Development of cultivation methods of *Ulva intestinalis* and *Laminaria ochroleuca*, native seaweed species with commercial value

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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Resumo

Na Europa, o interesse por algas tem aumentado continuamente durante a última década. O surgimento de novos usos tais como alimentos, suplementos alimentares, nutracêuticos e biocombustível, tem despertado a procura por biomassa pelo que, o desenvolvimento de métodos de cultivo sustentáveis tornas-se necessário para responder à procura existente e minimizar os riscos de sobreexploração.

Neste projeto, as espécies de algas nativas *Ulva intestinalis* e *Laminaria ochroleuca* foram estudadas, primeiramente para avaliar seu crescimento e composição bioquímica quando cultivadas num sistema de tanques em duas estações. No inverno, *U. intestinalis* demonstrou um comportamento oportunista, tendendo a esporular quando a intensidade da luz era baixa e a crescer quando a exposição à luz aumentava. No entanto, na primavera, as mesmas temperaturas que proporcionaram melhores resultados no inverno, parecem ter interagido com a luz e causado a esporulação. O teor de proteína diminuiu ao longo do tempo, o conteúdo fenólico foi maior no inverno, a atividade antioxidante via ABTS+• foi semelhante entre as duas estações e via DPPH• foi maior na primavera*. L. ochroleuca*, no inverno, consumiu nitrato eficientemente e os juvenis demonstraram sensibilidade a um aumento da intensidade luminosa. Na primavera, um aumento de fosfato, amónia e nitrato no fornecimento de água resultou num aumento significativo na biomassa mas, quando as temperaturas atingiram 30 ° C, os indivíduos sofreram *stress* térmico e degradadaram-se severamente. O teor de proteína foi significativamente menor no inverno, após o cultivo do tanque, enquanto que os fenóis foram maiores nos indivíduos obtidos do campo em ambas as estações. A atividade via ABTS + • diminuiu ao longo do tempo e via DPPH • foi significativamente maior na biomassa colhida no campo no inverno.

Além disso, foram realizados ensaios laboratoriais para testar, em primeiro lugar, o efeito de três temperaturas diferentes (12, 16 e 20 ºC). Em ambas as espécies, o crescimento não foi significativamente diferente entre as temperaturas testadas*.* No entanto, *Ulva intestinalis* exibiu maior esporulação mais a 20 ºC. O segundo ensaio testou o efeito de três densidades (5, 10 e 15 g / L) interagindo com duas intensidades luminosas (100 e 200 µmol m^{-2} s^{-1}). Para *U. intestinalis,* o crescimento foi significativamente maior a 200 µmol m^{-2} s^{-1} durante as primeiras três semanas, no entanto, também ocorreu maior esporulação. Nas últimas duas semanas, o crescimento em ambas as intensidades não foi significativamente diferente. Para *L. ochroleuca,* as densidades e intensidades de luz testadas não afetaram diferentemente o crescimento

da biomassa.

Os resultados obtidos demonstram que ambas as espécies possuem potencial para incorporação em aquacultura, no entanto, os sistemas precisam ser configurados de forma a superar os principais desafios para o cultivo dessas espécies: a reprodução assexuada no caso de *U. intestinalis* e a fotoinibição no caso de *L. ochroleuca*.

Palavras-chave: Aquacultura de algas, *Ulva intestinalis, Laminaria ochroleuca*, sazonalidade, bioatividade, conteúdo proteico, temperatura, intensidade luminosa, densidade.

Abstract

In Europe, interest in seaweed has been increasing steadily during the past decade. The emergence of new uses like feed, food supplements, nutraceuticals and biofuel has been sparking the demand for biomass and thus, the development of sustainable cultivation methods is necessary to aid this demand and counteract the risks of overexploitation.

In this project, native seaweed species *Ulva intestinalis* and *Laminaria ochroleuca* were studied, firstly to assess their growth and biochemical composition when cultivated in a tank system in two seasons. In winter, *U. intestinalis* demonstrated an opportunistic behaviour, tending to sporulate when light intensity was low and to grow when light exposure increased. However, in spring, the same temperatures that provided better results in winter, seemed to have interacted with light and caused sporulation. Protein content decreased overtime, phenolic content was higher in winter, antioxidant activity via ABTS+• was similar in both seasons and DPPH• was higher in spring. *L. ochroleuca,* in winter, consumed nitrate efficiently and juveniles demonstrated sensitivity to increases in light intensity. In spring, an increase of phosphate, ammonia and nitrate in the water supply resulted in a significant increase in biomass but, when temperatures reached 30 ° C, individuals suffered thermal stress and severely degraded. Protein content was significantly lower in winter after tank cultivation, while phenols were higher in individuals obtained from the wild for both seasons. The activity via ABTS+• decreased over time and via DPPH• was significantly higher in the biomass harvested from the field in winter.

Furthermore, laboratory trials were conducted to test, firstly, the effect of three different temperatures (12, 16 and 20 ºC). For both species, growth was not significantly different between the tested temperatures. However, *Ulva intestinalis* exhibited greater sporulation at 20 ºC. The second trial tested the effect of three densities (5, 10 and 15 g/L) at two different photon fluence rates (100 and 200 µmol m^{-2} s^{-1}). For *U. intestinalis*, growth was significantly higher at 200 µmol m^{-2} s^{-1} during the first three weeks of the trial, although it caused higher sporulation. In the last two weeks, growth at both photon fluency rates did not show significant differences. For *L. ochroleuca*, the tested densities and light intensities did not affect differently the growth of the biomass.

The obtained results demonstrate that both species have potential for aquaculture incorporation, however, systems need to be setup as to overcome the main

challenges for the cultivation of these species: asexual reproduction in the case of *U. intestinalis* and photoinhibition in the case of *L. ochroleuca*.

Keywords: Seaweed aquaculture, *Ulva intestinalis*, *Laminaria ochroleuca*, seasonality, bioactivity, protein content, temperature, light intensity, density.

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Introduction

Marine macroalgae, commonly known as seaweeds, also known as sea vegetables, are plant-like organisms that grow usually attached to rock or other hard substrata in coastal areas (Kolb *et al.,* 2004; Kılınç et al., 2013). These organisms produce very versatile products with high nutritional interest, that have been explored by societies throughout the centuries for a wide range of applications (Mamatha *et al.*, 2007; Khalil *et al*., 2017). Its use as food has been traced back to the 4th century in Japan and the 6th century in China (McHugh, 2003). Despite being consumed as food in Asian countries since ancient times, in Europe, the commercial exploitation of seaweeds started occurring after World Wars I and II, as a response to lack of resources (Kılınç et al., 2013; Anis *et al.*, 2017). This increased demand for protein drove the exploitation of alternative sources such as seaweeds (Kılınç *et al.,* 2013). Nowadays, China, Japan and the Republic of Korea are the largest consumers of seaweed as food (McHugh, 2003). Nonetheless, as citizens from these countries migrated to other countries, taking their cultural heritage with them, the demand for seaweed for food outside of Asia increased as well (FAO, 2004). In addition, evidence that consumption of algal food products may have health and nutritional benefits has been increasing, raising their popularity (Wells *et al*., 2016). Furthermore, with the growing trend for consumers to seek a healthier life style and include organically grown foods and unprocessed foods from clean environments in their diet, the popularity of seaweed is also expected to grow (Ibañez *et al*., 2012).

Firstly, fertilizing the fields with seaweeds was once a tradition and this practice has been regaining relevance in recent years because of its potential for sustainable agriculture, as a means to avoid excessive chemical fertilizer applications and increase mineral absorption (Sousa-Pinto, 1998; Cabral, 2005; Dhargalkar & Pereira, 2005). Moreover, seaweed extracts can also function as plant growth supplements and improve resistance to pests and diseases (Verkleij, 1992). Besides, seaweeds have also been reported to be a suitable ingredient for cattle feed. In fact, in the Orkney Island, sheep are fed almost exclusively on seaweed (Hansen *et al.,* 2003). Furthermore, seaweed addition to feed has been reported to improve animals' mineral status and decrease methane emissions (Rey-Crespo *et al.* 2014).

In addition, seaweeds have been historically explored for their medicinal properties (Khalil *et al*., 2017). Since the 1st century BC, Romans treated their joint pain by applying poultice extracted from *Fucus vesiculosus* (Anis & Hasan, 2017). Later, during the Tang Dynasty (6th to 9th centuries), kelp were used to treat iodine deficiency,

goiter and thyroid enlargement in inland China (Ping *et al.*, 1999). More recently, numerous studies have demonstrated the importance of antioxidants in the prevention and growth control of certain tumours, as well as in cardiovascular and degenerative diseases (Abe *et al.* 2007; Wang *et al.*, 2010). In thalassotherapy, seaweed pastes are used in treatments, which are said to provide relief for rheumatism and osteoporosis (FAO, 2004). Seaweed extracts also have a long history of usage as additives, emulsifying, gelling, and stabilizing agents (Khalil *et al.*, 2017), due to their rich content in sulphated polysaccharides (Pereira *et al.*, 2013). Sulphated polysaccharides are known to have several biological activities, such as anticoagulant, antiviral, anti-tumour, anti-inflammatory and immunostimulating activities (Pereira, 2011). Additionally, several seaweeds possess phycocolloids, used in the food industry as natural additives, to improve quality attributes and shelf-life (Saha & Bhattacharya, 2010).

In the cosmetic industry, extracts from seaweeds, such as alginate and carrageenan, are often used to improve the skin moisture retention properties of a product (FAO, 2004). Hyaluronic acid is a polysaccharide that can be found in plants and animals to aid hydration but, due to its limited availability, it is quite expensive in the market (Bakoš *et al.,* 1999). According to Wang and collaborators, polysaccharides extracted from seaweed exhibited better performance than hyaluronic acid, while being eco-friendly and a more economically viable option (Wang *et al.,* 2013). In addition, agar is a substance extracted from seaweed that is used as a thickening agent to control the viscosity and emollience of cosmetic products (Wang *et al.*, 2015). Moreover, the application of algal biomass for biofuel production, although still being developed, aims to produce viable “bioethanol”, which has the potential to produce low-carbon energy (Aizawa, 2007; Alvarado-morales *et al*., 2013; Jung *et al.,* 2017).

Considering all the applications mentioned, it is possible to predict that the natural resources of seaweed biomass will not be enough to sustain the World’s increasing need (McHugh, 2003). As the high demand started to threaten natural stocks, seaweed farming had to expand, undergoing a drastic development over the past 70 years, mainly in Asia, and recently in Europe and America (McHugh, 2003; Kim *et al.*, 2017). According to FAO (2018), in 2016, aquatic plants aquaculture production was estimated as 30.1 million tons, which provided a total value of US$11.7 billion. This production increase was led by Asian countries, mainly China and Indonesia. It is thus clear that seaweed aquaculture in Europe is, comparatively, not as developed and well embraced, with many challenges to overcome regarding both production techniques and social acceptability (Kim et al., 2017). In fact, in Northwestern Europe, developments in seaweed aquaculture are mainly related to research projects and the establishment of a small number of commercial farms, making seaweed aquaculture a still underdeveloped but

auspicious project for the future. Despite that, the development of seaweed farming in Europe is highly dependent of appropriate support from national and European policies. In recent years, projects have been seeking a deeper investigation of new species, cultivation methods and new applications (Werner *et al.,* 2004). Plus, increased emphasis has been placed on the evaluation of the commercial viability of seaweed aquaculture, having some small companies been emerging meanwhile (for example ALGAplus in Portugal). Additionally, most of the species produced in aquaculture in Asia are not native in Europe, which makes them interesting also for commercialization also in Asian countries. This raises the need to further investigate cost-efficient forms of producing high quality native seaweeds to provide biomass for products for the European market. This can be achieved by studying the impacts of seasonal variation on their nutritional value and biomass production, and by valuing the variety of available species from different geographical areas (Wells *et al*., 2016).

In this study, *Ulva intestinalis and Laminaria ochroleuca* were cultivated in a tank system in two distinct seasons, to test the influence of seasonal conditions on their growth and biochemical composition. Additionally, optimization trials were conducted in laboratory to test the best temperature, density and light conditions for the development of both species.

Ulva intestinalis

Ulva intestinalis (fig. 1), previously known as *Enteromorpha intestinalis*, belongs to the Phylum Chlorophyta, order Ulvales and to the algal genus *Ulva*, one of the most common genus of seaweeds in marine and brackish environments in the world (Blomster *et al.,* 2000; Ruangchuay *et al.,* 2012).

Fig. 1 - *Ulva intestinalis* in an intertidal pool (uniprot.org)

U. intestinalis typically grows as a tube of 1-3 cm width and may reach 60cm in length, being composed of irregularly arranged cells (Neto *et al.*, 2005; Ruangchuay *et al.,* 2007). The blade is smooth at its early stages of development (becoming gradually wrinkled as it grows) and the coloration also changes with growth, from an initial dark green to light green or yellow-green. Branching occurs near the holdfast, which is small and narrow (1 mm) (Prud'homme *et al.*, 2001; Ruangchuay *et al.,* 2007).

This species is characterized for being an opportunistic macroalgae in estuaries and coastal areas, forming dense mat-like structures (Fong *et al.,* 1998). As such, these macroalgae influence the nutrient flow in the ecosystem and provide high quality food for microbial and macrofaunal communities (Martins *et al.*, 1999).

Despite being a marine species, high concentrations of nitrogen have been reported to compensate the lack of salinity, allowing it to grow on brackish water (Kamer *et al.,* 2001). Its high adaptability and easy propagation are suspected to be due to its mode of reproduction, a diplohaplontic sexual cycle with biphasic isomorphic life stages (Bliding, 1963). This species alternates between two phases, a haploid (gametophyte)

and a diploid (sporophyte). Both male and female haploid gametophytes originate haploid biflagellate gametes, which, after fertilization, originate zygotes that develop into diploid sporophytes. Meiosis occurs in the sporophyte, in parallel with the formation of haploid quadriflagelated spores, that result in male and female haploid individuals (Thornber, 2006; Coelho *et al.,* 2007). Besides sexual reproduction, species from the genus *Ulva* have been reported to also reproduce asexually, originating clones (Bliding, 1963). Diploid individuals that are not able to undergo meiosis during spore production, create several viable diploid spores. Moreover, unfertilized gametes produced by haploid sporophytes have been described to germinate and grow into haploid thalli (Bliding, 1963; Larsen, 1981; Düwel, 2001). Low density and/or stressful environments may cause individuals to undergo asexual reproduction (Alström-Rapaport *et al.*, 2010).

Many species of Ulva are of particular interest for cultivation in several Asian countries, such as Japan, Korea, India and Indonesia (Prud'homme *et al.,* 2001). In Japan, *Ulva pertursa* and *Ulva prolifera* are economically important. In fact, *U. prolifera* serves as raw material for the production of highly nutritional food, due to its high mineral and vitamin content (Watanabe *et al.,* 1999). Additionally, *Ulva compressa* is used as an ingredient in the preparation of *pakoda*, a snack food consumed in India (Aguilera-Morales *et al.,* 2005; Mamatha *et al.*, 2007).

In Europe, there are records of *Ulva spp.* being used in poultry nutrition, notably to improve the egg yolk or meat colour (Indergaard & Minsaas, 1991). Furthermore, *U. intestinalis* has been used in Azores for the confection of "tortas" and as an ingredient for a gourmet version of the Portuguese dish *caldo verde*, demonstrating its potential as a novel food (Isabel Sousa Pinto, personal communication; Neto *et al*., 2014).

Some studies have demonstrated antioxidant properties in *Ulva lactuca*, comparable with those in commercial antioxidants and were found safe for topical use since they do not cause skin irritation (Morganti *et al.,* 2002; Balboa et al., 2014). Besides, reports stated the antioxidant capacity of *U. intestinalis* also makes it an interesting species for the cosmetic industry (Akköz *et al.,* 2011). This species demonstrates great potential for the European market since it has several positive traits inherent to the genus *Ulva*, allowing its application for a wide variety of uses, including cosmetics, medical use and food source, being even able to constitute a novel food for the crescent blue market. Additionally, its great capability of propagation, due to an opportunistic behaviour, constitutes a compelling factor that highlights the interest in this species.

Laminaria ochroleuca

Laminaria ochroleuca (fig. 2) is a Lusitanian species belonging to the order Laminariales. Species in this order, commonly referred to as kelp, are habitat structuring species (Angel & Ojeda, 2001), providing support to a great diversity of marine organisms by functioning as shelter and nursery (Bodkin, 1988; Graham, 2004; Steneck et al., 2002). Kelp forests form a three-dimensional habitat, serving as a key nutrient input in nearshore areas, by fixating high and steady carbon and nitrogen levels year-round (Duggins *et al.*1989; Fredriksen, 2003), having high primary productivity (Erlandson *et al*., 2007).

Fig. 2 - *Laminaria ochroleuca* in the northern Portuguese coast during a spring low tide (Pereira, 2014)

The geographical distribution of *L. ochroleuca* is comprised between the British Isles and the Moroccan coast (Birkett *et al.* 1998), being the main perennial forest forming species in the temperate Northeastern Atlantic, with optimum growth at 10-15ºC (Pereira *et al.*, 2011; Barbosa *et al*., 2017).

L. ochroleuca has a typical kelp life cycle, with a microscopic haploid gametophyte phase and a macroscopic diploid sporophyte phase (Dayton, 1985). The sporophyte is composed of three different parts: holdfast, stipe and blade (Nakahara & Nakamura, 1973). In the typical reproductive season (between summer and fall), blade growth either ceases or decreases drastically and sporangia are produced in the apical part of the blade. When it matures, the tissue disaggregates releasing the spores (Mann, 1973; Lüning, 1980; Buchholz & Lüning, 1999). Since studies on *L. ochroleuca* are sparse, many conclusions about its ecology have been made due to its similarity with *L. digitata* and *L. hyperborea* (Birkett *et al.*, 1998).

Kelp exploration can be traced as far back as the old empires (Chinese and Greek), in times of famine and war, mainly as a food source, becoming remarkably important in the 7th Century in Japanese cuisine (Newton, 1951; Ping *et al.,* 1999). Also, Scottish people used it to fabricate soap and gunpowder (Gittins, 1966). In the coast of Western Europe, *Laminaria* species were considered an important asset as fertilizers, although their usage has been decreasing with time (Braud, 1974). In Northern Portugal, a traditional way to exploit *Laminaria* sp. is through the collection of "sargaço" – a combination of marine algae (*Saccorhiza*, *Laminaria*, *Fucus*, *Codium*, *Palmaria*, *Gelidium* and *Chondrus*) that detach from rocks. These seaweeds are laid on the beach to dry and later applied as fertilizers (Cabral, 2005; Araújo et al., 2006).

Some kelps are suitable for extraction of alginate, a naturally occurring polysaccharide of high commercial value (DeLucca *et al.,* 1990). Alginate extracts are used both as food additives (E-400 to E-405) and as appetite suppressants (Gallardo *et al.*, 1990).

Laminaria sp. has been reported to possess anticancer, antioxidative, antiviral, antitherogenic, immunostimulatory and anti-inflammatory compounds (Reddy *et al.*, 1984; Reddy *et al.,* 1985; Yamamoto & Maruyama, 1985; Jeong *et al*., 2006; Oomizu *et al.,* 2006; Makarenkova *et al.*, 2010; Matanjun & Muhammad, 2010; Shiratori *et al.,* 2005;). There have also been described abilities of *Laminaria* sp. in the prevention of lifestyle-related diseases, such as obesity and diabetes (Shirosaki & Koyama, 2011). Furthermore, crude extracts from algae of this genus are rich in phenolic compounds, which have antimicrobial efficacy, as well as antioxidant properties (Kadam *et al.,* 2015).

Nowadays, an interesting market product for the European market is *kombu*, which is dried seaweed of the genus *Saccharina* and *Laminaria,* considered a delicacy in Asia (Davidson, 1999). In fact, in Japan, kombu is used in the preparation of several dishes (including fish, meat, soups and rice) and also used powdered to be added to sauces or to make infusions (Madhusudan *et al.,*2011). Additionally, this product has great protein and vitamin content, being also a good source of most minerals and iodine (Kolb *et al.,* 2004).

As for medical application, there have been successful reports of the use of *Laminaria* sp. tents as cervical dilators in treating dysmenorrhea and making the uterine cavity available for probing and examination (Hale & Pion, 1972).

Additionally, *Laminaria* sp. extracts are used in the cosmetic and pharmaceutical industries, due to their analgesic, anti-inflammatory and anti-fungal activity, for the treatment of ulcers, burns and skin grafts and as impression material for prosthetics (Attwood, 1989; Vázquez-Freire *et al.,* 1994; Hellio *et al.*, 2000; Paul & Sharma, 2004; Bonneville *et al*., 2007; Cheng *et al.,* 2010).

Europe's demand for alginates amounted to an estimated 10 thousand tons annually in 2010, steadily increasing throughout the years. European consumers have been buying more processed food (which often contain alginates as thickening agents) and importation of these extracts from other continents are small, as France and Norway are key suppliers (CBI – Ministry of Foreign Affairs, 2017). Despite that, European importers are open to new sources of brown seaweeds, since diversification of suppliers reduces the market's risk. Based on this, we can assume that *L. ochroleuca*, an abundant kelp species in Portugal, has the potential of playing an important role in the alginate supply industry and also as a new product, "Atlantic Kombu". Given the similarity between *L. ochroleuca* and other species in the same genus, and the shortage of existing information regarding this species, it is of great advantage to both the Portuguese economy and European markets, to further investigate this species, in order to diversify market products and increase alginate production.

Furthermore, for the production of biofuel, kelps are a good candidate as a biomass source, given their productivity, quick growth and easiness of production, not requiring land for that purpose (Aresta *et al.*, 2005; Adams *et al.*, 2009; Vivekanand *et al.*, 2012).

Objectives

The popularization of seaweed has created the need to explore sustainable ways of production, as to avoid the overexploitation of natural stocks. Since European seaweed aquaculture still has many obstacles to overcome, developing culturing methods for Portuguese seaweeds is of the utmost importance for Portugal to accompany Europe in the blue economy age.

This work focused on developing tank cultivation techniques for two native species from Northern Portugal: *Laminaria ochroleuca* and *Ulva intestinalis*. The aim was to assess the effects of seasonality, both in growth and biochemical composition in outdoor tests. These assessments provided data regarding the productivity of these two species. These trials were complemented with laboratory experiments to evaluate the most adequate conditions for seaweed growth, which will allow the further development of cultivation methodologies in tanks, increasing their efficiency, production and decreasing risk.

These results are expected to deepen the overall knowledge on seaweed aquaculture, aiding its development, and supporting its commercial application in Portugal, particularly in the Northern region.

Material and methods

Outdoor Cultivation

The purpose of this experiment was to assess seasonal productivity and biochemical composition variation of the native seaweed species *Ulva intestinalis* and *Laminaria ochroleuca*.

Wild specimens of *U. intestinalis* were collected from the rocky shore in Foz do Douro (41º09'49.23"N 8º41'14.35"O) and juveniles of *L. ochroleuca* were collected in rocky shores in northern PT, in Vila Chã (41º17'44.17"N, 8º44'12.79"W) and Viana do Castelo (41º41'53.79"N 8º51'19.57"O). Collection of *L. ochroleuca* was made in two different areas since juveniles are scarce in comparison to adult individuals, in order to avoid a large impact in the ecosystem.

A total of 2 trials per species were carried out in two distinct seasons: winter (slow-growth season) and spring (fast-growth season) (table 1). The slow-growth season was expected to have lower light intensity and lower temperatures. Rain was also expected to occur more frequently. On the contrary, the fast-growth season, with a longer photoperiod, was expected to have higher light intensity, higher temperatures and less occurrence of rain, meeting better conditions for development (Alström-Rapaport *et al.,* 2010; Pereira *et al.*, 2019).

Table 1 – Procedure of biomass collection and distribution for the outdoor trials

Ulva intestinalis	
Winter (slow-growth season)	**Spring (fast-growth season)**
Biomass collection: 02/02/2018	Biomass collection: 14/05/2018
700 g collected (10 g/L per tank)	700 g collected (10 g/L per tank)
Extra 300 g stocked for analysis	Extra 300 g stocked for analysis
Trial duration: February – April	Trial duration: May – June
Laminaria ochroleuca	
Winter (slow-growth season)	**Spring (fast-growth season)**
Biomass collection: 02/02/2018	Biomass collection: 03/05/2018
218 individuals collected (170 g = 70 individuals per tank)	89 individuals collected (170 g = 30 individuals per tank)
Extra 300 g stocked for analysis	Extra 300 g stocked for analysis
Trial duration: February – April	Trial duration: May – June

The collected biomass was firstly washed thoroughly with freshwater to remove invertebrates. Contrarily to *U. intestinalis*, biomass of *L. ochroleuca* was not washed with freshwater due to its high sensitivity to fresh water, which damages the tissue (personal observation). Afterwards, biomass was weighted and distributed in the tank system - three replicate tanks per species. Since the *L. ochroleuca* individuals obtained were too small to distribute them in a density of 10 g/L, they were instead separated according to size (small < 10 cm; 10 < average > 15 cm; big < 15 cm) and distributed by number, in a total weight of 170 g. Each trial was programmed to last 8 weeks (plus one initial week of acclimation).

Fig. 3 - Tank system located in Foz do Douro (Porto, Portugal)

The 70L tanks were part of an outdoor system working as a pilot-scale trial, located in Foz do Douro (Porto, Portugal) with independent tanks (fig. 3) allowing for independent replication. Each tank was equipped with a continuous aeration outlet at the bottom to avoid water stagnation, the accumulation of metabolites in the seaweed's surface and to improve nutrient and carbon dioxide availability and uptake. It also allows circulation of the biomass, which helps homogenize light exposure.

During the fast-growth season, the sides of the tanks were enveloped in a shadowing net to prevent photoinhibition and/or damage.

Fig. 4 - Nutrient addition and measurement of water parameters in outdoor trial

Water renovation was continuous, amounting to one full renovation every 2 days. The water supplied was collected from the sea in the Foz do Douro region and filtered with a 0.12 mm filter before entering the system and enriched with 0.16 µmol/L of PO_4^{-3} (supplied as sodium phosphate) and 1.28 µmol/L of NO_3^- (supplied as sodium nitrate) three times a week (fig. 4). Nutrient addition was meant to guarantee that the nutrients available in the seawater would not be limitative to the seaweed's growth. These concentrations were similar to the Von Stosch medium, but used in a 1:10 strength to avoid epiphytes and growth of other seaweeds in the walls of the tank, which could affect growth and quality of the cultivated seaweed. However, in the fast-growth season, nutrient addition in *U. intestinalis* was doubled because nutrient depletion might have contributed to a sporulation event in the slow-growth season trial.

During the trial, light and temperature were measured semi-continuously, every 30 minutes with Onset Hobo Pendant® Temperature / Light data loggers, placed in the tanks. Data collected was displayed as the weekly mean for maximum, mean and minimum temperatures and light intensity.

The biomass of each tank was weighed weekly (fresh weight) and restocked at the established density of 10g/L for *U. intestinalis*. Restock was not done for *L. ochroleuca* because it would imply indiscriminate cuts to the blade of the individuals. The algae obtained at the end of the trial for both species was stocked for further analysis.

To monitor environmental conditions, pH, dissolved oxygen and salinity values were measured three times per week with a multiparameter probe (fig. 4). Mean weekly values of these parameters were calculated.

Fig. 5 - Nutrient analysis of water samples with Palintest® Tablet Kit and Palintest® photometer

Weekly, water samples were collected for nutrient analysis (two water samples were collected from two random tanks – total of two replicate per species), to monitor nutrient availability in the tanks. The nutrients were analysed with a Palintest® Tablet Kit and a Palintest® photometer 7000 (fig. 5) to measure ammonia, nitrite, nitrate and phosphate content.

Induction of *Laminaria ochroleuca* sporulation

Since *Laminaria ochroleuca* reproduces by sporulation, an induction of this process was performed, using reproductive tissue collected in the field in Vila Chã (41º17'44.17"N, 8º44'12.79"W) in 02/02/2018 and Viana do Castelo (41º41'53.79"N 8º51'19.57"O) in 03/05/2018 and 17/05/2018.

Firstly, the reproductive tissue, called sori, was cleaned with paper to remove dirt and epiphytes, cut out and put in beaker with 200 mL of autoclaved seawater. Afterwards, the beaker was put in an ultrasonic cleaner during 5 min, to maximize spore release, and subsequently covered with aluminium foil and left at 5ºC during 2h. Then, the solution was filtrated (with a mesh cloth, to remove any fragmented tissue). The obtained spore solution was sprayed onto rope spools and left to dry during 1h before being put in plastic containers filled with autoclaved water and covered with cling film, and left to grow until 1cm recruits were obtained (fig. 6). The spools were put in a climatic chamber at 16 ºC under a light intensity of 100 µmol m^{-2} s^{-1}.

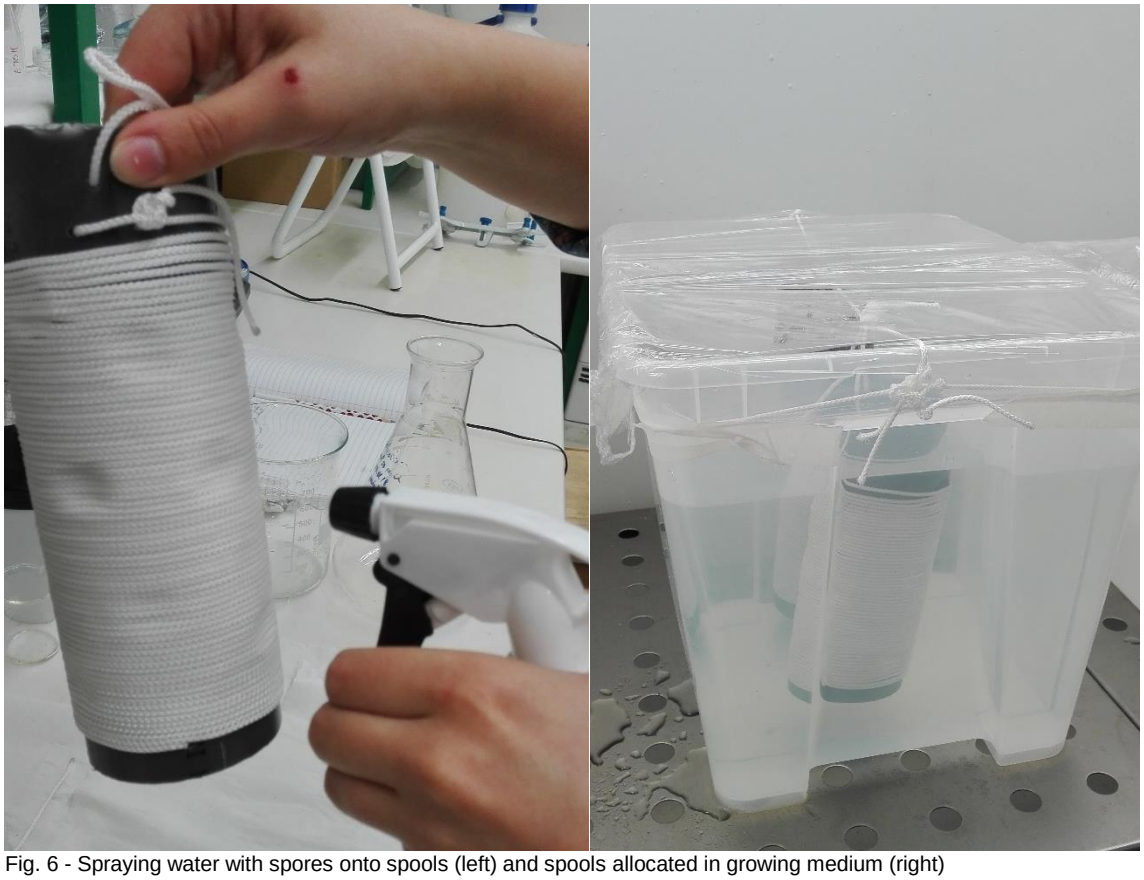


Fig. 6 - Spraying water with spores onto spools (left) and spools allocated in growing medium (right)

Biochemical analysis and assessment of bioactivity

An assessment of the biochemical profile of the algae biomass was performed with the initial and final samples of the outdoor cultivation experiments in the slow and fast-growth seasons, in order to determine how the species' composition varied throughout the year and to test tank cultivation affected the composition of the seaweeds.

All samples were dried at 60ºC for 48h and later analysed in order to determine protein and phenolic content, as well as antioxidant capacity. To extract the compounds, the dry biomass was grounded to a powder. For extraction, 15mg of dry biomass was homogenised with 1.5 mL of EtOH:H_2O (1:1, v/v) in a Precellys® Evolution homogeneizer (Bertin Corp., Rockville, USA) with twelve 30 seconds cycles at 8000 rpm, with zirconia beads. The extract was kept in cold (4º C) prior to analysis. All analysis methods were spectrophotometric. This solvent was chosen for its capacity to extract both polar and nonpolar components.

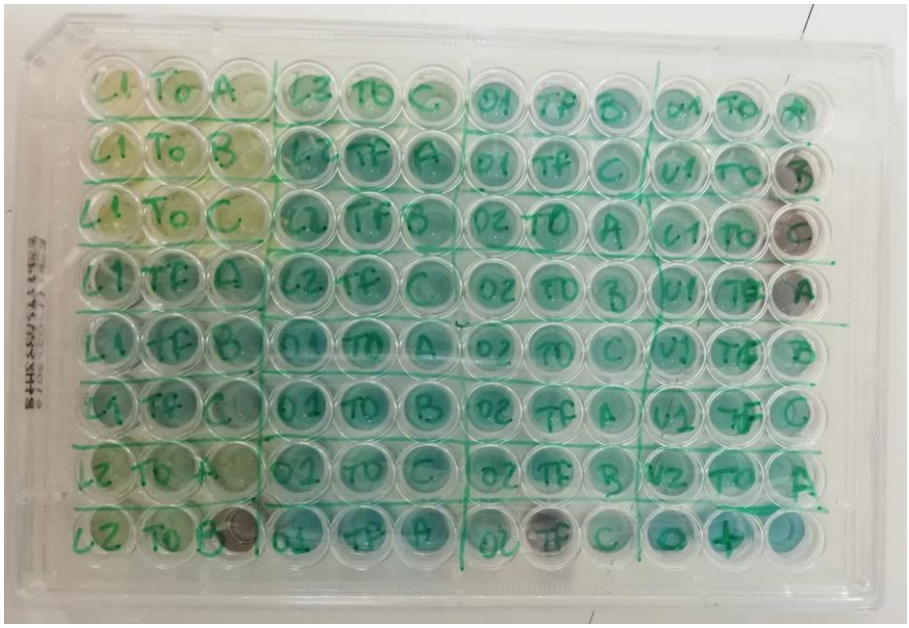


Fig. 7 – Microplate used for analysis of protein content

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 for 15 minutes. After the reaction, the microplate (fig. 6) was read at 595 nm. The results are expressed as percentage of dry weight ($\%_{DW}$) (Bradford, 1976).

Phenolic content quantification

Total phenolic content of the extract was quantified by the Folin-Ciocalteu method. In a 96-wells plate, 25 µL of sample were diluted in 125 µL of H_2O and reacted with 25 µL of Folin-Ciocalteu reagent during 5 minutes. Then, 75 µL of Na_2CO_3 (7%) was added. After 90 minutes 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 *et al.*, 1927).

Antioxidant Capacity

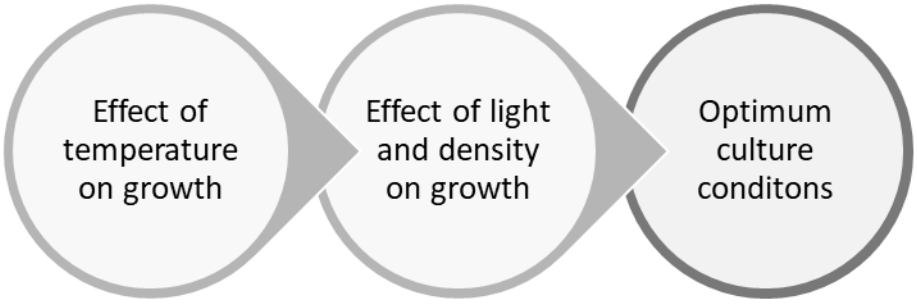
Since there is no universal antioxidant assay, and different groups of molecules are sensitive to different radicals, antioxidant capacity was assessed by two different assays ABTS+• and DPPH•. The radical-scavenging capacity of the extracts was evaluated trough the ABTS radical cation (ABTS+•) assay. In a 96-wells plate, 63 µL of sample were reacted with 180 µL of ABTS+• reagent (when read at 734 nm the reagent's absorbance must be between 0.680 and 0.720) for 6 minutes. After the reaction, the plate was read at 734 nm (Guedes *et al.,* 2013).

The DPPH radical assay (DPPH•) was also performed in a 96-wells plate, in which 63 µL of extract were reacted with 180 µL of DPPH• reagent (when read at 515 nm, the reagent absorbance must be between 0.800 and 0.900) for 30 minutes. After the reaction, the plate was read at 515 nm (Brand-Williams *et al.,* 1995).

For the 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 (Trolox Equivalents) per DW of biomass: $mgTE.gDW^{-1}$.

Lab cultivation optimization trials

The effects of temperature, density and light intensity on seaweed relative growth rates (RGR) and productivity were assessed for *Ulva intestinalis* and *Laminaria ochroleuca*, in laboratory. Wild specimens were collected at rocky shores in northern PT such as Foz do Douro (41º09'49.23"N 8º41'14.35"O) for *U. intestinalis* and Vilã Chã (41º17'44.17"N, 8º44'12.79"W) and Viana do Castelo (41º41'53.79"N 8º51'19.57"O) for juveniles of *L. ochroleuca*. Collection was done in 20/03/2018 for both species for the temperature trials and 29/06/2018 for *U. intestinalis* 17/05/2018 for *L. ochroleuca* for the light and density trials. Before the trial, biomass was carefully examined removal of invertebrates and, for *U. intestinalis*, biomass was additionally washed thoroughly with freshwater for the same purpose.



Factor 1: temperature
12 ºC
16 ºC
20 ºC

Factor 2: light intensity
100 µmol m^{-2} s^{-1}
200 µmol m^{-2} s^{-1}

Factor 3: density
5 g/L
10 g/L
15 g/L

Fig. 8 – Procedure of the laboratory culture trials

Effect of temperature on growth

Three levels of temperature were tested (12, 16 and 20 ºC) in three different FitoClima® chambers for a period of 5 weeks (including an initial week for acclimation) at a density of 10 g/L (fig. 8) for both species.

These experiments were performed under a 12h:12h light:dark photoperiod, since it is the typical photoperiod of the beginning of the fast-growth season – spring, with a photon fluency rate of 100 µmol m^{-2} s^{-1}. The water used was autoclaved and the salinity was adjusted to 35 psu – the typical Portuguese seawater salinity.

The algae were placed in 1 L Erlenmeyer flasks with 500 mL Provasoli Enriched Seawater (PES) (20 mL/L) and aeration. The PES medium includes micronutrients, which were supplied by seawater in the outdoor trials. Medium was changed twice a week. Once a week, biomass was weighted (fresh weight) and restocked to the original density for *U. intestinalis*. Restock was not done for *L. ochroleuca* because it would imply indiscriminate cuts to the blade of the individuals. For the weighting, the biomass was first placed in a salad spinner to allow removal of water without damaging tissue and allow the obtention of more concrete values of weigh. Values of pH, dissolved oxygen and salinity values were measured weekly with a multiparameter probe (fig. 9).

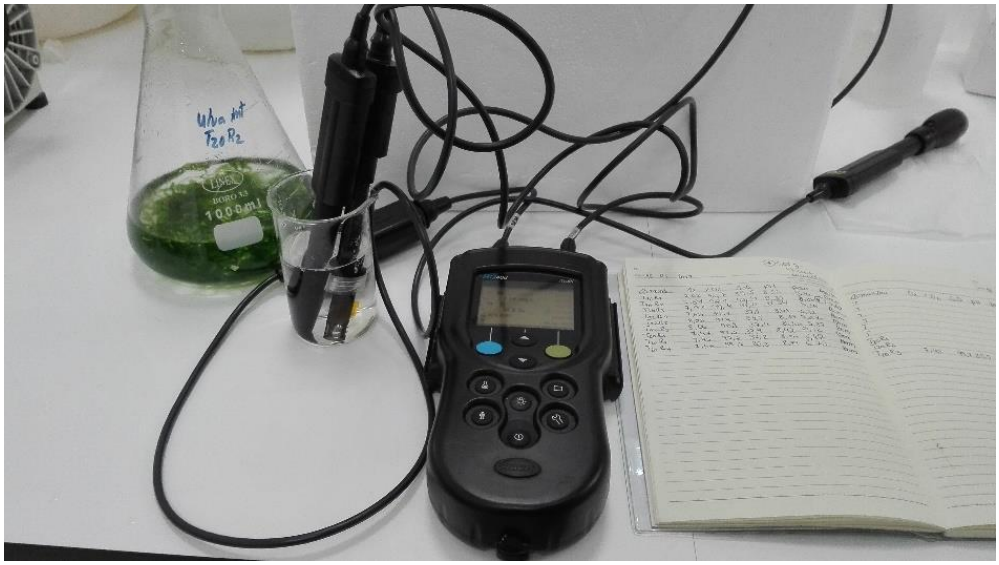


Fig. 9 - Measurement of water parameters in laboratory cultivation trial

Effect of Light and Density on Growth

Two levels of light intensities (100 and 200 µmol m^{-2} s^{-1}) were tested at three different stocking densities (5, 10 and 15 g/L), since these two factors influence each other (fig. 8), for a period of 5 weeks (including an initial acclimation week). The temperature to be used (16 º C) was chosen based on results from the previous trial or literature if obtained results in the previous trial were not conclusive.

Similarly to the previous trial, these experiments were performed under a 12h:12h light:dark photoperiod, salinity was adjusted to 35 psu and algae were placed in 1L Erlenmeyer flasks with 500mL of PES (20 mL/L). Once a week, biomass was weighed (fresh weight) and restocked at the original density for *U. intestinalis.* Once again, restock was not done for *L. ochroleuca* because it would imply indiscriminate cuts to the blade of the individuals. Values of pH, dissolved oxygen and salinity were measured weekly with a multiparameter probe (fig. 9).

Data Treatment

For all trials, growth rate was calculated using the following formula:

$$\mathrm{RGR}\ (\mathrm{g\ day^{-1}\ g^{-1}}) = \frac{(\mathrm{lnA} - \mathrm{lnB})}{t}$$

In which A and B are the final and initial biomass, respectively, and *t* is the number of days of cultivation. Data was analysed using SPSS Statistics (Version 24).

Since during the outdoor cultivation the same biomass was measured over time, growth data was analysed with RMANOVA (Repeated Measures ANOVA) to check if time points diverged significantly and, if so, if this difference could be explained by variation in an environmental factor (temperature, light, salinity, dissolved oxygen and pH). Nutrient content and biochemical content and activity data for *U. intestinalis* and *L. ochroleuca* were analysed independently using 1-way ANOVA, considering Time as factor. When results indicated a significant difference, a Tukey test was performed for pairwise comparisons. Since the design in the optimization trials, both to find the optimal growth temperature and optimal density and light intensity conditions, included the measurement of replicates over time, RMANOVA was performed, considering Time as Within-Subject Factor and either Temperature or Density and Light level as Between-

Subjects Variable. Tukey tests were performed for factors or factor interactions with p-values lower than 0.05.

Results

Outdoor Cultivation Trials - *Ulva intestinalis*

Winter - slow-growth season (February to April)

The winter trial occurred between 02/02/18 and 10/04/18, lasting a total of 8 weeks. Average relative growth rate ("RGR") was 4,33E-03 g day^{-1} g^{-1}.

On week 4 (fig. 10), biomass started to decline as a brown seaweed started to grow on the tank's walls and may have inhibited photosynthesis due to sunlight blockage and competed for nutrients, resulting in biomass loss. Afterwards, the tanks were carefully cleaned for removal of the brown seaweed. On week 6, a rise in light intensity occurred, accompanied by a considerable increase in growth. Afterwards, a sudden decrease in light (20000 lux) was accompanied by a decrease in growth rate (5,24E-02 g day^{-1} g^{-1}). Mean temperature was constant throughout the trial (≈ 12 ºC). Mean value of pH was 8.93 ± 0.25, salinity was 29.7 ± 2.2 psu and oxigen was 12.53 ± 2.02 mg L^{-1}.

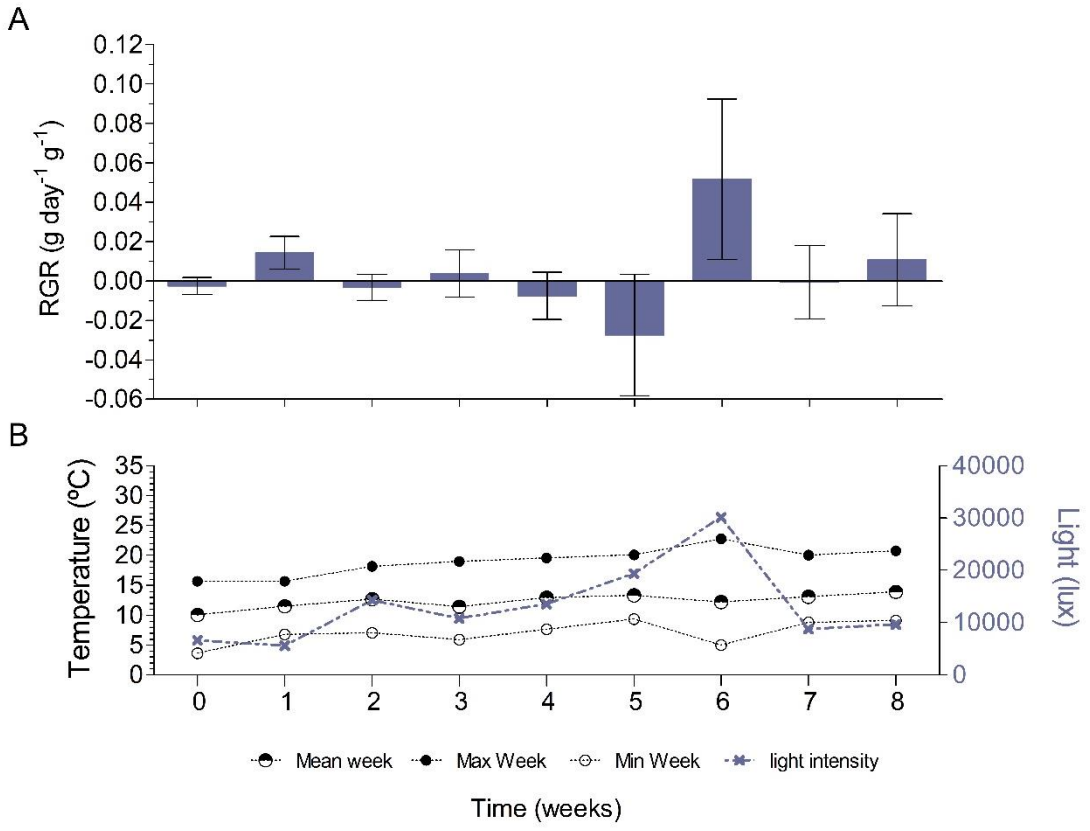


Fig. 10 - Relative growth rate ("RGR") (g $day^{-1}g^{-1}$) of *Ulva intestinalis* (A) and mean light intensity (lux), mean, minimum and maximum temperature (ºC) (B) for each week of the experiment in the slow-growth season. Period of 9 weeks (including acclimation week). Error bars are SD

Nutrient analysis detected a slight increase in ammonia in week 5 (fig. 11, A). In contrast, phosphate values decreased in week 5 (fig. 11, B). Nitrite was more abundant in water between weeks 2 and 4, being gradually consumed until it was fully consumed in week 8 (fig. 11, C). Nitrate had a peak in its values in week 6, the same week in which RGR increased greatly (fig. 11, D). Tukey test found significant differences in ammonia values with time, but not for phosphate, nitrite or nitrate. Ammonia content varied significantly between weeks 0 and 8.

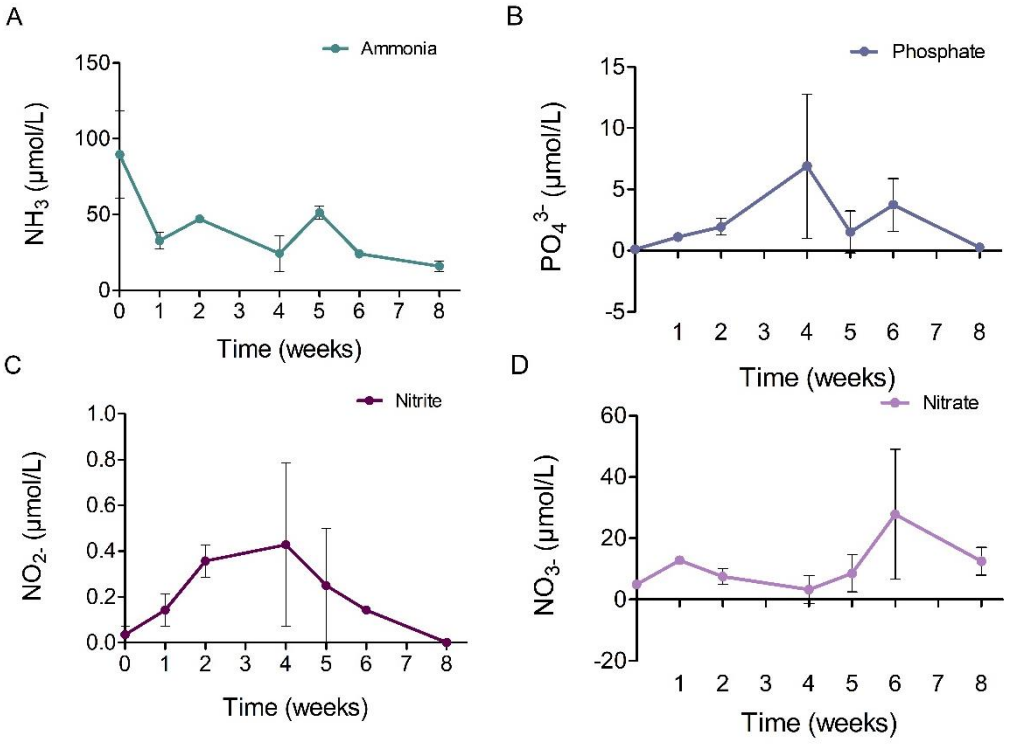


Fig. 11 - Concentration of ammonia (NH_3), phosphate (PO_4^{3-}), nitrite (NO_{2-}) and nitrate (NO_{3-}) in water samples of *Ulva intestinalis*, over time. Error bars are SD

Spring - fast-growth season (May to June)

The spring trial lasted a total of 7 weeks, between 14/05/2018 and 09/07/2018. Average relative growth rate ("RGR") was 1,43E-02 g day^{-1} g^{-1}.

In weeks 1 and 4 (fig. 12) a loss in biomass was observed. This happened alongside an increase in light intensity. In weeks 5, 6 and 7, RGR increased gradually, accompanied by a light intensity decrease and temperature increase. Mean temperature was constant throughout the trial (≈ 22 ºC). Mean value of pH was 9.86 ± 0.46, salinity was 32.3 ± 1.6 psu and oxigen was 12.24 ± 1.49 mg L^{-1}.

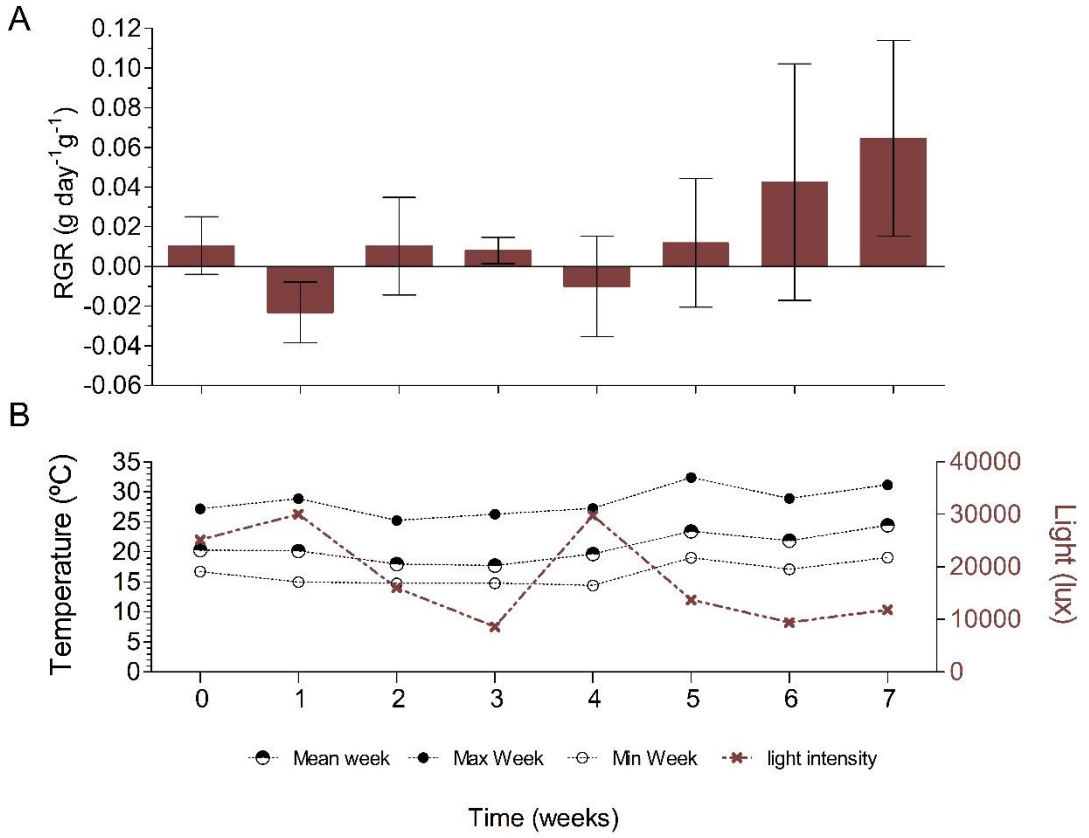


Fig. 12 - Relative growth rate ("RGR") (g $day^{-1}g^{-1}$) of *Ulva intestinalis* (A) and mean light intensity (lux), average, minimum and maximum temperature (ºC) (B) for each week of the experiment in the fast-growth season, Period of 8 weeks (including acclimation week). Error bars are SD

Ammonia values peaked in weeks 3 and 7 (fig. 13, A), phosphate values were overall constant (fig. 13, B), nitrite hit a peak in week 3 and again in week 7 (fig. 13, C) and nitrate remained overall constant, without significant peaks (fig. 13, D). Tukey test found significant differences with time for ammonia and nitrite contents, but not for phosphate and nitrate.

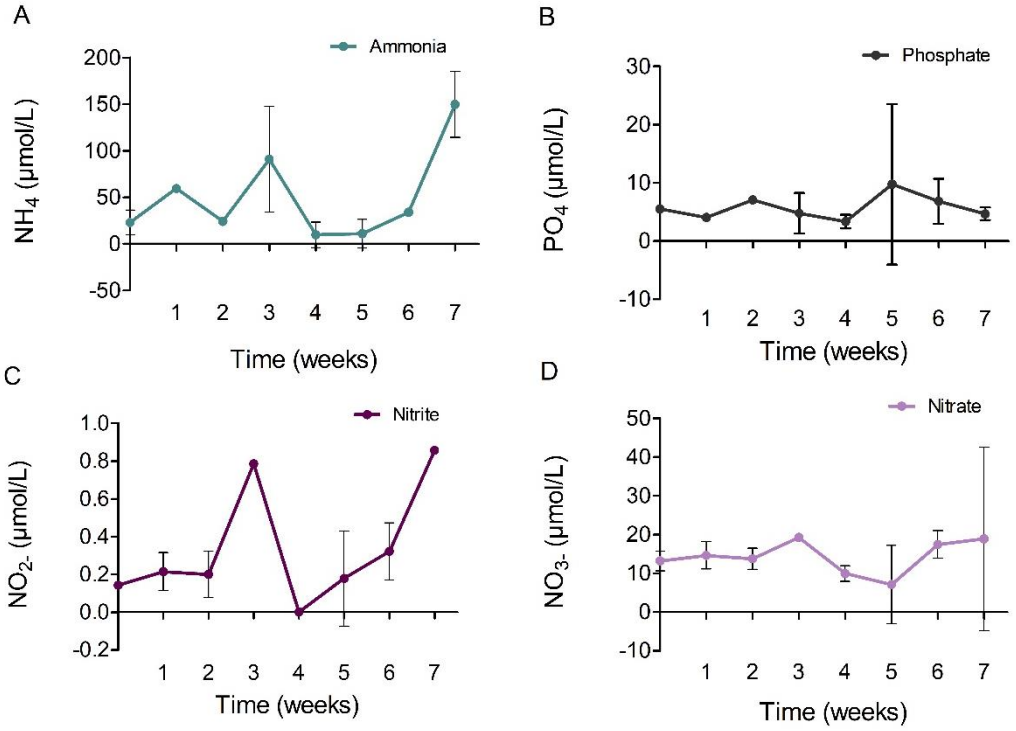


Fig. 13 - Concentration of ammonia (NH_3), phosphate (PO_4^{3-}), nitrite (NO_{2-}) and nitrate (NO_{3-}) in water samples of *Ulva intestinalis*, over time. Error bars are SD

Biochemical analysis and assessment of bioactivity of *Ulva intestinalis*

In the analysis of the samples obtained in both trials (fig. 14), "T_0" corresponds to biomass collected in the wild and "T_F" corresponds to biomass collected after tank cultivation. The numbers "0", "8", "13" and "20" depict the number of weeks since the beginning of collection, with 0 corresponding to 02/02/2018.

Results show that protein content varied significantly with time (1-way ANOVA, Time, p = 0.007, F(3) = 8.599, fig. 14, A) between week 0 of the slow-growth season and both time points of the fast-growth season. There were no significant differences between T_0 and T_F of the fast-growth season.

Phenolic content varied significantly with time (1-way ANOVA, Time, p = 0.008, F(3) = 8.362, fig. 14, B), showing highest values in week 8 of the slow-growth season and lowest values during the fast-growth season.

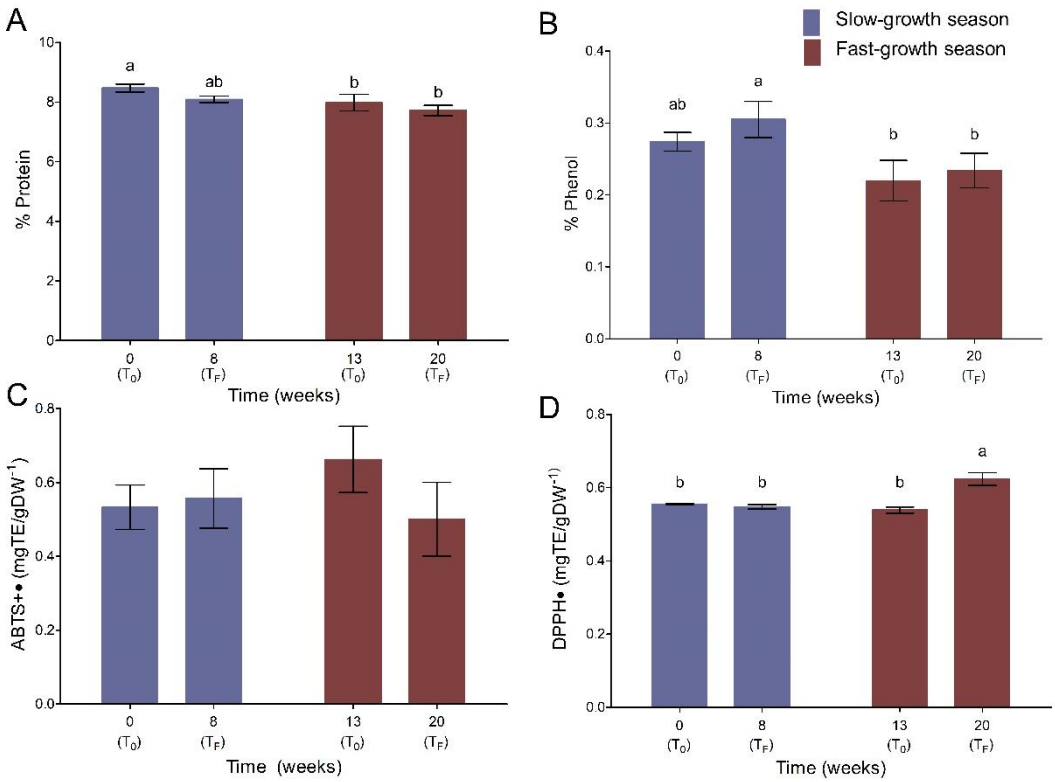


Fig. 14 - Protein content (%) (A), phenolic content (%) (B) and antioxidant scavenging activity via ABTS+• (C) and DPPH• (D) (mgTE/gDW^{-1}) of *Ulva intestinalis* at Time 0 ("T_0" – biomass from wild) and Final Time ("T_F" - end of trial) in the slow-growth and fast-growth seasons. Different letters indicate significant differences between seasons and time of collections of samples based on Tukey's multiple comparison test. Error bars are SD

Antioxidant activity, detected with the ABTS+• method, did not vary significantly with time (1-way ANOVA, Time, p = 0.056, F(3) = 3.876, fig. 14, C). Antioxidant activity,

detected with the DPPH• method, varied significantly with time (1-way ANOVA, Time, $p < 0.001$, $F(3) = 45.149$, fig. 14, D), showing greater values in week 20 of the fast-growth season.

Outdoor Cultivation – *Laminaria ochroleuca*

Winter - slow-growth season (February to April)

This trial occurred between 02/02/2018 and 10/04/2018, in a total of 8 weeks. Average relative growth rate ("RGR") was 1,16E-02 g day^{-1} g^{-1}.

During the trial (fig. 15), growth of the species was relatively constant. In week 6, a loss in biomass was accompanied by an increase in light intensity. Mean temperature values were overall constant during the trial (≈ 10 ºC). Mean value of pH was 8.81 ± 0.19, salinity was 29.7 ± 2.3 psu and oxigen was 11.27 ± 0.67 mg L^{-1}.

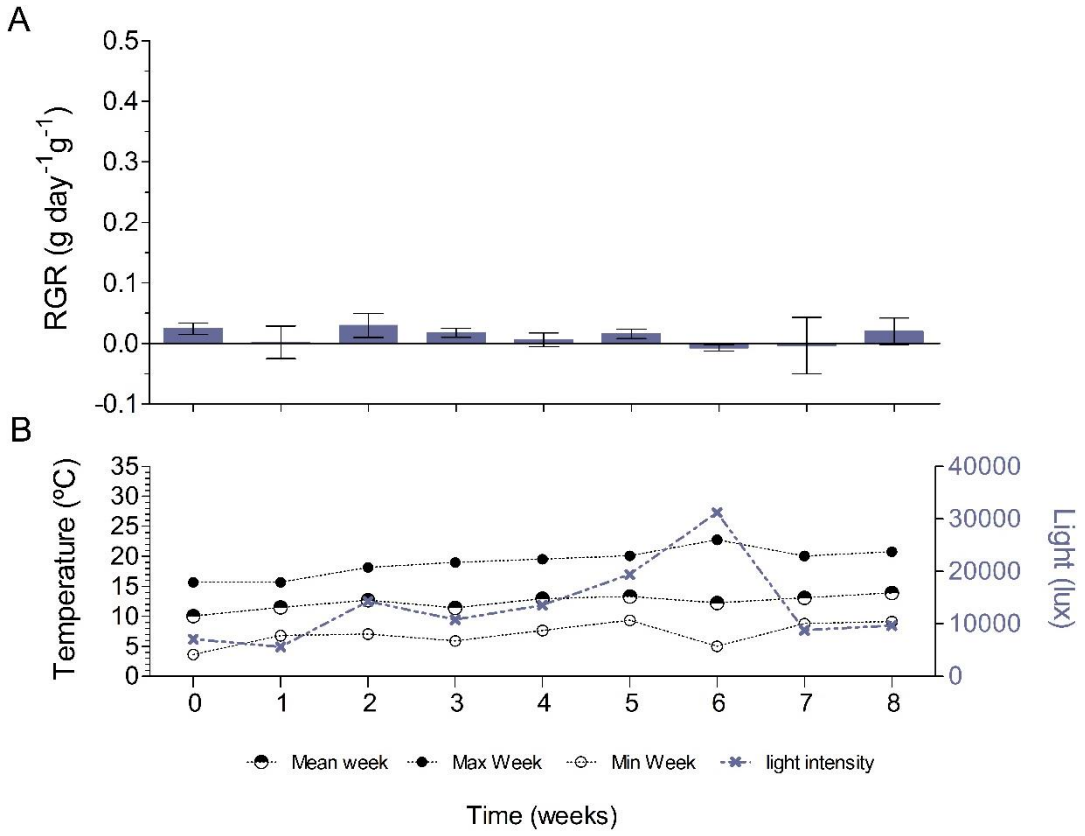


Fig. 15 - Relative growth rate ("RGR") (g $day^{-1}g^{-1}$) of *Laminaria ochroleuca* (A) and mean light intensity (lux), average, minimum and maximum temperature (ºC) (B) for each week of the experiment in the slow-growth season. Period of 9 weeks (including acclimation week), Error bars are SD

According to the Tukey test, nutrient concentration varied significantly with time only for phosphate, having had a peak in weeks 4 and 6 (fig. 16, B). A peak of ammonia occurred in week 6 (fig. 16, A), in nitrite in weeks 4 and 5 (fig. 16, C) and in nitrate in week 6 (fig. 16, D).

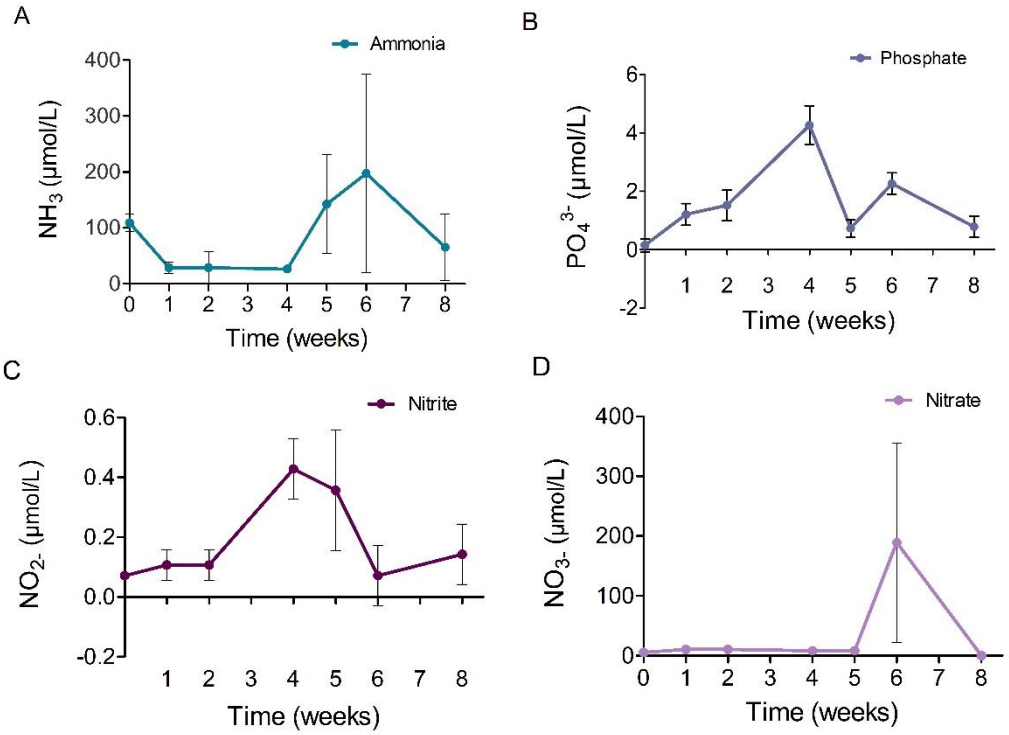


Fig. 16 - Concentration of ammonia (NH_3), phosphate (PO_4^{3-}), nitrite (NO^{2-}) and nitrate (NO_{3-}) in water samples of *Laminaria ochroleuca*, over time. Error bars are SD

Spring – fast-growth season (May to June)

The fast-growth trial occurred between 03/05/2018 and 25/06/2018, in a total of 6 weeks. Average relative growth rate ("RGR") was 4,18E-02 g day^{-1} g^{-1}.

RGR was relatively constant during the first 5 weeks of the trial (fig. 17) and growth values were similar to the values of the slow-growth season trial (0.05 g day^{-1} day^{-1}). In week 6, there was a great increase in RGR which occurred alongside a peak of phosphate and nitrite in water (fig. 18, B, D). After week 6, the prolongation of high temperatures, above the maximum described for *L. ochroleuca* (≈ 24 ºC) resulted in partial deterioration of the algae, which resulted in a premature ending of the trial. Mean temperature values were overall constant during the trial (≈ 20 ºC). Mean value of pH was 9.28 ± 0.29, salinity was 32.0 ± 0.97 psu and oxigen was 10.59 ± 0.54 mg L^{-1}.

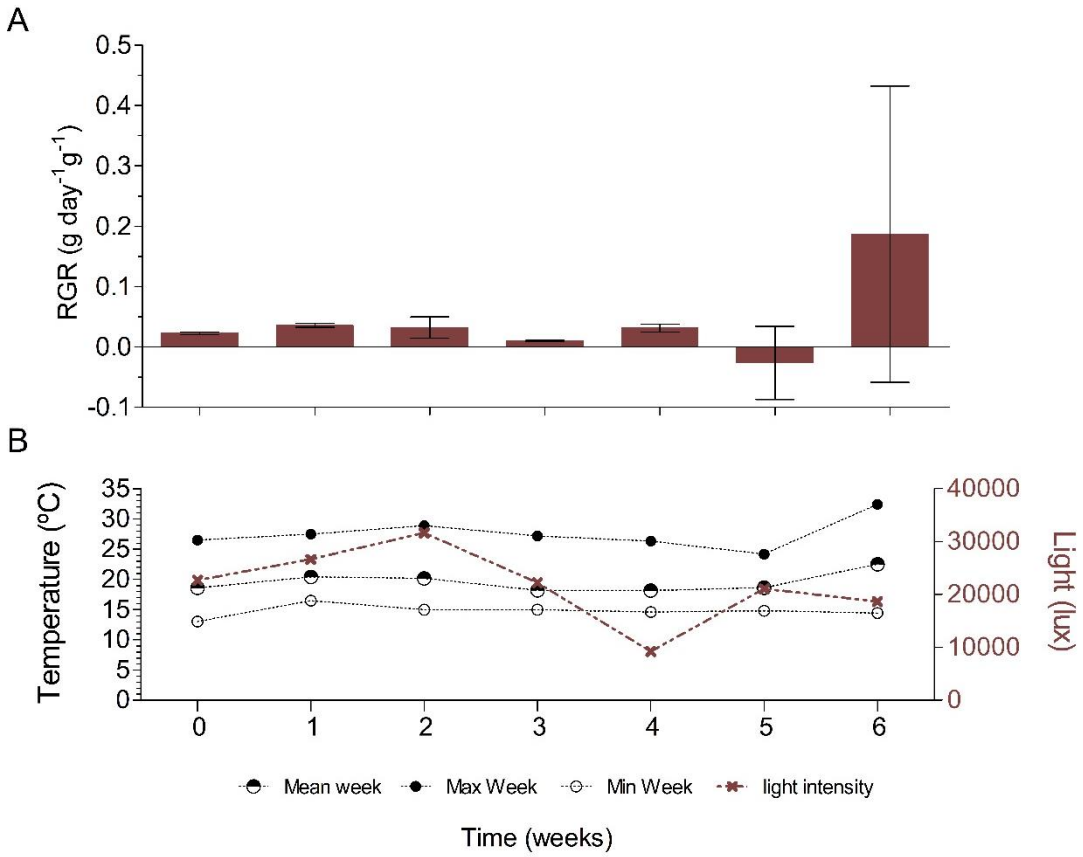


Fig. 17 - Relative growth rate ("RGR") (g $day^{-1}g^{-1}$) of *Laminaria ochroleuca* (A) and mean light intensity (lux), average, minimum and maximum temperature (ºC) (bottom) for each week of the experiment in the fast-growth season. Period of 7 weeks (including acclimation), Error bars are SD

Tukey test showed significant differences only in nitrite availability with time, between weeks 3 and 5. On the second half of the trial, there was an overall gradual rise in nutrient content in water. Ammonia started increasing in week 4 (fig. 18, A), phosphate

increased notably after week 3 (fig. 18, B), nitrite hit a peak in week 5 despite values being very low through all the trial (fig. 18, C) and nitrate increased after week 3, in which nitrate was totally absorbed (fig. 18, D). Nitrite values (fig. 18, C) however, was an exception, being almost totally consumed in week 6.

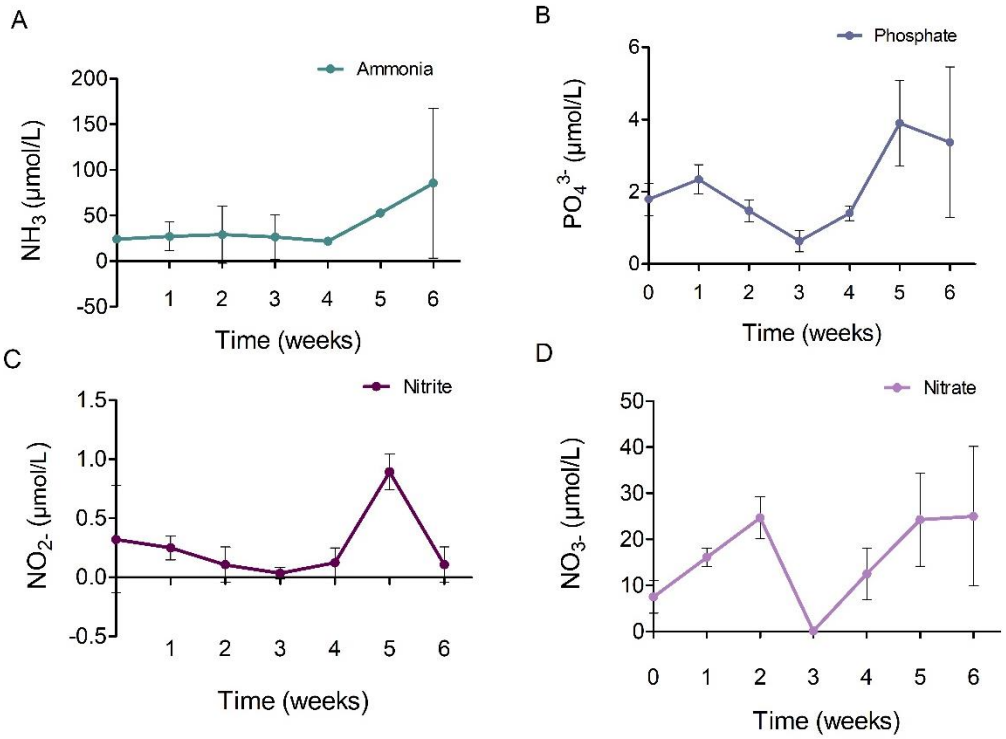


Fig. 18 - Concentration of ammonia (NH_3), phosphate (PO_4^{3-}), nitrite (NO^{2-}) and nitrate (NO_{3-}) in water samples of *Laminaria ochroleuca*, over time. Error bars are SD

Biochemical analysis and assessment of bioactivity of *Laminaria ochroleuca*

In the analysis of the samples obtained in both trials (fig. 19), "T_0" corresponds to biomass collected in the wild and "T_F" corresponds to biomass collected after tank cultivation. The numbers "0", "8", "13" and "20" depict the number of weeks since the beginning of collection, with 0 corresponding to 02/02/2018.

Results demonstrate that protein content varied significantly with time (1-way ANOVA, Time, $p < 0.001$, $F(3) = 14.516$, fig. 19, A) showing a decrease in week 17, after tank cultivation in the fast-growth season.

Phenolic content varied significantly with time (1-way ANOVA, Time, $p < 0.001$, $F(3) = 302.393$, fig. 19, B), showing a considerable decrease from specimens collected in the wild (weeks 0 and 11) to tank cultivated individuals (weeks 8 and 17).

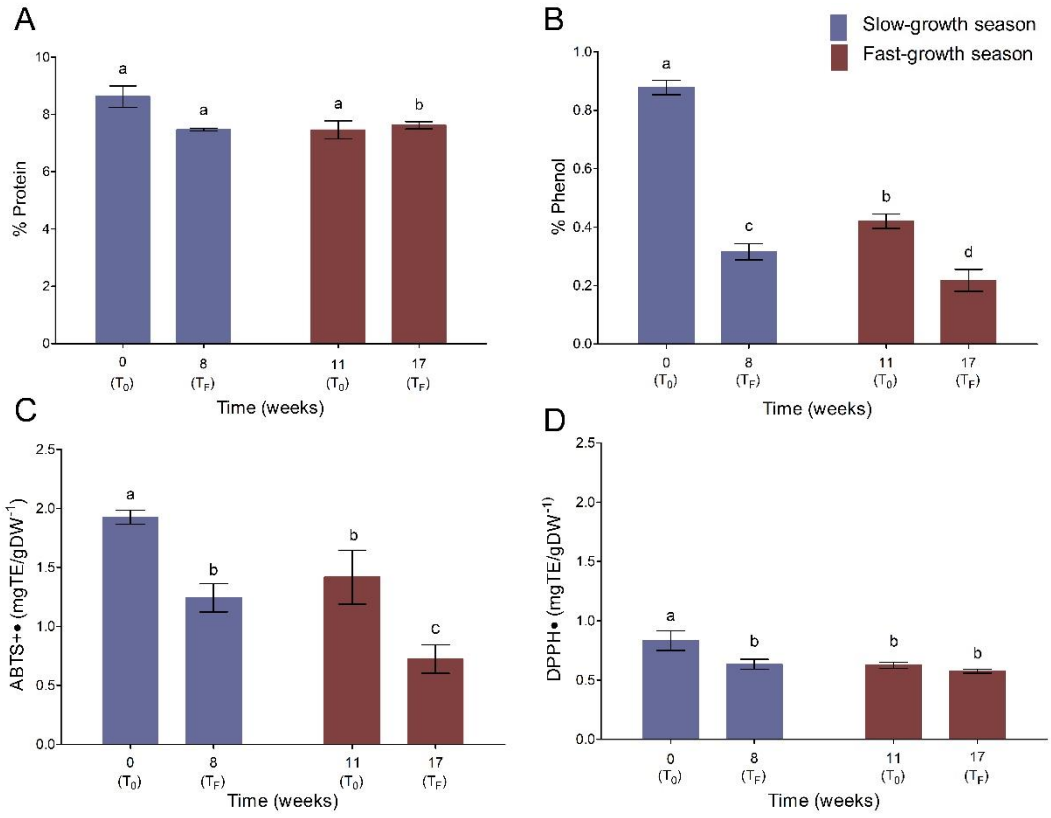


Fig. 19 - Protein content (%) (A), phenolic content (%) (B) and antioxidant scavenging activity via ABTS+• (C) and DPPH• (D) (mgTE.gDW^{-1}) in *Laminaria ochroleuca at* Time 0 ("T_0" – biomass from wild) and Final Time ("T_F" - end of trial) in the slow-growth and fast-growth seasons (A: $p<0.001$; B: $p<0.001$; C: $p<0.001$; D: $p=0.001$). Different letters indicate significant differences between seasons and time of collections of samples based on Tukey's multiple comparison test. Error bars are SD

Antioxidant activity, detected with the ABTS+• method, varied significantly with time (1-way ANOVA, Time, $p < 0.001$, $F(3) = 32.287$, fig. 19, C), showing a decrease

over time, with the highest activity in week 0 and lowest in week 17. No significant differences were observed between weeks 8 and 11.

On the other hand, antioxidant activity detected with the DPPH• method, varied significantly with time (1-way ANOVA, Time, $p = 0.001$, $F(3) = 15.921$, fig. 19, D), exhibiting overall much lower activity than the ABTS+• method. The highest activity occurred in week 0. No significant difference was observed between the remaining time points.

Induction of *Laminaria ochroleuca* sporulation

This experience did not have positive results, as no recruits grew in the spools. Despite several attempts of spore release having been made, the obtained results could have been due to the collected tissue not being mature enough at the time.

Lab cultivation optimization trials – *Ulva intestinalis*

Effect of temperature on growth

U. intestinalis growth varied significantly with Time and Temperature (RMANOVA, Time * Temperature, p = 0.017, F(8) = 6.591, fig. 20). However, Tukey test was unable to detect significant differences between points.

Under 20ºC, *U. intestinalis* suffered a considerable decrease in biomass in week 0 (acclimation). In week 1 a negative RGR occurred, not only at 20 ºC, but also at 16 ºC. Furthermore, in week 2, all temperatures had a decrease in biomass. In week 3, biomass in all temperatures demonstrated a positive RGR and, in week 4, only 12 ºC showed a negative growth rate.

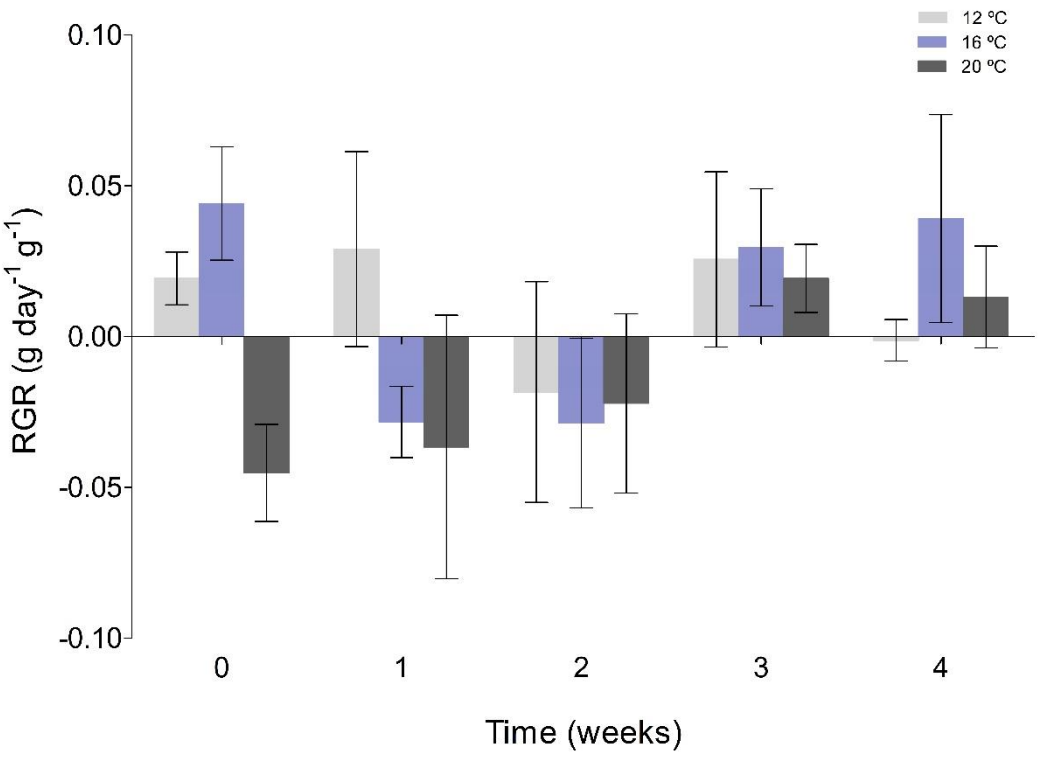


Fig. 20 - Relative growth rate ("RGR") (g day^{-1}g^{-1}) of *Ulva intestinalis* at three different temperatures (12, 16 and 20ºC). Photoperiod of 12h:12h light:dark, for a period of 5 weeks (including acclimation). Error bars are SD

Effect of Light and Density on Growth

U. intestinalis growth varied significantly with Time and Light Level (RMANOVA, Time * Light, $p < 0.039$, $F(4) = 4.010$, fig. 21) but not with Time and Density (RMANOVA, Time * Density, $p = 0.953$, $F(8) = 0.343$). As such, the tested densities did not affect differently the tested biomass.

Significant growth could be observed in week 2. Growth under light intensity of 100 µmol m^{-2} s^{-1} was significantly lower in acclimation and week 2 and equal to 200 µmol m^{-2} s^{-1} in weeks 1, 3 and 4. Overall, light intensity of 200 µmol m^{-2} s^{-1} showed higher growth values throughout the trial, even though these values decreased gradually after week 1.

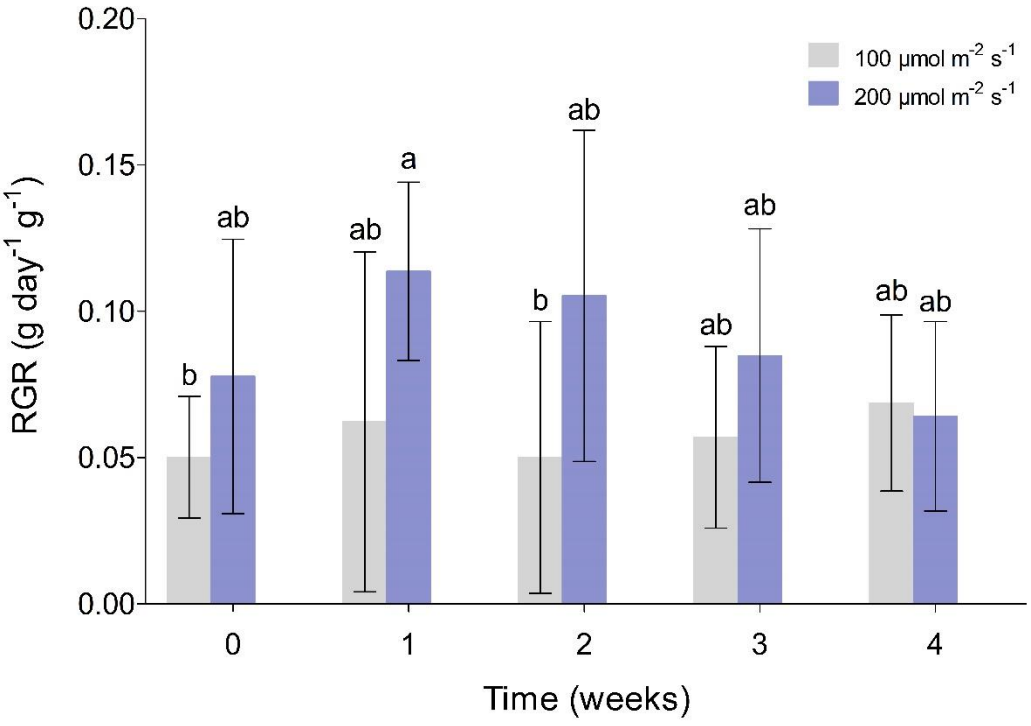


Fig. 21 - Relative growth rate ("RGR") (g $day^{-1}g^{-1}$) of *Ulva intestinalis* under a light intensity of 100 µmol m^{-2} s^{-1} and 200 µmol m^{-2} s^{-1}. Temperature of 16ºC, photoperiod of 12h:12h light:dark, for a period of 5 weeks (including acclimation). Different letters indicate significant differences based on Tukey's multiple comparison test ($p<0.039$). Error bars are SD

Lab cultivation optimization trials – *Laminaria ochroleuca*

Effect of temperature on growth

L. ochroleuca growth varied significantly with Time (RMANOVA, Time, p = 0.015, F(4) = 21.473, fig. 22) but not with Time and Temperature (RMANOVA, Time * Temperature, p = 0.187, F(8) = 2.124). As such, the tested temperatures did not affect differently the biomass.

RGR was negative only in week 1, however, obtained values were not significantly different from the previous and subsequent weeks. In weeks 0 and 2, growth rate was positive but significantly lower than in weeks 3 and 4.

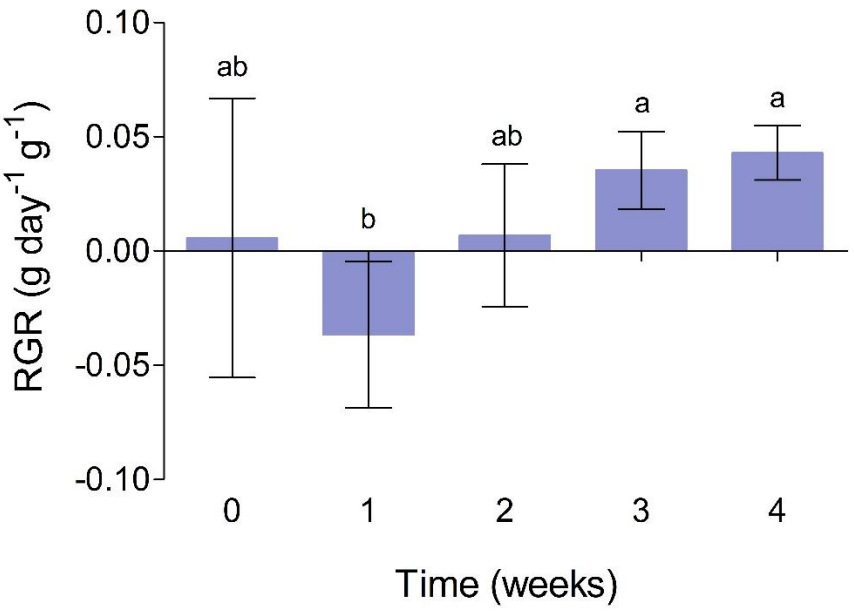


Fig. 22- Relative growth rate ("RGR") (g $day^{-1}g^{-1}$) of *Laminaria ochroleuca* at three different temperatures (12, 16 and 20ºC). Photoperiod of 12h:12h light:dark, for a period of 5 weeks (including acclimation). Different letters indicate significant differences based on Tukey's multiple comparison test (p=0.015). Error bars are SD

Effect of Light and Density on Growth

L. ochroleuca growth varied significantly with Time, Light Level and Density (RMANOVA, Time * Light * Density, $p < 0.001$, $F(18) = 2.133$, fig. 23).

Biomass put in a density of 5 g/L seemed to have had the tendency to grow the highest in all weeks and biomass and 15 g/L the lowest, despite the nonexistence of significant differences regarding density.

In biomass at 100µmol m^{-2} s^{-1}, RGR was positive in all weeks and, on the contrary, in the light intensity of 200µmol m^{-2} s^{-1} it was not. The only significant difference between light intensities was for density of 5 g/L in week 2 (*), in which a considerable biomass loss occurred, maintaining its negative growth values also in week 3. Biomass in 15 g/L suffered biomass loss in week 4. Biomass in 10 g/L density was the most stable density throughout this trial.

The large SD values registered in 5 g/L density of weeks 2 and 4 are due to the fact that one of the replicates had better growth rate in comparison to the other two.

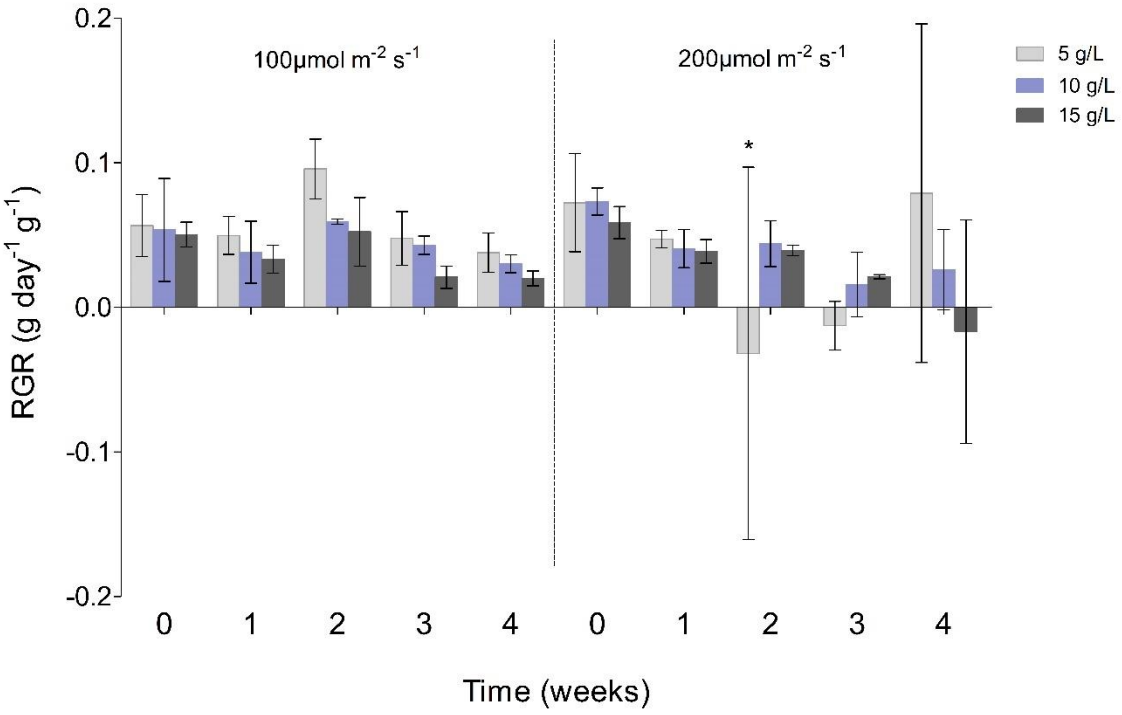


Fig. 23 - Relative growth rate ("RGR") (g $day^{-1}g^{-1}$) of *Laminaria ochroleuca* at three different densities (5, 10 and 15 g/L) under a light intensity of 100 µmol m^{-2} s^{-1} and 200 µmol m^{-2} s^{-1}. Temperature of 16ºC and a 12h:12h light:dark photoperiod for a period of 5 weeks (including acclimation). * marks the only a significant difference between light intensities. Error bars are SD

Discussion

Outdoor Cultivation Trials - *Ulva intestinalis*

Seaweed from the intertidal are periodically exposed to air, experiencing a variety of stressful conditions, such as high light exposure, high and low temperature, desiccation and osmotic stress and nutrient limitation (Davison & Pearson, 1996). As photosynthetic organisms, seaweed growth is strongly influenced by light exposure, which for species in the intertidal and shallow subtidal is a factor greatly influenced by tides (Saffo, 1987; Vergara *et al.*, 1997). *Ulva intestinalis* is an opportunist species that occupies the intertidal. It is able to grow rapidly and survive under stressful conditions (Fong *et al.*, 1998). Its growth is reported to be regulated mainly by light availability and temperature (Hernandez *et al.,* 1997). Tides highly influence not only light exposition but also the temperature to which the biomass is exposed (Pearson *et al.,* 2009).

In the slow-growth trial, the biggest growth rate registered occurred in week 6, associated with an increment in light intensity. This positive response indicates the species has a high light demand, typical of intertidal seaweeds (Gómez & Huovinen, 2011).

Loss of biomass, however, occurred in several weeks, such as 0, 2, 4, 5 and 7, which does not correlate with the variation in any single environmental factor, meaning it might be the result of an interaction between multiple factors. In culture conditions, *Ulva intestinalis* has been reported to only reproduce asexually (Ruangchuay *et al.,* 2012). The same was also previously reported for *Ulva prolifera* (Lin *et al.*, 2008). Furthermore*, Ulva* species have been reported to rely on asexual reproduction when in stressful conditions, which may imply that algae put in tanks experience a change in the environment that triggers a stress response. This type of reproduction is characterized by a physical fragmentation (Alström-Rapaport *et al.*, 2010). In fact, during the trial, it was possible to observe recruitment in the tank's wall, resulting from spores released in the water. As such, the biomass loss likely occurred due to sporulation, even though the exact conditions that induced sporulation were not clear. Even so, biomass seemed to have relied on spore release when light exposure was lower and later grew when light conditions were appropriate, depicting the opportunistic behavior of *Ulva intestinalis*.

Seaweed growth is dependent on nutrient availability (Harrison & Hurd, 2001). The water supplied to the tank system was rich in nutrients, and nutrients were added to

guarantee their availability would not be limiting. Nutrient content in water supply varied (fig. 1, Annex A) and was expected to influence the nutrient fixation of the seaweed.

Phosphate values in tank water were overall low. Experiments on *U. lactuca* have concluded that this species is an efficient biofilter for removal of phosphate in sewage treatment plants (Tsagkamilis *et al.,* 2010). Therefore, *U. intestinalis* was also expected to show good efficiency in fixating phosphate, explaining why its levels were mostly low. As for nitrite values, they appear to have been limitative in week 8, which may justify the observed decrease in growth. Lastly, nitrate values did not appear to be limitative in any of the weeks. Besides, a peak of nitrate in the water supply (fig. 1, D, Annex A) occurred simultaneously with a peak in nitrate content in the tanks in week 6. The fact that nutrient levels reached quite low values in some weeks of this trial motivated doubling the nutrient addition in the fast-growth season. Since a quicker growth was expected, higher demand for nutrients was also to be expected.

In the fast-growth trial, biomass loss occurred in weeks 1 and 4, occurring in parallel with higher light exposition (approximately 30000 lux – the same peak as in the slow-growth season). This would contradict results from the slow-growth season trial if light dependence was the only key factor for growth success. Despite shadowing nets being setup to obscure the tanks successfully enough in order for light intensity not to exceed values attained in the previous trial – and thus preventing photoinhibition - water temperature was not regulated, raising to as 19-23 ºC of mean temperatures and 33 ºC of maximum temperatures. In the slow-growth trial, mean temperature never exceeded 13ºC and maximum temperature 20ºC. In the wild, tides provide cold water to allow the algae to recover from any damage suffered during low tide heat exposure (Pearson *et al.,* 2009). In this trial, however, water renovation was continuous amounting only to a full renovation every 2 days. Given the sporulation response of *U. intestinalis* when stressful conditions occur, loss of biomass is expected to have been due to sporulation.

In fact, growth increased exponentially in weeks 5, 6, 7, with large quantities of biomass growing attached to the walls of the tanks. This demonstrates that loss of biomass could have been, in fact, due to sporulation as the spores released were able to recruit in the tanks' walls and grow successfully, since temperatures were appropriate for development.

Ammonia content was significantly different in week 7, in which ammonia levels increased. That most likely occurred due to an increase of ammonia levels in sea water. As for nitrite content, it was significantly different in week 4, which was seemingly stressful for the algae and caused sporulation, resulting in biomass loss. This decrease in nitrite happened alongside a rise in light exposure, the most probable explanation for this depletion, since nitrogen consumption is influenced by light, even though the nutrient

dose added to of *U. intestinalis* tanks was doubled in order to avoid limitation (Harrison & Hurd, 2001).

Salinity values were comparatively lower in the slow-growth season relatively to the fast-growth season, most likely due to the occurrence of rain. Furthermore, pH levels were considerably higher in the fast-growth season, which might be the results of higher growth rates in this trial.

Overall, *U. intestinalis* can be an interesting species for aquaculture, since it is capable of quick recruitment and seems to demonstrate good resilience to environmental conditions. However, the main challenge in this study for *U. intestinalis* cultivation was the prevention of sporulation of the species. As such, systems need to be setup as to not create conditions driving asexual reproduction, such as a higher water renovation rate to avoid temperatures from rising as much during summer. Still, further studies are needed to understand the exact conditions driving sporulation. Sporulation induction might, however, be multifactorial, and studies on the interactions between factors, such as temperature and light exposure, are important to develop methods for a viable application of *U. intestinalis* production in aquaculture.

Biochemical analysis and assessment of bioactivity of *Ulva intestinalis*

The biomass composition of seaweed varies according to several factors, such as species, growth conditions, environmental conditions and harvesting times (Aguilera-Morales *et al.,* 2005; Ibañez *et al.,* 2012; Hou *et al.*, 2015;). Furthermore, differences in sampling, drying and extraction methods can also influence biochemical composition and nutritional values of the algae (Chan *et al.,* 1997). In this project, despite being exposed to natural environmental conditions and water supplied being directly collected from the sea, tank cultivation conditions were different from the wild and may have had influence in development of seaweed.

Protein content in *Ulva intestinalis* varied between 7.72 ± 0.17 and 8.48 ± 0.13% of dry weight. Values were significantly higher in the slow-growth season, particularly in week 0. Comparing week 0 (beginning of February) to weeks 13 and 18 (mid May and beginning of July), there was a significant difference in protein content of around 0.5%. Since protein content decreased over time, irrespective of being wild or cultivated seaweed, this might indicate it was not due to tank cultivation, but a result of seasonality of the species. Haroon *et al.* (2000) reported protein content in *Ulva* spp. collected from

Gulf of Gdańsk coast, Poland, between April to October 1993 to have varied between 9.42 ± 4.62% and 20.60 ± 5.00%. The samples obtained in week 8 (beginning of April) presented a content of protein of 8.09 ± 0.10 %, being slightly lower than the results obtained by Haroon *et al.* On the other hand, Akköz *et al.* (2009), reported the protein content of *U. intestinalis* originated from Acıgöl Lake, in Turkey, to be of 15.02 ± 1.02%, which are results higher than the ones obtained in this experiment. This could have been either due to the time of collection, which is not stated in their report, or to the difference in salinity. Salinity stress is reported to affect protein production (Esteves & Suzuki, 2008). In our experiment, seaweed grew in seawater with a salinity of 35 psu, that is reported to stimulate sporulation more than lower salinities (Sousa *et al.*, 2007). Since sporulation is a biosynthetic process that, like protein production, is energy-requiring, it can be hypothesized that higher salinity levels may have affected protein synthesis in this experiment, making this production less effective than if salinity values were lower (Wang *et al*., 2016).

Phenolic content also seems to demonstrate higher values in the slow-growth season, particularly in week 8 after tank cultivation, with phenol content reaching 0.3% of dry weight. However, it was not significantly different from the initial sample collected in the field. In the fast-growth season, the percentage of this compound was significantly lower, not exceeding 0.25%. Trigui *et al.* (2013) reported that phenolic content in *U. rigida* was higher in extracts collected in late winter (February) and early spring (March). In the present study, content of samples demonstrated significantly higher phenolic content in early spring (beginning of April).

Antioxidant activity detected with the ABTS+• method did not show significant differences with time. The activity with the DPPH• method, on the other hand, showed best results in the fast-growth season in week 20 - T_F (beginning of July).

Reports have stated that a correlation between phenolic content and antioxidant activity can be found (Turumtay *et al.*, 2014). In the study conducted by Trigui *et al*. (2013) antioxidant activity was accompanied by higher phenolic content – February and March. However, negative correlations between the two have also been found, suggesting that phenolic compounds are not the only contributors to antioxidant activities (Terpinc *et al.*, 2012). In this study, no correlation between variation in antioxidant activity and phenolic content was found.

Overall, the obtained results demonstrate that protein content in *U. intestinalis* decreased overtime (a possible result of seasonality), phenolic content was higher in the slow-growth season, antioxidant activity via ABTS+• was similar between both seasons and DPPH• was higher in the fast-growth season. Despite seeming to have had little

influence, the obtained values do not allow to fully comprehend if results were due to the seasonality of the species or if tank cultivation had an influence in the cultivation.

Outdoor Cultivation Trials – *Laminaria ochroleuca*

Kelps can demonstrate resilience when submitted to different environmental conditions, adjusting their physiological processes to survive (Staehr & Wernberg, 2009; Wernberg *et al*., 2010). However, kelp juveniles under stress are much more sensitive than adults (Dean & Jacobsen, 1984). Susceptibility to light damage has been reported to affect more intensely younger individuals (Roleda *et al.,* 2004). Since this experiment was done with juveniles, it is expected that they would have shown higher sensitivity to high light exposure than if it was done with adults.

L. ochroleuca is a warm-temperate species and its growth has been reported as optimum between 10 and 15 ºC and slow at 5 ºC (tom Dieck, 1992; Birkett *et al.*, 1998). Sporophytes were reported to have a lower and upper temperature limits of 0 ºC and 23 ºC when exposed for two weeks to those temperatures (tom Dieck, 1992; tom Dieck & de Oliveira, 1993). Additionally, survival has been reported at 30 ºC when exposed to 1h heat shock (Pereira *et al.*, 2015).

In the slow-growth season, biomass loss occurred in week 6, most likely because of the increase in light intensity. Phosphate levels were significantly higher in week 4, reaching values of around 5 µmol/L, similar to the values found in the water supply in the same week (fig. 3, B, Annex A), which may indicate this peak was due to a rise in phosphate content in water supply. *L. ochroleuca* seemed to have consumed most of the nitrate available, especially in week 5, in which its value was close 0 µmol/L, which may have limited growth.

As for the fast-growth trial, increase in growth rate occurred in week 6 accompanied by a peak in phosphate and ammonia and a small increase in nitrate in the water supply (fig. 4, A, B, C, Annex A). In the water supply, nutrient content increased for phosphate and nitrate, but not ammonia. Therefore, the increased growth may have been boosted by phosphate and nitrate availability. Moreover, rapid decomposing apical tissue increases ammonia concentrations (Hargreaves J. A., 2004). Therefore, the peek in ammonia value, despite non-significant, it was most likely due to degradation of biomass that occurred in week 5.

Moreover, during the first four weeks of the trial, growth rates were similar to the slow-growth trial (≥ 0.05 g day^{-1} g^{-1}). Similarly to the slow-growth trial, nitrate content was low in week 3, appearing to have been depleted, which indicates that *L. ochroleuca*

consumed almost all the nitrates available.

Furthermore, since rain occurred more frequently in the slow-growth trial, just like in *Ulva intestinalis* cultivation, salinity was lower in the slow-growth season when compared to the fast-growth season. Moreover, pH levels were higher in the fast-growth season, as well as algae production. Maximum temperature in week 6 reached 30 ºC, which is above the upper tolerance limit of the species. This stress most probably caused serious stress in the individuals resulting in death. This severe disintegration of biomass motivated ending the trial prematurely, on week 7.

L. ochroleuca seems to be a potential species for aquaculture, especially because it seems to absorb nutrients efficiently. Growth of biomass was high and ≈ 5 times higher than *Ulva intestinalis*. In this trial, the main challenge was preventing photoinhibition (for which shadowing nets were used, although not having been very efficient) and controlling temperature. As such, systems need to be setup as to create conditions to allow better control of temperature and to avoid overexposure of individuals to light.

Biochemical analysis and assessment of bioactivity of *Laminaria ochroleuca*

Protein content was significantly higher in weeks 0, 8 and 11, reaching values of over 8% in week 0, which corresponds to biomass collected from the field, in February. Week 17 (late June – end of fast-growth trial) was significantly lower, amounting to around 7.8%. Northern Spanish *L. ochroleuca* collected in July has been reported to contain 7.5 % of protein (Sánchez-Machado *et al.,* 2004). As such, the obtained results are in agreement to the previously reported.

Phenolic percentage was higher in individuals obtained from the wild for both seasons, which may be due to tank cultivation. Since phenols are compounds produced by plants to avoid herbivore attacks, the cultivation in tanks may have limited the production of this compound since there were not herbivores in the tanks (Boeckler *et al.,* 2011). Content in phenols was highest in week 0 – around 0.9% – and lowest in week 9 – around 0.2%.

Both antioxidant assays, ABTS+• and DPPH•, demonstrate highest radical scavenging capacity in week 0. However, ABTS+• value for this sample was higher than DPPH• (1.92 ± 0.06 mgTE.gDW^{-1} and 0.83 ± 0.08 mgTE.gDW-1, respectively). Additionally, the value for ABTS+• was lowest in week 17 (0.72 ± 0.12 mgTE.gDW^{-1}), whilst with DPPH•, the other three samples did not have significant differences.

Overall, the obtained results demonstrate that protein content was significantly lower in the fast-growth season - after tank cultivation - in comparison to all other samples, being a possible result of seasonality of the species. On the other hand, the percentage of phenols was higher in individuals obtained from the wild for both seasons. The activity via ABTS decreased+• over time, which may indicate it was a result of seasonality. The activity via DPPH• was significantly higher in the biomass harvested from the field in the slow-growth season, also a possible result of seasonality. Despite seeming to have had a bit of influence, the obtained values do not allow to fully comprehend if results were due to the seasonality of the species or if tank cultivation had an influence in the cultivation.

Lab cultivation optimization trials – *Ulva intestinalis*

Effect of temperature on growth

Results demonstrated an initial loss of biomass for all temperatures, affecting biomass at 20 ºC in weeks 0 to 2, 16 ºC in weeks 1 and 2 and 12ºC in week 2. These negative values were mainly due to sporulation, which was observed as a green layer growing attached to the bottom of the flasks. Under culture conditions, this species has been observed to only reproduce asexually, which includes a total disintegration of the thalli, as the zoospores form in all its length (Ruangchuay *et al.,* 2012).

Ruangchuay *et al.* (2012) performed an *in vitro* experiment and found sporulation in *U. intestinalis* occurred earlier at 25 ºC in comparison to 20 ºC and 30 ºC, indicating that the species may have an ideal temperature range for sporulation. Furthermore, in the same experiment, maximum growth of *U. intestinalis* occurred at that same temperature (25 ºC), before sporulation (Ruangchuay *et al.*, 2012).

In this experiment, 20 ºC was the temperature that exhibited highest sporulation, although it was not preceded by the highest growth rates. Despite that, a straight comparison with Ruangchuay and collaborator's study cannot be made, since water salinity was different. In the study conducted by Ruangchuay, water salinity was 20 psu while in this trial salinity was on average 35 psu.

Additionally, optimal growth of the Korean strain of *U. intestinalis* was achieved at 15 ºC and 24 psu salinity (Kim & Lee, 1996). Results are in agreement with this report, since 16º C resulted in higher growth rate. Despite that, a direct comparison cannot be made since the salinities differed.

Effect of Light and Density on Growth

Growth was significantly higher under light intensity of 200 µmol m^{-2} s^{-1} during weeks 0, 1 and 2. From week 3 on, no significant growth over time was observed at this photon fluency rate. Increases in light intensity have been reported to stimulate sporulation (Sousa *et al.,* 2007). Therefore, a photon fluency rate of 200 µmol m^{-2} s^{-1} was expected to cause more sporulation. In fact, under these conditions, after week 3, it was possible to observe that the species had started to sporulate considerably, as large green layers started to grow in the bottom of the flasks, which justifies the evident decrease in growth rate. On the other hand, *U. intestinalis* exposed to 100 µmol m^{-2} s^{-1} also sporulated but less evidently, not causing significant differences in biomass variation, and only small patches of green attached to the bottom of the flask could be observed.

Furthermore, salinities of 35 psu have been reported to highly promote sporulation when compared to 20 psu, while at 5 psu sporulation is reduced (Sousa *et al.*, 2007). Martins *et al.* (1999) reported that the Portuguese strain grows best at salinities of 15-20 psu. However, that study was conducted in *U. intestinalis* collected in the Mondego estuary, which may be better adapted to brackish water, while in this study it was collected in the sea shore. As such, for this experiment, water collected from the sea was used in order to avoid hydric stress.

Lab cultivation optimization trials – *Laminaria ochroleuca*

Effect of temperature on growth

In Northern Portugal, seawater temperature is usually around 15ºC, while in tidal pools it may reach 30 ºC on warmer summer days (Engelen et al., 2008; Pearson et al., 2009). *L. ochroleuca* inhabits areas influenced by tides and the conditions they are exposed to can change quick and drastically, facing extreme conditions for short periods of time (Pereira *et al.,* 2015). Sporophytes of *L. ochroleuca* have a lower and upper temperature limit of 0 ºC and 23 ºC, respectively, and grow optimally at between 10 and 15 ºC (tom Dieck, 1992; tom Dieck & de Oliveira, 1993). Therefore, in this trial, sporophytes of *L. ochroleuca* were expected to grow better at 12 and 16 ºC. However, that did not occur, as there were no significant differences between temperatures. This might have to do with the generality of studies being done with population from the polar range of distribution. Individuals in the equatorial range of distribution, as is Northern

Portugal, are expected to perform better at higher temperatures than individuals from colder areas (Birkett *et al.,* 1998; Pereira *et al.*, 2015).

Even though there was a week 0 for acclimation of the biomass, that period may or may not have been enough for that. As such, in week 1, the biomass loss that occurred may have been due to the change in environmental conditions and the biomass not being yet adapted to the new conditions. Juveniles are expected to show higher susceptibility to temperatures in comparison to *L. ochroleuca* adults (Lüning, 1984; Müller *et al.,* 2009). Individuals seemed to have adapted in week 2, since growth rates gradually increased from there on.

Effect of Light and Density on Growth

Results for different light and density conditions showed only significant lower differences in week 2, in biomass in 5 g/L density at 200 µmol m^{-2} s^{-1}. This may have occurred because lower density resulted in higher light exposure of the young sporophytes, that may have caused photoinhibition to the smaller juveniles. In fact, reports about younger individuals being more affected by light damage have been found (Roleda *et al.,* 2004). Despite that, growth seemed not be affected by the light intensities and densities tested.

Additionally, in this trial, replicates in 10 g/L density at 100 µmol m^{-2} s^{-1} did not suffer significant biomass loss, contrarily to the biomass in the same density in the temperature trial. Growth of *L. ochroleuca,* in nature, occurs from winter to spring (Pereira *et al.*, 2019). Juveniles used in the temperature trial were collected in winter (end of March) and were smaller, whilst recruits for the light and density trial were collected in spring (mid June) and were relatively bigger in comparison. The difference in growth observed in both trials may have been due to the size of the individuals. Also, it has been previously reported that response to temperature varies throughout the year (Lüning, 1984; Müller *et al.*, 2009).

Conclusions

The present study aimed to provide information concerning two species with applicability in aquaculture, *Ulva intestinalis* and *Laminaria ochroleuca,* and developing culture methods that can be used as future reference for further studies.

U. intestinalis demonstrated an opportunistic behavior, sporulating when light intensity was low and growing when light exposure increased. The species also seemed to have been influenced by an interaction between light and temperature. Furthermore, phosphate and nitrate consumption were overall high, meaning the species fixated them efficiently. The fast-growth season (spring) was the season with higher growth (≈ 3 times superior to slow-growth season (winter). Protein content decreased in spring and phenolic content was overall higher in winter, which might indicate it was result of seasonality. In the temperature trial, *U. intestinalis* showed and overall higher growth at 16 ºC (although not significantly different) and sporulated the most at 20 ºC, indicating temperature is one of the factors regulating both sporulation and growth rate. In the light and density trial, growth at 200 µmol m^{-2} s^{-1} was overall higher but also caused higher sporulation. The main challenge for *U. intestinalis* cultivation was the prevention of sporulation of the species.

L. ochroleuca juveniles demonstrated susceptibility to increases in light intensity and to temperatures above 30 ªC, the latter having caused heath induced stress that resulted in severe disintegration of the biomass. Despite that, spring was the season with higher growth and, overall, this species grew ≈ 5 times more than *U. intestinalis*. Consumption of nitrate was efficient, occasionally depleting it, which might have limited further growth. Increases in phosphate, ammonia and nitrate in the water supply, overall resulted in significant biomass increase. The main challenge for the cultivation of *L. ochroleuca* was preventing photoinhibition and controlling temperature. Phenolic content was significantly higher in specimens from the wild - in both seasons - and antioxidant activity by ABTS+• decreased in spring. In the temperature trial, no significant differences of the tested temperatures (12, 16 and 20ºC) on growth of *L. ochroleuca* were observed. This was unexpected, as previous studies reported optimum growth between 10-15 ºC. This difference in response to temperature might result from the temperatures this species is exposed to in Northern Portugal, while previous studies have been mostly performed with individuals from higher latitudes. Therefore, the Portuguese strain is expected to sustain higher temperatures than the northern region strains, as shown in previous reports. In the light intensity and density trial, overall, growth did not seem to be affected by the densities and light intensities used.

Future Perspectives

In this study, temperature was tested individually, and light intensity and density were tested as interacting factors, since density affects the incidence of light on the tissue. However, light requirements and optimums also vary with temperature and, as such, testing this interaction would be interesting for both species. Additionally, in the *Ulva intestinalis* trials, the main difficulty was to prevent sporulation. Since one of the factors known to influence sporulation is salinity, a good follow up trial would be testing the effects of different salinities on these species, under the optimum temperature and light intensities defined in the previously proposed follow up trial.

For the biochemical analysis, it would be interesting to make an analysis of biomass year-round, with samples of biomass collected more frequently from the wild and from tank cultivation, in order to better understand the seasonality of the species and also if tank cultivation has an effect on it.

In an outdoor cultivation system, density should be maintained at an optimum, nutrient availability should never be limiting and water renovation should be adequate for elimination of metabolites and compensating the effect of evaporation, maintaining salinity constant. As such, a better control of temperature – for example, with increment in water renovation - would be important, especially for warmer days. Additionally, in future trials, it would also be advisable to include more replicates to avoid big standard deviation values and allow more reliable results. Also, as nutrient intake was not a focus in this study – nutrients were provided to guarantee that they were not limitative for growth, in future works it would be interesting to analyze the nutrient necessities of both seaweeds, as well as the N:P ratio, since it would be useful information for cultivating the species in monocultures.

For aquaculture application, a potential approach to growing *U. intestinalis* would be to firstly induce the sporulation of the species, in order to obtain spores for cultivation. Afterwards, testing if the species would grow more efficiently free floating or sown on a surface (ropes or sheets) would be a good follow up. Moreover, the obtained spores would then develop in a low salinity environment, around 15-20 psu, to drive vegetative growth while avoiding sporulation.

As for *Laminaria ochroleuca*, in all trials, individuals were free-floating. It could, however, be more efficient to have them grow in the bottom of the tank, decreasing photoinhibition. Therefore, in future experiments, sowing spores or gametophytes onto a surface or tying them to a rope might be more favorable. For industrial production of this species, it would be more adequate to have an initial nursery of recruits, obtained

from spores released from mature tissues, with a lower light exposure, as young sporophytes are more sensitive than adults. At a later stage, the juveniles obtained previously would be placed in a tank system where they would develop further, until meeting market needs.

For both species, in the outdoor trials, nutrient solutions were used, this, however, is not the most economically viable method for production of algae at an industrial scale. Nevertheless, this method may be regarded as a proxy for the incorporation of these species in integrated multi-trophic aquaculture (IMTA) systems, which would be much more cost effective. In IMTA systems, fed aquaculture (finfish or shrimp) is integrated with inorganic (seaweed) and organic (shellfish) aquaculture in order to create a balanced ecosystem with nutrient bioremediation capability and mutual benefits for the species (Chopin *et al.*, 2001). In trials, on both seasons, *Ulva intestinalis* and *L. ochroleuca* seemed to utilize nutrients differently, fixating them more efficiently at different time points. Ashkenazi *et al.* (2018) conducted a study, with an innovative experimental setting, that focused on integrating three marine macroalgae species (*Ulva rigida, Gracilaria conferta, Hypnea musciformis*) serially connected via two-stage seaweed culture tanks to a finfish culture, assessing and comparing the biofiltration and growth performance of the seaweeds. The design did not inhibit the performance of the individual species and improved overall productivity. Similarly, it may be advantageous to use *U. intestinalis* and *L. ochroleuca* together in an IMTA system, since, by using nutrients differently, they might complement each other, resulting in a system with a higher and more stable water quality. As such, future experiments to test how they interact in an IMTA system would be a good follow up to the work developed in this project. Additionally, analysis of composition and bioactivity should be conducted for seaweeds grown in IMTA and in a monoculture in the same time frame, to test possible differences created by the different growing conditions and determine the best approach to achieve the best compromise between value of the product and cost of production.

Overall, the obtained results emphasize the potential for aquaculture of both species and also highlight the need for additional studies, in order to better understand the conditions they require for growth, develop methods able to replicate such conditions in a cultivation system and determine the best timing for harvest, according to different objectives (for instance, biomass, bioactivity or protein content).

References

Abdul Khalil, H. P. S., Saurabh, C. K., Tye, Y. Y., Lai, T. K., Easa, A. M., Rosamah, E., Banerjee, A. (2017). Seaweed based sustainable films and composites for food and pharmaceutical applications: A review. *Renewable and Sustainable Energy Reviews*, *77*(April), 353–362.

Abe, K., Hattori, H., & Hirano, M. (2007). Accumulation and antioxidant activity of secondary carotenoids in the aerial microalga *Coelastrella striolata* var. *multistriata*. *Food Chemistry*, *100*(2), 656–661.

Adams, J. M., Gallagher, J. A., & Donnison, I. S. (2009). Fermentation study on *Saccharina latissima* for bioethanol production considering variable pre-treatments. *Journal of applied Phycology*, 21(5), 569–574.

Aguilera-Morales, M., Casas-Valdez, M., Carrillo-Domínguez, S., González-Acosta, B., & Pérez-Gil, F. (2005). Chemical composition and microbiological assays of marine algae *Enteromorpha* spp. as a potential food source. *Journal of Food Composition and Analysis*, 18(1), 79-88.

Aizawa, M., Asaoka, K., Atsumi, M., & Sakou, T. (2007). Seaweed Bioethanol Production in Japan - The Ocean Sunrise Project, 1–5. Aizawa, (2007, September). In *OCEANS 2007* (pp. 1-5). IEEE.

Akköz, C., Arslan, D., Ünver, A., Özcan, M. M., & Yilmaz, B. (2011). Chemical composition, total phenolic and mineral contents of *Enteromorpha intestinalis* (l.) kütz. and *Cladophora glomerata* (l.) kütz. seaweeds. *Journal of Food Biochemistry*, *35*(2), 513–523.

Alström-Rapaport, C., Leskinen, E., & Pamilo, P. (2010). Seasonal variation in the mode of reproduction of Ulva intestinalis in a brackish water environment. *Aquatic Botany*, *93*(4), 244–249.

Alvarado-morales, M., Boldrin, A., Karakashev, D. B., Holdt, S. L., Angelidaki, I., & Astrup, T. (2013). Life cycle assessment of biofuel production from brown seaweed in Nordic conditions. *BIORESOURCE TECHNOLOGY*, *129*, 92–99.

Angel, A., & Ojeda, F. P. (2001). Structure and trophic organization of subtidal fish assemblages on the northern Chilean coast : the effect of habitat complexity. *Marine Ecology Progress Series, 217*, 81–91.

Anis, M., Ahmed, S., & Hasan, M. M. (2017). Algae As Nutrition, Medicine and Cosmetic: the Forgotten History, Present Status and Future Trends. *World Journal of Pharmacy and Pharmaceutical Sciences*, (June), 1934–1959.

Araújo, R., Sousa-Pinto, I., Bárbara, I., & Quintino, V. (2006). Macroalgal communities

of intertidal rock pools in the northwest coast of Portugal. *Acta Oecologica*, *30*, 192–202.

Aresta, M., Dibenedetto, A., Carone, M., Colonna, T., & Fragale, C. (2005). Production of biodiesel from macroalgae by supercritical CO 2 extraction and thermochemical liquefaction. *Environmental Chemistry Letters*, 136–139.

Ashkenazi, D. Y., Israel, A., & Abelson, A. (2018). A novel two-stage seaweed integrated multi-trophic aquaculture. *Reviews in Aquaculture*, 1–17.

Attwood, A. I. (1989). Calcium alginate dressing accelerates split skin graft donor site healing. *Ournal of Plastic, Reconstructive & Aesthetic Surgery*, 42(4), 373-379.

Bakoš, D., Soldán, M., & Hernández-Fuentes, I. (1999). Hydroxyapatite-collagen-hyaluronic acid composite. *Biomaterials*, *20*(2), 191–195.

Balboa, E. M., Soto, M. L., Nogueira, D. R., González-López, N., Conde, E., Moure, A., Domínguez, H. (2014). Potential of antioxidant extracts produced by aqueous processing of renewable resources for the formulation of cosmetics. *Industrial Crops and Products*, *58*, 104–110.

Barbosa, M., Fernandes, F., Pereira, D. M., Azevedo, I. C., Sousa-Pinto, I., Andrade, P. B., & Valentão, P. (2017). Fatty acid patterns of the kelps *Saccharina latissima*, *Saccorhiza polyschides* and *Laminaria ochroleuca*: Influence of changing environmental conditions. *Arabian Journal of Chemistry*.

Birkett, D. A., Maggs, C. A., Dring, M. J., Boaden, P. J. S., & Seed, R. (1998). Infralittoral reef biotopes with kelp species. *Vol VII An Overview of Dynamic and Sensitivity Characteristics for Conservation Management of Marine SACs, 174.*

Birkett, DA, CA Maggs, MJ Dring, PJS Boaden, and R. S. (1998). Infralittoral reef biotopes with kelp species. *Vol VII An Overview of Dynamic and Sensitivity Characteristics for Conservation Management of Marine SACs, 174.*, *VII*(August), 1–174.

Bliding, C. (1963). A critical survey of European taxa in Ulvales. Opera Bot. 8, 5–160.

Blomster, J., Hoey, E. M., Maggs, C. A., & Stanhope, M. J. (2000). Species-specific oligonucleotide probes for macroalgae: molecular discrimination of two marine fouling species of Enteromorpha (Ulvophyceae). *Molecular Ecology*, *9*(2), 177–186.

Bodkin, J. L. (1988). Effects of kelp forest removal on associated fish assemblages in central California. *Journal of Experimental Marine Biology and Ecology, 117*, 227–238.

Bonneville, M., Saint-Mezard, P., Benetiere, J., Hennino, A., Pernet, I., Denis, A., & Nicolas, N. F. (2007). *Laminaria ochroleuca* extract reduces skin inflammation [14]. *Journal of the European Academy of Dermatology and Venereology*, 21(8), 1124-1125.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, (72(1-2)), 248–254.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. L. W. T. (1995). Use of a free radical method to evaluate antioxidant activity. LWT-Food science and Technology. *LWT-Food Science and Technology*, (28(1)), 25–30.

Braud, J. (1974). Etude de quelques parametres écologiques, biologiques et biochimiques chez une phéophycée des côtes bretonnes *Laminaria ochroleuca*. *Revue des Travaux de l'Institut des Pêches Maritimes, 38(2).*

Buchholz, C., & Lüning, K. (1999). Isolated, distal blade discs of the brown alga *Laminaria digitata* form sorus, but not discs, near to the meristematic transition zone. *Journal of Applied Phycology*, *11*(6), 579–584.

Cabral, J. P. (2005). A apanha de algas na ilha da Ínsua (Caminha) nos séculos XVII-XIX. Singularidades e conflitos. *Finisterra*, *40(80)*.

CBI – Ministry of Foreign Affairs. (n.d.) Retrieved December 2017 from https://www.Cbi.Eu/Sites/Default/Files/Market_information/Researches/Product-Factsheet-Europe-Alginates-2015.Pdf

Chan, J. C. C., Cheung, P. C. K., & Ang, P. O. (1997). Comparative studies on the effect of three drying methods on the nutritional composition of seaweed *Sargassum hemiphyllum* (Turn) C Ag. *Journal of Agricultural and Food Chemistry*, *45*(8), 3056–3059.

Cheng, Y., Dong, Y., Wang, J., Dong, M., Zou, Y., Ren, D., & Liu, W. (2010). Moisturizing and anti-sebum secretion effects of cosmetic application on human facial skin. I*nternational Journal of Cosmetic Science*, 32(1) , 81–83.

Chopin, T., Buschmann, A. H., Halling, C., Troell, M., Kautsky, N., Neori, A., Neefus, C. (2001). Integrating seaweeds into marine aquaculture systems: A key toward sustainability. *Journal of Phycology*, *37*(6), 975–986.

Coelho, S. M., Peters, A. F., Charrier, B., Roze, D., Destombe, C., Valero, M., & Cock, J. M. (2007). Complex life cycles of multicellular eukaryotes: New approaches based on the use of model organisms. *Gene*, *406(1),* 152-170.

Davidson, A. (1999). Kombu.

Davison, I. R., & Pearson, G. A. (1996). Stress tolerance in intertidal seaweeds. *Journal of Phycology*, *32*(2), 197–211.

Dayton, P. K. (1985). Ecology of Kelp Communities. *Annual Review of Ecology and Systematics*, *16*(1), 215–245.

Dean, T., & Jacobsen, F. (1984). Growth of juvenile *Macrocystis pyrifera* (Laminariales) in relation to environmental factors. *Marine Biology*, *311*, 301–311.

DeLucca, A. J., Connick, W. J., Fravel, D. R., Lewis, J. A., & Bland, J. M. (1990). The use of bacterial alginates to prepare biocontrol formulations. *Journal of industrial microbiology, 6(2)*, 129-134.

Dhargalkar, V. K., & Pereira, N. (2005). Seaweed : Promising Plant of the Millennium. *Source*, *71*(Table 2), 60–66.

Dos, B., Esteves, S., & Suzuki, M. S. (2008). Efeito Da Salinidade Sobre As Plantas. *Oecol. Bras*, *12*(4), 662–679.

Duggins, D. O., Simenstad, C. A., & Estes, J. A. (1989). Magnification of Secondary Production by Kelp Detritus in Coastal Marine Ecosystems. *Science, 245(4914)*, 170-173.

Düwel, L. (2001). Experimental studies on macroalgae along the salinity gradient in the Baltic Sea Area. PhD thesis, University of Copenhagen, Copenhagen.

Engelen, A. H., Espirito-Santo, C., Simões, T., Monteiro, C., Serrão, E. A., Pearson, G. A., & Santos, R. O. P. (2008). Periodicity of propagule expulsion and settlement in the competing native and invasive brown seaweeds, *Cystoseira humilis* and *Sargassum muticum* (Phaeophyta). *European Journal of Phycology*, *43*(3), 275–282.

Erlandson, J. M., Graham, M. H., Bourque, B. J., Corbett, D., Estes, J. A., & Steneck, R. S. (2007). The Kelp Highway Hypothesis : Marine Ecology , the Coastal Migration Theory , and the Peopling of the Americas. *The Journal of Island and Coastal Archaeology, 2(2)*, 37–41.

FAO (2004). Fisheries and Aquaculture topics. The State of World Fisheries and Aquaculture (SOFIA). Topics Fact Sheets. In: FAO Fisheries and Aquaculture Department [online]. Rome. [Cited December 2017] http://www.fao.org/fishery/

FAO (2018). Fisheries and Aquaculture topics. The State of World Fisheries and Aquaculture (SOFIA). Topics Fact Sheets. Text by Jean- Francois Pulvenis. In: FAO Fisheries and Aquaculture Department [online]. Rome. [Cited September 2018]. http://www.fao.org/fishery/

Folin, O., & Ciocalteu, V. (1927). On tyrosine and tryptophane determinations in proteins. Journal of biological chemistry. *Journal of Biological Chemistry*, (73(2)), 627–650.

Fong, P., Boyer, K. E., & Zedler, J. B. (1998). Developing an indicator of nutrient enrichment in coastal estuaries and lagoons using tissue nitrogen content of the opportunistic alga, *Enteromorpha intestinalis* (L. Link). *Journal of Experimental Marine Biology and Ecology*, *231*(1), 63–79.

Fredriksen, S. (2003). Food web studies in a Norwegian kelp forest based on stable isotope (δ 13 C and δ 15 N) analysis. *Marine Ecology Progress Series, 260,* 71-81.

Gallardo, T., Cobelas, M. A., Meneses, A. A. De, & Brasil, A. (1990). Current state of seaweed resources in Spain. *Hydrobiologia, 204(1)*, 287-292.

Guedes, A. C., Amaro, H. M., Gião, M. S., & Malcata, F. X. (2013). Optimization of ABTS radical cation assay specifically for determination of antioxidant capacity of intracellular extracts of microalgae and cyanobacteria. *Food Chemistry*, (138), 638–643.

Gittins, L. (1966). The manufacture of alkali in Britain, 1779–1789. *Annals of science, 22(3)*, *175*.

Gómez, I., & Huovinen, P. (2011). Morpho-functional patterns and zonation of south chilean seaweeds: The importance of photosynthetic and bio-optical traits. *Marine Ecology Progress Series*, *422*, 77–91.

Graham, M. H. (2004). Effects of Local Deforestation on the Diversity and Structure of Southern California Giant Kelp Forest Food Webs. *Ecosystems, 7*(4), 341–357.

Hale, R. W., & Pion, R. J. (1972). Laminaria: An Underutilized Clinical Adjunct. *Clinical Obstetrics and Gynecology*, *15*(3), 829–850.

Hansen, H. R., Hector, B. L., & Feldmann, J. (2003). A qualitative and quantitative evaluation of the seaweed diet of North Ronaldsay sheep. *Animal Feed Science and Technology*, *105*(1–4), 21–28.

Hargreaves J. A., T. C. S. (2004). Managing ammonia in fish pond. *SRAC Publication - Southern Regional Aquaculture Center*, (4608), 8.

Haroon, A. M., Szaniawska, A., Normant, M., & Janas, U. (2000). The biochemical composition of *Enteromorpha* spp. from the Gulf of Gdansk coast on the southern Baltic Sea. *Oceanologia*, *42*(1), 19–28.

Harrison, P. J., & Hurd, C. L. (2001). Nutrient physiology of seaweeds: Application of concepts to aquaculture. *Cahiers de Biologie Marine*, *42*(1–2), 71–82.

Hellio, C., Bremer, G., Pons, A. M., Gal, Y. Le, Bourgougnon, N., Rochelle, Â. D. La, & Rochelle, Â. D. La. (2000). Inhibition of the development of microorganisms (bacteria and fungi) by extracts of marine algae from Brittany, France. *Applied Microbiology and Biotechnology, 54(4)*, 543-549.

Hernandez, I., Peralta, G., Perez-Llorens, J. L., Vergara, J. J., & Niell, F. X. (1997). Biomass and growth dynamics of *Ulva* species in Palmones River estuary. *Journal of Phycology*, *33*, 764–772.

Hou, X., Hansen, J. H., & Bjerre, A. (2015). Integrated bioethanol and protein production from brown seaweed Laminaria digitata. *Bioresource Technology*, *197*, 310–317.

Ibañez, E., Herrero, M., Mendiola, J. A., & Castro-Puyana, M. (2012). Extraction and Characterization of Bioactive Compounds with Health Benefits from Marine Resources: Macro and Micro Algae, Cyanobacteria, and Invertebrates. In *Marine*

Bioactive Compounds (Vol. 9781461412, pp. 55–98). Boston, MA: Springer US.

Indergaard, Mentz, & Minsaas, J. S. (1991). Animal and human nutrition.

Jeong, H. Ã., Lee, S. Ã., Moon, P. Ã., Na, H., Park, R., Um, J., Hong, S. Ã. (2006). Alginic acid has anti-anaphylactic effects and inhibits inflammatory cytokine expression via suppression of nuclear factor- j B activation Clinical and Experimental Allergy, 785–794.

Jung, K. A., Lim, S.-R., Kim, Y., & Park, J. M. (2017). Opportunity and challenge of seaweed bioethanol based on life cycle CO 2 assessment. *Environmental Progress & Sustainable Energy*, *36*(1), 200–207.

Kadam, S. U., Donnell, C. P. O., Rai, D. K., Hossain, M. B., Burgess, C. M., Walsh, D., & Tiwari, B. K. (2015). Laminarin from Irish Brown Seaweeds *Ascophyllum nodosum* and *Laminaria hyperborea*: Ultrasound Assisted Extraction, Characterization and Bioactivity. *Marine drugs, 13(7),* 4270-4280.

Kamer, K., & Fong, P. (2001). Nitrogen enrichment ameliorates the negative effects of reduced salinity on the green macroalga *Enteromorpha intestinalis*. *Marine Ecology Progress Series, 218,* 87-93.

Kim, J. K., Yarish, C., Hwang, E. K., Park, M., & Kim, Y. (2017). Seaweed aquaculture: Cultivation technologies, challenges and its ecosystem services. *Algae*.

Kim, K., & Lee, I. (1996). The germling growth of *Enteromorpha intestinalis* (Chlorophyta) in laboratory culture under different combinations of irradiance and salinity and temperature and. *Phycologia*, *35*(4), 327–331.

Kılınç, B., Cirik, S., & Turan, G. (2013). Seaweeds for Food and Industrial Applications. In *Food Industry*. InTech.

Kolb, N., Vallorani, L., Milanović, N., & Stocchi, V. (2004). Evaluation of Marine Algae Wakame (*Undaria pinnatifida*) and Kombu *(Laminaria digitata japonica*) as Food Supplements. *Food Technology and Biotechnology*, *42*(1), 57–61.

Larsen, J. (1981). Crossing experiments with *Enteromorpha intestinalis* and *E. compressa* from different European localities. *Nordic Journal of Botany*, *1*(1), 128–136.

Lin, A., Shen, S., Wang, J., & Yan, B. (2008). Reproduction diversity of *Enteromorpha prolifera*. *Journal of Integrative Plant Biology*, *50*(5), 622–629.

Lüning, K. (1980). Critical levels of light and temperature regulating the gametogeneiss of three *Laminaria* species (Phaeophyceae). *Journal of Phycology*, *16*(1), 1–15.

Lüning, K. (1984). Temperature tolerance and biogeography of seaweeds: The marine algal flora of Helgoland (North Sea) as an example. *Helgoländer Meeresuntersuchungen*, *38*(2), 305–317.

Madhusudan, C., Manoj, S., Rahul, K., & Rishi, C. M. (2011). Seaweeds: A diet with

nutritional, medicinal and industrial value. *Research Journal of Medicinal Plant*, (5(2)), 153–157.

Makarenkova, I. D., Deriabin, P. G., L'vov, D. K., Zviagintseva, T. N., Besednova, N. N. (2010). Antiviral activity of sulfated polysaccharide from the brown algae *Laminaria japonica* against avian influenza A (H5N1) virus infection in the cultured cells. *Vopr. Virusol*, *55*, 41–45.

Mamatha, B. S., Namitha, K. K., Senthil, A., Smitha, J., & Ravishankar, G. A. (2007). Studies on use of Enteromorpha in snack food. *Food Chemistry, 101(4)*, 1707-1713.

Mann, K. (1973). Seaweeds: Their Productivity and Strategy for Growth. *Science*, *182(4116),*.

Martins, I., Oliveira, J. M., Flindt, M. R., & Marques, J. C. (1999). The effect of salinity on the growth rate of the macroalgae *Enteromorpha intestinalis* (Chlorophyta) in the Mondego estuary (west Portugal). *Acta Oecologica*, *20*(4), 259–265.

Matanjun, P., & Muhammad, K., Mustapha, N. M. (2010). Comparison of cardiovascular protective effects of tropical seaweeds, *Kappaphycus alvarezii*, *Caulerpa lentillifera*, and *Sargassum polycystum*, on high-cholesterol/high-fat diet in rats. *Journal of medicinal food, 13(4)*, 792-800.

McHugh, D. J. (2003). A guide to the seaweed industry (pp. 12-22). Rome: Food and Agriculture Organization of the United Nations.

Morganti, P., Cardillo, A., & Ciotto, P. Del. (2002). Role of topical and nutritional supplement to modify the oxidative stress. *International journal of cosmetic science, 24(6)*, 331-339.

Müller, R., Laepple, T., Bartsch, I., & Wiencke, C. (2009). Impact of oceanic warming on the distribution of seaweeds in polar and cold-temperate waters. *Botanica Marina*, *52*(6), 617–638.

Nakahara, H., & Nakamura, Y. (1973). Parthenogenesis , Apogamy and Apospory in *Alaria crassifolia* (Laminariales). *Marine Biology, 18(4),* 327–332.

Neto, A. I., Titley, I., & Raposeiro, P. (2005). Flora marinha do Litoral Dos Açores. Secretaria Regional do Ambiente e do Mar.

Newton, G. (1951). Seaweed Utilization Sampson Low. *London*, *pp188*.

Oomizu, S., Yanase, Y., Suzuki, H., Kameyoshi, Y., & Hide, M. (2006). Fucoidan prevents C e germline transcription and NF j B p52 translocation for IgE production in B cells. *Biochemical and biophysical research communications, 350(3),* 501–507.

Paiva, L., Lima, E., Patarra, R. F., Neto, A. I., & Baptista, J. (2014). Edible Azorean macroalgae as source of rich nutrients with impact on human health. *Food Chemistry*, *164*, 128–135.

Paul, W., & Sharma, C. (2004). Chitosan and alginate wound dressings: a short review.

Trends Biomater Artif Organs, *18*(1), 18–23.

Pearson, G. A., Lago-leston, A., & Mota, C. (2009). Frayed at the edges : selective pressure and adaptive response to abiotic stressors are mismatched in low diversity edge populations. *Journal of Ecology, 97(3)*, 450–462.

Pereira, Tânia R, Aschwin H Engelen, Gareth A Pearson, Ester A Serrào, Christophe Destombe, and M. V. (2011). 'Temperature effects on the microscopic haploid stage development of *Laminaria ochroleuca* and *Sacchoriza polyschides*, kelps with contrasting life histories. *CBM-Cahiers de Biologie Marine, 52(4),* 395.

Pereira, L. (2011). A review of the nutrient composition of selected edible seaweeds. *Seaweed: Ecology, nutrient composition and medicinal uses,* 15-47.

Pereira, L., Gheda, S. F., & Ribeiro-claro, P. J. A. (2013). Analysis by Vibrational Spectroscopy of Seaweed Polysaccharides with Potential Use in Food , Pharmaceutical and Cosmetic Industries, *2013*(vi), 1–8.

Pereira, T. R., Azevedo, I. C., Oliveira, P., Silva, D. M., & Sousa-Pinto, I. (2019). Life history traits of *Laminaria ochroleuca* in Portugal : The range-center of its geographical distribution. *Aquatic Botany*, *152*(September 2017), 1–9.

Pereira, T. R., Engelen, A. H., Pearson, G. A., Valero, M., & Serrão, E. A. (2015). Response of kelps from different latitudes to consecutive heat shock. *Journal of Experimental Marine Biology and Ecology*, *463*, 57–62.

Ping, C., Chen, P., & Xie, P. (1999). History and development of traditional Chinese medicine (Vol. I). *IOS Press*.

Prud'homme, V. R., & Trono, G. C. (2001). Plant resources of South-East Asia No. 15 (1): *Cryptogams: Algae. Backhuys Publishers.*

Reddy, B. S., Sharma, C., and Mathews, L. (1984). (1984). Effect of Japanese seaweed (*Laminaria angustata*) extracts on the mutagenicity of 7, 12-dimethylbenz [a] anthracene, a breast carcinogen, and of 3, 2′-dimethyl-4-aminobiphenyl, a colon and breast carcinogen. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 127(2),* 113-118.

Reddy, B. S., Numoto, S., & Choi, C. I. (1985). Effect of dietary *Laminaria angustata* (brown seaweed) on azoxymethane - induced intestinal carcinogenesis in male F344 rats.

Rey-Crespo, F., López-Alonso, M., & Miranda, M. (2014). The use of seaweed from the Galician coast as a mineral supplement in organic dairy cattle. *Animal*, *8*(4), 580–586.

Roleda, M. Y., Hanelt, D., Kräbs, G., & Wiencke, C. (2004). Morphology, growth, photosynthesis and pigments in *Laminaria ochroleuca* (Laminariales, Phaeophyta) under ultraviolet radiation. *Phycologia*, *43*(5), 603–613.

Ruangchuay, R., Lueangthuwapranit, C., & Pianthumdee, N. (2007). Apparent characteristics and taxonomic study of macroalgae in Pattani Bay. Songklanakarin Journal of Science and Technology, 29(4), 893-905.

Ruangchuay, R., Dahamat, S., Chirapat, A., & Notoya, M. (2012). Effects of culture conditions on the growth and reproduction of Gut Weed , *Ulva intestinalis* Linnaeus (Ulvales, Chlorophyta). *Songklankarin Journal of Science and Technology*, *34*(5), 501–507.

Saffo, M. B. (1987). Light on Seaweeds Recent studies have forced reassessment of the role of light- harvesting pigments in depth zonation of seaweeds. *Sciences-New York*, *37*(9), 654–664.

Saha, D., & Bhattacharya, S. (2010). Hydrocolloids as thickening and gelling agents in food: A critical review. *Journal of Food Science and Technology*, *47*(6), 587–597.

Sánchez-Machado, D. I., López-Cervantes, J., López-Hernández, J., & Paseiro-Losada, P. (2004). Fatty acids, total lipid, protein and ash contents of processed edible seaweeds. *Food Chemistry*, *85*(3), 439–444.

Shiratori, K., Ohgami, K., Ilieva, I., Jin, X., & Koyama, Y. (2005). Effects of fucoxanthin on lipopolysaccharide-induced inflammation in vitro and in vivo. *Experimental eye research, 81(4)*, 422–428.

Shirosaki, M., & Koyama, T. (2011). *Chapter 15 - Laminaria japonica as a Food for the Prevention of Obesity and Diabetes*. *Marine Medicinal Foods* (1st ed., Vol. 64). Elsevier Inc.

Sousa-Pinto. (1998). Seaweed resources of Portugal. In *Seaweed Resources of the World* (Ed Ohno & Critchley), JICA, Yokosuka. pp176-185.The seaweed resources of Portugal.

Sousa, A. I., Martins, I., Lillebø, A. I., Flindt, M. R., & Pardal, M. A. (2007). Influence of salinity, nutrients and light on the germination and growth of *Enteromorpha* sp. spores. *Journal of Experimental Marine Biology and Ecology*, *341*(1), 142–150.

Staehr, P. A., & Wernberg, T. (2009). Physiological responses of *Ecklonia radiata* (laminariales) to a latitudinal gradient in ocean temperature. *Journal of Phycology*, *45*(1), 91–99.

Steneck, R. S., Graham, M. H., Bourque, B. J., Erlandson, J. O. N. M., Estes, J. A., & Tegner, M. I. A. J. (2002). Kelp forest ecosystems : biodiversity , stability , resilience and future, *29*(4), 436–459.

Terpinc, P., Čeh, B., Ulrih, N. P., & Abramovič, H. (2012). Studies of the correlation between antioxidant properties and the total phenolic content of different oil cake extracts. *Industrial Crops and Products*, *39*(1), 210–217.

Thornber, C. S. (2006). Functional properties of the isomorphic biphasic algal life cycle.

Integrative and Comparative Biology, *46*(5), 605–614.

tom Dieck (Bartsch), I. (1992). North Pacific and North Atlantic digitate *Laminaria* species (Phaeophyta): hybridization experiments and temperature responses. *Phycologia, 31(2),* 147-163.

tom Dieck (Bartsch), I., & de Oliveira, E. C. (1993). The section Digitatae of the genus *Laminaria* (Phaeophyta) in the northern and southern Atlantic: crossing experiments and temperature responses. *Marine Biology*, *115*(1), 151–160.

Trigui, M., Gasmi, L., Zouari, I., & Tounsi, S. (2013). Seasonal variation in phenolic composition, antibacterial and antioxidant activities of *Ulva rigida* (Chlorophyta) and assessment of antiacetylcholinesterase potential. *Journal of Applied Phycology*, *25*(1), 319–328.

Tsagkamilis, P., Danielidis, D., Dring, M. J., & Katsaros, C. (2010). Removal of phosphate by the green seaweed *Ulva lactuca* in a small-scale sewage treatment plant (Ios Island, Aegean Sea, Greece). *Journal of Applied Phycology, 22(3)*, 331–339.

Turumtay, E. A., Islamoğlu, F., Çavuş, D., Şahin, H., Turumtay, H., & Vanholme, B. (2014). Correlation between phenolic compounds and antioxidant activity of Anzer tea (Thymus praecox Opiz subsp. caucasicus var. caucasicus). *Industrial Crops and Products*, *52*, 687–694.

Vázquez-Freire, M. J., Lamela, M., & Calleja, J. M. (1994). *Laminaria ochroleuca*: A preliminary study of its effect on the central nervous system. *Phytotherapy Research*, *8*(7), 422–425.

Vergara, J. J., Pérez-Lloréns, J. L., Peralta, G., Hernández, I., & Niell, F. X. (1997). Seasonal variation of photosynthetic performance and light attenuation in *Ulva* canopies from Palmones river estuary. *Journal of Phycology*, *33*(5), 764–772.

Verkleij, F. N. (1992). Seaweed extracts in agriculture and horticulture: A review. *Biological Agriculture and Horticulture*, *8*(4), 309–324.

Vivekanand, V., & Eijsink, V. G. H. (2012). Biogas production from the brown seaweed *Saccharina latissima* : thermal pretreatment and codigestion with wheat straw. *Journal of Applied Phycology, 24(5),* 1295–1301.

Wang, H., Lin, A., Gu, W., Huan, L., Gao, S., & Wang, G. (2016). The sporulation of the green alga *Ulva prolifera* is controlled by changes in photosynthetic electron transport chain. *Scientific Reports*, *6*(July 2015), 1–9.

Wang, H. M. D., Chen, C. C., Huynh, P., & Chang, J. S. (2015). Exploring the potential of using algae in cosmetics. *Bioresource Technology*, *184*, 355-362.

Wang, H. M., Pan, J. L., Chen, C. Y., Chiu, C. C., Yang, M. H., Chang, H. W., & Chang, J. S. (2010). Identification of anti-lung cancer extract from *Chlorella vulgaris* C-C by

antioxidant property using supercritical carbon dioxide extraction. *Process Biochemistry*, *45*(12), 1865–1872.

Wang, J., Jin, W., Hou, Y., Niu, X., Zhang, H., & Zhang, Q. (2013). Chemical composition and moisture-absorption/retention ability of polysaccharides extracted from five algae. *International Journal of Biological Macromolecules*, *57*, 26–29.

Watanabe, F., Takenaka, S., Katsura, H., Masumder, S. A. M. Z. H., Abe, K., Tamura, Y., & Nakano, Y. (1999). Dried green and purple lavers (Nori) contain substantial amounts of biologically active vitamin B12 but less of dietary iodine relative to other edible seaweeds. *Journal of Agricultural and Food Chemistry*, *47*(6), 2341–2343.

Wells, M. L., Potin, P., Craigie, J. S., Raven, J. A., Merchant, S. S., Helliwell, K. E., Brawley, S. H. (2016). Algae as nutritional and functional food sources : revisiting our understanding. *Journal of Applied Phycology*, *29(2),* 949-982.

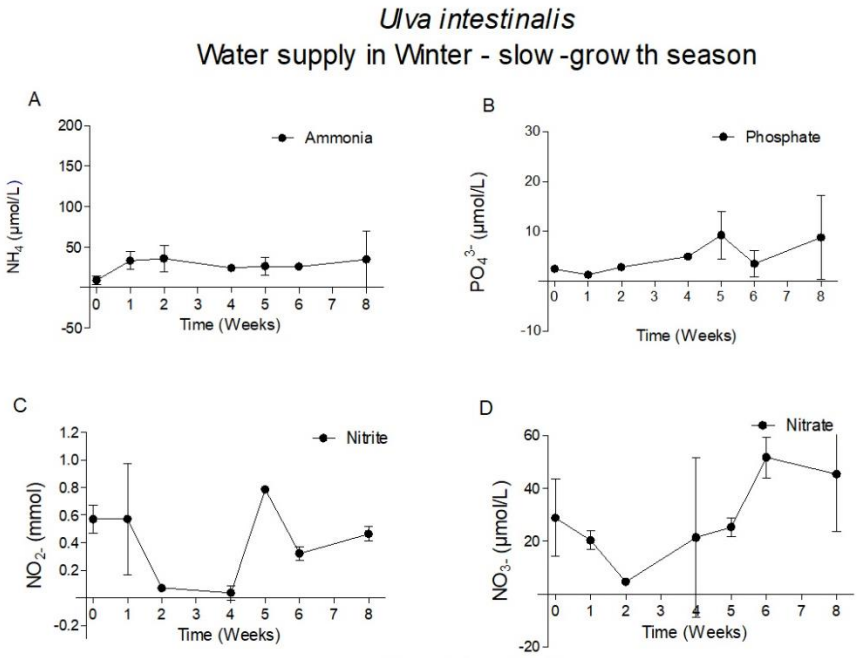
Wernberg, T., Thomsen, M. S., Tuya, F., Kendrick, G. A., Staehr, P. A., & Toohey, B. D. (2010). Decreasing resilience of kelp beds along a latitudinal temperature gradient: Potential implications for a warmer future. *Ecology Letters*, *13*(6), 685–694.

Werner, A., Clarke, C., & Kraan, S. (2004). *Strategic review of the feasibility of seaweed aquaculture in Ireland. NDP Marine RTDI Desk Study Series*. DK/01/008.

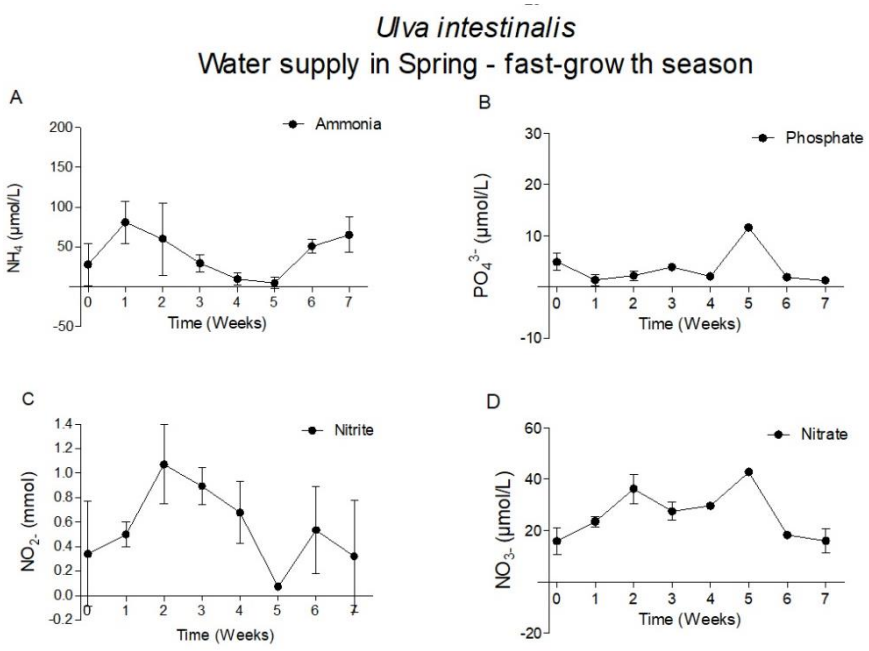
Yamamoto, I., & Maruyama, H. (1985). Effect of dietary seaweed preparations on 1,2-dimethylhydrazine-induced intestinal carcinogenesis in rats. *Cancer Letters*, *26*, 241–251.

Annex

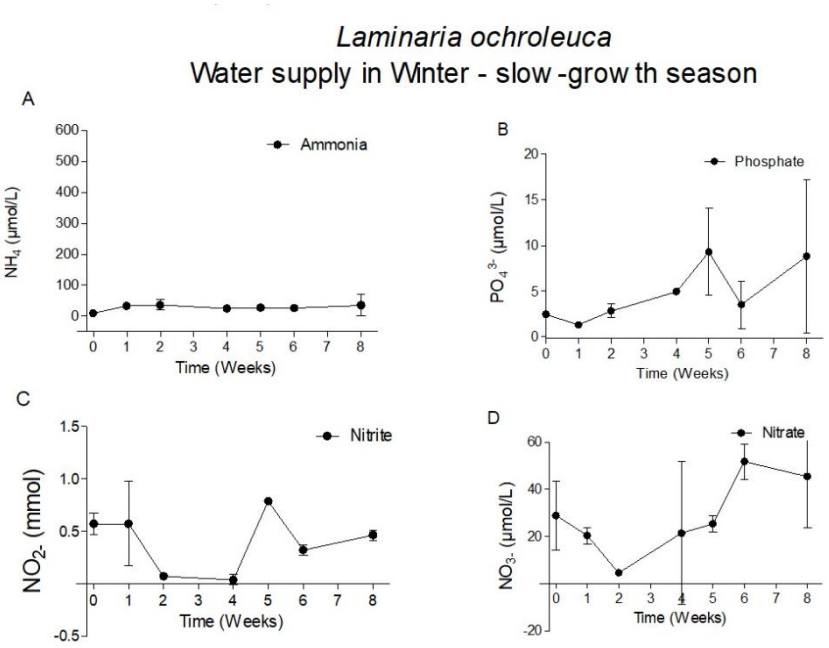
A



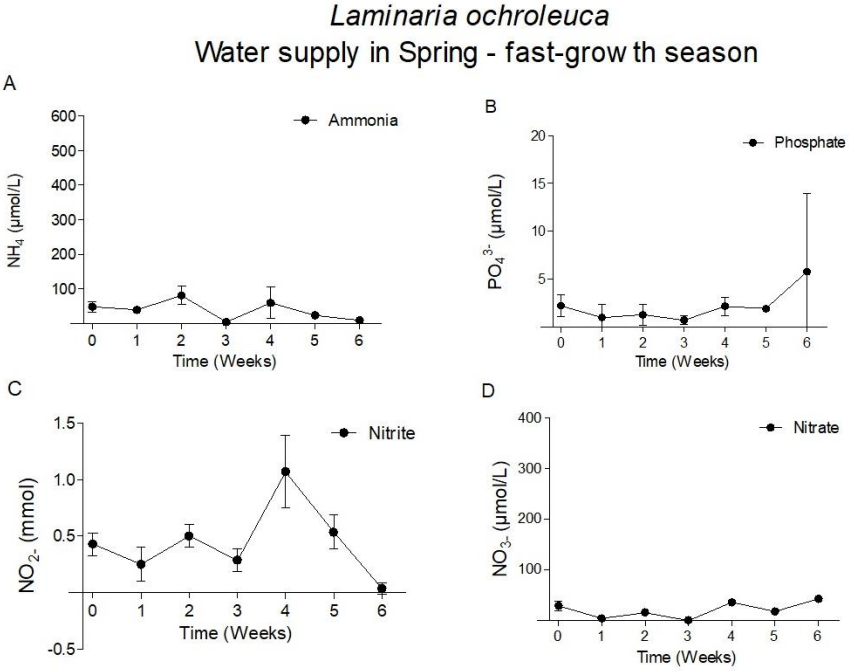
Supplementary Fig. 1 - Nutrient content (ammonia, phosphate, nitrite and nitrate (µmol/L)) in water supply of outdoor trials of *Ulva intestinalis* in Winter - slow-growth trial



Supplementary Fig. 2 - Nutrient content (ammonia, phosphate, nitrite and nitrate (µmol/L)) in water supply of outdoor trials of *Ulva intestinalis* in Spring - fast-growth trial



Supplementary Fig. 3 - Nutrient content (ammonia, phosphate, nitrite and nitrate (µmol/L)) in water supply of outdoor trials of *Laminaria ochroleuca* in Winter - slow-growth trial



Supplementary Fig. 4 - Nutrient content (ammonia, phosphate, nitrite and nitrate (µmol/L)) in water supply of outdoor trials of *Laminaria ochroleuca* in Spring - fast-growth trial