and weighed, every 7 days, each tank was cleaned as explained before and treatment was randomly placed. Before weighing, the excess superficial water was removed using a paper towel then biomass was weighted on a digital balance, and the process repeated until no differences in weight existed (fresh weight). The excess biomass from each unit received the same process as in the first trial, to obtain the dry weight.

2.3. Calculation of growth parameters

The productivity of the tanks was calculated by the equation adapted from Abreu and co-workers (2011): (g DW m^{-2} wk^{-1}) = (DW / FW) x [(F_{FW} – I_{FW}) / A], here wk stands for week., F_{FW} is the final fresh weight, I_{FW} the initial fresh weight, DW/FW the dry weight/ fresh weight ratio (0.143 for trial 1, 0.221 for trial 2, and 0.209 for trial 3) and A is 0.08 (m^2) the surface area of the tank. The cumulative yield is the accumulation of biomass produced overtime in each treatment, expressed in g DW m^{-2} as above.

2.4. Characterization of seaweed biomass

2.4.1. Carbon and nitrogen tissue analysis

For each trial Initial Biomass samples of *Plocamium* (n = 3), trials 1 and 2, and *Sphaerococcus* (n = 3), trial 3 were processed and analysed for initial tissue carbon (C) and nitrogen (N) content. After 9 weeks in culture for trial 1, and 6 weeks for trials 2 and 3, 2 samples from each density treatment were collected, processed and analysed for C and N

content. After collection and cleaning with saltwater, the samples were oven-dried (60 ºC) for several days until no change in weight was observed and maintained in a dry place until analysis. Before being send to final analysis, the seaweed samples were dried (60 ºC) again for a day, to eliminate any humidity gained, and ground to a fine powder using a mortar.

The analyses were carried out in an Elemental Analyzer (EA), Flash 2000 model, Organic Elemental Analyzer (Thermo Scientific), coupled to an Isotopic Ratio Mass Spectrometer (IRMS) (Delta V Advantage), at Marinnova facilities in October 2020. The analytical quality control was ensured by reading a sample of urea and *Chlorella sp.*, and the elemental composition was performed based on the caffeine calibration curve. The results are presented in percentage of N and C.

2.4.2. Samples preparation

To determine the protein, lipid, ashes and total polyphenolic content and antioxidant activity, samples (initial biomass and density 2, 4, 6 and 8 g L^{-1} from trials 2 and 3; and initial biomass from trial 1 to determine the total polyphenolic content) were prepared. To have success in the analyses the samples must be as homogeneous as possible to obtain more reliable results due to the fact that the sampling is more representative, for this, the samples were crushed and homogenized for 20 seconds using a Retsch MM 400 mill with a frequency of 25 s^{-1}, the samples were previously kept at -20 ºC, then lyophilized and kept at -80 ºC before and after they were ground. This analysis was performed in IPMA, Lisbon.

2.4.3. Determination of the protein content

The determination of protein content (% dw) was carried out by the Dumas method (Saint-Denis & Goupy, 2004), in a nitrogen analyser (LECO FP-528, Leco Corporation, EUA) calibrated with EDTA (ethylenediaminetetraacetic acid), and a conversion factor of nitrogen to protein of 6.25.

2.4.4. Determination of the total lipid content

The total lipid content was determined by the Bligh and Dyer method (1959), for this, 0.5 g of sample was weighted and transferred to 20 mL tubes, to this tubes 5 mL of methanol:

chloroform solution (2:1) were added, homogenization was performed using a polytron for 1 minute at 20 000 rpm using a medium rod (IKA Ultra-Turrax), after homogenization, 1 mL of saturated sodium chloride (NaCl) solution was added and stirring was continued for 30 seconds. 2 mL of chloroform was added, and homogenization continued for another 30 seconds; 2 mL of Milli-Q water was added and 1 more minute of homogenization, this time at 15 000 rpm. Tubes were centrifuged (Kubota 6 800) for 10 minutes at 4 ºC at 5 000 rpm (3 phases appear). The aqueous phase (upper) was discarded, and the organic phase (bottom) was removed and filtered through a column of cotton and anhydrous sodium sulphate to pear-balloons previously weighted. To the previous tubes were added 4 mL of chloroform and the polytron was used again for 1 minute at 20 000 rpm, tubes were then centrifuged for 3 minutes at 5 000 rpm at 4 ºC. Once again, the organic phase (bottom) was collected and filtrated through a column of cotton and anhydrous sodium sulphate to the pear-balloons. To the column of cotton and anhydrous sodium sulphate, 3 x 1 mL of chloroform was passed to drag leavings from the column. Chloroform was evaporated in a rotary evaporator (Heidolph 4011 Digital) in a bath at 45 ºC and 120 rpm. The samples were dried with a little nitrogen until they did not smell like chloroform. At the end, the weight of the pear-balloon was recorded, and the percentage (%) of lipids was obtained through the following equation:

$$\text{Total lipid content on dry basis (\% by weight)} = [(W_2-W_1) / m] \times 100$$

Where W_2 = Weight of the pear-balloon with the organic phase (g); W_1 = weight of the pear-balloon (g); m = weight of the sample (g).

2.4.5. Determination of the ashes content

The ashes content was determined by incinerating 1.5 g of sample in a muffle furnace gradually heated to 500 ºC (Heraeus KR 170E) following the official AOAC method (AOAC 920.153). After obtaining whitish ash, the capsule was cooled in a desiccator, followed by weighing, the process of heating, cooling, and weighing was repeated at half-hour intervals until a constant weight was obtained. Results are expressed as a percentage (%) of ash in dry weight, and were calculated by the following equation:

$$\text{Total ash on dry basis (\% by weight)} = [(W_2 – W_1) / m] \times 100$$

Where, W_2 = weight of the capsule with the ash (g); W_1 = Weight of empty capsule (g) and m = Weigh of the sample (g).

2.4.6. Extract preparation for evaluation of total polyphenolic content and antioxidant activity

The evaluation of the total polyphenols content and antioxidant activity was determined in two extracts, one using water as solvent and another one using ethanol 96 %. The extracts were prepared using the initial biomass of trials 1, 2, and 3 and final biomass of density 4 and 8 g L^{-1} of trial 2 and 3 (Figure 2.2).

Figure 2.2: a) Water (left) and ethanol 96 % (right) extracts of initial biomass from trial 1; b) Water extracts of initial biomass and densities 4 and 8 g L^{-1} from trial 2; c) Ethanol 96 % extracts of initial biomass and densities 4 and 8 g L^{-1} from trial 2; d) Water extracts of initial biomass and densities 4 and 8 g L^{-1} from trial 3 and e) Ethanol 96 % extracts of initial biomass and densities 4 and 8 g L^{-1} from trial 3.

To a sample of 1.25 g of freeze-dried and ground seaweed was added 25 mL of Milli-Q water or ethanol 96 %, this mixture was homogenized in a polytron at 30 000 rpm for 1 minute (IKA Ultra-Turrax) and put in an orbital (IKA-vibrax-VXR) overnight with 300 rpm

agitation, after that the samples were centrifuged at 4 ºC during 10 minutes at 5000 rpm (Kubota 6 800). The liquid phase was collected, and the volume adjusted to 25 mL.

2.4.7. Total polyphenolic content

The total polyphenols compounds in the aqueous and ethanolic extracts were determined by the spectrophotometric method, using the Folin-Ciocalteu reagent (FCR). According to the methodology previously described by Singleton and Rossi (1965), to a 5 mL tubes were added 100 µL from each extract, 600 µL of Milli-Q water and 150 µL of Folin-Ciocalteu Reagent diluted 2x and agitated in the vortex (IKA vortex 3). The tubes were covered with foil paper so that the reaction would take place in the dark for 5 minutes at room temperature (24 ºC). After the 5 minutes, 750 µL of a solution of sodium carbonate (Na_2CO_3) 2 % was added to the tubes, vortexing was done again and the tubes were incubated in the dark for an hour and half at room temperature (24 ºC). The absorbance value of each sample (n = 2) was obtained at 750 nm. The content of total polyphenols was obtained through a calibration curve using known concentrations of Gallic acid, the results were expressed in gallic acid equivalents (GAE) in mg GAE per 100 g of seaweed (dw).

2.4.8. Antioxidant activity

For the evaluation of antioxidant activity, three tests were carried out, according to previously described methodologies (Benzie & Strain, 1996; Martins et al., 2013; Re et al., 1999; Miliauskas et al., 2004).

2.4.8.1. FRAP method

The method consists in reducing the Fe(III)/ferricyanide [$FeCl_3/K_3Fe(CN)_6$] to Fe(II), ferrous form, by the antioxidant compound in the solution. Thus, the antioxidant power was determined by reducing the ferric ion, according to the methodology previously described by Benzie and Strain (1996) and Martins and co-workers (2013), using a ferrous sulphate calibration curve.

To a 5 mL tubes were added 100 µL from each extract and 3 mL of a FRAP solution (10:1:1, v/v/v of acetate buffer 0.3 M, pH 3.6, TPTZ solution 10 mM and ferric chloride ($FeCl_3 \cdot H_2O$)

20 mM;), the tubes were agitated in the vortex (IKA vortex 3) and incubated in the dark for 30 minutes at 37 ºC. Absorbance values of each sample (n = 2) were obtained at 595 nm (Unicam Helios alfa). The antioxidant activity was obtained through a calibration curve using known concentrations of iron sulphate, the results are presented in equivalents iron sulphate (ISE) equivalents in µmol ISE per 100 g of seaweed (dw).

2.4.8.2. ABTS method

The method described gives a measure of the antioxidant activity of the range of carotenoids and phenolics, determined by the decolorization of the $ABTS^{\bullet+}$, that is reduced in presence of such hydrogen-donating antioxidants, through measuring the reduction of the radical cation as the percentage inhibition of absorbance at 734 nm. The antioxidant activity was assessed as described by Re and co-workers (1999); to a 5 mL tubes were added 20 µL from each extract and 2 mL of a solution of ABTS (7 mM), the tubes were agitated in the vortex (IKA vortex 3) and incubated in the dark for 6 minutes at 30 ºC. Absorbance values of each sample (n = 2) were obtained at 734 nm (Unicam Helios alfa). The antioxidant activity was obtained through a calibration curve using known concentrations of Trolox, the results are presented in Trolox equivalents in µmol Trolox per 100 g of seaweed (dw).

2.4.8.3. DPPH method

$DPPH^{\bullet}$ is a stable radical used to assess the free radical reducing capabilities of various antioxidants, through the absorbance loss measured at 517 nm. This determination was performed accordingly to the method previously described for Miliauskas and co-workers (2004).

To a 5 mL tubes were added 1 mL from each extract and 2 mL of a solution of DPPH (0.15 M), the tubes were agitated in the vortex (IKA vortex 3) and incubated in the dark for 30 minutes at room temperature. Absorbance values of each sample (n = 2) were obtained at 517 nm (Unicam Helios alfa). The antioxidant activity was obtained through a calibration curve using known concentrations of ascorbic acid, the results are presented in ascorbic acid (AA) equivalents in mg AA per 100 g of seaweed (dw).

2.5. Statistical data analysis

Statistical analysis was done to a significance level (α) of 0.05, whenever *p* was lower than α, statistical differences were identified. In order to test normality and variance homogeneity, the Shapiro-Wilk's test and Levene's F-test, respectively, were used. When these assumptions were fulfilled, the parametric tests ANOVA and two-way t-test were used to test for differences between means (2 or more samples). When the required assumptions were not met transformation of the data was attempted, natural logarithmic, logarithm base ten, the inverse and square rout. If transformed data met the assumptions the parametric tests were used. If the data still didn't fulfil these assumptions, the nonparametric Kruskal-Wallis and Wilcoxon (2 samples) tests were used. Significant differences were analysed *a posteriori* with a Tukey HSD and Games Howell test, respectively after ANOVA and non-parametric tests with same n, and ANOVA with different n. Statistical analyses were performed using IBM SPSS statistical software, version 21.0 (IBM Corporation, Armonk, New York, EUA).

3. Results

3.1. Environmental parameters

Environment parameters measured during the trials, namely irradiance, water temperature, water pH, dissolved nutrients, are reported below in detail. Salinity, also measured, was constant and had the same value during the experiments (35 ppm), except for a week (10/07/2020 – 17/10/2020) during trial 2 where it reached 37 ppm.

3.1.1. Irradiance

The daily mean irradiance (Figure 3.1) were 182 ± 45.5, 451 ± 24.0, and 391 ± 52.9 µmol m^{-2} s^{-1}, for trials 1, 2, and 3 respectively. The highest daily mean irradiance was in June (480 µmol m^{-2} s^{-1}) and the lowest value in December (89 µmol m^{-2} s^{-1}). Non parametric analysis demonstrated significant differences on irradiance levels between trials [Z = -8.273; Z = -5.756; p < 0.001], the irradiance levels on trial 2 were significantly higher than the

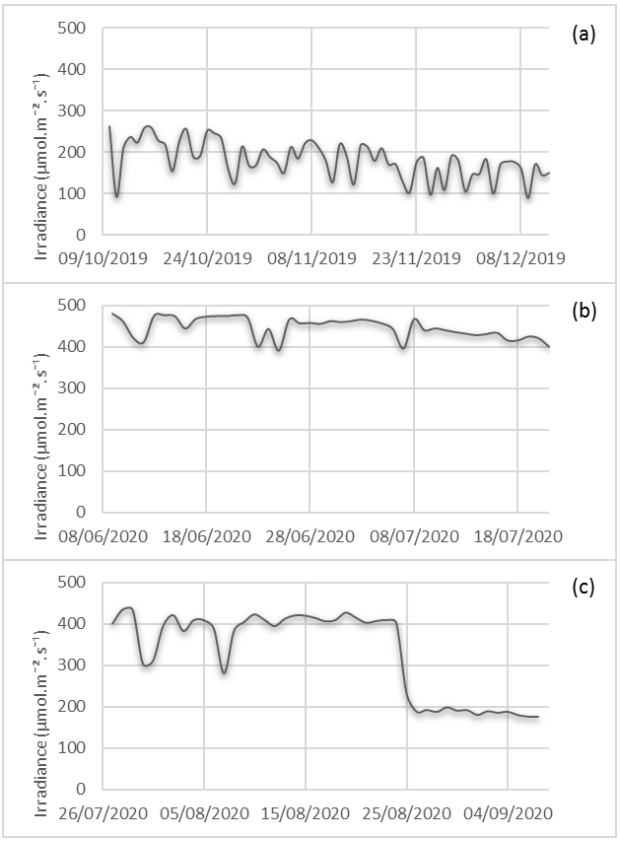

Figure 3.1: Daily mean surface irradiance measured at the seaweed system a) on trial 1, b) on trial 2 and c) on trial 3.

irradiance levels measured during trial 1 and were also higher during trial 2 when compared to trial 3.

3.1.2. Water temperature

The temperature variation between and during trials is presented in Figure 3.2. Where it is visible that in trial 1, from the third week onwards there was a drop in the water temperature, remaining lower until the end of the experiment. In trial 2, the water temperature increased from the beginning until the end of the experiment. In trial 3, the water temperature remains relatively constant throughout the experiment. Extreme temperature values were registered in November (12.9 ºC) and July (26.4 ºC).

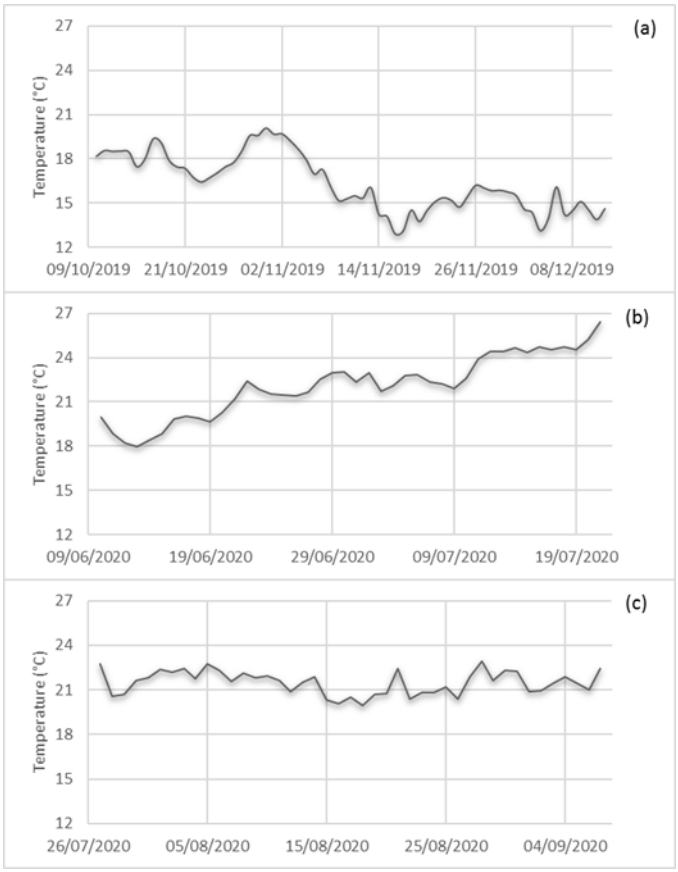

Figure 3.2: Mean temperature in the seaweed cultivation system during a) trial 1, b) trial 2 and c) trial 3, (n =3).

Mean temperatures for each trial were 16.4 ± 1.94 ºC (trial 1), 22.1 ± 2.10 ºC (trial 2) and 21.5 ± 0.80 ºC (trial 3). Kruskal-Wallis non-parametric analysis demonstrated significant differences on the water temperature between trials [$X^2(2) = 104.157$; $p < 0.001$]. The comparisons between pairs showed that water temperature on trial 1 (*P. cartilagineum* in winter) is significantly lower than water temperature on trial 2 (*P. cartilagineum* in spring),

but the water temperature on trial 2 does not differ significantly from trial 3, which were expected since trials 2 and 3 were performed on the same season.

3.1.3. Water pH

In trial 1, the pH water levels of the inflow was tested, since it was measured twice a day (morning and afternoon), and demonstrated that measurement at different times of the day does not interfere with the pH water levels [$t(14) = 0.269$; $p > 0.05$]. Also, differences between the different densities were tested, verifying that the different densities do not interfere with the pH water [$F(8, 102) = 1.492$; $F(8, 188) = 1.482$; $p > 0.05$, morning and afternoon, respectively]. When testing for significant differences between the inflow and the outflow from the different densities tested (morning and afternoon), the test showed a difference, despite this result the post-hoc does not identify where there are the significant differences. The average pH water levels oscillated between 7.5 and 8.2 (Figure 3.3 a).

On trials 2 (Figure 3.3 b) and 3 (Figure 3.3 c), the pH in water were only measured once a day (morning), during weekdays. On trial 2, according to Kruskal-Wallis test [$X^2(4) = 37.464$; $p < 0.001$] and post-hoc, showed that the inflow pH water was significantly lower than pH water outflow from every density, but no significant differences between the outflows from the density treatments were observed. The average water pH were 8.01 ± 0.076 for the inflow and 8.13 ± 0.076 from the outflow. Similar statistical analysis showed that the pH on trial 3, in inflow waters were significantly lower than the all measured outflows, but no significant differences were found between the different densities [$X^2(4) = 62.445$; $p < 0.001$]. The average pH water levels were 7.91 ± 0.055, 8.04 ± 0.055 for inflow and outflow respectively.

The comparison of inflow water pH between trials 1 and 2 (*P. cartilagineum*, different season), performed using the Wilcoxon test, showed that the inflow pH in trial 2 was significantly higher than in trial 1 [$Z = -3.581$; $p < 0.001$], and the comparison of the inflow

pH water levels from trial 2 and trial 3 (same season, two different species), showed that the pH from trial 2 was significantly higher than in trial 3 [Z = -3.416; $p < 0.05$].

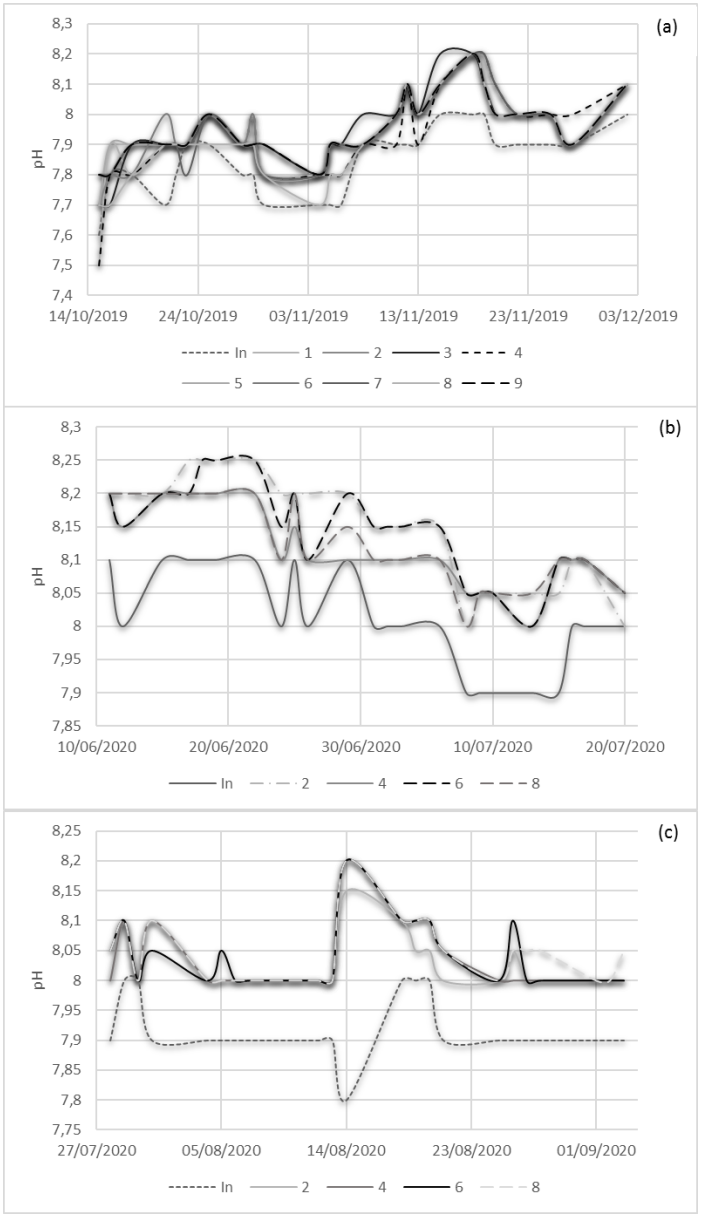

Figure 3.3: Water pH at the seaweed system during a) trial 1, mean of morning and afternoon measurements, b) trial 2, and c) trial 3 (In: Inflow water; 1 to 9: Outflow water, respectively from density treatment 1 to 9 g L^{-1}), n = 2.

3.1.4. Dissolved nutrients

The dissolved nutrients analysed were nitrogen as nitrate ($N\text{-}NO_3^-$) and total ammonia nitrogen (TAN: $N\text{-}NH_3+NH_4^+$), and phosphorus as phosphate ($P\text{-}PO_4^{3-}$). To investigate nutrient supply differences between trials, a Kruskal-Wallis test was performed and showed

that the levels of $N\text{-}NO_3^-$, TAN and $P\text{-}PO_4^{3-}$ differ from trial 1 (real fish waste) to trial 2 (artificial fish waste) [$X^2(2) = 18.505$; $X^2(2) = 31.324$; $X^2(2) = 39.876$; $p < 0.001$, respectively], but no significant differences between trial 2 and trial 3 were found. The levels of $P\text{-}PO_4^{3-}$ on trial 1 were higher than on trial 2; on the other side, the levels of TAN and $N\text{-}NO_3^-$ on trial 1 were lower than on the trial 2. The mean nutrient concentrations are present in the Table 3.1.

Table 3.1: Dissolved nutrients ($N\text{-}NO_3^-$, TAN and $P\text{-}PO_4^{3-}$) present in the water during the trials (mean ± S.D.). Means sharing a letter do not differ significantly ($p > 0.05$).

	$N\text{-}NO_3^-$ (µM)	TAN (µM)	$P\text{-}PO_4^{3-}$ (µM)
Trial 1	4.06 ± 1.616 (a)	20.71 ± 4.320 (a)	5.42 ± 1.261 (a)
Trial 2	10.50 ± 3.402 (b)	32.50 ± 10.785 (b)	2.76 ± 1.733 (b)
Trial 3	20.21 ± 17.323 (b)	45.61 ± 10.612 (b)	7.87 ± 11.754 (b)

3.2. Seaweed growth

3.2.1. Dry and Fresh weight

For trials 1, 2, and 3 the Kruskal-Wallis test was performed to see if there were any significant differences between the relationship of dry to fresh weight (DW/FW) of the initial biomass and final biomass of different densities (2, 4, 6, and 8 g L^{-1}) after two weeks in cultivation, and for all the trials there were no statistical differences between the initial biomass and the different densities tested ($p > 0.05$). Next, a one-way ANOVA was performed to see if the time of collections (initial biomass trials 1 and 2) affected the percentage of water content, and the content from trial 1 (88.76 ± 1.161 %) was significantly higher than on trial 2 (83.29 ± 0.360 %). Also, comparison between the water percentage content of the two investigated species showed no significant differences when comparing the initial biomass from trial 2 to trial 3 (81.41 ± 0.958 %).

3.2.2. Seaweed growth

Plocamium cartilagineum was the species that was kept in culture for a longer time, but it did not show consistent growth.

On trial 1, the highest values of productivity registered (121.45 and 96.74 g m^{-2} wk^{-1}) were observed at the end of October for *P. cartilagineum* cultivated under a density of 9 and 4 g L^{-1}, respectively. For this first trial a two-way ANOVA tested if the productivity differed along weeks (time) and differed according to the different density stocking, according to this test, the different densities had no significant effect [$F(8, 56) = 0.426$; $p > 0.05$], but different weeks had a significant effect on productivity [$F(8, 56) = 6.303$; $p < 0.05$], as shown in Figure 3.4 a. Seaweed at the lower densities, 1, 2 and 3 g L^{-1} did not survive until the end of the first trial (Figure 3.4).The productivity of *P. cartilagineum* in this trial for 8 g L^{-1} was more

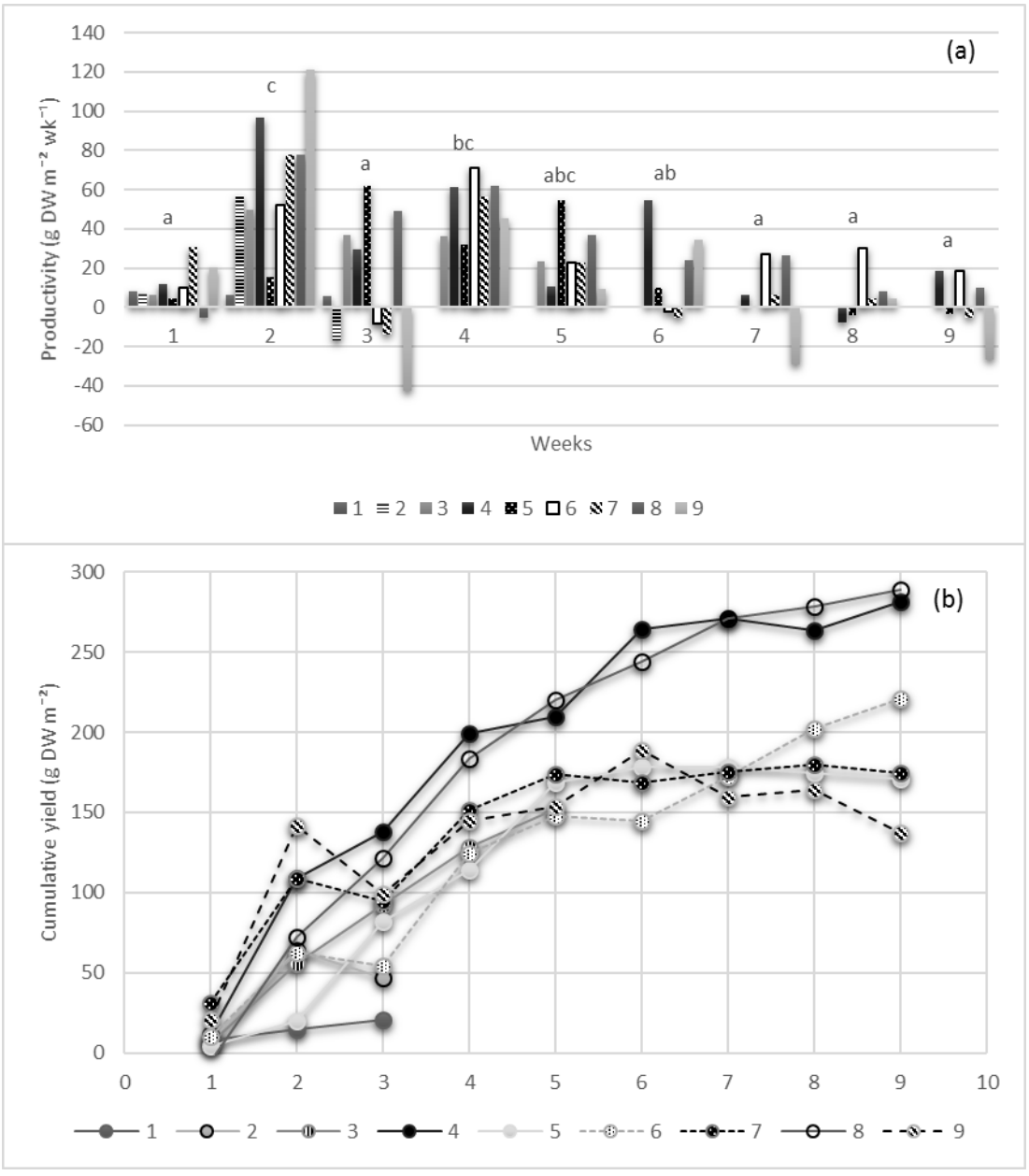

Figure 3.4: (a) Productivity of *P. cartilagineum* during trial 1. The bars represent the productivity of each density (1 to 9 g L^{-1}, n = 1). Means sharing a letter do not differ significantly ($p > 0.05$). (b) Cumulative yield of *P. cartilagineum* in trial 1 for each tested density. Each data point represents cumulative seaweed yield in an individual tank stocked in the indicated density.

constant throughout the experiment, and even though the different densities did not present significant differences, density 4 g L^{-1} obtained, on average, higher yield. For better visualization the cumulative yield is presented. The total cumulative yield was not significantly different for the densities tested had no significant differences [$X^2(8) = 8.000$; $p > 0.05$] (Figure 3.4 b) having on average 141.54 ± 117.070 g DW m^{-2}.

On trial 2 (Figure 3.5 a), the highest value of productivity registered (73.20 g DW m^{-2} wk^{-1}) was observed in the middle of June for *P. cartilagineum* cultivated under a density of 4 g L^{-1}. According to two-way ANOVA, the different stocking densities had no significant effect on productivity [$F(3, 44) = 0.965$; $p > 0.05$], but time had a significant effect [$F(5, 42) = 8.154$;

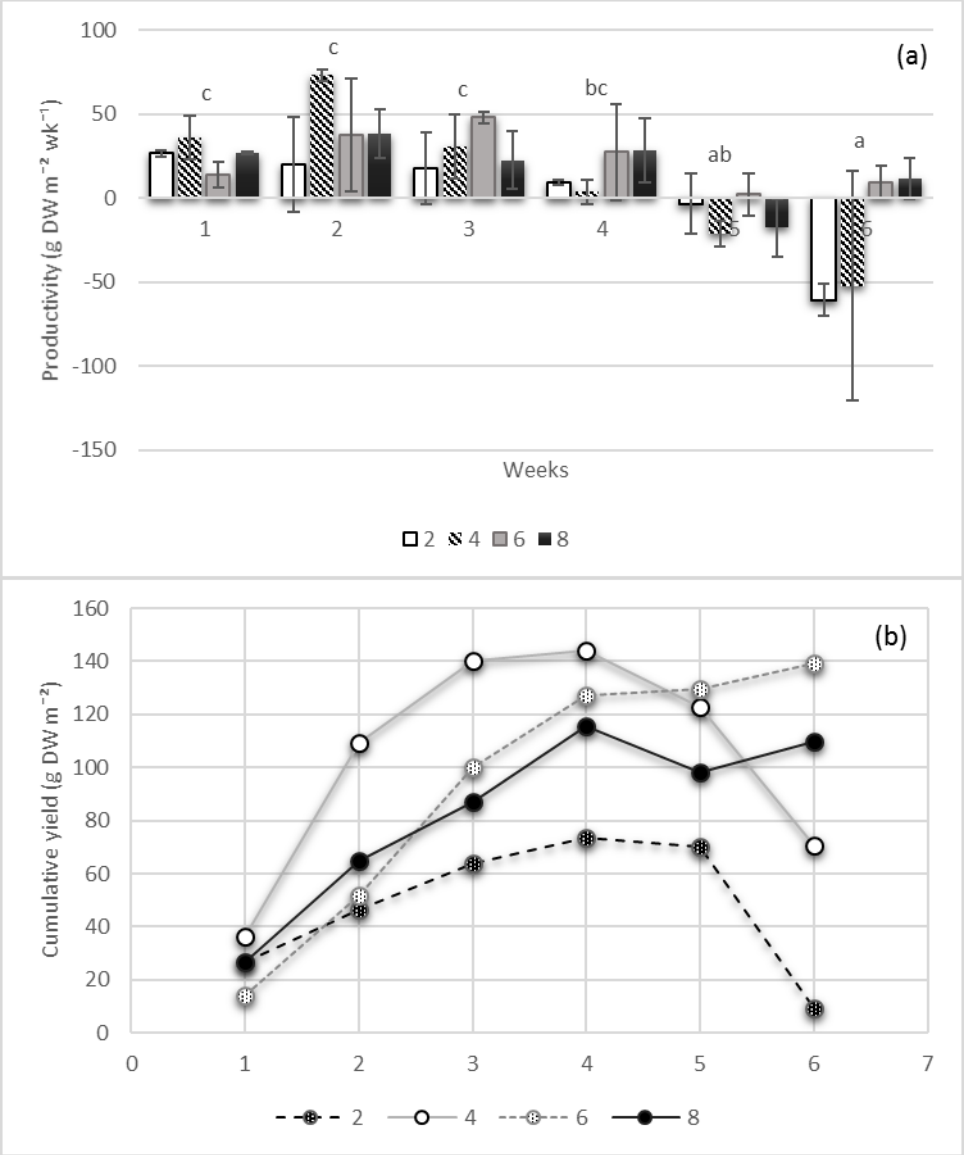

Figure 3.5: (a) Productivity of *P. cartilagineum* during trial 2. The bars represent the productivity of each density (2, 4, 6 and 8 g L^{-1}, n = 2). Means sharing a letter do not differ significantly ($p > 0.05$). (b) Cumulative yield of *P. cartilagineum* in trial 2 for each tested density. Each data point represents cumulative seaweed yield in an individual tank stocked in the indicated density.

$p < 0.05$], as shown in Figure 3.5 a. Even though there were no significant differences between the different densities, 6 g L^{-1} was the one, in terms of productivity, that had, on average, higher values and fewer variations during the time in cultivation. The different stocking densities were also tested to see if they had any effect in the total cumulative yield. In this trial the different densities also showed no significant differences [$X^2(3) = 3.500$; $p > 0.05$]; despite the test results, it is possible to see from Figure 3.5 b that density 2 g L^{-1} (9.45 ± 81.216 g DW m^{-2}) had lower values, and density 4 g L^{-1} the higher cumulative yield (70.43 ± 118.682 g DW m^{-2}) (Figure 3.5 b).

On trial 3 (Figure 3.6 a), the highest values of productivity (61.35 and 54.12 g DW m^{-2} wk^{-1}) for *S. coronopifolius* were observed under 8 and 6 g L^{-1} density cultivation, in the middle of August. In order to see if there was any effect of the time and stocking densities on productivity, a two-way ANOVA test was done and showed that biomass yield had significant differences only with the effect of time [$F(5, 42) = 9.786$; $p < 0.05$]. Even though there were no significant differences between the different densities, density 8 g L^{-1} was the one that had a better performance during the time in cultivation, with higher productivity values (19.74 ± 30.513 g DW m^{-2} wk^{-1}) and a more constant behaviour. The effect of the different stocking densities in the total cumulative yield was also tested; the different densities also showed no significant differences [$X^2(3) = 5.500$; $p > 0.05$], despite that, the Figure 3.6 b shows that by the end of the experimental period, density 8 g L^{-1} had the highest value (118.43 ± 64.163 g DW m^{-2}) (Figure 3.6 b).

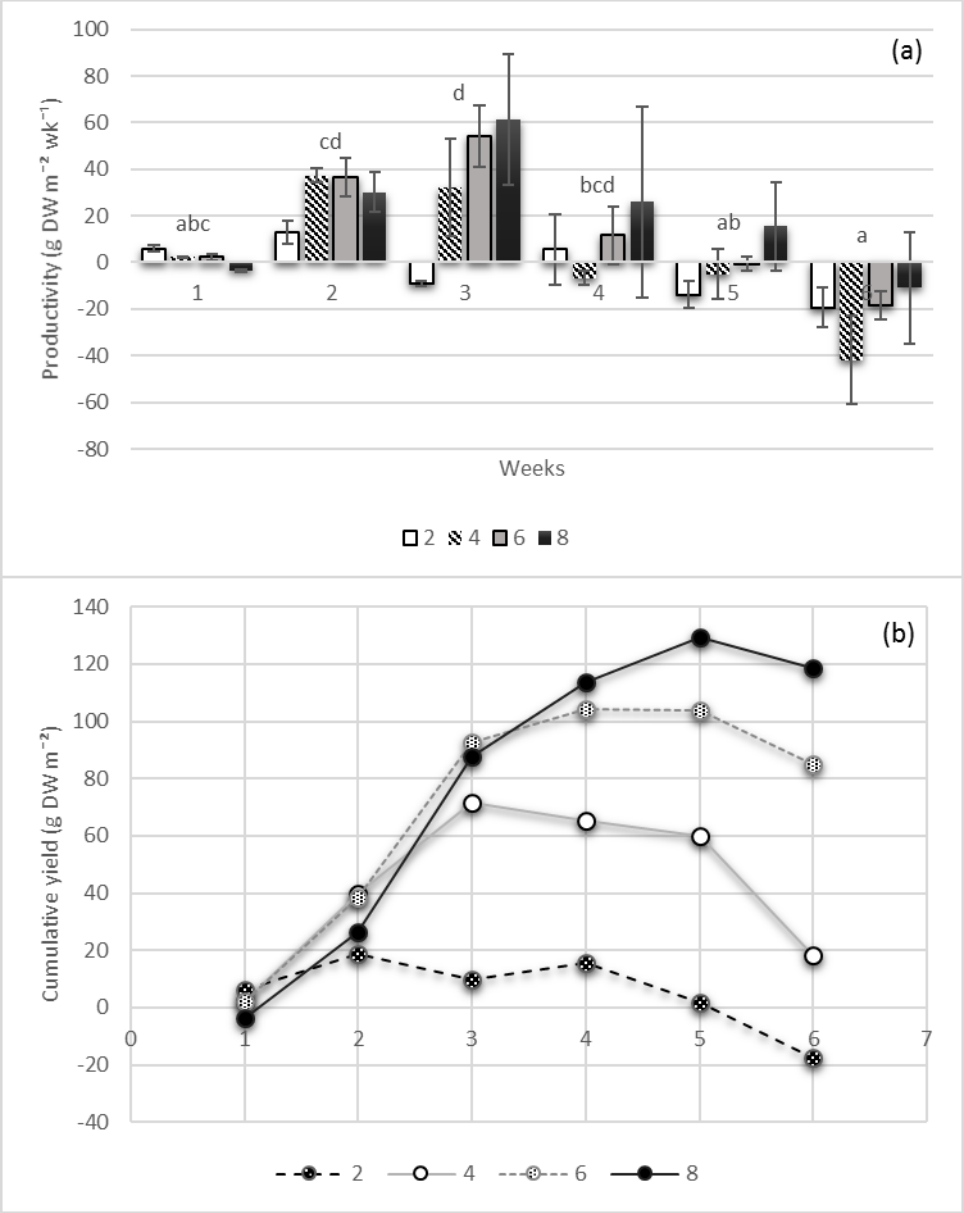

Figure 3.6: (a) Productivity of *S. coronopifolius* during trial 3. The bars represent the productivity of each density (2, 4, 6 and 8 g L^{-1}, n = 2). Means sharing a letter do not differ significantly ($p > 0.05$). (b) Cumulative yield of *S. coronopifolius* in trial 3 for each tested density. Each data point represents cumulative seaweed yield in an individual tank stocked in the indicated density.

According to the Wilcoxon test, the different trials performed during summer (trial 2 and trial 3) had no significant effect on the productivity of the two species [$Z = -1.918$; $p > 0.05$], despite that, the mean productivity for each trial showed that trial 2 had better productivity when compared with trial 3, which means that the *P. cartilagineum* was more productive than *S. coronopifolius* when tested in the same conditions.

At the end of each experimental trial with *P. cartilagineum* and *Sphaerococcus coronopifolius*, there were a lot of contamination with other seaweed species, green and brown species (see figure in Appendix A).

3.3. Seaweed biomass characterization

The seaweed biomass was characterized in terms of tissue carbon and nitrogen content, protein, lipids and ashes content, and so the total polyphenols content and antioxidant activity, that are described below in detail.

3.3.1. Tissue carbon and nitrogen content

The tissue nitrogen (N) (% dw) from the initial biomass of *P. cartilagineum* were 4.27 ± 0.163 % (trial 1; n = 3) and 4.25 ± 0.176 % (trial 2; n = 3), and for the *S. coronopifolius* 3.28 ± 0.098 % (trial 3, n = 3). Results from ANOVA and post-hoc Tukey test analysis show that the *P. cartilagineum* N content from the initial biomass in trial 1 (winter) is not significant different from that of trial 2 (spring); however the N content from the *P. cartilagineum* initial biomass from trial 2 is significantly higher when comparing to that of *S. coronopifolius* (trial 3, same season).

For trial 1, the unbalanced ANOVA and post-hoc Tukey test showed significant differences between the initial biomass and the final biomass from the different densities tested (4, 5, 6, 7, 8, and 9 g L^{-1}), the initial biomass had lower N content than the seaweed final biomass, also some significant differences between the final biomass in the different stocking densities were observed as shown in Figure 3.7 a [$F(6, 11) = 283.109$; $p < 0.001$].

For trial 2, the unbalanced ANOVA and post-hoc Tukey test used to evaluate the influence of cultivation and the different densities tested on seaweed N content showed significant differences [$F(4, 17) = 104.825$; $p < 0.001$], being that the N content increased in final biomass from all the different densities tested (2, 4, 6 and 8 g L^{-1}) when compared to the initial biomass content. When comparing final biomass among the different densities, only a significantly lower N content in density 6 g L^{-1} than 8 g L^{-1} was found (Figure 3.7 b).

For trial 3 the same tests were performed, and also showed that the N content from the initial biomass was significantly lower than in final biomass from the different densities tested (2, 4, 6, and 8 g L^{-1}). Besides final biomass from density 4 g L^{-1} had significantly higher values than the N content than from density 8 g L^{-1} (Figure 3.7 c).

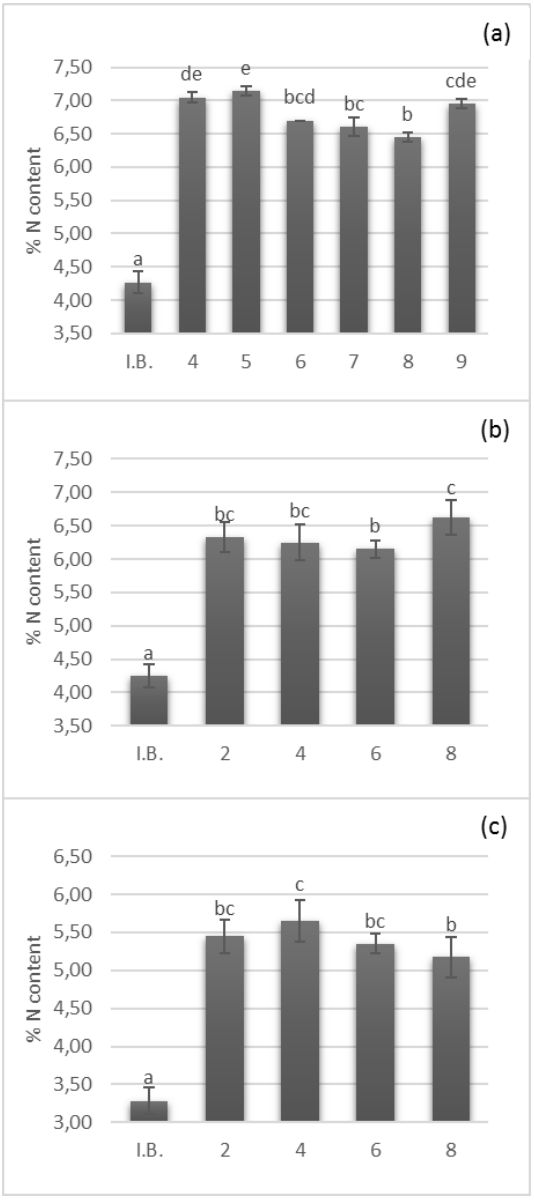

Figure 3.7: Mean (± SD) nitrogen concentration (% dw) in tissues of *P. cartilagineum* on (a) trial 1, n = 2, and (b) trial 2, n = 4; and *S. coronopifolius* (c) trial 3, n = 4 from the natural population (I.B.: Initial biomass) and after the time in cultivation (trial 1: 9 weeks, trial 2 and 3: 6 weeks) under densities (2, 4, 5, 6, 7, 8, 9 g L^{-1}). Means sharing a letter do not differ significantly ($p > 0.05$).

For the tissue carbon (C) content (% dw), the same statistical tests were used, unbalanced ANOVA for trial 1 [$F(6, 11) = 117.864$], trials 2 [$F(4, 17) = 42.995$] and 3 [$F(4, 17) = 3.386$], showing that the time in cultivation had a significant effect on the C content on trial 1, 2 and 3 ($p < 0.001$); on trial 1 and 2 the C content of the initial biomass was significantly lower

than in the final biomass from the different densities tested (2, 4, 6 and 8 g L^{-1}), on trial 1 there were significant differences between the different densities (Figure 3.8 a), but for trial 2 no significant differences were observed between the different densities (Figure 3.8 b). In trial 3, the post-hoc performed was different from the previous ones, the Games-Howell test, since that for the C content it did not pass the homogeneity variance test (Levene test), in this trial the C content from the initial biomass had a significantly lower value than the density 4 g L^{-1}, but density 8 g L^{-1} also differs from density 4 g L^{-1} and had the lower value registered (Figure 3.8 c).

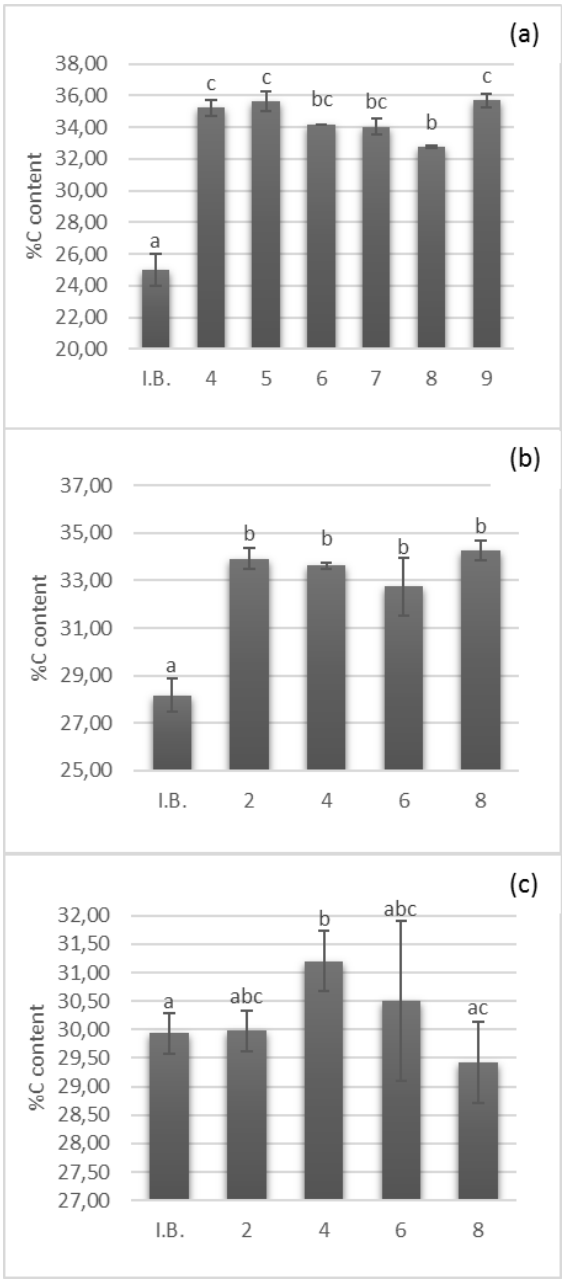

Figure 3.8: Mean (± SD) carbon concentration (% dw) in tissues of *P. cartilagineum* on (a) trial 1, n = 2, and (b) trial 2, n = 4; and *S. coronopifolius* (c) trial 3, n = 4 from the natural population (I.B.: Initial biomass) and after the time in cultivation (trial 1: 9 weeks, trial 2 and 3: 6 weeks) under densities (2, 4, 5, 6, 7, 8, 9 g L^{-1}). Means sharing a letter do not differ significantly ($p > 0.05$).

The C content (% dw) from the natural population of *P. cartilagineum* oscillated between 25.00 ± 0.986 % (trial 1; n = 3) and 28.38 ± 0.624 % (trial 2; n = 3), and for the *S. coronopifolius* the value was 29.93 ± 0.362 % (n = 3), in this case, the ANOVA and post-hoc Tukey test, showed that the C content from the initial biomass from trial 1 was significantly lower than the C content from the initial biomass from trial 2, the same happens when comparing the C content from the initial biomass from trial 2, that was significantly lower than the C content from the initial biomass from trial 3 [$F(2, 15) = 76.767$;$p < 0.001$].

When analysing the C to N ratio (C:N), presented in Figure 3.9, the unbalanced ANOVA, and post-hoc Games-Howell test showed that only the ratio from the initial biomass (trial 1: 5.86 ± 0.237; trial 2: 6.68 ± .153; trial 3: 9.12 ± 0.241) had significantly higher values than

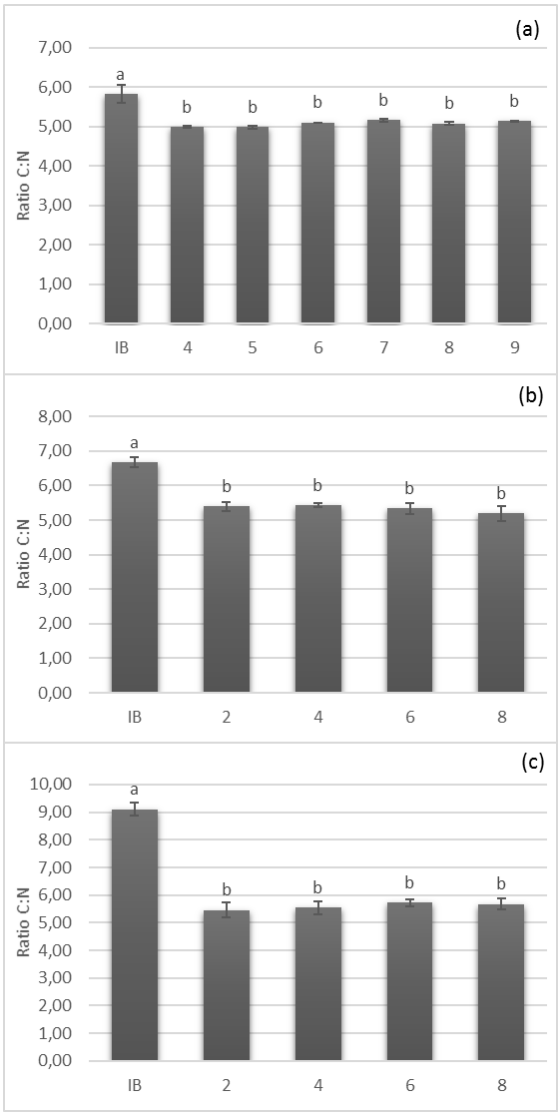

Figure 3.9: Mean (± SD) C:N ratio in tissues of *P. cartilagineum* on a) trial 1, n = 2, and b) trial 2, n = 4; and *S. coronopifolius* c) trial 3, n = 4 from the natural population (I.B.: Initial biomass) and after the time in cultivation (trial 1: 9 weeks, trial 2 and 3: 6 weeks) under densities (2, 4, 5, 6, 7, 8, 9 g L^{-1}). Means sharing a letter do not differ significantly ($p > 0.05$).

the final biomass from the densities tested for trial 1, 2 and 3 (5.10 ± 0.071; 5.37 ± 0.159; 5.63 ± 0.210, respectively).

The nutrient removal capacity of the *P. cartilagineum* and *S. coronopifolius* was analysed in terms of the N removed in the seaweed biomass produced, calculated from N content and seaweed yield, and referred as N-yield. As showed in Figure 3.10 a, during trial 1 the density 8 g L^{-1} showed higher results when compared to the other densities tested (4, 5, 6, 7, 9 g L^{-1}), despite that, the Kruskal-Wallis test showed no significant differences [$X^2(5)$ =

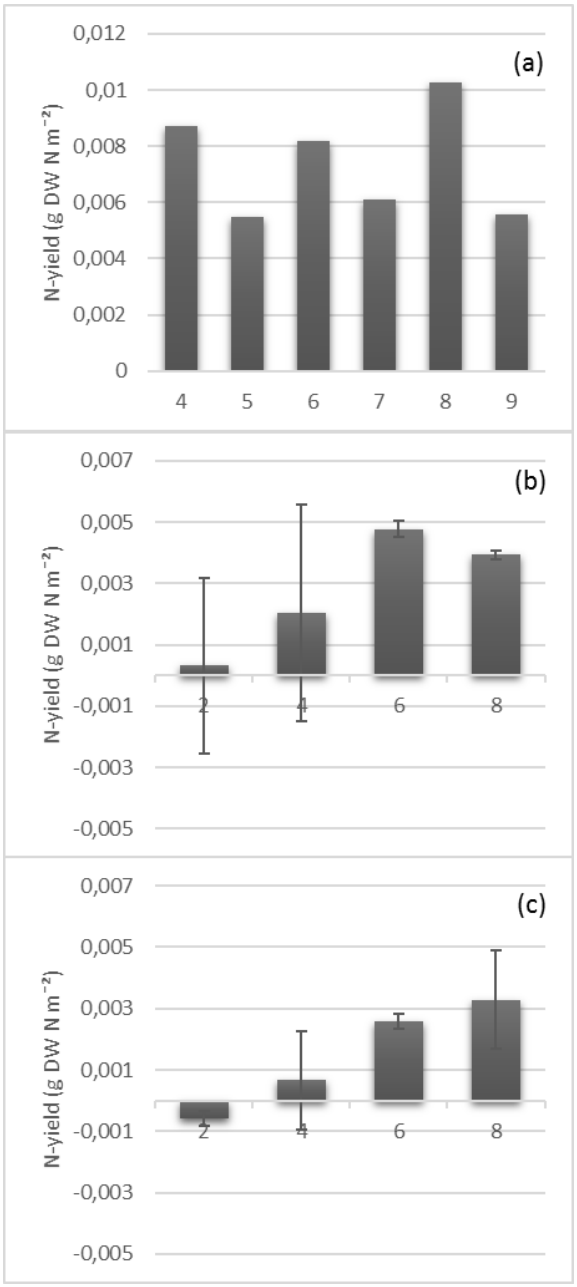

Figure 3.10: N-yield of produced biomass from (a) trial 1, (b) trial 2, and (c) trial 3. Data presented are from the cumulative biomass produced during the entire experiment and seaweed tissue N-content from the final biomass from the different densities tested (2, 4, 5, 6, 7, 8, 9 g L^{-1}). Mean (± SD; n =2).

5.000; p > 0.05] between the N-yield from the different densities tested (7.38 x 10^{-3} ± 1.962 x 10^{-3} g N DW m^{-2}). For trial 2, the density 6 g L^{-1} showed to be more efficient in terms of N removal, but like on trial 1, the test showed no significant differences [$X^2(3) = 5.167$; p > 0.05] (2.77 x 10^{-3} ± 2.522 x 10^{-3} g N DW m^{-2}) (Figure 3.10 b). During trial 3 (Figure 3.10 c), the seaweeds were capable to remove N from the water (1.49 x 10^{-3} ± 1.853 x 10^{-3} g N DW m^{-2}), as it happened during trials 1 and 2, and in this case, the density 8 g L^{-1} was the one with higher values of N-yield, even though the statistical test showed no significant differences between the different densities tested (2, 4, 6, 8 g L^{-1}). When comparing the performance during the different trials, the N-yield of *P. cartilagineum* did not showed significant differences between the two seasons tested, and *S. coronopifolius* did not showed significant differences when compared to *P. cartilagineum* tested in the same season, even though it is possible to see that *P. cartilagineum* had higher mean values during trial 1 (Figure 3.10).

The negative results on the three trials are explained by the negative values obtained with the cumulative yield, since in some weeks, there was no biomass production, in fact the values obtained were lower than the initial biomass of each tank, as previously described Figure 3.7.

3.3.2. Protein content

The protein content (% dw) was only measured in the initial biomass and final biomass from densities 2, 4, 6, and 8 g L^{-1} from trials 2 and 3. Kruskal-Wallis test showed that the protein content did not change with time in cultivation, both in trial 2 and trial 3 [Trial 2: X2(4) = 4.681; trial 3: X2(4) = 4.000; p > 0.05], despite the results of the test, it is possible to see a slight increase in the protein content during the time in cultivation (Table 3.2). A Wilcoxon test, showed that the protein content in trial 2 was significantly higher than the content in trial 3 (Z = -2.371; p < 0.05), which means that *P. cartilagineum* (36.83 ± 1.156 %) has a superior protein content than *S. coronopifolius* (29.40 ± 1.156 %) during the same season.

As said previously in methods, the protein content was obtained with a nitrogen-to-protein conversion factor of 6.25 that was proposed by the laboratory where this analysis was performed. On the basis of early determinations, the average nitrogen content of proteins was found to be about 16 %, which lead to use of the calculation N x 6.25 (1/0.16 = 6.25) to convert nitrogen content into protein content (Sáez-Plaza et al., 2013). On the basis of the results obtained, the average N content of proteins was found to be about 17.21 % for

trial 2, and 18.75 % for trial 3, which led to calculation of the factor to convert nitrogen into protein content of 5.80 and 5.33 for trials 2 and 3, respectively.

3.3.3. Total lipid content

Also the total lipid content (% dw) was only measured using the initial biomass and final biomass from densities 2, 4, 6, and 8 g L-1 from trials 2 and 3. The results showed that the total lipid content did not change with time in cultivation, and even the different densities showed no interference in the final content during trial 2 and trial 3 [Trial 2: [$X^2(4) = 7.309$; $p > 0.05$]; trial 3: [$X^2(4) = 7.855$; $p > 0.05$; (n = 2)], according to Kruskal-Wallis test; despite the lack of statistical significance, it is possible to observe a small increase in the lipid content from initial to final biomass (Table 3.2). When testing if there were any significant differences between trials 2 and 3, which means if there were any differences between the total lipid content in the two different seaweed species, the paired-samples t-test showed that there were no significant differences [$t(9) = -2.234$; $p > 0.05$], despite that, it is possible to see that the average of lipid content on trial 3 (4.80 ± 0.822 %) was slightly higher than the lipid content on trial 2 (4.50 ± 0.743 %).

3.3.4. Ashes content

Based on the same test performed in total lipid content, using the initial biomass and final biomass from the different densities (2, 4, 6 and 8 g L^{-1}) from trials 2 and 3 [Trial 2: $X^2(4) =$

Table 3.2: Total lipids, ashes and protein content of the analyzed seaweed. Values are expressed as the mean ± SD (n = 2).

Samples **(Trial 2)**	Protein (% dw)	Total Lipids (% dw)	Ashes (% dw)
Initial Biomass	21.40 ± 0.00	3.61 ± 0.03	36.60 ± 0.01
Density 2 g L^{-1}	38.40 ± 0.00	5.61 ± 0.45	25.62 ± 0.00
Density 4 g L^{-1}	36.15 ± 1.63	5.39 ± 0.18	25.50 ± 2.00
Density 6 g L^{-1}	37.00 ± 0.57	5.11 ± 0.11	27.10 ± 1.73
Density 8 g L^{-1}	36.55 ± 1.20	4.30 ± 0.19	25.00 ± 0.06
(Trial 3)			
Initial Biomass	16.30 ± 0.00	3.55 ± 0.02	31.42 ± 0.01
Density 2 g L^{-1}	29.30 ± 0.07	5.18 ± 0.16	29.59 ± 0.02
Density 4 g L^{-1}	30.90 ± 2.83	5.35 ± 0.33	28.49 ± 0.38
Density 6 g L^{-1}	28.90 ± 0.99	4.21 ± 0.29	27.82 ± 1.01
Density 8 g L^{-1}	28.55 ± 0.78	4.21 ± 0.07	29.04 ± 0.40

5.600; $p > 0.05$; trial 3 [$X^2(4) = 7.418$; $p > 0.05$; ($n = 2$)], also showed that the time in cultivation does not change the percentage of ashes (Table 3.2).

3.3.5. Total polyphenolic content

The content of total polyphenols was obtained through two extracts, water and ethanol 96%, for the initial biomass (trial 1, 2 and 3) and the final biomass (trial 2 and 3) from densities 4 and 8 g L^{-1}.

When comparing the initial biomass collected for trials 1 and 2, the independent-samples t-test showed significant differences on the content of total polyphenols in *P. cartilagineum*; on ethanol extract the average of total content of polyphenols on trial 1 (64.32 ± 6.451 mg GAE/100 g) was higher than in trial 2 (42.85 ± 1.738 mg GAE/100 g). While on water extracts trial 2 (82.47 ± 3.336 mg GAE/100 g) had a higher average total content of polyphenols than trial 1 (75.71 ± 2.263 mg GAE/100 g).

For trial 2, the ANOVA test showed that the time in cultivation affected the total polyphenols content. The Tukey post-hoc test showed that phenolic content in the initial biomass was significant lower than in final biomass for the densities 4 and 8 g L^{-1}, and differences between the two different densities (4 g L^{-1} > 8 g L^{-1} > Initial biomass) for the water [$F(2,9) = 386.786$; $p < 0.05$] and ethanol 96% extract [$F(2,9) = 98.395$; $p < 0.05$] (Table 3.3).

For trial 3, the ANOVA test showed that cultivation at different densities affected the total polyphenols content, in ethanol 96% extract (4 g L^{-1} > 8 g L^{-1}) according to the Tukey post-hoc test [$F(2,9) = 7.991$; $p < 0.05$], and also time in cultivation had an effect in this content, in water extract, the initial biomass had a significant lower value of the polyphenols content when compared to the final biomass from the two densities tested [$F(2,9) = 28.130$; $p < 0.05$] (Table 3.3).

The results obtained demonstrate that the total concentration of polyphenols in seaweeds varies according to the species.

Table 3.3: Results of the evaluation of phenolic compounds and antioxidant activity of the analyzed seaweed. Values are expressed as mean ± SD (n = 4).

Samples	FRAP (µmol ISE/100 g)		ABTS (µmol Trolox/100g)		DPPH• (mg AA/100 g)		Total polyphenols (mg GAE/100 g)	
Extract	Water	Ethanol 96 %	Water	Ethanol 96 %	Water	Ethanol 96 %	Water	Ethanol 96 %
(Trial 1)								
Initial biomass	-	-	-	-	-	-	75.71 ± 2.26	64.32 ± 6.45
(Trial 2)								
Initial biomass	50.19 ± 33.49	0	1616.99 ± 177.76	1687.74 ± 561.11	8.28 ± 2.97	35.62 ± 1.05	82.47 ± 3.34	42.85 ± 1.74
Density 4 g L^{-1}	505.24 ± 72.93	3.00 ± 4.92	4434.78 ± 262.04	3336.20 ± 169.89	21.26 ± 4.75	25.89 ± 6.73	207.86 ± 10.30	100.37 ± 7.22
Density 8 g L^{-1}	525.67 ± 125.07	0	4296.30 ± 531.97	2310.50 ± 305.50	45.28 ± 9.36	41.09 ± 0.59	189.73 ± 5.02	62.99 ± 6.98
(Trial 3)								
Initial biomass	152.50 ± 2.83	14.57 ± 29.14	2829.80 ± 128.09	1738.13 ± 539.44	43.60 ± 2.03	61.53 ± 4.65	83.32 ± 2.51	95.46 ± 10.11
Density 4 g L^{-1}	356.12 ± 8.86	59.53 ± 9.72	4488.79 ± 243.57	3778.14 ± 579.95	57.81 ± 1.48	28.67 ± 10.84	154.73 ± 5.52	109.07 ± 13.72
Density 8 g L^{-1}	214.93 ± 129.61	31.71 ± 22.07	4137.03 ± 250.60	3274.19 ± 493.93	54.26 ± 4.00	45.72 ± 1.23	158.58 ± 27.01	79.40 ± 6.38

3.3.6. Antioxidant activity

The antioxidant activity was tested using three different methods and was obtained through two different extracts, water and ethanol 96 %, only the initial biomass and the final biomass from densities 4 and 8 g L^{-1} (trial 2 and 3) were analysed.

3.3.6.1. FRAP method

On trial 2 a transformation of the data (square root) was performed, ANOVA and post-hoc Tukey test showed only significant differences between the initial biomass and the final biomass from the two densities on water extracts (initial biomass had significantly lower activity than final biomass from densities 4 and 8 g L^{-1}) [$F(2,9) = 40.739$; $p < 0.05$]. While on ethanol 96% extract there were no significant statistical differences between the tested samples [$F(2,9) = 2.217$; $p > 0.05$] (Table 3.3).

For trial 3, the results for the ethanol 96 % extract showed, according to an ANOVA test, that the initial biomass had a significantly lower antioxidant activity only comparing to final biomass at density 4 g L^{-1} [$F(2,9) = 4.321$; $p < 0.05$]. On water extracts, a Kruskal-Wallis test showed the same results [$X^2(2) = 8.028$; $p < 0.05$] (Table 3.3).

It is observed that the species *S. coronopifolius* was the one with the higher reducing power (FRAP) with 152.50 ± 2.830 µmol ISE/100 g on water extract on the initial biomass.

3.3.6.2. ABTS method

According to a multivariate ANOVA, and the post-hoc Tukey test, the trial 2 showed that the initial biomass had significant lower value than final biomass from densities 4 and 8 g L^{-1}, for water extracts [$F(2,9) = 78.998$; $p < 0.05$]. For ethanol 96 % extracts, final biomass from density 4 g L^{-1} had significant higher activity than density 8 g L^{-1} and initial biomass [$F(2,9) = 19.025$; $p < 0.05$].

For trial 3 the same tests were applied, and for the two extracts the initial biomass had significant lower antioxidant activity than final biomass from densities 4 and 8 g L^{-1} [Water: $F(2,9) = 66.192$; ethanol: $F(2,9) = 15.552$; $p < 0.05$] (Table 3.3).

It is observed that the species *S. coronopifolius* has a higher reducing power (ABTS) with 2829.80 ± 128.088 µmol Trolox /100 g on water extract and 1738.13 ± 539.437 µmol Trolox/100 g on the initial biomass.

3.3.6.3. DPPH method

On trial 2 two different tests were used to test for significant differences. For water extracts a ANOVA test and post-hoc Tukey test showed that all the samples tested (initial biomass < final 4 g L^{-1} < final 8 g L^{-1}) differ from each other [$F(2,9) = 35.520$; $p < 0.05$]. While on ethanol 96 % extracts a non-parametric test, Kruskal-Wallis, showed that final biomass density 4 g L^{-1} had significant lower activity from density 8 g L^{-1} [$X^2(2) = 9.846$; $p < 0.05$] (Table 3.3).

According to a multivariate ANOVA, and the post-hoc Tukey test, trial 3 showed to had significantly higher values for the initial biomass than the two different final densities tested (4 and 8 g L^{-1}), and density 4 g L^{-1} had significantly lower values than density 8 g L^{-1} on

ethanol 96% extracts [$F(2,9) = 23.064$; $p < 0.05$]; for water extracts only the initial biomass had significantly lower activity from the two final different densities (4 and 8 g L^{-1}) [$F(2,9) = 29.464$; $p < 0.05$] (Table 3.3).

And again, the species *S. coronopifolius* was the most capable of reducing the DPPH radical, obtaining a maximum inhibition value of 61.53 ± 4.649 % on ethanol 96 % extract and 43.60 ± 2.029 % on water extract on the initial biomass.

4. Discussion

Plocamium cartilagineum and *Sphaerococcus coronopifolius* had a poorly performance in terms of grow and removal of nitrogen throughout the time of the experiments in a pilot-scale seaweed biofilter tank system, when compared to other species. Despite the overall low productivity, the slightly best results, in terms of productivity and N-yield, were achieved at a stocking density of 4 and 6 g L^{-1} for *P. cartilagineum* (trial 1 and 2, respectively), and 8 g L^{-1} for *S. coronopifolius*. At these conditions, the system produced 31.30 ± 33.249 g DW m^{-2} wk^{-1} and removed 8.73 x 10^{-3} g DW m^{-2} during trial 1, producing 0.28 kg DW m^{-2}. And in trial 2 produced 23.21 ± 22.103 g DW m^{-2} wk^{-1} removing 4.77 x 10^{-3} ± 2.572 x 10^{-4} g DW m^{-2} of nitrogen, producing 0.14 kg DW m^{-2}. During trial 3 and under density 8 g L^{-1}, it was produced 19.74 ± 30.513 g DW m^{-2} wk^{-1} removing 3.29 x 10^{-3} ± 1.603 x 10^{-3} g DW m^{-2} of nitrogen, at the end of the experiment 0.12 kg DW m^{-2} was produced. While *P. cartilagineum* was more productive than *S. coronopifolius*, these species had much lower values than *G. vermiculophylla* (0.7 ± 0.05 kg DW m^{-2} $month^{-1}$, at 3 kg m^{-2}) (Abreu et al., 2011), *A. armata* tetrasporophyte (120 g DW m^{-2} d^{-1}, at 5 g L^{-1}), and *Ulva rotundata* (48 g DW m^{-2} d^{-1}, at 2 g L^{-1}) (Mata, 2008). According to the functional-form model proposed by Littler and co-workers (1983), a somehow lower productivity of both species was expected, since coarsely branched species have lower performance when compared to filamentous species (*Falkenbergia rufolanosa*, the tetrasporophyte phase of *A. armata*) and sheet-like species, like *Ulva spp*., but this model does not explained the main differences in productivity between, for instance species like *Gracilaria* and the two tested species. The hypothetical morphological adaptations of opportunistic *vs* persistent macroalgae have been suggested by Littler and Littler (1980), suggesting that the opportunistic species had a high intrinsic growth rate in the opposite of the others, suggesting an adaptation in response to environmental stress and disturbances, making the persistent species, more resilient due to the allocation of resources at the expense of photosynthetic tissue, and so less growth, however, these predictions were limited for a number of species tested in a temperate system. Opportunistic green seaweed species were observed in all trials, which is a common problem in red seaweed aquaculture, considered persistent (Bidwell et al., 1985; Demetropoulos & Langdon, 2004; Friedlander, 1992).

While the salinity had a nearly constant value, of 35 (the high value for the salinity is explained since the water is collected from a lagoon where there is more evaporation) previous studies showed that *G. vermiculophylla* grew faster in an environment with a salinity close to 26, and a lower growth rate in 35 (Nyberg & Wallentinus, 2009), on the other hand, *Palmaria mollis* showed better growth at a salinity close to 30 ‰

(Demetropoulos & Langdon, 2004), making this parameter an important one in the growth of the seaweed and possibly a limiting one in this study. This parameter has not been tested, but by the results it opens the door to a possible new study, to try to understand its influence on those two species.

These trials were performed in a system, where the water temperature was partially controlled, but it was also exposed to the changing weather conditions, and while temperature requirements for *P. cartilagineum* and *S. coronopifolius* have not been previously reported, the capacity of these species to sustain growth even with large temperature variations (winter and spring/summer) was in accordance with laboratory studies and a similar cultivation system carried out with *G. vermiculophylla* (Abreu et al., 2011; Yokoya et al., 1999), showing the tolerance of *P. cartilagineum* and *S. coronopifolius* to a wide range of environmental conditions, with some limits. When comparing the water temperatures from Albufeira, the collecting site, during the winter time (trial 1), the mean experimental temperature was lower (16.4 ± 1.94 ºC), comparing to the mean temperature of the ocean (20.4 ºC to 16.9 ºC, October to December); during spring/summer, the mean ocean temperature (19.2 ºC to 20.4 º C, June to September) was lower than the water temperature present during the experiments (22.1 ± 2.10 ºC and 21.5 ± 0.80 ºC, for trials 2 and 3, respectively) (Site 4); as previously mentioned, the cultivation system was exposed to the weather conditions, and so when the temperature of the air decreased during winter, the water temperature also decreased, and when the air temperature increased the water temperature had the same behaviour, As the temperatures to which the seaweeds were subjected were below (winter) and above (summer) that from the collection site it is possible it was outside the species ideal temperature ranges. On winter trial (1), when the water temperatures started to decrease, the growth of this species started to alter for all the different stocking densities, the further the temperatures went down, less and negative growth values were registered; on trial 2, when the temperature started to increase, the productivity in all stocking densities started to decrease, showing that the temperature may have had an impact on the growth of the seaweed. On trial 3, the variation of temperature did not have extreme values as in trial 2, and so this environmental parameter did not seem to be the limiting factor. As seen in the first two trials, the variation of temperature seems to have an influence in the growth of the species, which makes this parameter an interesting one to perform more studies using this species.

Besides temperature, light also plays a role in conditioning the performance of seaweeds affecting growth, nutrient uptake, and composition of the seaweed (Lobban & Harrison, 1997; Lüning, 1991; Figueroa et al., 2003; Pliego-Cortés et al., 2019). However is a difficult

parameter to study when comparing growth rates under different tank culture conditions, since its intensity is affected by depth and stocking density (Demetropoulos & Langdon, 2004). Understanding the requirements for each species is one of the challenges to implement in a land-based seaweed culture system, Robbins (1978) showed that light saturation for *P. palmata* occurs at approximately 212 µmol photons m^{-2} s^{-1}, this saturation level is lower than the values measured during trial 2 and 3, however, during trial 1 the levels were lower than that value, which may influence the photosynthesis and so the growing process, but the mean productivity during this trial had the higher value. The lower the density, the more light penetrates the water column, which would lead to an increase in productivity up to a certain limit; in the case of trial 1, the productivity was higher, although the levels of light were lower when compared to trial 2; during trial 1, the density 4 g L^{-1} showed higher productivity values, but the lower densities (1, 2 and 3 g L^{-1}), which would receive more light, ended up dying, but in trial 2, these densities were able to survive and growth, even exposed to higher levels of light. The density with better results was density 6 g L^{-1} for trial 2, and 8 g L^{-1} during trial 3, higher densities, these results showed that the intensity, even if affected by the depth and stocking, was not enough to affect growth, and probably lower light intensity may be more advantageous for these species.

The two species thrive in the intertidal areas and are adapted to severe changes in environmental parameters that are present in that area (Nyberg et al., 2009), one adaptation that can make them so resistant is related to the ability to overcome limiting conditions of dissolved C (Andría et al., 2001). *Gracilaria* species, same phylum as *P. cartilagineum* and *S. coronopifolius*, showed that when farmed in seawater with high pH water levels can use other forms of carbon (HCO_3^-) besides CO_2 in photosynthesis, however, if the pH water levels are higher than 9.0, it compromises the photosynthesis and biomass production (Zou & Gao, 2009; Zou et al., 2004). During the three trials, the pH water levels never went above that critical value and no significant differences in pH water levels were found between the different densities, which can be an indicator of non-limitation by C, as seen in *Gracilaria* species (Israel et al., 1999; Zou et al., 2004). This is also in accordance with the fact that no significant differences were found in growth between the different densities tested. Overall results indicate that temperature, light and salinity were likely to be limiting factors of the seaweed growth, the first two environmental parameters are usually the most important factors affecting growth and nutrient uptake of seaweed (Lobban & Harrison, 1997; Lüning, 1991). Israel and co-workers (1999) showed that for *Gracilaria tenuistipiata* in an outdoor cultivation system the higher salinities (39 ‰) can be antagonize by the

abundant irradiance and constant mixing through aeration and seawater flow, since that an outdoor system, it is hard to control salinity or pH like in laboratory conditions.

Previous studies with seaweeds have examined the assimilation of nitrogen, and different sources of nitrogen. Fries (1963) and Iwasaki (1967) found that nitrate was the best source of nitrogen for the growth of several seaweed species and ammonia utilized only at low concentrations. Waite and Mitchell (1972) showed that the carbon fixation by *Ulva lactuca* was inhibited by ammonia concentrations higher than 60 µM, and in the three trials, the values recorded never went above that value (20.71 ± 4.320 µM, 32.50 ± 10.785 µM and 45.61 ± 10.612 µM, trials 1, 2 and 3 respectively). Also, Rui and co-workers (1990) showed that for *K. alvarezii*, levels between 35 - 50 mM of ammonia can produce adverse effects on the growth of this species. Prince and Kingsbury (1973), by observation of *Chondrus crispus* in nature, suggested that *C. crispus* was able to utilize ammonia when nitrate was depleted from coastal marine waters. In the studies performed by Prince (1974), *C. crispus* and *F. vesiculosus* had the same growth behaviour using nitrogen in the form of either ammonia or nitrate, his study also showed that high concentrations of ammonia were apparently toxic for *F. vesiculosus* when using lower cultivation stocking densities. The growth of the two species used in the trials (1, 2, and 3) was probably not inhibited by the concentration of ammonia since the water samples collected at the time of the experiments had relatively low ammonia concentration.

According to Hanisak (1987), the nitrogen critical value required for maximal growth of *G. tikvahiae* is 2 %, and considering that value, the growth of *P. cartilagineum* and *S. coronopifolius* does not seem to be nitrogen-limited in its natural environment (4.27 ± 0.160 %; 4.25 ± 0.176 %; 3.28 ± 0.098 %, trial 1, 2 and 3, respectively) and at the cultivation system, that had higher values, the increase in the N content shows that the seaweed was able to accumulate this nutrient while maintaining growth, despite that, these two species may have a different critical value, another point that can be further study. The N content always increased after the cultivation period at both systems which goes in agreement with the results obtained by Mata (2008) where the N content of *A. armata* and *U. rigida* also increased with time in cultivation in an IMTA system and had slight variations during that time (May had lower values than December for both species). Abreu and co-workers (2011) that used *Gracilaria* in their experiments, showed that the C content had slightly increased with time in an IMTA system, on the other hand, Schuenhoff and co-workers (2006) showed that the C content in *A. armata* did not change with time in cultivation.

The C:N ratios determined, also confirmed that the two species were growing in N saturated conditions, since they are below 13.5, the ratio suggested by Hanisak (1990). The fact that the seaweeds continued to accumulate nitrogen when cultured might indicate the improved growing conditions and the continuous supply of ammonia (Hurd, 2000; Smit, 2002). (Peralta-García and co-workers (2017) showed that the C:N ratio was lower in cultured *R. pseudopalmata* when compared to the wild species as happened in the trials performed in this study, but Abreu and co-workers (2011) had different results with *Gracilaria vermiculophylla*, the time in cultivation led to an increase in the C:N ratio.

In terms of N removal capacity, the best results were achieved at a stocking density of 8 and 6 g L^{-1} for *P. cartilagineum*, trials 1 and 2 respectively, removing 1.02 x 10^{-2} g DW m^{-2} and 4.77 x 10^{-3} ± 2.572 x 10^{-4} g DW m^{-2}, of N on trial 1 and 2, respectively; and for *S. coronopifolius* the best results were achieved with a density of 8 g L^{-1}, removing 3.29 x 10^{-3} ± 1.603 x 10^{-3} g DW m^{-2} of N. These results showed that densities 6 and 8 g L^{-1} are the best to use for these two species since they have the higher productivity and, therefore, higher N removal. Red seaweeds are already present in several studies showing their ability to be biofilters for integrated, inland, fish and seaweed aquaculture (Abreu et al., 2011; Mata, 2008; Schuenhoff et al., 2006); and even though, the seaweeds showed to have the ability to remove nutrients in the experiments performed, they were far behind the values obtained with *A. armata* (5.9 g N DW m^{-2} d^{-1}, with similar water temperature, water exchange and stocking density of 5 g L^{-1}) (Schuenhoff et al., 2006) and even the *Ulva rigida* (3.6 g N DW m^{-2} d^{-1}, with similar water temperature, water exchange and stocking density of 4 g L^{-1}) (Mata, 2008). The results obtained for N removal were very low, but as explained in the results section, this is due to the lower biomass that was produced, when comparing only the N content, it is possible to see that higher production rates can be achieved by finding the ideal cultivation conditions, this species could be capable to remove nutrients more efficiently. Mata (2008) obtained a N content, for *A. armata* and *U. rigida,* of 6.04 ± 0.07 % and 5.25 ± 0.18 % after the time in cultivation, that was lower than the percentage obtained for *S. coronopifolius* (5.41 ± 0.097 %), and *P. cartilagineum* (6.82 ± 0.044 %, 6.34 ± 0.204 %, for trials 1 and 2 respectively); in his experiments he reported the higher biofiltration for *U. rigida* ever obtained in an integrated fish/seaweed aquaculture, this results were probably due to the characteristics of the cultivation system, that had higher light availability that lead to a high amount of biomass produced resulting in a high nutrient uptake.

The protein content in seaweed is generally low, but the protein quality of seaweeds had shown to be superior to most terrestrial plants (Mæhre et al., 2014). Higher protein contents are recorded for red seaweed, proteins can represent up to 35 % and 47 % of dry matter in

Palmaria palmata and *Porphyra tenera*, respectively (Morgan et al., 1980; Arasaki & Arasaki, 1983), these levels are comparable to those found in high-protein vegetables such as soybeans, in which proteins make up to 40 % of the dry mass (Murata & Nakazoe, 2001). Protein content found in the seaweed in this study seems to be on the same range reported in other studies for red algae (Marinho-Soriano et al., 2006; Tibbetts et al., 2016; Morgan et al., 1980; Arasaki & Arasaki, 1983), the slight differences may occur by geographical and seasonal variation (Hagen et al., 2004) but also by methodological differences, the method used was Dumas method that determines the total nitrogen that is converted to protein using a nitrogen-to-protein conversion factor set to 6.25, however, an alternative conversion factor has been suggested for seaweeds, 4.92 (Lourenço et al., 2002). This factor is the most practical way of determining protein content, but the accuracy of protein determination by this method depends on the establishment of the conversion factor specific to individual species, and as seen in this study, two species of seaweed, belonging to the same group (red algae) showed slightly different values (5.80 and 5.33 for *P. cartilagineum* and *S. coronopifolius*, respectively), reinforcing that this factor needs to be better explored and that the traditional factor (6.25) is unsuitable for seaweeds. Also, in this study there was a slight increase in the protein content with time in cultivation, as happened in a study with *Palmaria Palmata* (Mishra et al., 2009), however, Shuuluka and co-workers (2013) shown that different species of wild *Ulva spp.* had higher protein content during February to June, but farmed *Ulva spp.* had no seasonal pattern during the time in cultivation and had lower values when compared to wild samples in the same period.

It is known that the higher the ash content, the lower the organic matter content, the ash content also gives a rough estimate of the total mineral content and trace elements (Mæhre et al., 2014). The ash content in seaweed is higher when compared to vegetables (Murata & Nakazoe, 2001). In the red algae, the values normally find on ash content have a high range of values (Holdt & Kraan, 2011). In this study the mean ash content for *P. cartilagineum* was 28.23 ± 4.99 % and 29.27 ± 1.396 % for *S. coronopifolius* with no variations with time in cultivation, but according to Mishra and co-workers (2009) the cultured strain showed an increased accumulation of ash in comparison to the wild strain of *Palmaria palmata*, and Morgan and co-workers (1980) showed some variations on the wild species (12 - 37 %) probably owing to differences in methodology and differences in plants, arising from the site, seasonal, and population variations. Robledo and Freile Pelegrín (1997) evaluated the ash content from natural populations in the spring of 1994 of *Gracilaria cornea* showing an ash content of 29 %, on the other hand, Marinho-Soriano and co-workers (2006) showed different values of ash content for *Gracilaria cervicornis* (8 %).

These seasonal and environmental variations in the composition of seaweed make generalizations impossible (Holdt & Kraan, 2011).

Although seaweeds are not a conventional source of lipids (the quantified values of total lipids are below 6 %), their small lipid fraction may represent an interesting added value in some samples of seaweeds. Lipids are a broad group of naturally occurring molecules which includes fats, waxes, sterols, fat-soluble, vitamins (such as vitamins A, D, E, and K), mono-, di- and triacylglycerols, diglycerides, phospholipids, and others (Holdt & Kraan, 2011). Like the other biochemical components, the lipid content varies with season and other environmental factors, for example, *Laminaria* species has maximum content of lipids in winter (Haug & Jensen, 1954), however on *Fucus sp*. this happened during summer (Kim et al., 1996). *Gracilaria* species also showed that when attached its lipid content was higher than when not attached, and no differences in the fatty acid composition were observed (Khotimchenko & Levchenko, 1997; Kim et al., 1996). In this study it was possible to see a slight increase with time in cultivation, Peralta-García and co-workers (2017) are in agreement with the results obtained in this study, using *Rhodymenia pseudopalmata*, a red seaweed that is already successfully farmed.

Phenolic compounds are considered to be one of the most important groups to contribute to antioxidant activity, which gives them value for the cosmeceutical and pharmaceutical industry, and they also showed other effects such as radiation protection, antibiotic and antidiabetic (Holdt & Kraan, 2011; Ganesan et al., 2008). The antioxidant properties consist of delaying the oxidation of various compounds, inhibiting the initiation or propagation of chain reactions (Amarowicz et al., 2004). From preventing oxidation reactions in foods, medicines, and cosmetics to the role of reactive oxygen species (ROS) in diseases such as cancer, cardiovascular, inflammatory, neurodegenerative, and autoimmune diseases, antioxidant activity is essential to delay any of these cases (Magalhães et al., 2008). Epidemiological studies have demonstrated a direct relationship between consumption of products rich in antioxidants and decreased morbidity and mortality (Huang et al., 2005). The evidence that implies oxidative stress in the development of various diseases and imbalances leads to recognition of the role of antioxidants in the preservation of human health and the prevention and help in the treatment of diseases (Niki, 2010). Published reports on the total antioxidant activity of all seaweed extracts are not available, however, a total antioxidant activity of 245-376 mg AA/g extract has been reported in higher plant extracts (Kumaran & Karunakaran, 2007). Certain polyphenols work as preventive medicines for problems such as cardiovascular diseases, cancer, arthritis, and autoimmune disorders by helping to protect tissues against oxidative stress (Garbisa et al., 2001; Kang

et al., 2003; Maliakal et al., 2001). In addition, polyphenols are found as an anti-inflammatory agent and have an anti-allergic effect and antibacterial activity together with other broad therapeutic perspectives (Li et al., 2009; Sailler & Glombitza, 1999; Zubia et al., 2008). Red seaweed have low concentrations of phenols compared to brown species (Mabeau & Fleurence, 1993). Phenol content has a wide range of values (Connan et al., 2004; Chandini et al., 2008; Ganesan et al., 2008), and the results obtained for either extract were in the range previously obtained in different studies. Concentrations of polyphenols exhibit seasonal variations, but also vary within the different parts of thalli, basal part or fronds (Johnson & Mann, 1986), and also depends on variables such as the environmental conditions of the place of origin and the time of harvesting (Quirós et al., 2010). In this study, on trial 2 and 3 the density 4 g L^{-1} was the one that stood out the most in terms of phenolic content, and the content on the initial biomass had the lowest values showing an increase during the time in cultivation, reinforcing that the environmental conditions have an impact on phenolic content (Quirós et al., 2010). When comparing the initial biomass from trials 1 and 2 (same species, different season), the water extract showed a higher phenolic content on trial 2 (spring/summer). During spring and summer there is an increase in hours of light and intensity, which can lead seaweeds to produce more phenolic compounds for a photo-protective role (Flodin et al., 1999; Pedersen et al., 1996), Connan and co-workers (2004) tested different brown seaweeds during time for phenolic contents, and showed that for all of the seaweeds tested, the phenolic content varied seasonally, *Fucus vesiculosus* had the highest values during summer, but *Laminaria digitata* had the lower values during the same period, showing differences from species to species, making it hard to make generalizations, also Ronnberg and Ruokolahti (1986) and Steinberg and Van Altena (1992) showed maximum values during spring and summer. In ethanol extracts, the phenolic content was lower on trial 2, Ganesan and co-workers (2008) showed that when using water extracts, the species *E. kappaphycus* had the higher phenolic content, but when using methanol extract the higher content was obtained on *Gracilaria edulis*, showing that the solvent used also interferes with the results.

The levels of antioxidants present in seaweed can also be affected by a number of other parameters such as location and salinity, Sampath-Wiley and co-workers (2008) examined the effect of sun exposure and emersion on *Porphyra umbilicalis* and found that seaweed located in upper intertidal regions during summer contained higher levels of antioxidants than submerged seaweed, reinforcing that the seaweeds, when in stressful situations, like light exposure, have an increase in antioxidant compounds.

S. coronopifolius showed higher values of antioxidant activity in the three methods used when compared to *P. cartilagineum*. In FRAP method there were null values on ethanol extracts, FRAP reagent cannot detect compounds that act by radical quenching (Cerretani & Bendini, 2010). FRAP assay measures the reducing potential of an antioxidant, and the results obtained are in accordance with the results reported by Costa (2014). It is noted that the obtained results from the ABTS method were higher than those obtained by DPPH or FRAP method, as happened in Rajurkar and Hande (2011) work, also the results from this study had higher values than the plants used by them. DPPH radical is a stable radical that can be reduced by an antioxidant (Brand-Williams et al., 1995). Kumar and co-workers (2008) showed that when, using water and ethanol extracts both had equivalent results in *K. alvarezii* seaweed, as happened in this study, also the same study showed again that antioxidant activity depends on the species analyzed. Li and co-workers (2007) also shown that the red alga *P. urceolata* had a DPPH-scavenging ability.

Preliminary studies using the species *Fucus* showed that the water extracts had the highest values compared to ethanol extracts (Farvin & Jacobsen, 2013; Farvin et al., 2010), Kumar and co-workers (2008) using *K. alvarezii*, showed again the influence of the solvent, but in this case, the ethanol extract had higher values than the water extract. As it happened with the phenolic content, the solvents used bring variations into the results, also it has been reported that solvents used for extraction affect the chemical species (Yuan et al., 2005).

One of the great difficulties when comparing the values obtained for the phenolic content and antioxidant activity with the values obtained from other studies is the lack of uniformity of the standards used in the various techniques, as well as the techniques that can be used with the same purpose, also as Regal and co-workers (2020) showed, the drying process, storage conditions and time can have an impact in the biochemistry of the seaweeds.

Some results could have been better explained by knowing the composition of fatty acids present in the biomass of the seaweeds, which was planned for this work, but due to delays, it was not possible to obtain the results in time to include them in this work.

5. Conclusion

P. cartilagineum and *S. coronopifolius* showed to have characteristics to be considered as a seaweed biofilter of inland aquaculture practices. Although both tested species did not have high production or high N removal values, they showed a higher N content, and so these two species deserve further experiments to find the best parameters to obtain their maximum productivity or the best parameters to obtain certain metabolites with commercial value. The environmental conditions during the cultivation trials (temperature, light and salinity) are likely to have an impact on the performance of the two species, and so further experimental works is required, in particular testing salinity, since in an inland system like the one used, it could be adjusted. On an inland system, the light will always vary throughout the year and even throughout the day, just as temperature even with a cooling system, and so these parameters must be taken into consideration and more studies should be done, but probably in a different type of system. Another point to take into account is handling, for example, it is possible that collection and cleaning has damaged the seaweed contributing to the poor growth observed.

The lower values for the productivity of the two species, can also be related to the system itself, and other hypotheses can be chosen, like get the seaweed attached instead of being free in the water column, or maybe change to an offshore instead of an inland system.

The biomass of *P. cartilagineum* and *S. coronopifolius* produced in the system revealed to be of excellent quality for some applications: good nutrient content, and possibly as a source of phenolic compounds and antioxidants for the pharmaceutic, medic, and cosmetic industry. Recent studies showed that bromoterpenes from *S. coronopifolius* have an antitumor potential in fibroblast and lung malignant cells (Alves et al., 2020), Smyrniotopoulos and co-workers (2010) showed that this species also had an antibacterial activity that helps against a panel of bacteria including multidrug-resistance and methicillin-resistant. Many of the halogenated monoterpenes isolated from the genus *Plocamium* have been proven to be active in pharmaceutical screening possessing mutagenic, cytotoxic activities as well as in agrochemical testing (König et al., 1999; König et al., 1990; Williard & Grab, 1984), being the largest variety isolated from *Plocamium cartilagineum* (Abreu & Galindro, 1996, 1998; Díaz-Marrero et al., 2002), it also showed the most potent anti-insect activity (Kladi et al., 2004). Sabry and co-workers (2017) also showed that *P. cartilagineum* can be active as a cytotoxic agent in human lung cancer and other cell line assays against leukaemia and human colon cancers. Fratini and co-workers (2019) demonstrated another use of *S. coronopifolius* in the restoration of painting and paper artworks and consolidation

of matt paintings, showing how diverse the extracts of the seaweeds can be. The production of seaweeds in IMTA can also be beneficial for cultured fish health (Bansemir et al., 2006; Dashtiannasab et al., 2016).

Bringing all these economic aspects together with the environmental benefits of IMTA is crucial in order to make the different participants involved, like industries, scientists, managers, and politicians to communicate and develop real measures to turn aquaculture into a more sustainable and socially accepted industry, integrating seaweeds in the animal aquaculture industry.

6. References

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Appendix A – Examples of contamination present in the experiments.