Cultivation of *Osmundea pinnatifida* and *Codium tomentosum*, native seaweed species with commercial potential

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O Presidente do Júri,

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Resumo

Com a procura de um modo de vida mais saudável, os europeus vêem a procurar alternativas como são as algas como forma de obter os nutrientes de que necessitam e também para obter suplementos alimentares, farmacêuticos e biocombustível. Com o aumento do interesse por este recurso aumenta o risco da sua sobre-exploração, por isso são necessárias alternativas sustentáveis para produzir a biomassa.

Neste projeto estudou-se *Codium tomentosum* e *Osmundea pinnatifida*, algas da costa portuguesa, para determinar as melhores condições para o seu cultivo. Foram realizados ensaios de otimização no laboratório em que foram avaliados diferentes valores de temperatura (12ºC, 16ºC e 20ºC), e a interação entre densidade (5, 10, 15 g / L) e intensidade luminosa (100 e 200 µmol m-2 s-1). Ao mesmo tempo, o cultivo dessas algas foi realizado num sistema de cultivo ao ar livre durante duas estações diferentes do ano (inverno e verão).

Para *Codium* nos ensaios de otimização, os melhores resultados foram obtidos a 16ºC e 200 µmol m-2 s-1 de intensidade luminosa. Nos ensaios de cultivo, o verão monstra as maiores taxas de crescimento. Apesar do melhor crescimento desta alga no verão, esta época também foi a maior presença de epífitas nos tanques que competem com as algas por luz e nutrientes. As análises bioquímicas desta alga mostram que o processo de cultivo não afeta negativamente os componentes estudados e, no caso dos fenóis, há um aumento destes valores devido ao cultivo. Os resultados dos ensaios de otimização mostraram que a melhor temperatura para crescimento de *Osmundea* foi de 16ºC. No entanto nos ensaios de interação de luz e densidade ocorreu perda de biomassa. Nos ensaios de cultivo durante o inverno a taxa de crescimento foi próxima de 0, enquanto que no verão a alga começou a degradar após 2 semanas, devido às temperaturas elevadas. Para as análises bioquímicas, o processo de cultivo não afeta a atividade antioxidante e o teor de proteína. Os componentes fenólicos tiveram variação sazonal, decrescendo no período de verão.

Em conclusão, *Codium* é uma espécie com potencial para a aquacultura, embora ainda seja necessário continuar a estudar esta alga para obter uma melhor compreensão das melhores condições para realizar o cultivo desta. Quanto a *Osmundea* os resultados não são tão animadores, sendo que a principal dificuldade no cultivo dessa alga é a sensibilidade desta à temperatura mais elevada, exigindo estudos adicionais para o desenvolvimento de seus métodos de cultivo.

Palavras-chave: Algas; *Codium tomentosum*; *Osmundea pinnatifida*; aquacultura; bioatividade

Abstract

In the search for a healthier way of life, Europeans are seeing alternatives such like seaweeds as a way of obtaining not only the nutrients they need in a much healthier form but also food supplements, nutraceuticals and biofuel. As always, when there is an increase in demand, the risks of overexploiting the natural resources are very high so sustainable alternatives to produce the biomass are needed.

In this project *Codium tomentosum* and *Osmundea pinnatifida*, seaweeds from the Portuguese coast, were studied to determine the best conditions for their cultivation. One part of this study consisted in optimization trials in the laboratory. In these trials it was evaluated different values of temperature (12ºC, 16ºC and 20ºC), and the interaction between density (5, 10, 15 g/L) and light intensity (100 and 200 µmol m-2 s-1). At the same time, cultivation of these seaweeds was performed in an outdoor cultivation system during 2 different seasons of the year (winter and summer).

For *Codium tomentosum* in the optimization trials the best results were obtained at 16ºC and 200 µmol m-2 s-1 of light intensity. The trial that demonstrate the highest growth rates was the summer trial. Despite the best growth of this seaweed in the summer, this season also had the highest presence of epiphytes in the tanks, that compete with the seaweeds for light and nutrients. The biochemical analyses of this seaweed show that the cultivation process didn't affect negatively the components studied and, in the case of phenols, there is an increase of the values due to the cultivation. The optimization trials results showed that the best temperature to grow *Osmundea pinnatifida* was 16ºC. There was biomass loss on the trials of interaction of light and density. In the slow growth season (winter) *Osmundea pinnatifida* growth rate was close to 0, whereas in the high growth season (summer) the seaweed biomass started to degrade after 2 weeks, due to the elevated temperatures. For the biochemical analyses the cultivation process didn't affect the antioxidant activity and the protein content. The phenol content had a seasonal variation, decreasing in the summer period.

In conclusion *Codium tomentosum* is a species with potential for aquaculture, although there is still the need to continue to study this seaweed to obtain a better understanding of the best conditions to perform the cultivation. As for *Osmundea pinnatifida* the results are not so encouraging, with the principal difficulty identified for the cultivation of this seaweed being the sensibility to higher temperature, calling for further studies to develop its cultivation methods.

Key words: Seaweed; *Codium tomentosum*; *Osmundea pinnatifida*; tank cultivation; bioactivity

Content

List of figures

List of tables

Introduction

The world population is growing more and more every day and at the same rhythm also the need for natural resources, to support not only food supply but also to produce all types of medicines, cosmetic and other products essential to daily life. Past human errors show us that exploiting a resource to its maximum capacity did not to solve this problem, so the scientific community is trying its best to explore alternative sources of these products, being one of the alternatives the use of seaweeds.

As the world is mostly occupied by seawater, the study of the marine ecosystems is very important. In the coastal areas, especially the rocky shores, the dominant autotrophic biomass is seaweeds, that play a central structural and functional role in the habitats (Dayton, 1975). These ecosystems can be a source of numerous biologically active natural products, some of which aren't found in the terrestrial habitats (Carte, 1996).

Since the 4th century, seaweeds are used as food by humans, mostly in the Asian countries. In Europe, there are present approximately 1550 species of seaweeds, and this number tend to increase with the development of molecular tools (Arenas et al., 2014). In Portugal, seaweeds were traditionally harvested in Northern Portugal and their harvests was regulated in 1308 by king D. Dinis (CD Trowbridge et al., 2006). Some companies (i.e. Algaplus, WEdotech, AlgaFuel, among others) are developing activities to empower the biotechnological potential of the marine flora present in Iberian Peninsula (Cardoso et al., 2014).

Traditionally in Europe, seaweeds are used in the human diet, as feed and biofertilizer since the XVIII century. But the interest for this resource increased during Word Wars I and II when, because of the lack of other resources, seaweeds were used for food, animal feed and to help with throat irritation of soldiers who suffered gas attacks (Hasan, 2017).

Seaweeds are photosynthetic aquatic organisms that belong to the Eukaryote Domain and the Kingdoms Plantae (the green and red algae) and Chromista (brown algae). Seaweeds are primary producers, which means that they are at the base of the food chain of aquatic ecosystems, because they produce oxygen and organic compounds and support highly productive benthic communities (Mann, 1973; Pereira, et al., 2009; Pereira, 2010).

The use of the coastal areas for activities such as fishing, tourism, maritime traffic and exploitation of natural resources causes an increase of the anthropogenic stress (Arenas et al., 2014).

Unlike the situation globally, where seaweed produced in aquaculture represent 96% of all seaweeds used, the most common form to obtain seaweeds in Europe and in Portugal is by harvesting (Bixler & Porse, 2011). According to FAO Yearbook of Fishery and Aquaculture Statistics (FAO, 2016) the percentage of seaweeds produced by aquaculture corresponds to 20% of the total world marine aquaculture. It is estimated that in 2016 the total aquaculture production of seaweeds reach 30,1 million tons, with an economical value of 11,6 billion $ (FAO, 2018). The commercial harvesting of seaweeds occurs in about 35 countries spread over the globe (FAO, 2014).

The intensity of harvesting to obtain the necessary biomass may cause over exploitation of the costal ecosystems that lead to the destruction of the natural habitats, pollution and the increase of sedimentation (Walker & Kendrick, 1998; Claudet & Fraschetti, 2010; Munday,et al., 2013). The collection of seaweeds from the ocean aquaculture is dependent of harvest technology, labour availability and weather conditions that can be unpredictable and cause large losses in the investment. Also the water quality is unstable, influencing the seaweed biomass characteristics (Stengel, et al., 2011). In land-based aquaculture some of these problems didn't occur because the aquaculture facilities are placed in sheltered areas where, although technology and specialized labour are needed, it is possible to control the water quality thus reducing the concern with food safety (Hafting, et al., 2012).

The number of species that are currently commercially explored is low, but most of the biomass that is consumed is obtained from ocean-based cultivation. In the ocean-based methods, anchored lines or nets are used which can be seeded or have individuals tied for them to grow-out (de Góes & Reis, 2011). One of the reasons why only a few species are cultivated is that seaweeds need to be resistant to epiphytes (Hafting et al., 2012). The highest limitations of ocean-based cultivation are the environmental conditions (such as currents and nutrient availability) and the adverse effects of unfavourable weather and ocean conditions (Hafting et al., 2012). The cultivation of seaweeds provides ecosystem services for example by removing nutrients upon harvest when integrated in an IMTA aquaculture (Kim, et al., 2017).

In land-based cultivation these limitations didn't exist, thus at these sites a wider range of species can be produced. In these, the water is agitated by air diffusion to keep the

algae in circulation and improve exposure to light and nutrients, which are used more efficiently. The seawater is obtained by pumping water from the shore. In some cases, the water can be obtained from a secondary source like fed aquaculture, integrating fed and extractive organisms, increasing the water nutrient content with advantages in terms of seaweed growth (Chopin et al., 2001).

Seaweeds are now viewed as a resource that can be used for numerous uses, from agriculture as fertilizer, to biomedical applications. One of the more recent applications of the seaweed is for the cosmetic and well-being industries as well as for the production of biofuels and biogas (Dahiya, 2015; Pereira & Correia, 2015). This diversity of uses is due to the unique properties of seaweeds, and in recent years the interest for the study of their natural biologically active products is rising (Carte, 1996; McHugh, 2003). Also, seaweeds are a safe and abundant natural source of iodine, being the consumption of iodine beneficial in metabolic regulation and growth patterns (Mouritsen, 2013).

They are use in food industry is one of the more important applications of seaweeds because they are a source of fibre, minerals, polyunsaturated fatty acids, essential amino acids and vitamins A, B, C and E (Rajapakse & Kim, 2011). The interest in seaweeds is especially high the population that seeks a healthier lifestyle because seaweeds provides not only a highly nutritive food product but also has physiological benefits (Ibañez, et al., 2012). Seaweed components such as protein and phenolic compounds demonstrated strong antioxidant activities (Yuan & Walsh, 2006) as well as other compounds like carotenoids, ascorbic acid, among others (Celikler, et al., 2009). A constant supply of these compounds provides an antioxidant support for diseases prevention (Ribeiro, et al., 2007). The phenolic compounds are stress-induced compounds and are involved in mechanisms of chemical protection against biotic factors like contamination due to bacteria and abiotic factors like for example metal contamination (Llewellyn & Airs, 2010; Stengel et al., 2011). The protein extracts are also a high value component of the seaweeds because they can be applied in the form of phycobiliproteins which make the value of protein compounds high enough to justify its exploration (Fleurence, 2016). Several studies show us that seaweeds can also be used for the extraction of compounds with antiviral, antitumoral and antibacterial activities and also purification of residual waters (Pereira et al., 2009; Pereira, 2011).

One of the most important components derived from algae are polysaccharides like carrageenan, agar and alginates (European registration numbers - E407, E406 and E400, respectively) (Pereira, 2010).

According to Michalak & Chojnacka, (2015) these polysaccharides are presently the most important products produced from seaweeds from an economic perspective. The carrageenan was discovered in 1862, by the British pharmacist Stanford, and was extracted from *Chondrus crispus* (Pereira et al., 2009). The species used for this production can be found around the globe and the more frequently used belong to the genera Eucheuma, Gigartina and Hypnea (Hasan, 2017). The carrageenan are gelling agents and are stable both in suspension or emulsions, which is important for the pharmaceutical industry (Goswami & Naik, 2014). Carrageenan are one of the major texturizing ingredients used by the food industry. They are also important for the cosmetics and medical industries (Goswami & Naik, 2014).

In order to sustain the increase in population and consequent growing need for food and other products without overexploiting the natural populations, sustained ways of seaweed production are needed. Therefore, development of cultivation methods to answer the increased demand is mandatory.

In this work *Osmundea pinnatifida* and *Codium tomentosum* were studied since they are a "new species" for aquaculture and present a high potential for use as food and other applications.

Codium tomentosum

Also known as "chorão-do-mar" (Portuguese), "miru" or "limu" (Japanese) and "aala-ula" (Hawaiian), *C. tomentosum* (fig. 1) is a marine green macroalgae (phylum Chlorophyta) of the family Codiaceae (Pereira, 2016). *Codium* Stackhouse is one of the widest distributed marine genus around the world (González & Santelices, 2004) and embrace around 150 species (Choi, et al, 2013).

Codium tomentosum can be found in Western Ireland, Orkney Islands, southern England, Channel Islands, Netherlands, southward to Morocco, Azores Islands and Algeria (Silva, 1955).

Morphologically, individuals present a cylindrical thallus, regularly dichotomous with spongy and elastic consistency and a dark green colour (Silva, 1955). This seaweed can grow up to 30 cm in length and the branches, thin and cylindrical, can growth up to 8 10 millimetres in diameter (SIA, 2013). Terminal segments are often long with apices rounded or slightly pointed (Silva, 1955). It commonly presents numerous hairs (or hair

scars) below the apex (Silva, 1955). In culture, water movement is very important to regulate the formation of spongy thalli (Ramus 1972; Park & Son, 1992)

This seaweed can be confused with the invasive species *Codium fragile.* These two species can be distinguish by morphological characteristics as the utricles, which in *Codium tomentosum* are non-mucronated (Trowbridge et al., 2014).

Figure 1 Codium tomentosum in the field (OMARE, 2017)

Codium tomentosum is a perennial alga and its reproduction and growth peaks are coincident, which occur during the late summer and beginning of autumn. (Kang,et al., 2008)

According to previous studies (Arasaki, 1955; Borden & Stein, 1969) this genus has a diploid life cycle with gametic meiosis (Miravalles, et al., 2012). In sexual reproduction, zygotes are obtained by the merge of the haploid gametes (Lobban & Harrison, 1997; Nanba, et al., 2005).

In its natural environment, this species occurs not only in sheltered conditions but also in more exposed ones, in the upper and lower intertidal areas and in tidal pools (Pereira et al., 2009). Also, this species is normally observed as a host of the following epiphytes: *Ectocarpus, Ceramium, Ulva* and *Chaetomorpha* (González & Santelices, 2004). The principal consequences of epiphytes are changes in the nutrient uptake, gas exchanges

and shadowing, which causes a drop in the growth rate and can also increase the drag forces causing the seaweed affected to be ripped from the substrate (Lobban & Harrison, 1997; Graham & Wilcox, 2000).

Although intensive studies about *C. tomentosum* are scarce, *Codium fragile i*s used in a variety of fields such as food, biomedicine (Kang et al., 2008), as well as in cosmetic and pharmaceutic industries (Guiry & Guiry, 2018a). In Portugal, *C. tomentosum* is already used in some dishes (Sousa Pinto, personal communication).

Codium tomentosum is known to be a source of sulphated galactans compounds (Wang, et al., 2014) with bioactive properties as antigenotoxic, antibacterial (Christabell et al., 2011), anti-coagulant (Shanmugam, et al., 2002) antioxidant, antitumor and hypoglycaemic activities, n-carboxylic (Yildiz, et al., 2014), novel sterols, arachidonic acid, palmitic acid (Andrade et al., 2013), carotenoids, halogenated metabolites and other bioactive compounds (Dembitsky, et al., 2003; Andrade et al., 2013). Samples collected from the Portuguese coast display high levels of 16:0, 16:3n-3, 18:1n-9, 18:2n-6 and 18:3n-3 in their fatty acids pool (da Costa et al., 2015). Rodrigues et al., (2015) reported that for this seaweed the protein content was 18,8g/100g DW (dry weight), the linolenic acid (C18:3 C6 C9 C12) content of 2,84g/100g fat and a sodium content of 204,52mg/g DW. Also, an arachidonic acid value of 2,86g/100g fat that is associated with inflammatory processes was reported (Rodrigues et al., 2015).

There are few study's in the bibliography about the optimum cultivation conditions for *Codium tomentosum*. Based on bibliography consulted for *Codium fragile* ssp. tomentosoides, the peak of growth and reproduction is obtained with temperatures ranging from 18ºC to 24ºC, salinity between 24-30‰ and 16h of light a day. For this species the thallus didn't survive at temperatures above 33ºC (Hanisak, 1979). On studies performed for the species *C. fragile* ssp, the ideal profundity to cultivate this seaweed in open sea was 1m deep (Hwang, et al., 2008) and for this species a light intensity above 3000 lux is critical for the formation of spongy thalli (Nanba et al., 2005). Hwang et al (2008) concluded that for a more stable cultivation of this seaweed the most economic and effective method is artificial seeding of isolated medullary filaments. There is one study showing that the optimum growth of *C. tomentosum* happens at 20ºC, with relative growth rates of 0,22-0,4g day-1 (fresh weight) at a salinity of 30,6 ‰ (Yang, et al., 1997).

Osmundea pinnatifida (Hudson) Stackhouse

The genus *Osmundea* was re-established by Nam, et al (1994), including species that previously belonged to the genus Laurencia. *Osmundea* differs from Laurencia by the presence of spermatangial depressions, and in that the tetrasporangia are produced from apical and epidermal cells instead of pericentral ones. This genus is included the phylum Rhodophyta, class Florideophyceae, order Ceramiales, family Rhodomelaceae and tribe Laurenciae (Guiry & Guiry, 2018b).

O. pinnatifida (fig 2) is known to have a distinct spicy flavour which gives this seaweed the common names of pepper dulce (Guiry & Guiry, 2018b) or “erva malagueta” (in Portuguese). This seaweed is mostly used as a food condiment, namely in Scotland, Ireland and Portugal (Azores islands). In the Azores, it is consumed dried, but can also be pickled in vinegar with onions and eaten with fried fish (Patarra, et al., 2011).

O. pinnatifida is found among the intertidal flora of the north-western coast of the Iberian Peninsula (Cardoso et al., 2014), where it is present throughout the year in the intertidal rocky shores. *O. pinnatifida* is considered a perennial alga that can be found in the substratum attached by stoloniferous branches and a basal crust (Pereira et al., 2014). *O. pinnatifida* species can be found in the North-East and South-West Pacific, the Atlantic and the Mediterranean Sea (Machin-Sanchez et al., 2012).

Morphologically, this seaweed is characterized by an erect thallus with a cartilaginous texture and a brownish-purple colour, the branching is alternate with branches becoming smaller when reach the apex of the seaweed and the extremity became round (Machin-Sanchez et al., 2012) The colour is an aspect very dependent of the part of the shore where it is collected. High shore seaweeds have a more yellow colour because they are exposed to levels of sunlight superior to the ones found on the low part of the shore where they present a more brownish-purple colour (Pereira et al., 2009; Pereira, 2014).

Figure 2 - Osmundea pinnatifida (Hudson) Stackhouse (AlgaeBase, 2017)

According to Machín-Sánchez et. al (2012), the male reproductive structures are branches with pocket-shaped spermatangial pits, located at bifurcations of ultimate branchlets or laterally in series. The pocket-shaped structure has a narrow apical pore. The feminine gametophyte has a cystocarp located laterally, mainly in the second-order branches, sessile, slightly ovoid whit a non-protuberant ostiole. The tetrasporangia are born randomly on cortical cells (Machin-Sanchez et al., 2012).

This species is known to be a source of fibber, protein, fatty acid such as linoleic acid (Patarra et al., 2013), vitamins A, E (alfa-tocopherol and γ-tocopherol), K (K1 e K2) and minerals (sodium, potassium, magnesium, caesium and calcium) (Paiva et al., 2014). Also, it has antioxidant properties, and its fatty acid profile is adequate for use as food and in food supplements. They are also useful for the pharmaceutical industry (Paiva et al., 2014). According with Rodrigues et al., (2015) this seaweed has also an inhibitory potential against α-glucosidase. The α-glucosidase inhibitors (AGIs) are used in the treatment of patients with type 2 diabetes and it provides beneficial effects on the glycaemic control and post load insulin methods (Van De Laar et al., 2005).

Rodrigues et al., (2015) reported that for *Osmundea pinnatifida* collected from Portuguese shores the values of calcium and potassium for this seaweed are 54,1 mg per DW and 261,0 mg per DW respectively. The values of PUFA and MUFA for this seaweed are respectively 25,0% and 17,3% per total fatty acid content for seaweeds harvests in Azores coast (Paiva et al., 2014). The same author reported that *O.*

pinnatifida presented 41,6% of EAA/protein, with values of leucine, proline and aspartic acid of 16,5 mg/g, 13,69 mg/g and 12,2 mg/g of protein, a soluble carbohydrates content of 17,6% of DW and a total sugar content of 32,4% DW (Rodrigues et al., 2015). The protein value is highly variable but for the *O. pinnatifida* found in the north and centre shores of Portugal the value is around 23,8% DW. According to Andrade et al. (2013), the *O. pinnatifida* found in Portugal has high levels of mannitol, that is a naturally occurring polyol (sugar alcohol) that can be use, for example in the food and the pharmaceutical industry (Saha & Racine, 2011).

Cultivation studies for this species are very scarce and information concerning its growth conditions and productivity were not found in the literature.

Objectives

The purpose of this work was to contribute to the development of cultivation methods for *Osmundea pinnatifida* and *Codium tomentosum* by:

- Assessing the influence of temperature, light intensity and density on growth at a laboratory scale for each species;

- Assessing growth rates and biochemical composition (antioxidant activities, protein and phenol content) variability in an outdoor system at two different seasons: winter and summer

Material and methods

Laboratorial cultivation optimization trials

The biological material (both species) used in the optimization trials was collected between February to June from the following rocky shores: Vila Chã (41º17'44.17"N, 8º44'12.79"W) Belinho (41º34'53ºN 8º48'16W) and Viana do Castelo (41º41'53.79"N 8º51'19.57"O). After collection, seaweeds were transported to the laboratory in isothermal boxes and cleaned thoroughly with fresh water to remove invertebrates and the epiphytes were removed manually. After the cleaning the seaweed were kept in aerated flasks and with a light intensity of 100 µmol m^{-2} s^{-1}.

Before the beginning of the trials, all the material (seawater, nutrient media, flasks and aeration systems) was prepared, and light, temperature, and photoperiod were set in the climatic chambers (fig. 3).

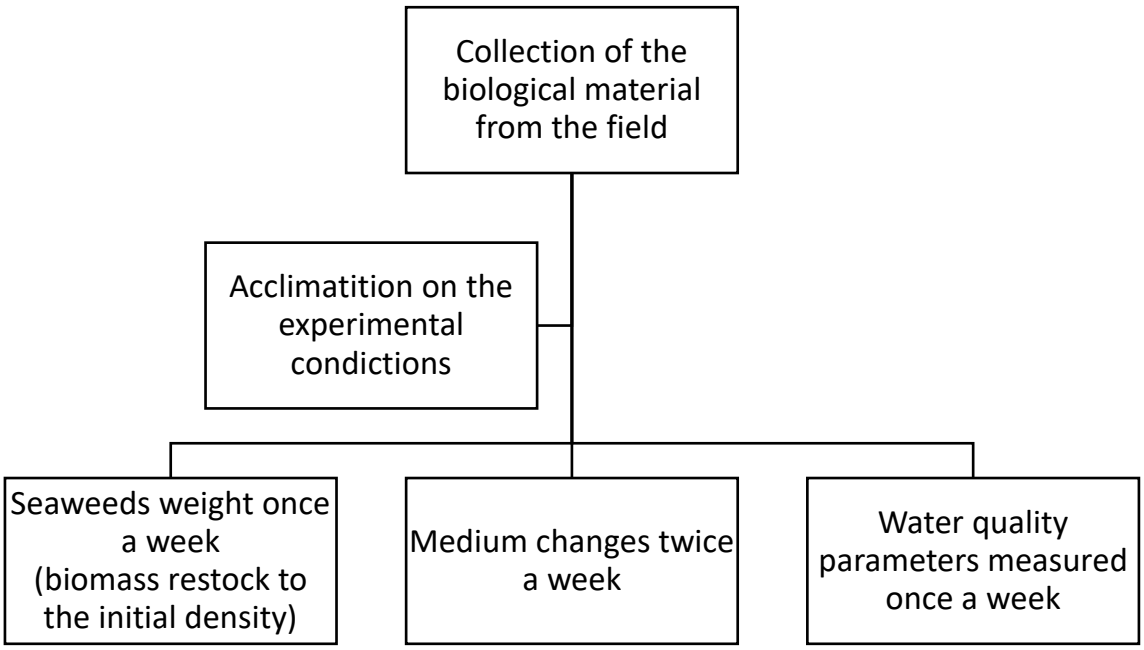

Figure 3 Procedure of the laboratorial cultivation optimization trials

Effect of temperature

This trial occurred between 20 of March to 26 of April 2018 for *O. pinnatifida* both species. This experiment was performed for both target species, and each trial had a 4 week duration with 3 replicates per specie and the seaweeds were placed in the system 1 week prior of the trial for acclimation (fig. 3). Temperature levels tested were 12, 16, and

20 ºC. The experience was performed with a light intensity of 100 µmol m^{-2} s^{-1} and a photoperiod of 12:12 light: dark with a density of 10 g/l. Seaweeds where place in 1L Erlenmeyer's with a culture volume of 500mL.The nutrient media, Provasoli Enriched Seawater (PES) (Starr & Zeikus, 1993) was changed twice a week. The assessment of the biomass was made once a week and the densities were reset to the initial values (except if biomass loss occurred) (fig 5). The presence of epiphytes was recorded and categorized as "absent", "low" or "high". The water quality parameters as dissolved oxygen, salinity and pH were also measured using a multiparametric probe (HACH).

Effect of light intensity and density

This trial occurred between 2 of May to 7 of June for *C. tomentosum* and 29 of June to 3 of August for *O. pinnatifida* (fig.3). This experiment was also performed for both species and each trial had a 4 week duration with 3 replicates per specie and the seaweeds were placed in the system 1 week prior of the trial for acclimation. The purpose of this work was to evaluate the effect of light intensity and cultivation density on growth as well as the potential interaction between these 2 factors. Two levels of light (100 and 200 µmol m^{-2} s^{-1}) and 3 levels of density (5, 10 and 15 g/l) were tested (fig. 4). The photoperiod, volume and nutrient medium was the same as in the previous trial and the temperature was 16ºC, the best result of the previous trial. The nutrient media was changed twice a week. The assessment of the biomass was made once a week and the densities were reset to the initial values (fig. 5). The presence of epiphytes was recorded and categorized as "absent", "low" or "high". The water parameters as dissolved oxygen, salinity and pH were also measured using a multiparametric probe (HACH) (fig. 6).

Figure 4 Climatic chamber with seaweeds

Figure 5 Weighing of the seaweeds

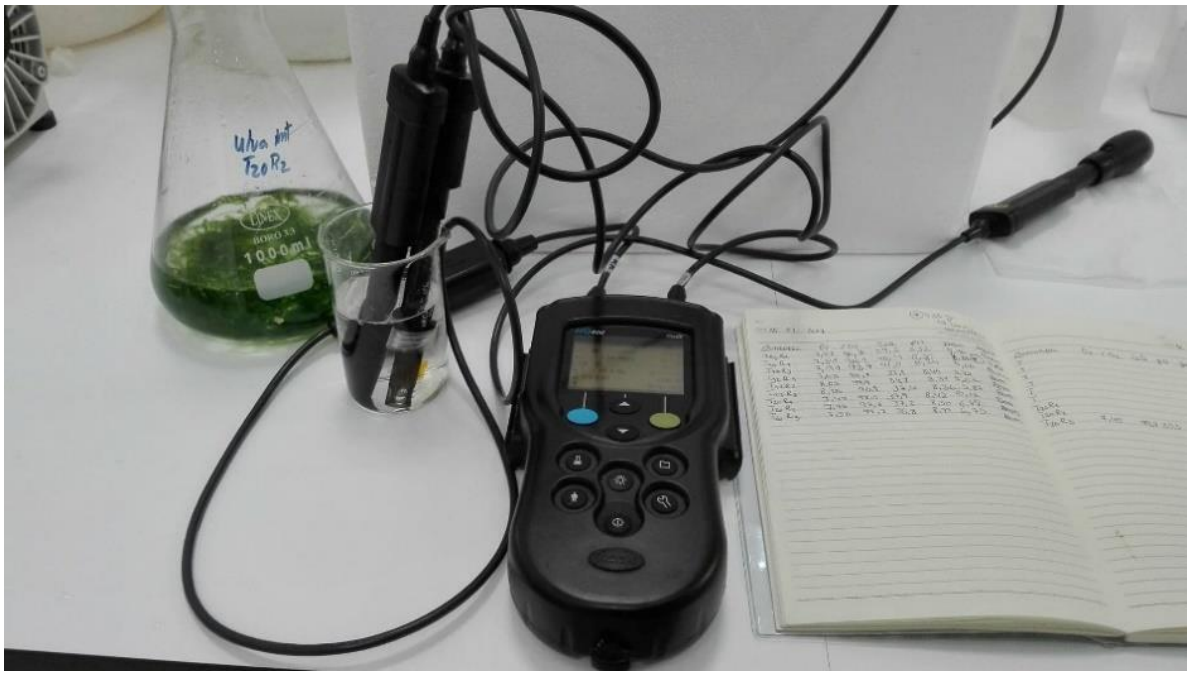

Figure 6 Multiparametric probe for measuring the physical chemical parameters

Outdoor cultivation trials

The target species of this study were collected during January 2018 and June 2018 in rocky shores in northern PT Vila Chã (41º17'44.17"N, 8º44'12.79"W) Belinho (41º34’53ºN 8º48’16W) and Viana do Castelo (41º41'53.79"N 8º51'19.57"O). After collection, seaweeds were transported to the laboratory in isothermal boxes and cleaned

thoroughly with fresh water to remove invertebrates and the epiphytes were removed manually.

The experiment was performed for both species of the study and each trial had a 2 month duration. This trial was conducted at Estação Zoológica Marítima Dr. Augusto Nobre.

The purpose of these trials was to compare the growth of the seaweeds in study during two seasons of the year. The trials were performed under winter conditions, between February and March 2018 and summer conditions, during May and July 2018 (fig.7).

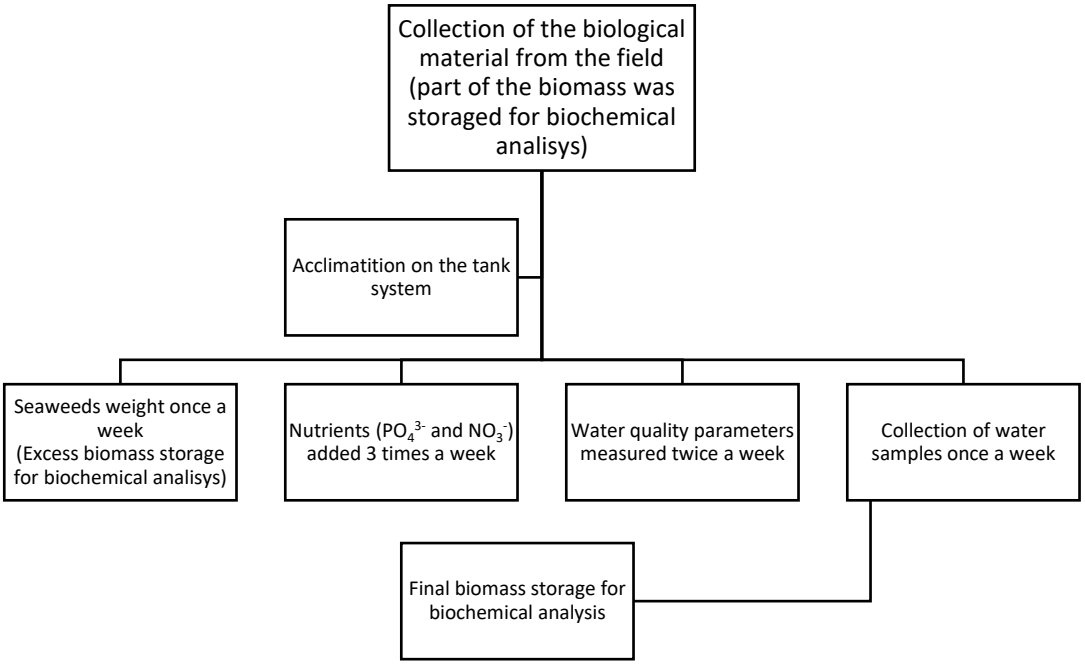

Figure 7 Procedure of the outdoor cultivation trials

The seaweeds were maintained in an outdoor system in 70L tanks (fig. 8) with aeration (to make the seaweeds circulate in the water column to ensure that not only the exposure to light was the same for all individuals but also to improve the nutrient uptake and to prevent the supersaturation of oxygen produced photosynthetically (Craigie & Shacklock, 1995). Three replicate tanks were used for each species (6 tanks in total). A continuous seawater flow allowed a renewal of the tank volume every 2 days. The nutrients were added at a final concentration of 0,16 µmol/L of $PO4^{3-}$ (supplied as sodium phosphate) and 1,28 µmol/L of NO_3^- (supplied as sodium nitrate) three times a week to compensate the water renovation. (fig. 9). In the summer season to prevent photoinhibition the tanks were protected with a shadowing net.

Figure 8 Tank cultivation system

Figure 9 Left: measurement of the water quality parameters; Middle: Weighting of the seaweeds; Right: Addition of nutrients

The seaweeds collected were maintained 1 week in tanks prior to the trials. To determine the initial weight, excess water was removed by centrifuging and the biomass was distributed among the tanks to obtain a 10g/l density in each tank.

During the trial, light and temperature were measured every 30 minutes with Onset Hobo Pendant Temperature / Light data loggers. Each week the biomass was removed with fishing nets from the tanks and the epiphytes were manually removed. Afterwards, the

seaweeds were centrifuged and weighed (fig.9) and placed in the tanks at the initial density. When growth occurred, excess algae was dried and stored for chemical analyses. Water quality parameters (pH, temperature, dissolved oxygen and salinity) were measured twice a week using a multiparametric probe (HACH).

Once a week, water samples were collected from each tank and analysed for nutrients (ammonia, phosphate, nitrite and nitrate) using a Palintest® photometer 7000 and Palintest® tablet kits (fig. 10).

Figure 10 Analysis of the water parameters

Besides the biomass that was storage during the trial, also biomass samples were collected at the beginning and end of each trial to evaluate the biochemical composition and antioxidant bioactivity. These samples were dried at 60 ºC, which retards microbial growth, helps to preserve the desirable qualities, and reduces storage volume.

Biochemical analysis

Biochemical composition and Antioxidant capacity

In order to evaluate a possible commercial application of the seaweed biomass, assessment of biochemical profile is crucial.

Thus, protein and phenolic compounds were determined, as well as antioxidant capacity of *Osmundea pinnatifida* and *Codium tomentosum*. Because there is not a universal antioxidant assay, and different groups of molecules are sensitive to different radicals, antioxidant capacity was assessed by two different assays ($ABTS^{\bullet+}$ and $DPPH^{\bullet}$).

Extraction of compounds

First, dry biomass of seaweed was grounded and reduced to powder. For extraction, 15mg of biomass was homogenized with 1,5 mL of EtOH: H2O (1:1, v/v) in a Presells® Evolution (Bertin Corp., Rockville, USA) with 12 cycles of 30 seconds at 8000 rpm, with zirconia beads. Extract was kept in the cold (4º C) prior to analysis. This solvent was chosen for its capacity to extract both polar and nonpolar components.

Antioxidant Capacity

As referred before, antioxidant scavenging activity was ascertained via two different assays for total activity ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$).

The ABTS $^{\bullet+}$ radical cation ($ABTS^{\bullet+}$) assay was conducted in a 96-wells plate, where 63 µL of sample were reacted with 180 µL of $ABTS^{\bullet+}$ reagent for 6 minutes. After the reaction, the plate was read at 734 nm (at an absorption between 0.680 and 0.720) (Guedes, 2013).

For $DPPH^{\bullet}$ radical assay, also in a 96-wells plate, 63 µL of extract were reacted with 180 µL of $DPPH^{\bullet}$ reagent for 30 minutes, and after the reaction, the plate was read at 515 nm (at an absorption between 0.800 and 0.900) (Brand-Williams *et al*., 1995).

For quantification of the antioxidant capacity, a calibration curve, using a known antioxidant – Trolox, was established for both methods, so antioxidant capacity was expressed as TE per DW of biomass mg TE/g_{DW}^{-1}.

Phenolic content quantification

Quantification of total phenols was performed by the spectrophotometric by the Folin-Ciocalteu method. In a 96-wells plate, 25 µL of sample were reacted with 125 µL of H2O and 25 µL of Folin-Ciocalteu reagent during 5 minutes, then it was added 75 µL of Na2CO3 (7%) and left reacting during 90 minutes, after the reaction, the plate was read at 760 nm. Gallic acid was used as reference, and the results are expressed as percentage of dry weight (%DW) (Folin & Ciocalteu, 1927).

Protein content quantification

Protein content was quantified by the Bradford protein assay, using bovine serum albumin as standard. In a 96-wells plate, 25 µL of extract were reacted with 200 µL of Bradford reagent during 15 minutes, after the reaction, the plate was read at 595 nm. The results are expressed as percentage of dry weight (%DW) (Bradford, (1976).

Data analysis

Growth assessment

The growth rate, expressed as growth per day, was calculated using the following equation:

$$\text{RGR (g g}^{-1}\text{ day}^{-1}\text{)} = \frac{(LnA - LnB)}{n}$$

Where A is the final fresh weight (g) and B is the initial fresh weight (g) of seaweed; n corresponds to the number of days in cultivation.

Statistical methods

All the obtained data were statically analysed using SPSS (version 24). For the lab optimization trials RGR (relative growth data) was analysed with RMANOVA (Repeated Measures ANOVA), considering Time as Within-Subject Factor and Temperature, Density and Light level as Between-Subjects Variable and for the biochemical analysis a 1-way ANOVA with time as factor was used. Tukey tests were performed for factors with p-values lower than 0,05.

Results

Laboratory optimization trials

Effect of temperature on *Codium tomentosum*

Codium tomentosum growth rates varied significantly with Time (RMANOVA, Time, p = 0,04, F (4) = 10,688, Fig.11 (A)) and with temperature (RMANOVA, Temperature, p = 0,002, F (2) = 20,309, Fig. 11 (B)) but the 2 factors didn't interact. About the effect of time (fig. 9(A)), growth decreased in the first week after the acclimation period and recovered in the following weeks. As for the effect of temperature (fig.11 (B)), at 16 ˚C RGR was approximately 0,025 g day^{-1} g^{-1} at 12 and 20 ºC, RGR was similar and approximately 0,015 g day^{-1} g^{-1}. Throughout this trial the epiphytes maintained a "low" level except for the last week at 20˚C when the level of epiphytes was "high", and the principal epiphyte was *Ulva* spp.

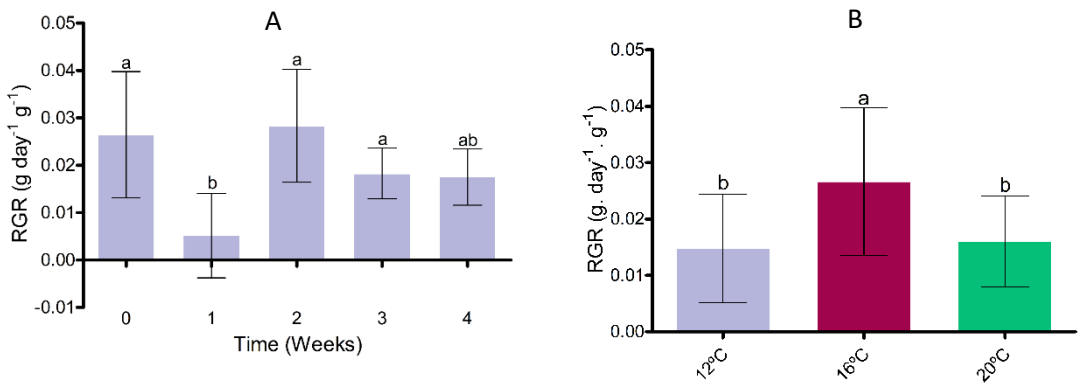

Figure 11 Effect of time (A) and temperature (B) on growth of Codium tomentosum. Week 0 corresponds to the acclimation period and the following ones correspond to the trial. Different letters were attributed to significantly different points based on Tukey test. Data are expressed as mean ± SD (n=15)

Effects of density and light intensity on *Codium tomentosum*

Codium growth varied significantly with Time and Light Level (RMANOVA, Time * Light, p = 0,009, F (4) = 6,742, Fig. 12) but not with Time and Density (RMANOVA, Time * Density, p = 0,552, F (8) = 1,161). Tukey test found no significant differences between measurements.

For the intensity 100 µmol m^{-2} s^{-1} the growth rate decreased from the acclimation week until the 3rd week of the trial and presented an increase in the last week (fig. 12). For the intensity 200 µmol m^{-2} s^{-1}, growth was similar along the trial although lower than the value obtained in the acclimation week. Epiphyte level was "low" during the acclimation week and during the rest of the trial maintained a "high" level. The intensity 200 µmol m^{-2} s^{-1} was the one with the highest presence of epiphytes. The principal epiphyte was *Ulva* spp. and *Ectocarpus* spp.

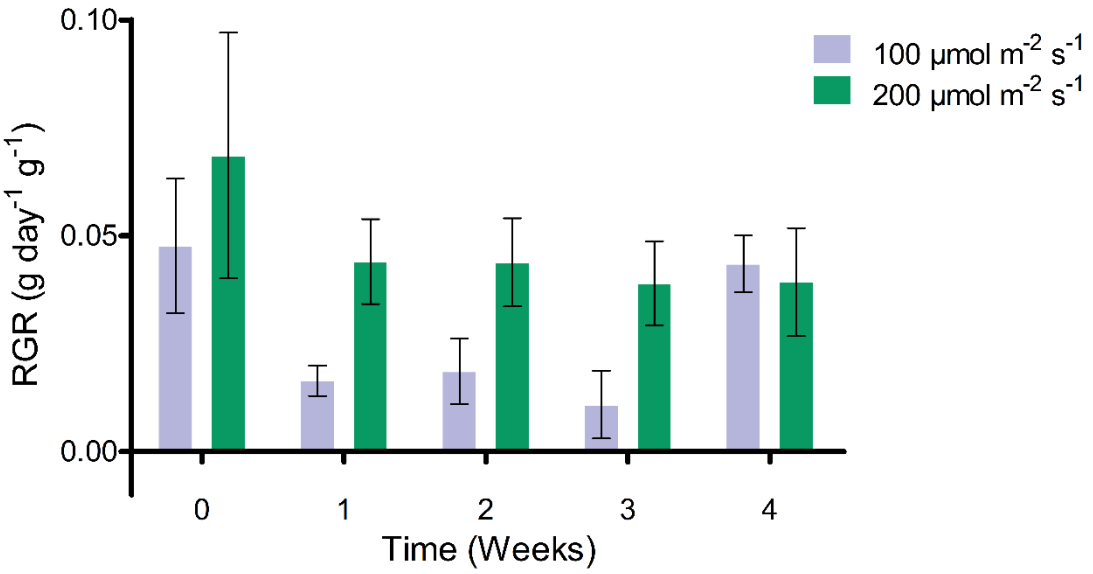

Figure 12 Effect of light intensity on Codium tomentosum growth rate throughout time. The week 0 correspond to the acclimation week and the following ones correspond to the trial. Different letters were attributed to significantly different points based on Tukey test. Data are expressed as mean ± SD (n=9).

Effect of temperature on *Osmundea pinnatifida*

Osmundea pinnatifida growth rates varied significantly with both Temperature and Time, and a significant interaction was observed between these factors (RMANOVA, Time * Temperature, p = 0,008, F (8) = 8,651, fig. 13). The highest growth rates (0,03 g g^{-1} day^{-1}) were observed at 16 ˚C during the first and second weeks after acclimation (Fig. 13). At this temperature, a significant decrease was observed in the third week, followed by a recovery in the growth rate in the last week. At 12ºC, in the first week after acclimation a large decrease in growth was observed, although some recovery was observed in the following weeks. At 20ºC, *O. pinnatifida* growth rates were similar throughout time, although higher values were observed in the first week after acclimation and lower values were observed in the third week when the growth was significantly lower. Throughout this trial the epiphytes maintained a "low" level, and the principal epiphyte was *Ulva* spp.

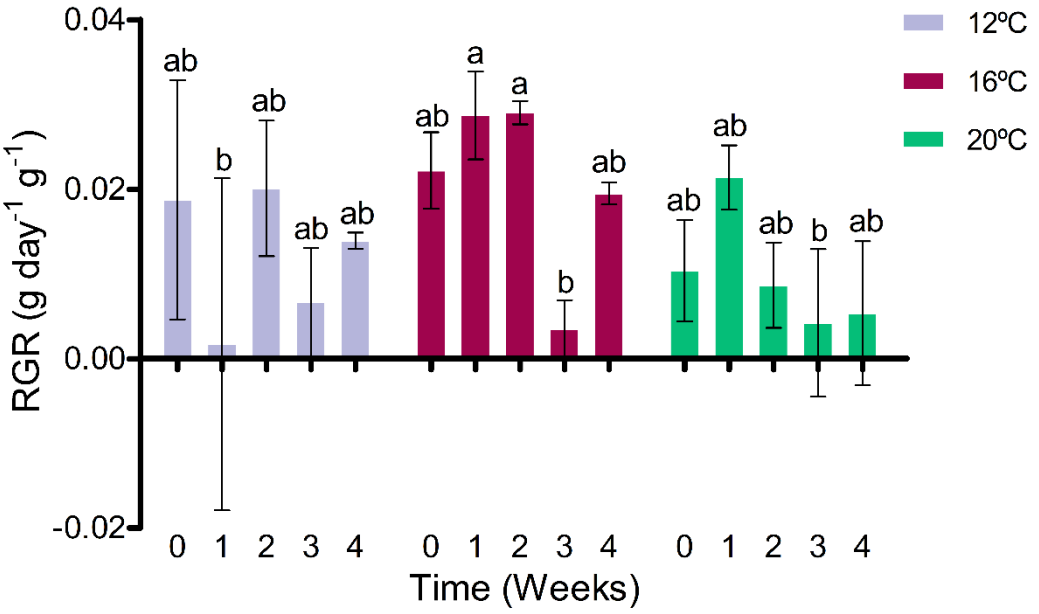

Figure 13 Effect of temperature (12ºC, 16ºC and 20ºC) on the growth rate of Osmundea pinnatifida throughout time. Week 0 corresponds to the acclimation week. Different letters were attributed to significantly different points based on Tukey test results. Data are expressed as mean ± SD (n=3)

Effects of density and light intensity on *Osmundea pinnatifida*

In this trial, conducted at 16 ºC which was the temperature that yielded the best results in the previous trial, the growth of *Osmundea pinnatifida* was almost always negative, resulting from biomass loss, which was higher at the lower light intensity (Fig.14). Significant differences were found for Time and Light Level (RMANOVA, Time * Light, p = 0,035, F (4) = 4,171, Fig.14) but not with Time and Density (RMANOVA, Time * Density, p = 0,422, F (8) = 1,075). In this trial the epiphytes maintained a “low” level in the first 2 weeks, and afterwards increased to “high”, and the principal epiphyte was again *Ulva* spp. Part of the epiphytes were growing in the walls of the culture flasks.

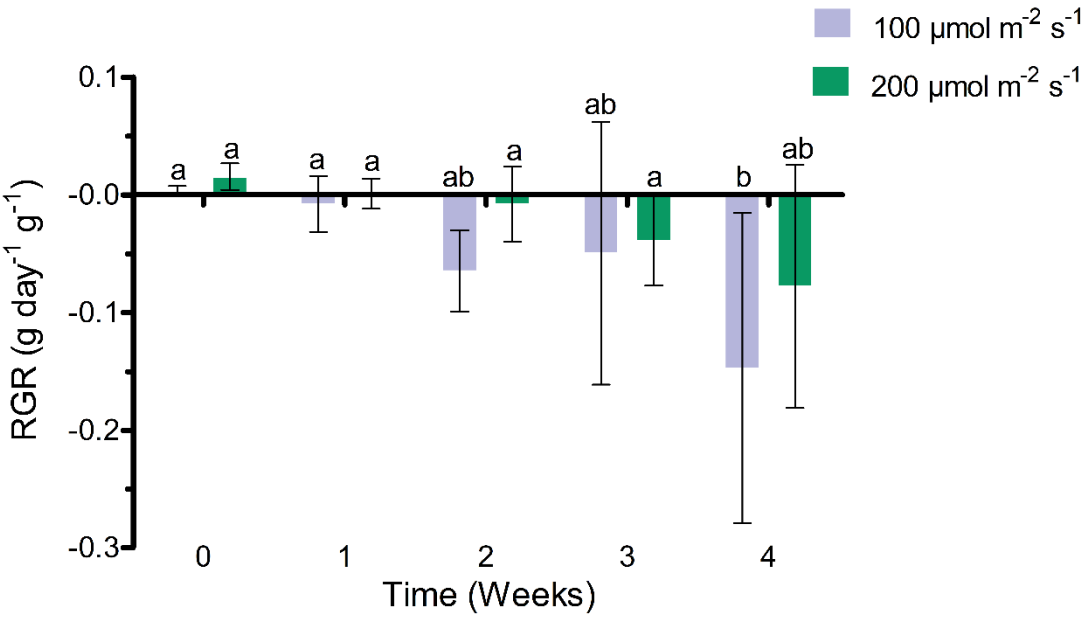

Figure 14 Effect of light intensity on the growth rate of Osmundea pinnatifida throughout time. The week 0 correspond to acclimation period. Different letters were attributed to significantly different points based on Tukey test results. Data are expressed as mean ± SD (n=9)

Outdoor trials – *Codium tomentosum*

During the winter season *C. tomentosum* showed a positive RGR during the entire trial, with similar values, except for the week 7 when the value of RGR was negative (Fig.15).

Temperature varied between 3˚C and 22 ˚C, with a mean value of 12 ˚C. The values of light intensity were similar during the trial except for the high light increase that occur during week 6 (fig. 15). Additionally, during this week the system suffered from a malfunction of the air pump, which caused a decrease in the air input to the tanks.

For this trial the pH values varied between 8,6 ± 0,08 and 9,3 ± 0,08, the salinity between 27,3 ± 0,30‰ and 33,9±0,74‰ and the dissolved oxygen between 11,2 ± 0,58 mg/L and 12,6 ± 0,90 mg/L (Table 1).

Table 1 Values of pH, Salinity and Dissolved oxygen for the species C. tomentosum throughout the winter trial

	W_0	W_1	W_2	W_3	W_4	W_5	W_6	W_7	W_8	W_9
pH	8,7 ± 0,03		8,9 ± 0,09	8,7 ± 0,02	8,8 ± 0.03	9,0 ± 0,11	8,9 ± 0,15	8,6 ± 0,08	9,1 ± 0,02	9,3 ± 0,08
Salinity (‰)	32, 8 ± 0,32		33,9 ± 0,74		27,4 ± 1,09	29,6 ± 3,53	28,4 ± 0,10	29,3 ± 0,36	27,3 ± 0,30	28,8 ± 1,91
Dissolved oxygen (mg/L)	12,4 ± 0,23		11,4 ± 0,54	11,8 ± 0.05	11,5 ± 0,09	12,2 ± 0,99	12,1 ± 0,95	11,8 ± 0,37	11,2 ± 0,58	12,6 ± 0,90

Considering nutrient availability, the concentration of ammonia, phosphate and nitrate didn't show significant differences along the time. Ammonia concentration in the tanks ranged from 11,9 ± 0,00 to 31,5 ± 1,56 µmol/L, except on the week of acclimation when a peak of 134,2 ± 53,0 µmol/L was registered; phosphate from 0,3 ± 0,05 to 9,2 ± 3,95 µmol/L and nitrate from 4,3 ± 0,00 to 32,8 ± 20,70 µmol/L (fig. 16). In the case of nitrite, the values ranged between 0,04 ± 0,036 and 0,2 ± 0,04 µmol/L and had a peak at week 5 of 0,6 ± 0,14 µmol/L, which was significantly higher than the rest of the weeks (fig. 16). This value is coincident with a large increase of the nitrite values, 0,8 ± 0,00 µmol/L of the seawater inflow (Anexo A, fig 1).

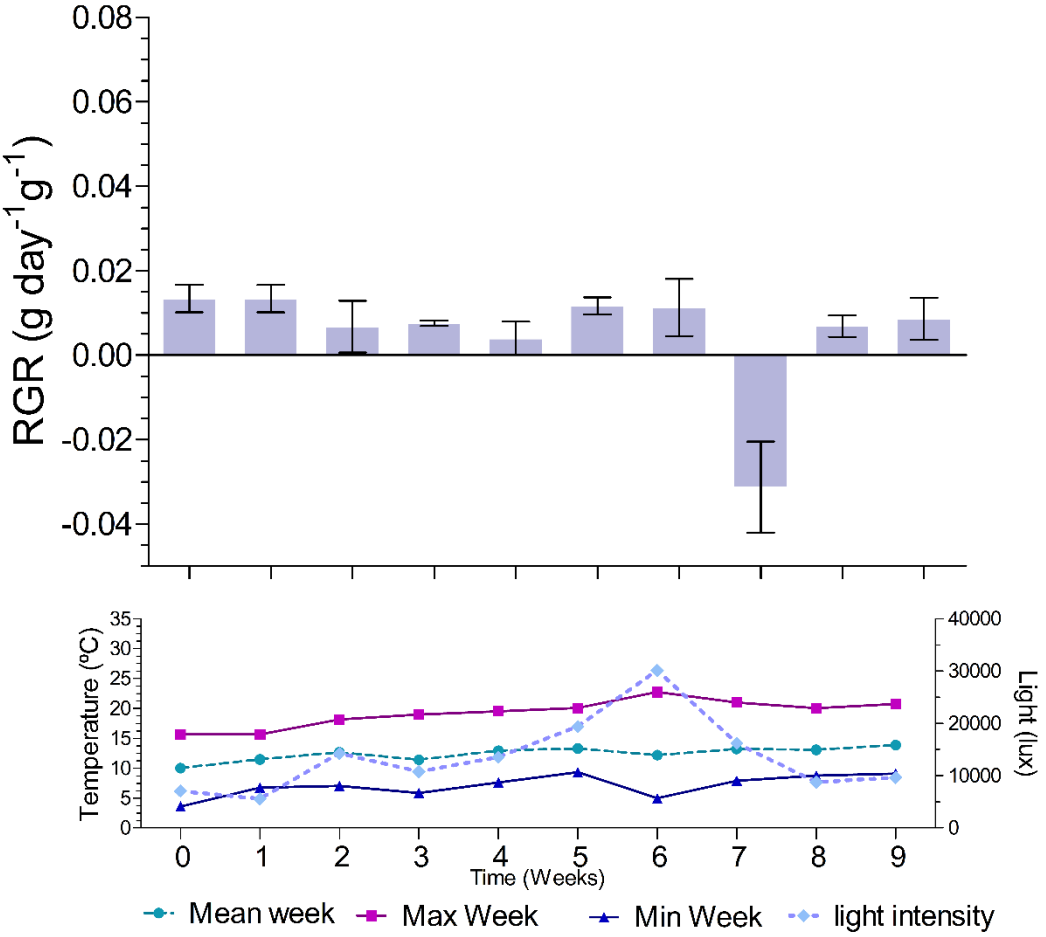

Figure 15 Growth rate of Codium tomentosum throughout the winter trial. Week 0 corresponds to the acclimation week and the following ones correspond to the trial. The graphic bellow shows the values of temperature and light intensity registered throughout the same period. Data are expressed as mean ± SD (n=3)

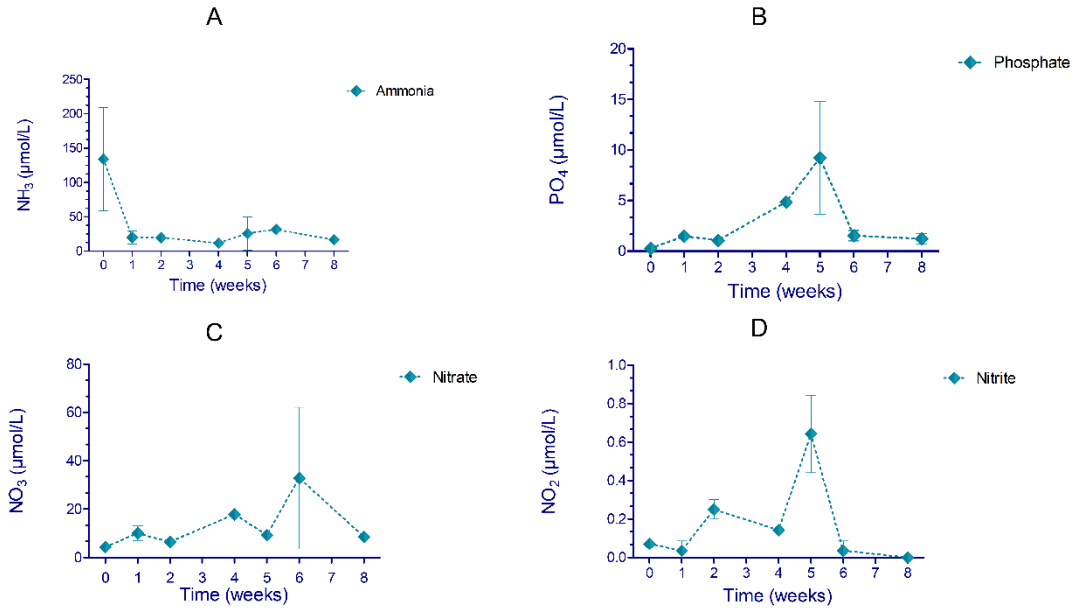

Figure 16 Nutrient concentration of ammonia (A), phosphate (B), nitrate (C) and nitrite (D) in the water of Codium tomentosum tanks throughout the winter trial. Data are expressed as mean ± SD (n=2).

During the summer trial, the values of RGR remained positive during the entire trial with the higher growth rate occurring at weeks 2 and 4 and the lower RGR occurring at weeks 3 and 5 (fig. 17).

During this trial the temperatures varied between 13˚C and 32˚C, with a mean value of 19,5˚C. To prevent over exposure to light, which may cause photo inhibition, shadowing nets were placed on the tanks during week 4. Therefore, the higher value of light intensity occurred during week 3 and the lower at week 4 as show by fig. 17.

For this trial the pH values varied between 9,0 ± 0,03 and 9,9 ± 0,42, the salinity between 30,5 ± 0,28 ‰ and 33,9 ± 0,00 ‰ and the dissolved oxygen between 9,5 ± 0,82 mg/L and 11,4 ± 0,25 mg/L (table. 2).

Table 2 Values of pH, Salinity and Dissolved oxygen for the species C. tomentosum throughout the summer trial

	W_0	W_1	W_2	W_3	W_4	W_5	W_6	W_7	W_8
pH	9,0 ± 0,16	9,1 ± 0,04	9,0 ± 0,03	9,0 ± 0,03	9,0 ± 0,07	9,1 ± 0,06	9,1 ± 0,07	9,9 ± 0,42	9,4 ± 0,09
Salinity (‰)	32,1 ± 0,20	33,0 ± 0,68	31,9 ± 0,12	30,5 ± 0,28	33,7 ± 0,65	33,0 ± 0,08	33,9 ± 0,00	33,0 ±3,07	32,6 ± 3,26
Dissolved oxygen (mg/L)	10,1 ± 0,70	10,4 ± 0,47	10,5 ± 0,28	10,2 ± 0,25	11,4 ± 0,25	10,4 ± 0,22	9,6 ± 0,42	9,5 ± 0,82	10,8± 3,00

Considering nutrient concentration, no significant differences were found for phosphate, nitrate and nitrite along the trial. Phosphate concentration in the tanks ranged from 0,5 ± 0,52 to 7,4 ± 0,00 µmol/L, nitrate from 11,4 ± 4,28 to 78,5 ± 42,84 µmol/L, and nitrite from 0,04 ± 0,036 to 0,7 ± 0,18 µmol/L (fig. 18). As for ammonia the values ranged from 4,4 ± 0,00 to 32,5 ± 2,50, with a peak at week 8 of 48,7 ± 4,37 µmol/L that was significantly higher than the rest of the weeks (fig. 18).

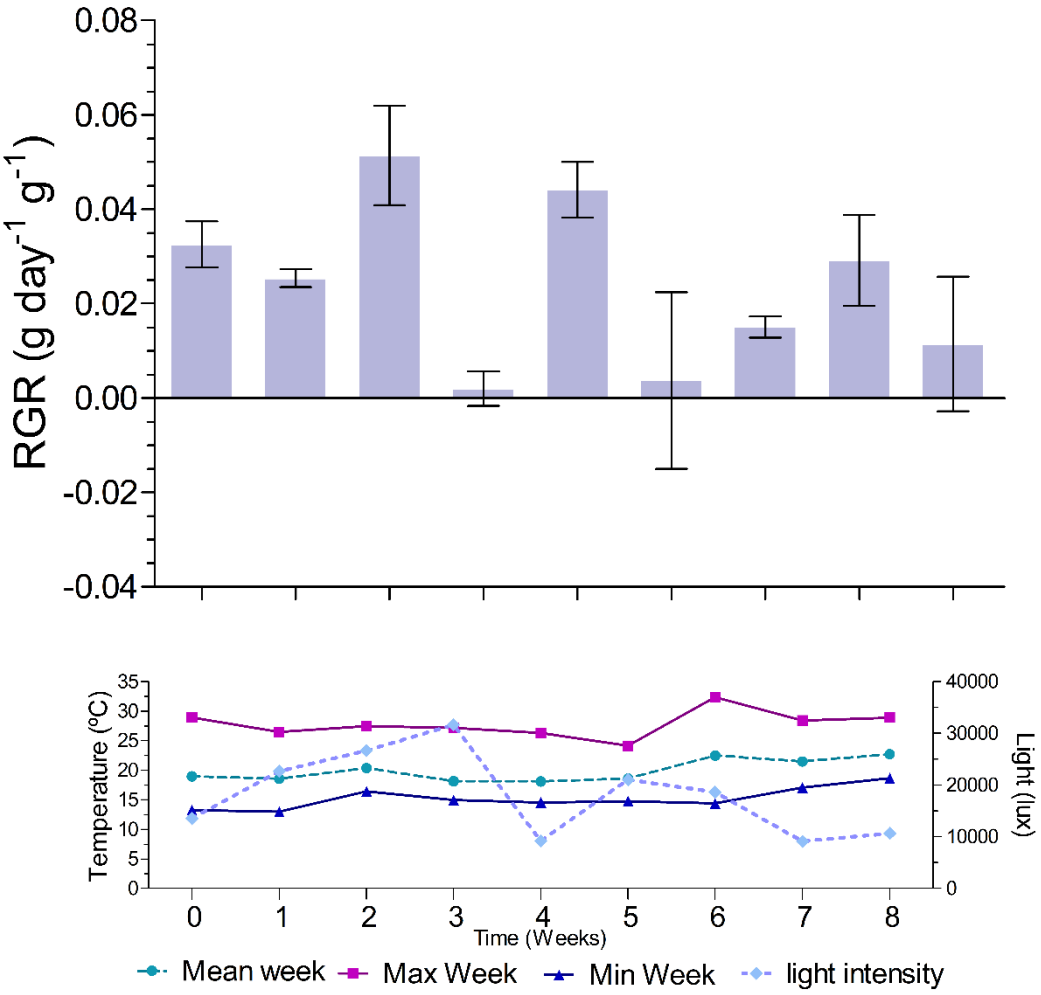

Figure 17 Growth rate of Codium tomentosum throughout the summer trial. Week 0 corresponds to the acclimation week and the following ones correspond to the trial. The graphic bellow shows the values of temperature and light intensity throughout the same period. Data are expressed as mean ± SD (n=3)

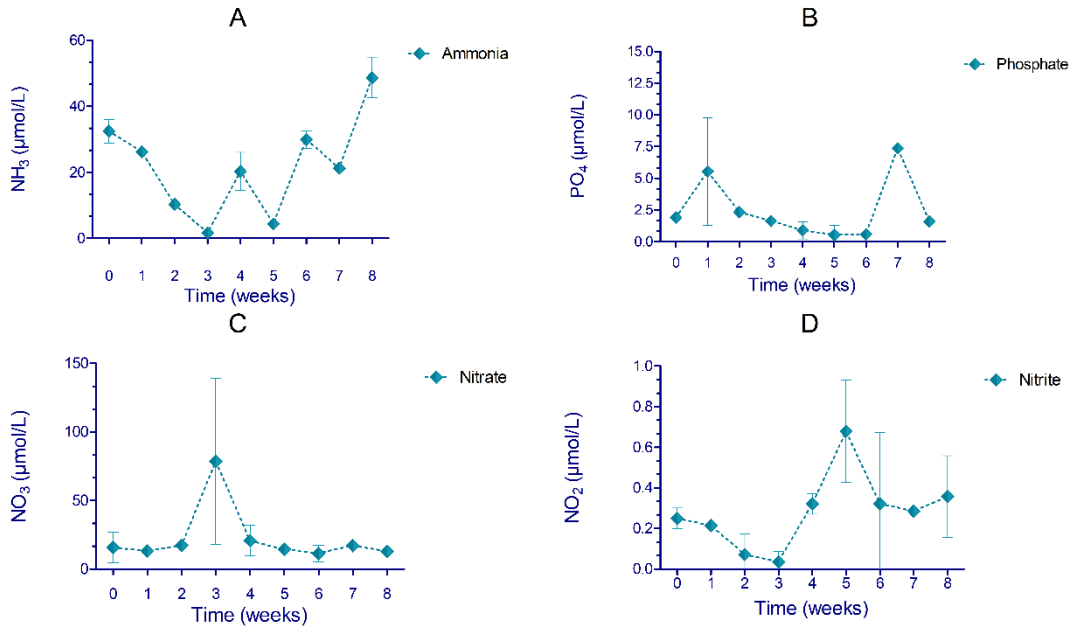

Figure 6 Nutrient concentration of ammonia (A), phosphate (B), nitrate (C) and nitrite (D) in the water of Codium tomentosum tanks throughout the summer trial. Data are expressed as mean ± SD (n=2).

Outdoor trials – *Osmundea pinnatifida*

Growth rate during the winter trial was very low, with positive values in weeks 1, 2, 5 and 8 and presenting tissue loss in weeks 3,5 and 6. The highest RGR was 0,01 g day^{-1} g^{-1} in week 5 (fig 19).

During this trial the temperatures varied between 3˚C and 23˚C, with a mean value of 12˚C. The higher value of light intensity occurred during week 6 and the lower at week 5 (fig. 19).

For this trial the pH values varied between 8,67 and 8,90, the salinity between 28,4‰ and 33,7‰ and the dissolved oxygen between 10,45 mg/L and 12,90 mg/L (table 3).

Table 3 Values of pH, Salinity and Dissolved oxygen for the species O. pinnatifida throughout the winter trial

	W_0	W_1	W_2	W_3	W_4	W_5	W_6	W_7	W_8
pH	8,9 ± 0,20	8,7 ± 0,12	8,8 ± 0,16		8,9 ± 0.07	8,7 ± 0,02	8,8 ± 0,02	9,0 ± 0,08	8,8 ± 0,04
Salinity (‰)	29,3 ± 3,07	32,6 ± 2,10	32,3 ± 0,46		33,7 ± 0,65		28,4 ± 0,69	28,3 ± 0,38	29,2 ± 8,55
Dissolved oxygen (mg/L)	11,8 ± 0,62	11,9 ± 0,33	12,5 ± 0,28		11,4 ± 0,25	11,6 ± 0,01	11,3 ± 0,11	10,5 ± 0,31	12,9± 0,41

As for the nutrient concentrations, the values of ammonia nitrate and nitrite didn't demonstrate significant differences along the trial (fig. 16). Ammonia concentration in the tanks showed peaks at weeks 1 (107,0 ± 98,92 µmol/L) and 7 (131,4 ± 112,02 µmol/L) and the rest of the weeks ranged from 20,9 ± 1,56 to 32,5 µmol/L, nitrate from 3,2 ± 0,36 to 13,2 ± 2,50 µmol/L and nitrite from 0,1 ± 0,11 to 0,6 ± 0,25 µmol/L. In the case of phosphate values ranged between 0,8 ± 0,16 and 3,4 ± 0,05 µmol/L with a peak at week 8 of 10,1 ± 2,05 µmol/L, which is significantly different from the rest of the weeks (fig. 20). This value is coincident with a large increase of the phosphate values, 9,3 ± 3,37 µmol/L of the seawater inflow (Anexo A, fig 3).

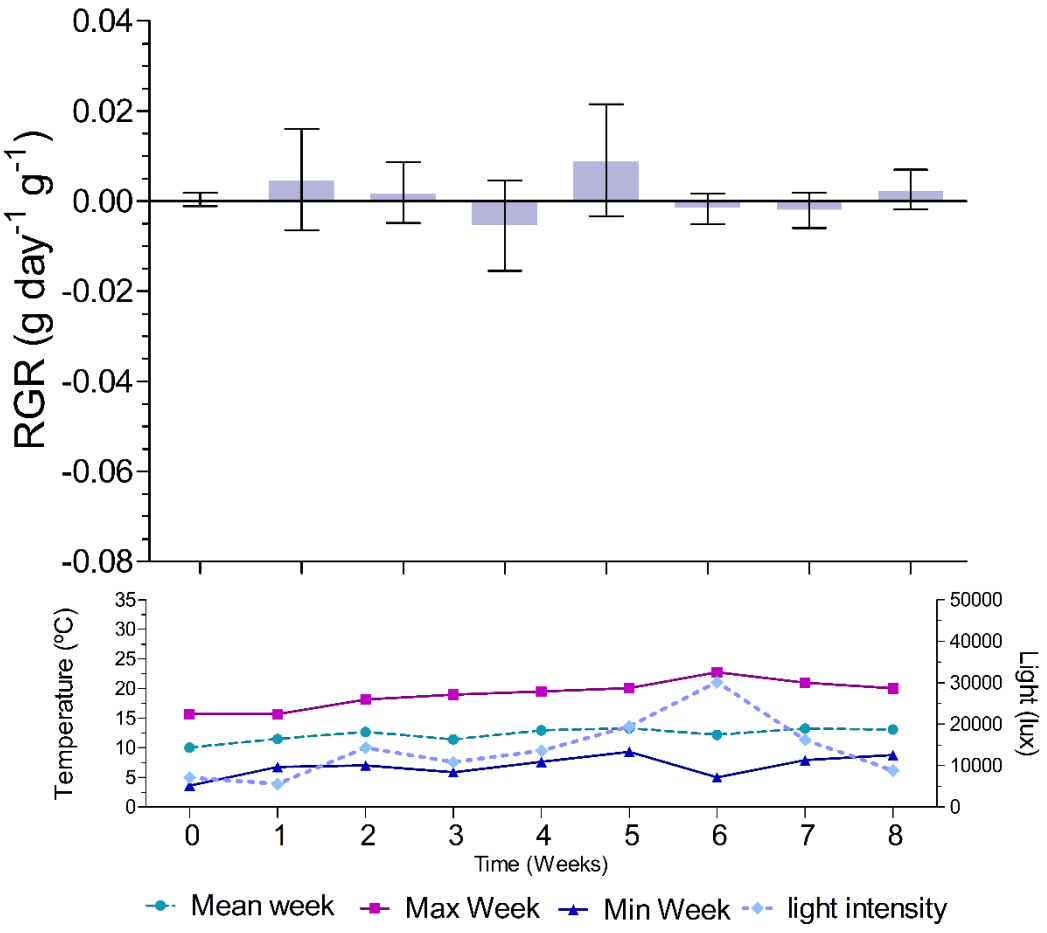

Figure 79 Growth rate of Osmundea pinnatifida throughout the winter trial. Week 0 corresponds to the acclimation week and the following ones correspond to the trial. The graphic bellow shows the values of temperature and light intensity throughout the same period. Data are expressed as mean ± SD (n=3)

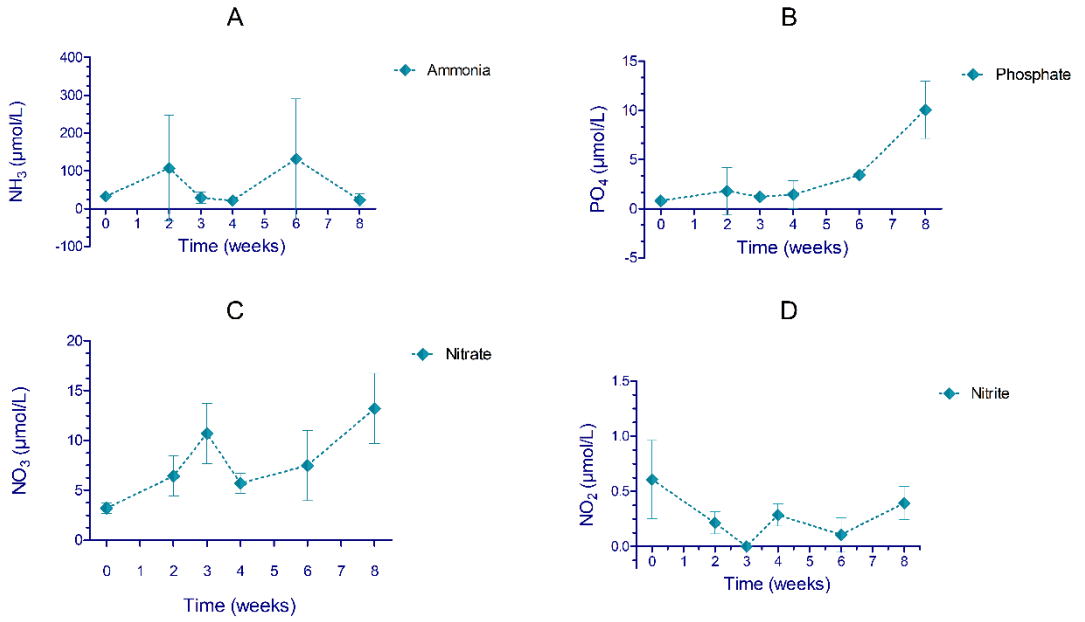

Figure 20 Nutrient concentration of ammonia (A), phosphate (B), nitrate (C) and nitrite (D) in the water of Osmundea pinnatifida tanks throughout the winter trial. Data are expressed as mean ± SD (n=2).

Results obtained for *O. pinnatifida* in the summer trial are presented in the figure 19. Two consecutive trials were conducted from 30 of April to 28 of May, and from 4 of June to 25 of June. Both trials were finalized earlier due to biomass loss. The high degradation of the seaweeds is coincident with high temperatures, with maximum temperatures around 30ºC observed for the majority of the trial (fig. 21).

During this trial the temperatures varied between 11˚C and 32˚C, with a mean value of 18,5˚C. To prevent over exposition of light, shadowing nets were placed on the tanks before the beginning of the second attempt that in fig, 19 correspond to week 5. Therefore, the higher value of light intensity occurred during week 3 and the lower at week 6 (fig. 21).

For this trial the pH values varied between 8,16 and 9,60, the salinity between 29,2 ‰ and 36,6 ‰ and the dissolved oxygen between 8,16 mg/L and 12,23 mg/l (table 4).

Table 4 Values of pH, Salinity and Dissolved oxygen for the species O. pinnatifida throughout the summer trial

	W_0	W_1	W_2	W_3	W_4	W_5	W_6	W_7	W_8
pH	9,2 ± 0,20	9,0 ± 0,01	8,9 ± 0,09	9,0 ± 0,03	8,8 ± 0.08	9,0 ± 0,05	9,1 ± 0,01	9,2 ± 0,24	8,9 ± 0,58
Salinity (‰)	30,7 ± 1,32	32,1 ± 0,26	32,8 ± 0,89	30,5 ± 0,28	30,5 ± 0,08	31,6 ± 0,97	31,6 ± 0,09	33,4 ±0,54	36,0 ± 0,40
Dissolved oxygen (mg/L)	11,0 ± 0,89	10,6 ± 0,20	9,8 ± 0,40	10,2 ± 0,25	9,8 ± 0,09	10,0 ± 0,11	11,7 ± 0,66	9,4 ± 0,22	

For this trial the pH values varied between 8,16 and 9,60, the salinity between 29,2 ‰ and 36,6 ‰ and the dissolved oxygen between 8,16 mg/L and 12,23 mg/L (table 4).

As for the nutrient analyses, the values of ammonia, phosphate, nitrate and nitrite didn't present significant differences over time (fig 22). Ammonia concentration in the tanks ranged from 20,6 ± 1,87 to 321,4 ± 277,73 µmol/L, phosphate from 0,5 ± 0,32 to 6,5 ± 3,32 µmol/L nitrate from to 6,4 to 21,4 ± 2,14 µmol/L and nitrite from 0,07 to 0,8 ± 0,21 µmol/L (fig. 22).

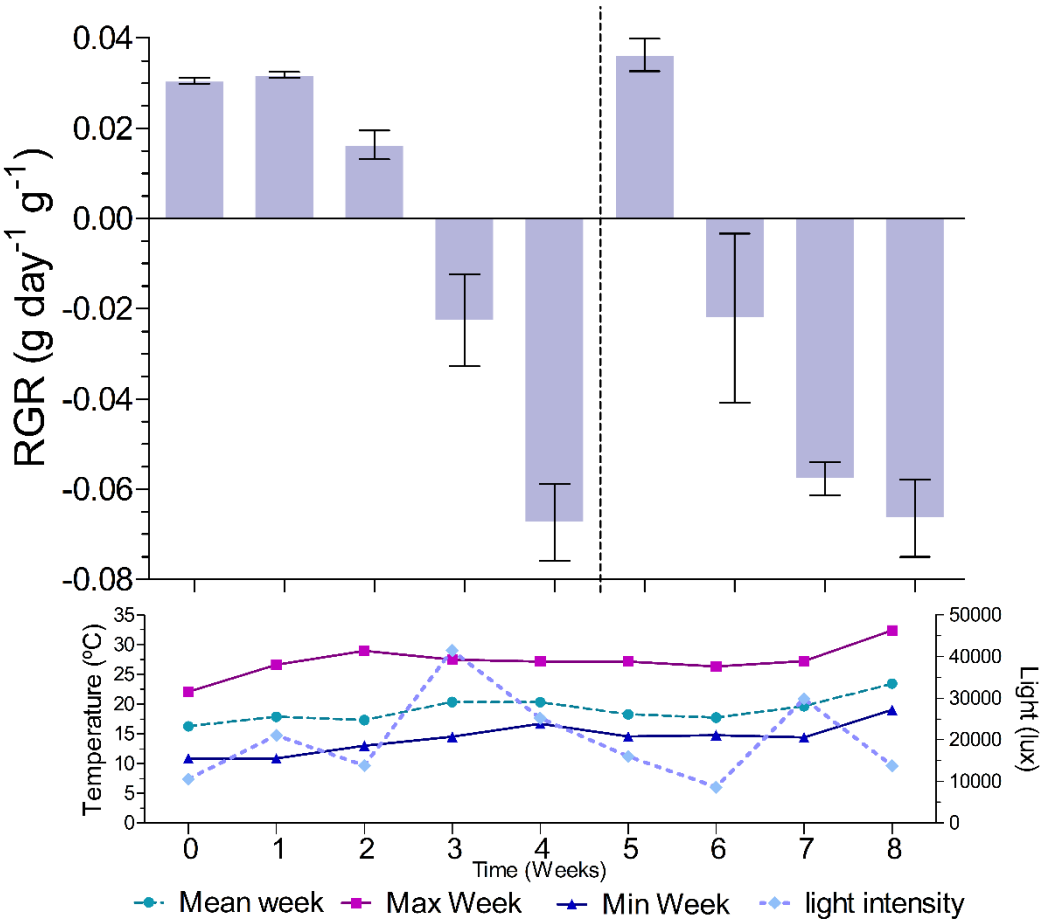

Figure 21 Growth rate of Osmundea pinnatifida throughout the summer season. The week 0 correspond to the acclimation week and the following ones correspond to the trial. The graphic bellow shows the values of temperature and light intensity throughout the same period. Data are expressed as mean ± SD (n=3)

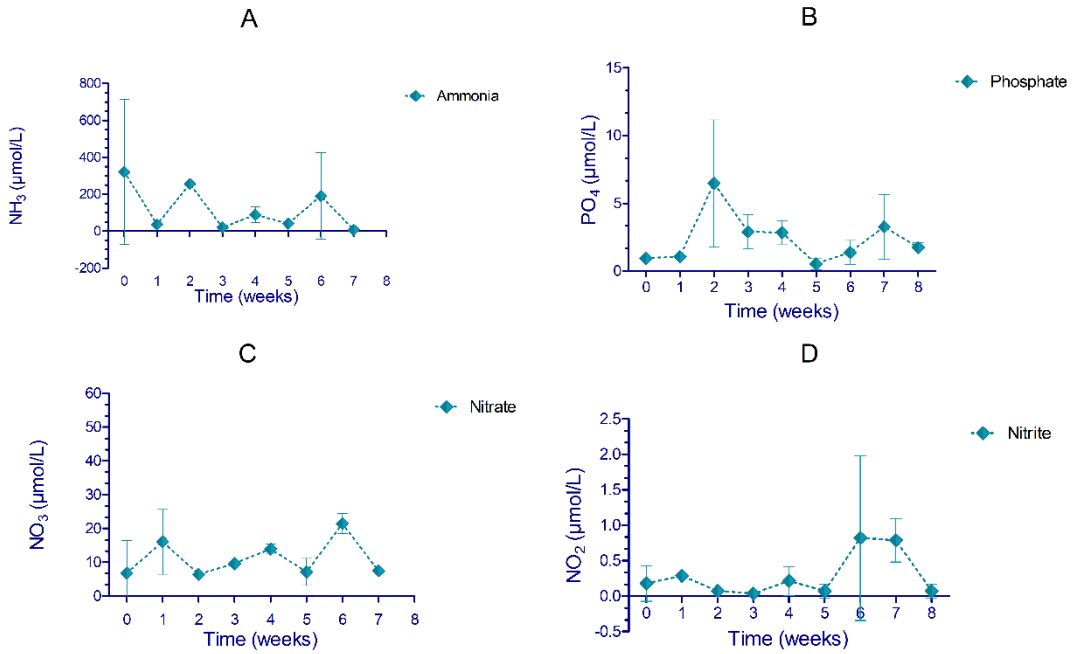

Figure 22 Nutrient concentration of ammonia (A), phosphate (B), nitrate (C) and nitrite (D) in the water of Osmundea pinnatifida tanks throughout the summer trial. Data are expressed as mean ± SD (n=2).

Biochemical analysis - *Codium tomentosum*

Antioxidant capacity, evaluated by the $ABTS^{\bullet+}$ method varied significantly with Time (1-way ANOVA, Time, p = 0,001, F (9) = 5,160 fig. 23 (A)). In the winter, the wild biomass presented significantly lower antioxidant capacity than the biomass in the acclimation week. No significant differences were found during the rest of the trial. In the summer trial the wild biomass didn't show significant differences compared to the rest of the trial. Comparing the two trials, no significant differences were obtained for the antioxidant capacity of the seaweed biomass at the end of the trials.

Considering the antioxidant activity evaluated by the $DPPH^{\bullet}$ method, a significant effect of Time was obtained (1-way ANOVA, Time, p = 0,041, F (9) = 2,518, fig. 21 (B)). Nevertheless, no significant differences were found between points based on the Tukey test.

Phenol content varied significantly with Time (1-way ANOVA, Time, p < 0,001, F (9) = 32,862, fig. 21(C)). In both trials the wild seaweed presented lower values than the cultivated biomass. In the winter trial, an increase was observed from the first week and after the second week, the biomass presented significantly higher content of phenols than the wild biomass. In the summer trial, the wild biomass also presented significantly lower phenol content than values obtained in the rest of the trial. The highest value considering both trials was obtained in the acclimation week of the summer trial. At the end of the summer trial a significantly higher phenol content was obtained, compared to the winter trial.

Protein content varied significantly with Time (1-way ANOVA, Time, p = 0,022, F (9) = 2,931, fig. 21 (D)). In the winter trial there were no significant differences in protein content between the wild and cultivated biomass during the trial. In the summer trial significant differences were found in protein content between the wild biomass and biomass cultivated, the latter being the highest.

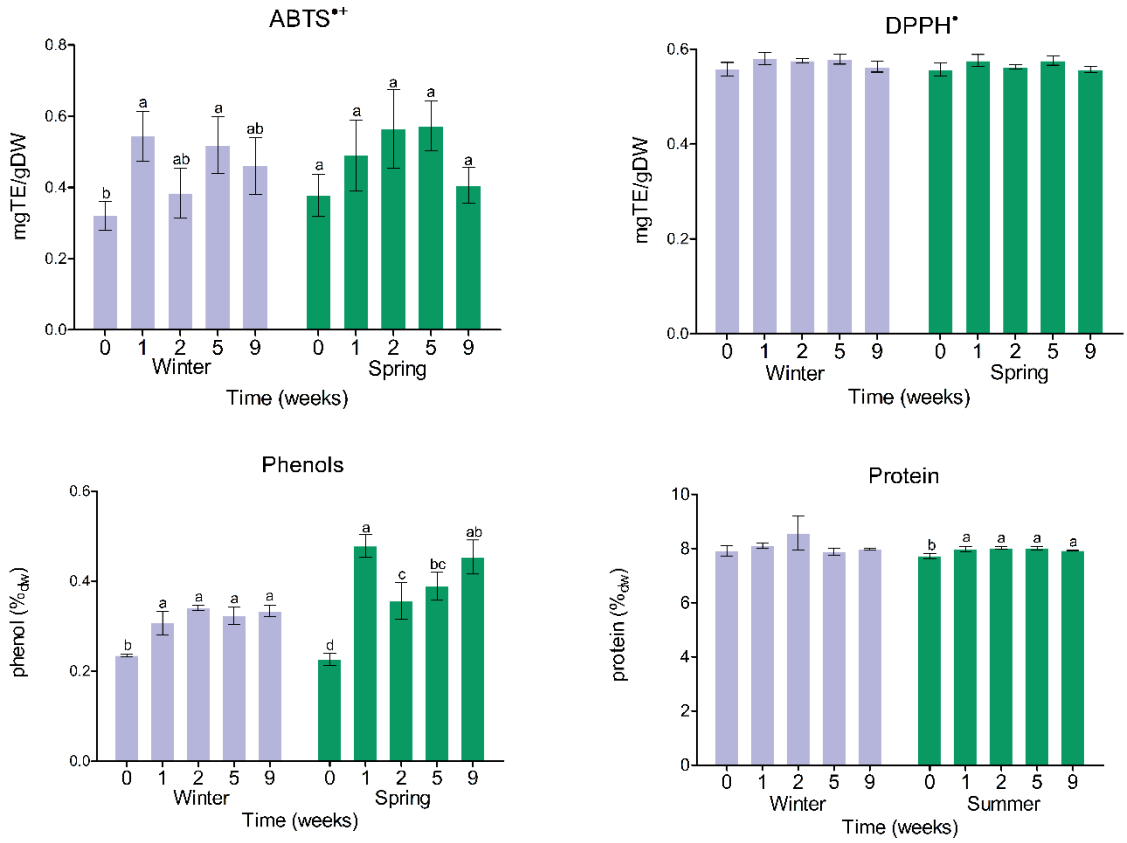

Figure 23 Antioxidant capacity by ABTS•+ (A) and DPPH• (B) methods, Phenolic content (C) and Protein content (D) variation along time for C. tomentosum. T0 and Ft correspond to the wild biomass and to the biomass at the end of the trial, respectively. The purple corresponds to the first season and the green the second season. Different letters were attributed to significantly different points based on Tukey test results. Data are expressed as mean ± SD (n=3)

Biochemical analysis – *Osmundea pinnatifida*

Since biomass loss occurred in both trials and some of the biomass was degraded, the biomass at the end of the trials was carefully chosen so that degraded biomass was not analysed.

Antioxidant capacity, evaluated by the ABTS$^{\bullet+}$ method varied significantly with Time (1-way ANOVA, Time, p = 0,017, F (3) = 6,313, Fig.21 (A)). In the winter trial no significant differences were found between the wild biomass and the biomass at the final of the trial. The same was obtained in the summer trial. Comparing the two trials, there are significant differences on the antioxidant capacity between the end of the second trial and the biomass analysed in the first trial.

Considering the antioxidant activity, evaluated by the DPPH$^{\bullet}$ method, a significant effect of time was observed (1-way ANOVA, Time, p = 0,029, F (3) = 5,092, Fig. 21 (B)).

Nevertheless, no significant difference between points based on the Tukey test was detected.

Phenol content varied significantly with Time (1-way ANOVA, Time, $p < 0,001$, $F(3) = 147,583$, Fig 21 (C)). In the winter trial the biomass at the end of the trial presented significantly higher content of phenols than the wild biomass. In the summer trial on the contrary, the wild biomass presented significantly higher phenol content than the biomass at the end of the trial. The highest value was registered for the acclimation week of the summer trial. At the end of the summer trial the phenol content was similar to the wild biomass in the winter trial.

Protein content did not vary significantly with time (1-way ANOVA, Time, $p = 0,394$, $F(3) = 1,129$, Fig. 21 (D)).

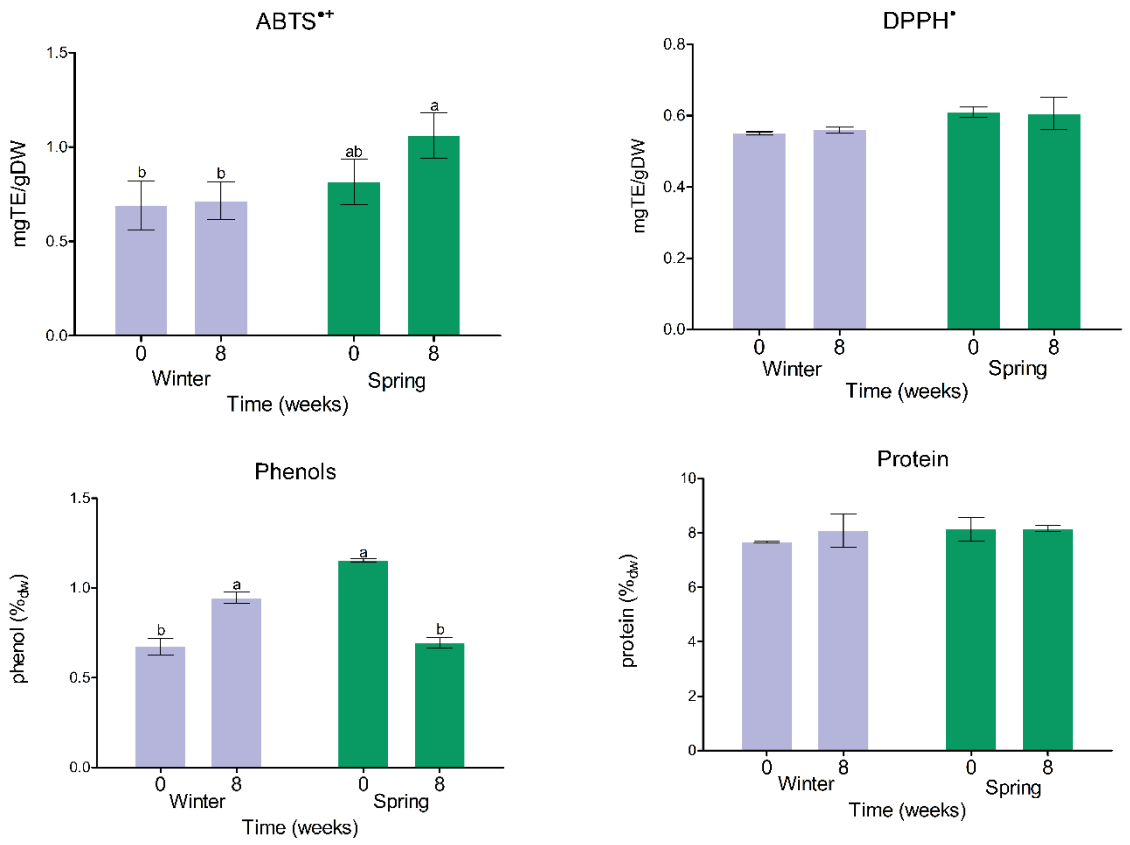

Figure 24 Antioxidant capacity by ABTS•+ (A) and DPPH• (B) methods, Phenolic content (C) and Protein content (D) variation along time for O. pinnatifida. T0 and Tf correspond to the wild biomass and to the biomass at the end of the trial, respectively. The purple corresponds to the first season and the green the second season. Different letters were attributed to significantly different points based on Tukey test results. Data are expressed as mean ± SD (n=3)

Discussion

Optimization trials

For the development of seaweed cultivation methods, the knowledge of the conditions that provide the best growth of the targeted seaweed species is essential. In this study the effect of temperature, density and light intensity, were evaluated since this information is essential for seaweed performance in aquaculture. The target species *O. pinnatifida* and *C. tomentosum*, were selected because of their nutritional characteristics, which makes them interesting to aquaculture (Rodrigues et al., 2015). Also, because previous studies on the effect of those factors on the growth of these two species are scarce. For *C. tomentosum*, the species C. fragile was used as a reference species, since the two have similar morphology and may share a habitat (Trowbridge et al., 2004).

Optimization trials for *Codium tomentosum*

For *C. tomentosum*, the trial to evaluate the effect of temperature on the growth of this seaweed, indicates that this species can grow at temperatures ranging between 12ºC and 20ºC, with the best results obtained at 16ºC. Although the studies for this species are scarce, there are a few studies for the genus *Codium* that shows that some species of this genus are able to grow in a wide range of temperatures (Hanisak, 1979; Park & Son, 1992; Yang, et al., 1997b; Hwang et al., 2008; P. Silva & Abreu, 2014). Yang et al., (1997) described that the best temperature to grow *C. tomentosum* was 20ºC, which disagrees with the results obtained. Nevertheless, the study of Yang et al., (1997) was made with filamentous forms of *C. tomentosum* grown at 20ºC, therefore perhaps the reason for this difference lingers in this fact. Also, growth can be affected by other factors such as salinity and photoperiod. For example, Hanisak, (1979) determined that for the species C. fragile ssp., the salinity range for higher growth/reproduction was 24-30‰ and the photoperiod was 16H:8H. The decreased growth rate obtained in the first week probably means that a longer acclimation period was needed since the seaweed were able to recover on the following week so this decrease of growth probably did not affect the seaweeds integrity. The tolerance to this range of temperatures and the fact that it was able to recover after the acclimation week can be explained by the fact that several seaweeds from temperate regions are resistant to temperature stress by increased tolerance or activation of recovery mechanisms (Eggert, 2012). There are few studies of these mechanisms, but it is known that thermal tolerance is associated with processes that changed the cellular metabolism. The studies available so far show several

mechanisms such as the heat shock proteins, which protect cellular proteins from protein misfolding and degradation by accumulated functions as molecular chaperones (Sorensen et al., 2003). Also, Collen et al., (2007) used cDNA microarrays to assess the effects of heat stress on the transcriptome of Chondrus crispus. This study demonstrated that high temperatures leads to an alteration on the gene expression and resource allocation, including detoxifying enzymes and antioxidant proteins. As for the tolerance of low temperatures, it was observed that the cells of Caulerpa racemosa increase the proportions of polyunsaturated fatty acids coincidently with the decrease of the water temperature in winter (Blazina et al., 2009). Other studies show that the membrane fluidity is increased by the unsaturation of fatty acids, which provides the cells with tolerance to cold stress allowing them to survive to low temperatures (Murata and Los, 1997).

The average RGR obtained during this trial for the best temperature was 0,027 ± 0,013 g day^{-1} g^{-1}, and although no studies were found for this species for comparison, there is a study using C. fragile ssp tomentosoides that, for 18ºC and a salinity of approximately 30 ‰, a similar growth was reported (Hanisak, 1979).

Considering the effect of density and light intensity, *C. tomentosum* showed to be able to grow at densities between 5 and 15 g/L, and no significant interaction was obtained between this factor and light intensity. Although the Tukey test couldn't detect any significant differences between the 2 light intensities, results showed that during weeks 1, 2 and 3 the best growth was obtained on the higher intensity. Nevertheless, at this light intensity more epiphytes occurred thus maybe a lower light intensity (between 100 µmol m^{-2} s^{-1} and 200 µmol m^{-2} s^{-1}) would be more advantageous to produce clean cultures. This strategy is supported also by the results since although during the first weeks the lower light intensity resulted in a smaller growth, in the last week no difference was observed between the two light intensities.

The average RGR obtained during this trial for the best light intensity was 0,043 ± 0,020 g day^{-1} g^{-1}, and although no studies were found for this species, a study using C. fragile ssp. tomentosoides with a light intensity of 90 µmol m^{-2} s^{-1} and the same photoperiod that was used in the present study registered an RGR of 0,15 g day^{-1} g^{-1}(Hanisak, 1979). In the same study, one of the conclusions was that for this species the increase in light hours increased the growth, not because of a photoperiod effect, but because of the increase in total daily irradiance. Therefore, in a future study it should be interesting the

observation of the effect of different light intensities and photoperiods to see if *C. tomentosum* also shows this preference.

Optimization trials for *Osmundea pinnatifida*

Results showed that at laboratory scale, the best temperature to cultivate *O. pinnatifida* is 16 ºC. This might be because this is the temperature closer to the field conditions. Nevertheless, the biomass loss observed in the third week at 16 ºC and 20 ºC suggest that other factors may be influencing the results. The temperature 12 ºC is the only one with a decrease of growth in the first week, which may be due to the need of a longer period of acclimation to these temperature conditions.

The density levels tested showed no effect on growth, suggesting that densities ranging between 5 and 15 g/L are not limiting for cultivation in free floating conditions. Although occurred biomass loss for both light intensity's tested, this loss was lower on the higher lights, suggesting that excess light was not a that factor contributing to the negative results obtained for this species at the laboratory trials. In nature this species grows during spring when light intensity is higher than winter and day length is increasing. No studies were found in the published literature to compare with the results of the present study, thus continuing efforts are needed to find the suitable conditions for the cultivation of this species. Prathep (2003) observed an increase in abundance of the seaweed during autumn, winter and spring when the peak of growth occurs, and afterwards a decrease with the increase of the number of hours of the day and temperature of the water. Since the seaweeds used in this study were collected during the summer, they probably were already in the stage of biomass decrease, therefore influencing the results obtained. Considering the results described above, this species needs further research on these and other cultivation parameters, namely photoperiod and nutrient media.

Outdoor cultivations

These trials served as preliminary studies to evaluate the performance of the seaweeds *O. pinnatifida* and *C. tomentosum* in a pilot scale system, during two different seasons (winter and summer). The importance of this study lingers in the fact of understand the response of the target species when exposed to variable weather conditions, such as temperature and light, and rain which may cause salinity fluctuations. Again, few studies on the outdoor tank cultivation of these two species are scarce. As above, a similar species, C. fragile was used for comparison since these two species can be found in the same locations and have some similar morphological characteristics (Trowbridge, 2001).

Outdoor cultivation – *Codium tomentosum*

During winter *C. tomentosum* RGR values did not vary much and were never above 0.02 g day -1 g-1 with only one exception on week 7 when biomass loss occurred. Within the parameters measured during the trial a possible cause for this loss of biomass may have been the sharp increase of light intensity on week 6 whose result was only observed on week 7, which may have reached photoinhibition levels.

During the summer trial, growth was observed during all weeks of the trial, with the higher RGR values reaching 0,05 g day^{-1} g^{-1}. Although temperature was higher than in winter, light availability in the water column was not higher due to the placement of the shadowing net to prevent excess light and photoinhibition. Intertidal seaweeds present a higher stress tolerance than those from the sublittoral, which have lower resistance to desiccation and freezing (Dring and Brown 1985, Davison et al., 1989). On the other hand, intertidal seaweeds are susceptible to photoinhibition because of the alternation between the submersion period of the day when the light levels can limit photosynthesis, and the immersion period when they became exposed to direct sunlight (Dring, 1987). These seaweeds have an optimum photosynthesis at lower light levels when the seaweeds are submersed (Fork and Oquist 1981). When exposed to air, there is a decline on the photosynthetic rates since the inorganic carbon supply is greatly restricted (Looban et al., 1985). Additionally, when exposed to high light intensities the photosynthetic system of the seaweeds can be damaged, which contribute to the decrease of both quantum efficiency and maximum photosynthetic rates. During these periods of stress, production of reactive oxygen species (ROS) by seaweeds increase significantly (Sampath-Wiley, et al., 2008). The principal defence mechanisms that seaweeds and higher plants develop to protect the tissues against ROS are superoxide dismutase (SDOs), peroxidases and catalases (Asada, 1999). Although the light intensity was similar to the winter trial, the number of hours that the seaweeds were exposed to light was superior. Thus, photosynthesis occurred during a longer period, resulting in a larger growth (Ogren, 1992).

Comparing the two experimental periods, medium growth rate was higher during the summer trial (0,024 ± 0,018 g day^{-1} g^{-1}) than during the winter period (0,005 ± 0,014 g day^{-1} $g^{-1)}$, as expected. Despite that, during the summer trial higher epiphyte growth was observed. These epiphytes compete with the target seaweed for nutrients and light intensity, so the conditions for *C. tomentosum* may not be the ideal (Neori et al., 2004). Since this seaweed usually presents epiphytes in its natural environment it is difficult to

control their development (González & Santelices, 2004). The fact that growth was observed during both the winter and the summer periods can be explained by the fact that this species is found in a broad geographical area, from the Mediterranean to the north Europe, which suggests that they have a high tolerance to temperature and light variability, which is characteristic of species occurring in temperate habitats (Eggert, 2012).

These results agree with a report by Silva & Abreu (2014) for tank cultivation of *C. tomentosum*, reporting higher growth rates during May, when higher temperatures and light intensity were observed.

Harrison & Hurd (2001) states that the quantity of nutrients is related with the growth of the seaweeds. The quantity of nutrients that the seaweeds are able to capture can be controlled by a diversity of factors, like the growth rate, productivity and epiphytes. Also, the quantity of nutrients available can influence the quantity of products produced by the seaweeds. Other factor that is quite important for the nutrient capture of the seaweeds is the water renewal (Gerald 1982; Wheeler, 1982; Hurd et al., 1996; Hurd, 2000). To follow the variability of nutrient availability in the tanks, the concentration of ammonia, nitrate, phosphate and nitrite were measured in the water entering the system and in the tanks. Some of the factors that influence the nutrient uptake by the seaweeds are nutritional history, type of tissue (the younger tissues present a higher nutrient uptake (Thomas et al., 1985), surface areas and even the production of hairs (Harrison et al., 1986). To obtain the best performance of the seaweeds produced in aquaculture it is important to determine the ideal amount of nutrients needed for a maximum growth and thus obtain higher economic gains (Harrison & Hurd, 2001). As such, in this trial nutrients were added to the water supply to assure that the nutrients would not be a limiting factor.

Harrison & Hurd, (2001) found that seaweeds with hairs, as is the case of *C. tomentosum*, have a higher nutrient uptake capacity. During the winter trial the values of ammonia, phosphate and nitrate did not vary during the trial. Since the growth rate also did not demonstrate a high variation during the trial, except for week 7, these values are in agreement with the growth rate. As for the nitrites they have a peak at week 5 of 0,6 ± 0,14 µmol/L that can be explain by a peak of this nutrient in the seawater inflow (Anexo A, fig. 1). As for week 7 there are no variations on the nutrients that could justify the loss of biomass. For the summer trial the values for phosphate, nitrate and nitrite were also stable. As for the ammonia values, a fluctuation was observed throughout the week, with the higher values occurring at weeks 6 and 8. This couldn't be explained by degradation

of the biomass of *C. tomentosum* since the growth rate remained positive during all the weeks of the trial. Comparing the two seasons no significant differences were observed, although the concentrations of nutrients usually are higher during winter than in summer (Harrison & Hurd, 2001).

The culture of macroalgae can suffer from contamination and overgrowth by epiphytes introduced with the material collected from the field and the surrounding environment (Lüning and Pang 2003). This contamination can cause several problems as reducing seaweed growth as well as the quality of biomass produced (Kerrison et al., 2016). To prevent the appearance of epiphytes in future studies, the manipulation of some of the culture conditions should be performed like test higher density, lower light or high pH (Lüning and Pang 2003). Also, chemical treatments have been utilised to remove these contaminants, but in this case the success of these treatments is dependent of careful control of the dosing to make sure that the quantities used are effective in the removal of the contamination but do not become harmful for the seaweeds in culture (Hoshaw and Rosowski 1973; Guillard 2007). There are no studies found in the literature that demonstrated the effect of chemical treatment on *C. tomentosum*.

Outdoor cultivation – *Osmundea pinnatifida*

Considering the winter trial *O. pinnatifida* showed a low performance, since no significant growth was observed during this period. As Guerra-García et al. (2011) described, most of the seaweed reached their growth peak in spring, therefore lower growth is expected during winter. Also, during the course of this trial the outdoor tanks were exposed to very adverse environmental conditions. Throughout the trial, successive storms occurred that decreased salinity and water temperature of the tanks, causing stress on the seaweeds. No apparent relationship was observed between growth and light intensity or temperature, but this may be due to the adverse conditions referred above that affected growth.

During the summer period two attempts were performed to conduct the trial but seaweed degradation occurred in both. In the first attempt biomass loss occurred after 4 weeks, and after a full restocking of the biomass for the second attempt, this biomass also degraded after 3 weeks. According to Prathep (2003), *O. pinnatifida* is more abundant in the spring and starts decreasing in the field with the increase of light hours and temperature, corroborating the results obtained in the present study. With this in mind, one reason for this loss of biomass may be the high values of temperature observed during this trial. Despite that, the acclimation week (for both attempts) and the week 1

and 2 for the first attempt shows a higher value of RGR than in the winter trial. This shows that the seaweed can tolerate higher light and temperature in a tank, which is expected since in the field the seaweed is exposed to these conditions a few hours a day. In the field, in high temperature periods at the end of the summer, seaweed lost its colour, as observed in fig. 25, suggesting that high temperatures lead to biomass degradation and/or lower growth, as described by Prathep et al. (2003).

Figure 25 Osmundea pinnatifida in the field in September (Borges, 2018)

These results suggest that the best period for growing this seaweed would be the end of winter and spring. In the summer period the seaweed starts losing consistency and degradation occurs. This degradation can be caused by excess exposure to direct solar light, thus shadowing of the tanks should be provided to prevent overexposure to excess light, causing photoinhibition. Also, temperature was likely another factor that influenced the results since in this study temperature reached over 30 ºC, and several studies in different regions demonstrate that for temperatures above 30ºC most seaweeds lose biomass (Eggert, 2012). In the field, this seaweed species is found on the intertidal where it is exposed to air and severe temperatures changes of 10-20ºC during the low tides, but this only occurs during a short period of the day and seaweed have mechanisms that protect them from such short-term thermal stress, although some growth limitation might occur (Davison and Pearson, 1996; Asada, 1999; Hellmuth and Hofmann, 2001). Thus, in tank cultivation, temperature is a key factor to take into account, especially when ambient temperature increases, and water renovation is insufficient to avoid large increases in temperature in the tanks. Besides that, seaweeds growth can also be controlled by the variation of light regime and the availability of nutrients (Kain, 1989; Wiencke et al., 2009).

Since seaweeds can be used as biofilters, further studies are needed on the effects of nutrients on the growth of these seaweed species. Most of the bibliography available concern studies of nutrient uptake by seaweeds such as *Gracilaria* spp. and *Ulva* spp., which are fast growing species. These species are already described as having high nutrient uptake capacity on an industrial scale (Martinez-Aragon et al., 2002). Pedersen & Borum, (1997) described that fast-growing species have higher demands of N compared with the slow growing seaweeds.

As for the winter period the values of ammonia, nitrate and nitrite did not demonstrate a variation during the trial, which is in agreement with the growth rate results, also without a high variation during the trial. As for the phosphates, the peak occurring at week 8, coincided with a peak in the seawater inflow. For the summer period there are no significant differences between the weeks on the values of ammonia, phosphate, nitrate and nitrite. Between the two seasons the only nutrient that showed significant differences was nitrate that was higher in the summer trial. As for the ammonia, peaks were observed at week 6 for the winter trial and weeks 2 and 6 in the summer trial. Since the seaweeds lost biomass in the weeks after those peaks these values could be justified by the degradation of the biomass of the seaweeds (Hargreaves et al., 2004).

Although epiphytes were not so abundant for *O. pinnatifida*, they were still present. As for the removal of epiphytes with the application of chemical treatments, Kerrison et al. (2016) described that to remove Ectocarpus siliculosus of *O. pinnatifida*, a 25% methanol for 1-5 minutes treatment has a positive effect. To remove *Ulva intestinalis* some treatments could be applied like for example a 10 minute exposure to 0,1% Sodium hypochlorite (NaClO), a 1 minute exposure to 1 % sodium hypochlorite (NaClO) or a 10 minute exposure to 0,5 % potassium iodide (KI). (Kerrison et al., 2016)

Biochemical analysis

During recent years the interest in compounds with antioxidant properties derived from natural sources, like seaweeds, has increased. This is because these compounds are beneficial for human health (Guedes et al., 2013). The biological activities present in the seaweeds are increasingly considered as interesting due to it's potential to become a source of pharmaceutical agents (Celikle et al., 2009) and is also interesting as an incentive for the conservation of biodiversity (Paiva et al., 2014).

Seaweeds living in intertidal areas are intermittently exposed to immersion and emersion due to tidal fluctuations that origins a variety of potentially stressful environmental

conditions such as desiccation, osmotic stress, unstable temperature, intense light and nutrient limitation that create free radicals and other oxidising agents to protect them from not only the environmental conditions but also from UV damage (Burritt et al., 2002; Zubia et al., 2007). The phenolic compounds are antioxidant molecules and antioxidant activity of seaweed phenols depends on their structure and especially on the degrees of polymerisation of phloroglucinol (Nakamura et al., 1996). Also, Mabeau & Fleurence (1993) described that the phenolic contents of green and red seaweeds are lower in comparison with brown seaweeds.

Certain green and reds seaweeds have relatively high protein contents, ranging from 9-26 % (w/w) dry weight for green seaweeds (Fleurence, 2016). It is expected that the levels of protein are determined by seasonality, with higher contents observed during winter (Fleurence, 2016; Khairy & El-Shafay, 2013). The phenol and protein values and other chemical constituents vary depending on factors like environmental conditions, harvesting, geographical distribution and habitat (Rodríguez-Bernaldo et al., 2010; Ibañez, et al., 2012).

Several studies show a correlation between elevated metabolism of antioxidants and increased tolerance to environmental stresses for green (Malanga and Puntarulo, 1995; Collen and Davison, 1999a, b; Choo et al., 2005; Dring, 2006), and red seaweeds (Collen and Davison,1999c, Burritt et al., 2002).

Biochemical analyses - *Codium tomentosum*

In the case of *C. tomentosum* the cultivation process did not affect significantly the antioxidant activity and protein content when compared to the wild biomass, but the phenol content increases with the cultivation process. No significant differences were obtained between seasons for both $ABTS^{\bullet+}$ and $DPPH^{\bullet}$, which suggests that the differences in the environmental factors did not affect the production of the antioxidant compounds. The value obtained with this study for the protein content was 8,2 ± 0,63% of DW. Therefore, this value is concordant with the study of Wijesekara and Kim (2015) that states that the values of protein content for green and red algae were between 10-47% of DW, but not concordant with Rodrigues et al. (2015) that state that the protein content of *C. tomentosum* is 18,8 % DW. Nevertheless, phenols were generally higher during summer, which was not translated in a clear increase in antioxidant activity. The phenol, protein and other chemical constituents values depending on factors like

environmental conditions, harvesting, geographical distribution, water temperature, salinity and habitat (Rodríguez-Bernaldo et al., 2010; Ibañez, et al., 2012).

Biochemical analyses – *Osmundea pinnatifida*

O. pinnatifida cultivation did not affect the antioxidant activity, phenol and protein content. The antioxidant capacity analysed by the $ABTS^{\bullet+}$ method was higher in the summer, suggesting that the differences in the environmental factors affected the production of the antioxidant compounds. Nevertheless, no significant differences were obtained between seasons for $DPPH^{\bullet}$, which may indicate that the compounds that contribute to this antioxidant effect were not influenced by season. In the summer period, although the $ABTS^{\bullet+}$ results were higher for the biomass at the end of the trial comparing to the wild biomass, the phenol content showed an opposite trend, indicating that phenols were not responsible for the observed increase in antioxidant capacity. (Celikler et al., 2009). The fact that the values of $DPPH^{\bullet}$ were low is concordant with Barreto et al. (2012), that also show that the extracts of *O. pinnatifida* are weak $DPPH^{\bullet}$ radical scavengers. Wijesekara and Kim (2015) described that the content of protein for green and red algae is between 10-47 % of DW. The value obtained with this study for the protein content was 8,2 ± 0,63% of DW, therefore this value is concordant with the study of Wijesekara and Kim (2015), but not with Paiva et al. (2014) that states that the protein content of *O. pinnatifida* is 20,79 ± 0,12% of DW.

Conclusion

Information acquired with this work is an important contribution for the establishment of aquaculture methods for *O. pinnatifida* and *C. tomentosum* or to support further studies aiming the development of those methods. Both the species in this study are considered "new species" for aquaculture, therefore all the work done throughout this project can be used as future reference.

For *C. tomentosum* the best temperature was 16ºC, the light intensity that showed the higher growth was 200 µmol m^{-2} s^{-1} and no significant differences were found between the 3 densities tested. As for the outdoor trial for both winter and summer the growth rate was positive. As for the biochemical analysis the values of antioxidant activities were similar between the seasons. The value of protein was lower than the ones obtained in other published studies. As for the phenols, for both trials the values of the cultivated seaweeds were higher than in the wild biomass.

O. pinnatifida was more of a challenge during this project, being the specie with less encouraging results. Despite that, it was possible to gather important information for the future cultivation of this seaweed concerning the effects of temperature and light intensity on its growth. For the trials performed in laboratory the best temperature was 16ºC, the light intensity that presents the lower biomass loss was 200 µmol m^{-2} s^{-1} and there were no significant differences between the 3 densities tested. For the outdoor cultivation trial was possible to observe a distinct difference between the periods studied and evaluate the principal impediments on the cultivation of this seaweed and develop strategies to overcome these difficulties. As for the biochemical analysis the values of antioxidant activities were similar between the seasons. The value of protein is lower than the one obtained in other studies. The phenols values were higher in the summer period. Also, for the phenols the cultivation process was beneficial during the winter period but on the summer period these values decreased at the end of the trial.

For the future, it is important to try to improve some of the techniques that were used in these trials. As for the laboratorial trials should be interesting to test a wider range of temperatures, light intensities and densities and also test new parameter such as pH, photoperiod, concentrations of nutrient medium and salinity

For *C. tomentosum* a good option to test could be the use of rectangular and long tanks to try to provide a better water circulation and the possibility of use a higher seaweed

density. As for *O. pinnatifida* the use of darker tanks and the increase of water renovation is recommended to prevent the water temperature from rising to 30ºC. For both species there should also be performed trials with higher nutrient concentrations than the ones used in this study. It could also be interesting to test how these seaweeds perform on an IMTA system since these systems can be more economical sustainable than the monoculture ones. Finally, it's also important to continue this study in the remaining seasons of the year to obtain a complete cycle of how the different seasons affect the seaweeds growth.

In the future was also important to continue the study of the nutrient intake for both the seaweeds and to overcome the high SD values that can be observed on results by increasing the number of replicates for both trials.

It is also important the continuation of the studies of bioactive compounds for both the seaweeds with the evaluation of more biochemical activities because as showed on this and previous studies they have a big potential. It should also be interesting to evaluate the biochemical characteristics of the biomass cultivated during the remaining seasons of the year to obtain a wider knowledge of how seasonality affects these components.

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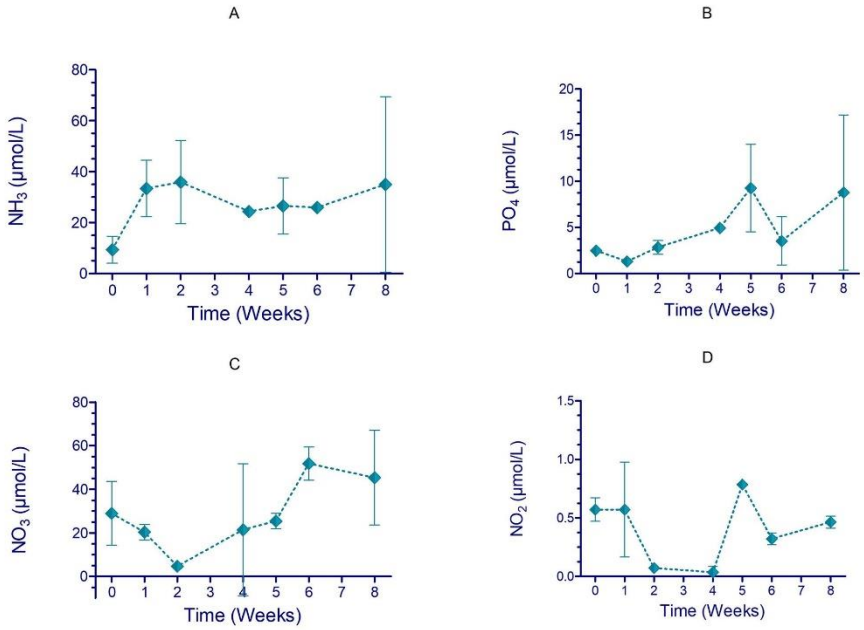
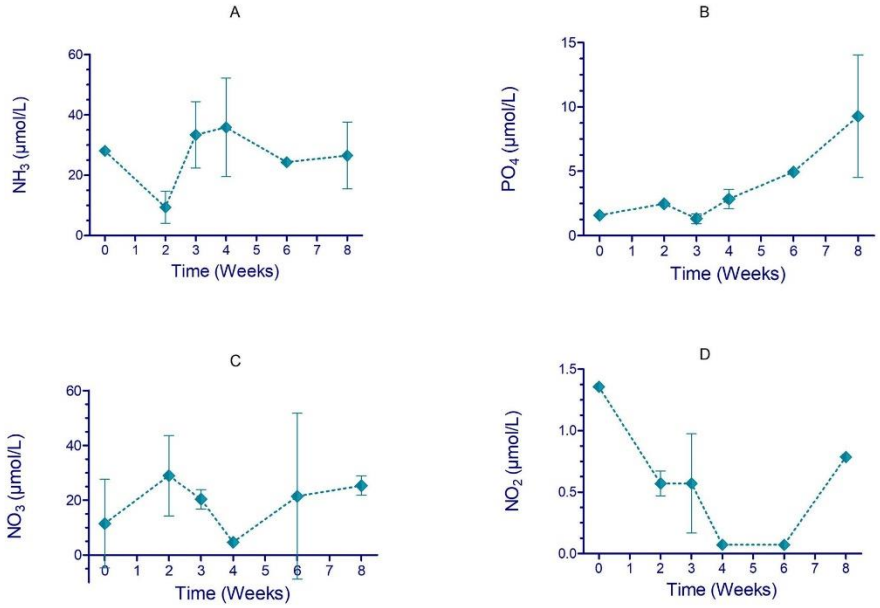
Annex

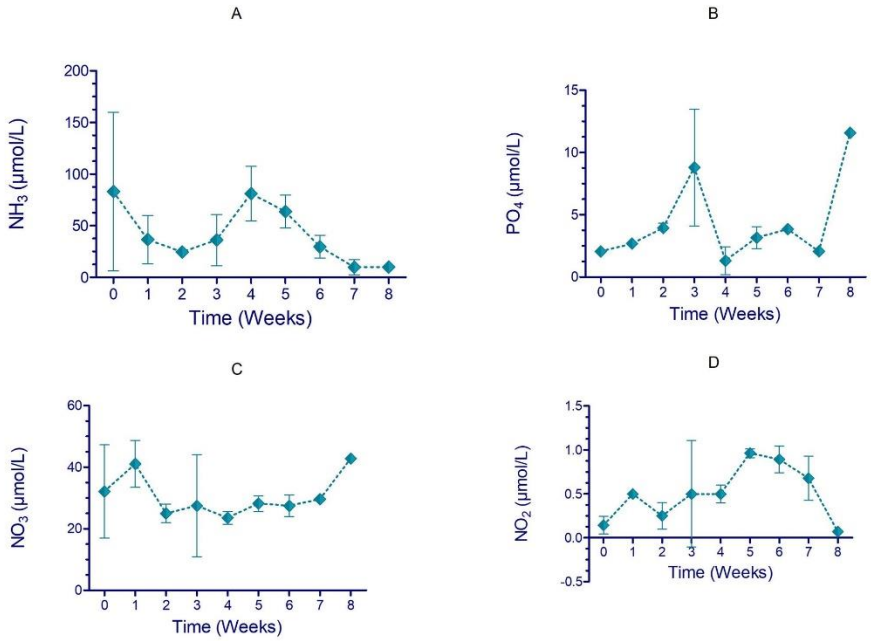
A



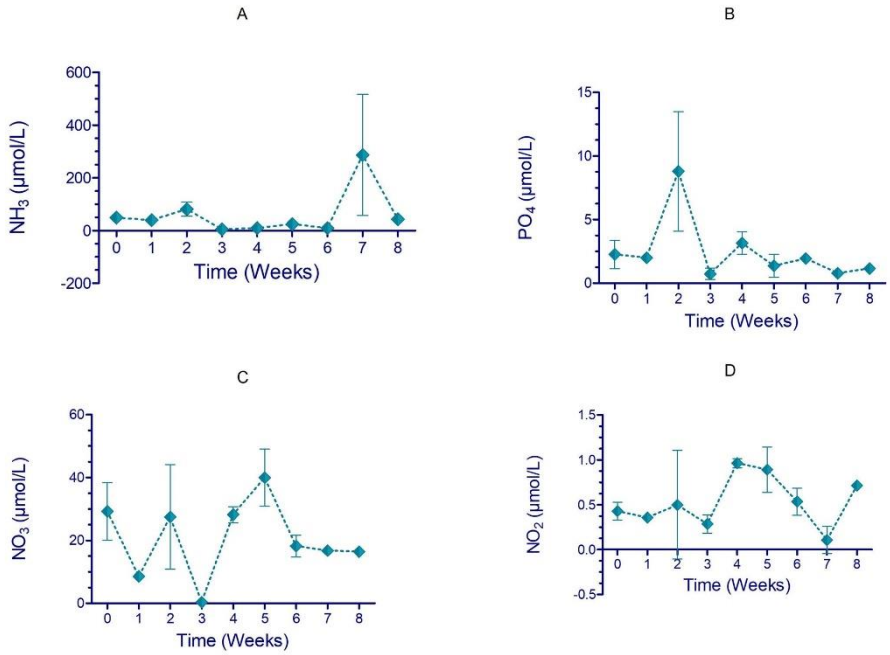
Supplementary figure 1. Nutrient concentration of ammonia (A), phosphate (B), nitrate (C) and nitrite (D) in the water supply of Codium tomentosum throughout the winter trial. Data are expressed as mean ± SD (n=2).



Supplementary figure 2 Nutrient concentration of ammonia (A), phosphate (B), nitrate (C) and nitrite (D) in the water supply of Codium tomentosum tanks throughout the summer trial. Data are expressed as mean ± SD (n=2).



Supplementary figure 3 Nutrient concentration of ammonia (A), phosphate (B), nitrate (C) and nitrite (D) in the water supply of Osmundea pinnatifida tanks throughout the winter trial. Data are expressed as mean ± SD (n=2).



Supplementary figure 4 Nutrient concentration of ammonia (A), phosphate (B), nitrate (C) and nitrite (D) in the water supply of Osmundea pinnatifida tanks throughout the summer trial. Data are expressed as mean ± SD (n=2)