U.PORTO

MESTRADO CIÊNCIAS DO MAR- RECURSOS MARINHOS

ESPECIALIZAÇÃO EM AQUACULTURA E PESCAS

Internship at ALGAplus, Ltd: Production of conchocelis

Inês Santos Oliveira

M

2021

U.PORTO

Inês Santos Oliveira. Internship at ALGAplus, Ltd: Production of conchocelis

M.ICBAS **2021**

Internship at ALGAplus, Ltd: Production of conchocelis

Inês Santos Oliveira

INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

Inês Santos Oliveira

Internship at ALGAplus, Ltd: Production of conchocelis

Estágio de Candidatura ao grau de Mestre em Ciências do Mar – Recursos Marinhos submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador
Doutora Maria Helena Trindade de Abreu
Categoria – Diretora Geral
Afiliação – ALGAplus, Lda.

Coorientador
Professor Doutor José Fernando Magalhães Gonçalves
Categoria – Professor Auxiliar
Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

Agradecimentos

À ALGAplus, pela oportunidade de concretizar este estudo e contactar diretamente com a realidade de produção de macroalgas em contexto empresarial. Por me permitir e criar condições para levar a cabo um trabalho prático in loco durante uma situação pandémica.

Ao professor José Fernando Gonçalves e à Helena Abreu pela orientação e disponiblidade, tanto durante o estágio e no seu planeamento, como durante a elaboração do trabalho escrito.

À equipa da ALGAplus, em particular à Andreia Rego, Madalena Mendes, Andreína Azevedo, Bárbara Pitarma e Jefferson Simas, pela excelente integração e por todo o apoio durante o meu estágio.

À equipa do Centro de Espetofotometria de Massa do Departamento de Química da Universidade de Aveiro, em especial à professora Rosário Domingues, Susana Aveiro e Tiago Conde pela disponibilidade e ajuda na análise bioquímica.

Aos meus colegas de estágio Solène Ropers, Marcos Figueiredo, João Mata e Inês Gonçalves pelo companheirismo e por serem um raio de luz nos dias mais cinzentos.

À minha família e amigos por serem o meu porto de abrigo nos momentos mais difíceis. Por nunca desistirem de mim e nunca permitirem que eu o faça. Por serem a força quando a minha própria falha.

Por ultimo, aos meus pais, pela incessante dedicação à minha formação como ser humano e académica; por colocarem os meus sonhos à frente dos seus e, com grande sacrifício, me proporcionarem seguir estudos nesta área. Nunca terei palavras para descrever o tamanho do obrigada que lhes devo.

Declaração de Honra

Declaro que o presente relatório é de minha autoria e não foi utilizado previamente noutro curso ou unidade curricular, desta ou de outra instituição. As referências a outros autores (afirmações, ideias, pensamentos) respeitam escrupulosamente as regras da atribuição, e encontram-se devidamente indicadas no texto e nas referências bibliográficas, de acordo com as normas de referenciação. Tenho consciência de que a prática de plágio e auto-plágio constitui um ilícito académico.

Inês Santos Oliveira

Abstract

Porphyra (=*Pyropia*) is one of the largest and most valuable groups of red seaweed, characterized by two distinct stages: blade and conchocelis phases. Little is still known about the conchocelis phase and its potential as a source of bioactive compounds. During a curricular internship at ALGAplus, Ltd an experiment was carried out within a confidential service provided to a customer and intended to optimize and upscale the vegetative growth of conchocelis of *Porphyra umbilicalis* in a land-based integrated multitrophic aquaculture (IMTA) system, establishing viable protocols of cultivation, in indoor photobioreactors (PBR) and outdoor tanks (T).

After three preliminary cultivation trials (one indoors and two outdoors), six outdoor (3T to 8T) and four indoor trials (3PBR to 6PBR) of four weeks each were carried out, with medium stock density and stock density adjustment at T_{2weeks}, using *P. umbilicalis* conchocelis (P3 SD and P3 LD). Water renewals were performed three times per week outdoors (twice of them partially), along with nutritional supplementation and abiotic conditions recording, and at T_{2weeks} indoors (by increasing the water column volume) with weekly nutritional supplementation and abiotic conditions recording. Relative growth rate (RGR ($\%.d^{-1}$)), yield ($g.l.d^{-1}$), quality of the biomass (qualitative analysis), lipid and ash content were studied, along with the influence of the origin of biomass, application of treatment x (confidential internal protocol), environment (indoors/outdoors) and abiotic conditions.

Regarding growth, the results showed better yield (F = 7.213, with 3 df) and RGR (F = 11.957, with 3 df) in indoor not treated conditions (Pvalue < 0.010), with the best results belonging to P3 LD not treated (5PBR, yield = 0.64 ± 0.07 $g.l.d^{-1}$and RGR = 6.91± 0.36 $\%.d^{-1}$), corroborating that treatment x constitutes distress for the cultures. Abiotic conditions did not show to influence growth outdoors, but it was verified a good positive correlation between pH and RGR in indoor trials (r^2 = 0.695, Pvalue = 0.002) which might be related to a higher/lower photosynthetic activity over the weeks. The indoor trials had better performances (RGR and Yield) in the first two weeks, but not outdoors, indicating that indoor weight assessment might result in biased results.

The quality of the biomass was influenced by the origin of the culture starter, at T_0, and influenced by both origin and environment at T_{4weeks}. The biomass from P3 LD and indoors trials had better quality results probably due to photoperiod stability at values over 12h and culture maintenance at more sterile conditions. Independently of the biomass used, quality at T_{4weeks} was “Good” indoors, while outdoors biomass quality

seemed to be influenced by abiotic conditions, although only two seasons were studied, for P3 SD.

Ash content was over 50% and higher for P3 LD, which might suggest that conchocelis have higher mineral content than red macroalgae (up to 50%), with great potential for bioremediation. Higher ash content at the end of trials suggested the culture medium is richer than the one used in the company protocols. Lipid content found in conchocelis suited the range described for red macroalgae (0.5-5%), showing no influence of the studied variables.

Alternative weight assessment methods indoors should be studied (image processing or conchocelis area), considering them for outdoors also. For stronger results outdoors, 12 months experiments should be carried out in the future, comparing different variables simultaneously, as studying its upscale and other variables (culture time, isolated conchocelis strains, different light wavelengths), applicable to indoors too. Testing the use of PBRs in outdoor conditions, as structures like green wall panels or horizontal PBRs can also be a cost-effective option in the future. N, P and C determination would have given important results about nutrient assimilation, nutritional profile and bioremediation potential, enabling the correlation between N determination and the colors of quality and contamination scale, assessing N content by rapid classification of cultures coloration and validating the created scale.

With this internship, I enlarged my knowledge in IMTA systems, seaweed biology and cultivation protocols. Working in this area in a company context was a unique experience, providing me with a different perspective than academic research work. There is still scarce work in conchocelis, but it sustains, along with this study, their potential. A lot of data may not be published: as important data for nori aquacultures, it is possible that some companies keep their work unpublished and not patented for confidentiality, mostly in Asian countries, where nori cultivation has centuries of history.

Keywords: *Porphyra*, conchocelis, IMTA, ALGAplus, relative growth rate, yield, lipidome.

Resumo

Porphyra (=*Pyropia*) constitui um dos groups de maior valor das macroalgas vermelhas, caracterizado por dois estágios distintos: lâminas e conchocelis. O conchecimento sobre conchocelis e o seu potencial como fonte de compostos bioativos ainda é escasso. Durante um estágio curricilar na ALGAplus, Lda. foi conduzido um estudo para responder a um serviço confidencial por parte de um cliente, objetivando a optimização de protocolos de produção e aumento de escala de produção de conchocelis de *Porphyra umbilicalis,* num sistema de aquacultura multitrófica integrada (IMTA) em tanques de terra, estabelecendo protocolos viáveis de cultivo em fotobiorreatores (FBRs) interiores e tanques exteriores (T).

Após três ensaios preliminares (um interior e dois exteriores), foram conduzidos seis estudos em exterior (do 3T ao 8T) e quatro no interior (do 3PBR ao 6PBR) de quatro semanas cada, com uma densidade de cultura média e acertos de densidade às duas semanas, usando biomassa P3 SD e P3 LD. Foram feitas renovaçoes de água três vezes por semana no exterior (duas delas parciais) juntamente com adição de um meio nutricional e registo de condições abióticas, e uma às duas semanas no interior (por subida da coluna de água), com suplementação nutiricional e registo de condições abióticas semanais. Foram estudados a taxa de crescimento relativo (RGR($\%.d^{-1}$)), yield ($g.l.d^{-1}$), qualidade da biomassa (análise qualitativa) e teor lipídico e de cinzas, assim como a influênica da origem de biomassa, uso do tratamento x (protocolo confidencial), ambiente (interior/exterior) e condições abióticas nestes parâmetros.

Os resultados revelaram Yield (F = 7.213, com 3 g.l.) e RGR (F = 11.957, com 3 g.l.) mais elevados no interior em biomassa não tratada (Pvale < 0.010), com os melhores resultados registados em P3 LD não tratada (5PBR yield = 0.64 ± 0.07 $g.l.d^{-1}$ e RGR = 6.91± 0.36 $\%.d^{-1}$), corroborando com a ideia de que o tratamento x constitui um fator de stress para as culturas. As condições abióticas não mostraram influencar o crescimento no exterior, porém, no interior, foi encontrada uma boa correlação positiva entre o pH e o RGR (r^2 = 0.695, Pvalue = 0.002), o que pode estar relacionado com maior/menor atividade fotossintética ao longo das semanas de ensaio. A performance de crescimento (yield e RGR) no interior foi melhor na primeira quinzena, mas o mesmo não se verificou no exterior, indicando que o método de estimativa da biomassa total pode estar a enviesar os resultados.

A qualidade da biomassa em T_0 foi influenciada pela origem da biomassa e às quatro semanas pela origem e ambiente de cultivo. A biomassa P3 LD nos ensaios no

interior teve melhores resultados na qualidade, provavelmente pelo fotoperíodo constante e acima das 12h e condições de cultivo mais estéreis. Independentemente da biomassa usada no interior, os resultados foram “Bom” no final dos ensaios, enquanto no exterior a qualidade da biomassa parece ser infleuncada pelas condições abióticas, embora só tenha sido testada duas épocas do ano diferentes para P3 SD.

O teor de cinzas obtido (acima de 50%) e superior em P3 LD sugere que os conchocelis apresentam um teor mineral mais elevado que os valores para as macroalgas vermelhas (até 50%) e um grande potencial em biorremediação. O teor em cinzas mais elevado no final dos ensaios sugere ainda que o meio de cultura usado nos ensaios é mais rico em minerais do que o usado nos protocolos da empresa. O teor lipídico registado em conchocelis encontra-se dentro dos padrões descritos para macroalgas vermelhas (0.5-5%), não tendo sido encontrada uma influência das variáveis estudadas no mesmo.

No futuro, a estudar metodologias alternativas de cálculo da biomassa total no interior (nomeadamente processamento de imagem e cálculo da área de conchocelis), considerando a sua aplicação no exterior. Para resultados no exterior mais fiáveis, realizar estudos de 12 meses, comparando variáveis diferentes em simultâneo, estudando também volumes maiores e outras variáveis (tempo em cultura, lotes isoaldos, luz com diferentes comprimentos de onda), aplicável também no interior. Uma opção que se pode tornar lucrativa é a utilizaação dos FBRs no exterior, assim como estruturas como os “green walls panels” ou FBRs horizontais. A determinação do teor de azoto, fósforo e carbono teria fornecido dados importantes sobre a assimilação de nutrientes, perfil nutricional e potencial de biorremediação, permitindo criar a relação entre o teor de azoto e as cores da escala de qualidade e contaminação, com um acesso rápido ao teor de azoto e validando a escala criada.

Com este estágio, pude alargar os meus concheimentos sobre sistemas IMTA. biologia e produção de macroalgas marinhas. Poder trabalhar nesta área em contexto empresarial foi uma experiência ímpar, que me providenciou uma perspetiva diferente do trabalho em contexto académico. O trabalho desenvolvido em conchocelis é ainda escasso, mas sustém, juntamente com o deste estudo, o seu potencial. Porém, muitos dados podem não estar publicados: sendo uma área de especial interesse para a aquacultura de nori, é possível que as empresas mantenham o seu trabalho confidencial e por publicar/patentear, principalmente na Ásia, onde o cultivo de nori é secular.

Keywords: *Porphyra*, conchocelis, IMTA, ALGAplus, taxa de crescimento relativo, yield, lipidómica.

Abbreviations and acronyms

1PBR: preliminary trial in indoor conditions

1T: first preliminary trial in outdoor conditions

2T: second preliminary trial in outdoor conditions

3PBR: trial in indoor conditions with conchocelis of *P. umbilicalis* from the short-day chamber without treatment x

3T: trial in outdoor conditions with conchocelis of *P. umbilicalis* from the short-day chamber without treatment x

4PBR: trial in indoor conditions with conchocelis of *P. umbilicalis* from the short-day chamber with treatment x

3T, 7T: trials in outdoor conditions with conchocelis of *P. umbilicalis* from the short-day chamber without treatment x

4T, 8T: trials in outdoor conditions with conchocelis of *P. umbilicalis* from the short-day chamber with treatment x

5PBR: trial in indoor conditions with conchocelis of *P. umbilicalis* from the long-day chamber without treatment x

5T: trial in outdoor conditions with conchocelis of *P. umbilicalis* from the long-day chamber without treatment x

6PBR: trial in indoor conditions with conchocelis of *P. umbilicalis* from the short-day chamber without treatment x

6T: trials in outdoor conditions with conchocelis of *P. umbilicalis* from the long-day chamber with treatment x

ASW: Autoclaved seawater

C: Carbon

CO_2: Carbon dioxide

COVID-19: Coronavirus disease

DM: Dry matter

EOA: Experimental Outdoor Area

FAO: The Food and Agriculture Organization

FSW: Filtered seawater

Fw: Fresh weight

HCO_3^-- Bicarbonate

IMTA: Integrated multitrophic aquaculture

LD: Long-day

MAAs: Mycosporine-like amino acids

MUFA: monounsaturated fatty acids

N: Nitrogen

NH_4^+: Ammonium

$(NH_4)_2HPO_4$): Ammonium phosphate monobasic

NO_3^-: Nitrate

O_2: Oxigen

P: Phosphorus

P3 LD: Conchocelis of *P. umbilicalis* from the long-day chamber

P3 SD: Conchocelis of *P. umbilicalis* from the short-day chamber

PBR: Photobioreactor

PFD: Photon flux density

PO_4^{3-}: Phosphates

PUFA: polyunsaturated fatty acids

RDI: Research, Development and Innovation

RGR: Relative Growth Rate

SA/V: Surface area to volume ratio

SD: Short-day

SFA: saturated fatty acids

T_0: Time of the beginning of the trials

T_{2weeks}: Time after two weeks of trial

T_{4weeks}: Time after four weeks of trial/end

UV: Ultraviolet

Wf: final weight

Index

Index of Figures

Index of Tables

Introduction

In a current conjecture of an exponentially growing population, with a misleading idea of the abundance of resources, sustainability is one of the watchwords of our century, leading the attention of the scientific community to the exploitation of natural resources (Biris-Dorhoi *et* al., 2020). Following the same line of thought, algae are an element to regard: from their very interesting nutritional profile up to the evidence of anti-oxidant, anti-diabetic, anti-hypertensive, anti-obesity, anti-microbial, anti-inflammatory and anti-cancer properties of their extracts (Biris-Dorhoi *et* al., 2020). The low caloric value (containing all the essential amino acids, substantial amounts of polyunsaturated fatty acids, and a large portion of carbohydrates belonging to dietary fiber) and potential as bioactive compounds source sustain the increasing interest in the last years in the subject, with many research groups dedicated to their study (Biris-Dorhoi *et* al., 2020; Gómez-Zorita *et* al., 2020).

The term algae comprise a wide consortium of organisms different in morphology, complexity and size (Gallardo, 2015; Pereira & Correia, 2015). With the capacity to transform luminous energy into chemical one, capturing carbon dioxide, these primary producers assume the base of the aquatic food chains, providing organic matter, with the release of more than half of the oxygen supply on earth, assuming nowadays the role of “lungs of the planet”, previously assigned to the Amazon rainforest (Gómez-Zorita *et* al., 2020; Pereira & Correia, 2015). When considering the marine environment, marine algae are usually divided into microalgae (unicellular organisms only visible recurring to ampliation instruments) and macroalgae (or seaweed): multicellular macroscopic (at least in one stage of their life) organisms, categorized into phyla *Chlorophyta* (green algae), *Ochrophyta* (brown algae) and *Rhodophyta* (red algae) (Pereira, 2009; Pereira & Correia, 2015).

Porphyra (=*Pyropia*) is one of the largest and most valuable groups of red seaweed (FAO, 2018; Pimentel *et* al., 2020). The life cycle is characterized by two distinct stages: a microscopic filamentous sporophyte, known as the conchocelis phase, and the macroscopic gametophyte blade phase, the “adult *Porphyra*” individuals (Drew, 1949). Traditionally known for its applications in the food sector, blade-derived components have been increasingly used also in sectors like nutraceuticals, pharmaceutics and cosmetics (da Costa *et* al., 2018). A lot of work has been developed focusing on the blade phase of *Porphyra* spp.; yet, there is still little information about

the conchocelis phase and its potential as a source of bioactive compounds (da Costa *et* al., 2018; Pimentel *et* al., 2020).

With a global consumption above one million tonnes annually, world requirements in biomass are in majority supplied by south-east Asia seaweed farms (da Costa *et* al., 2018; Pimentel *et* al., 2020). In Europe, more than 95% of Nori is imported from these countries; however, the interest in exploring the market for local "sea vegetables" has been growing, mostly supplied by wild harvesting of local species – "Atlantic Nori" – mainly in France (Pimentel *et* al., 2020).

After the recent close-up of the production cycle of two species of "Atlantic Nori", (*P. umbilicalis* and *P.dioica)* in land-based integrated multitrophic aquaculture (IMTA) at the Portuguese company ALGAplus, the company supplies nori biomass to the European food and cosmetics markets, maintaining the natural banks in ecological balance (Pimentel *et* al., 2020). Since it is sustained in the manipulation of the growth conditions, the cultivation of these species also allows a yearly supply of Atlantic Nori (Pereira & Yarish, 2008; Pimentel *et* al., 2020).

During a curricular internship at ALGAplus, Ltd., within the scope of acquiring a master's degree in Marine Sciences – Marine Resources, specialization of Aquaculture and fisheries, it was intended to contact and participate in tasks in an IMTA system, in a company context; to acquire knowledge about different seaweed aquaculture systems and techniques, and to learn more about phycology, different groups of seaweed and their biological features.

Leaning on the potential of conchocelis and lack of work in the field, an experiment was carried out during this internship, aiming for the optimization of the protocols of biomass production of conchocelis of *Porphyra umbilicalis*, in indoor and outdoor conditions, as the viability of producing conchocelis as a final product. To accomplish that, the viability of the vegetative growth in indoor (80 L photobioreactors), outdoor (15 L tanks) and the quality of biomass were studied; some hypotheses were verified:

- The origin of conchocelis influences the vegetative growth and quality of the biomass;
- Conditions of mass production (indoor/outdoor) influence vegetative growth and quality of the biomass;
- Seasonality influences the vegetative growth and quality of the biomass, in outdoor conditions.

- There is a relationship between biochemical analysis (ashes and lipid content) and origin, conditions of mass production and seasonality.

This study intends to disclose important data about conchocelis potential, production methodology, the influence of abiotic factors and the production capacity of the company using the available resources. It will be the first step for further studies on the economic viability of this production, more extensive biochemical analysis, validation of production protocols/revision of methodologies and upscale at the company.

This report is divided into four parts:

I) Introduction: General context about taxonomy, biological and physiological aspects, economic value and production of *Porphyra* spp., focusing on *P. umbilicalis*;

II) Internship: a description of the company, facilities, work developed and activities elaborated as an intern;

III) Production of conchocelis: description of the experimental work developed, methodologies, results and discussion;

IV) Final considerations about the experiment and the internship experience.

I.Seaweed: General Review

Algae englobes a wide range of simple organisms, physiologically and biochemically similar to terrestrial plants (Gallardo, 2015). The possibility to group in the same division organisms from different taxa, in a way that nowadays systematics does not, sustains the use of the term by the scientific community, even though it does not have taxonomic value anymore (Gallardo, 2015; Pereira & Correia, 2015). From prokaryotes to eukaryotes, uni and multicellular organisms, but all with rudimentary conducting tissues, algae comprise a series of species mostly occurring in water (freshwater, marine, or brackish) or humid environments, all of them with chlorophyll α and the accessory pigment β-carotene (Pereira & Correia, 2015).

Transforming luminous energy into chemical one from sunlight, carbon dioxide and water, algae play a big role in the aquatic ecosystems, functioning in most habitats as the primary producers of the food chains (Pereira & Correia, 2015). During the photosynthetic process, they also form oxygen in the process, crucial for the metabolism of the consumer organisms. Responsible for more than half of the oxygen supply on earth, algae are the “lungs of the planet” of nowadays, a designation previously assigned to Amazon forests (Gómez-Zorita *et* al., 2020; Pereira & Correia, 2015).

Typically photosynthetic autotrophic producers, a few have lost their photosynthetic ability, living as saprophytes or parasites, or evolved to heterotrophy, justifying the uncertainty of systematics on the number of phyla of algae (Gallardo, 2015). Varied in their habitats, morphological features and size, reproductive pathways, and complexity of the structures, it is common to categorize algae according to some of their characteristics (Gallardo, 2015; Pereira & Correia, 2015). Ubiquitously distributed in very diverse environments, one of the classifications used in algae is based on their environment habitat, dividing algae into marine algae and freshwater algae (Gallardo, 2015).

In the marine environment, marine algae are usually divided into microalgae (unicellular organisms only visible recurring to ampliation instruments sized between a millimeter to several centimeters) and macroalgae (or seaweed): macroscopic (at least in one stage of their life) algae, reaching sizes up to sixty meters (Pereira, 2009; Pereira & Correia, 2015). Seaweeds usually live attached to substrates; the majority can be found growing on the rocky shores or attached to seashells (lithophytic), but there are also epiphytic or endophytic species, living attached to or within other algae, respectively, endozoic (within small animals), or parasitic (Baweja *et* al., 2016; Pereira, 2009; Pereira

& Correia, 2015). With a wide range of colorations, sizes and shapes, it is estimated that the number of species of seaweed is between 7500 and 10000 (Gallardo, 2015).

From food, feed and soil fertilization, to cosmetics and pharmaceuticals, seaweeds are used for centuries valued for both their interesting nutritional profile and high content of bioactive compounds (Dhargalkar & Kavlekar, 2004; Silva *et* al., 2020) Phycocolloids like alginates, agar and carrageenan are the main application of seaweed, used as thickeners, gelling agents and stabilizers due to their peculiarity of forming colloidal solutions (Pereira, 2018). The potential bioactivity as anti-oxidant, anti-diabetic, anti-tumoral, anti-hypertensive, anti-obesity, anti-microbial and anti-inflammatory of seaweeds' extracts have been the focus of many research teams in the last few years (Biris-Dorhoi *et* al., 2020; Gómez-Zorita *et* al., 2020).

With a low caloric and fat content and an interesting profile of polyunsaturated fatty acids, the commonly known as "sea-vegetables" are an important source of protein, with a similar profile to egg protein (Pereira, 2018). Mainly constituted by polysaccharides of long-chain (fibers), playing a crucial role in regulating the intestinal transit, seaweeds are an important source of sulfated polysaccharides (where the phycocolloids are inserted), which have been described with high levels of bioactivity, largely associated with the protection of the gastrointestinal tract (anti-tumoral and anti-diabetic potential) (Pereira, 2018). Combining these with their profile of anti-oxidants (from vitamins, pigments and phenolic composts) of great impact in preventing degenerative diseases and anti-aging, seaweeds are considered one of the superfoods of most interest of the last decade, (Pereira, 2018; Silva *et* al., 2020).

The seaweed industry is worth almost 6 billion dollars per year and more than 30 tonnes of biomass fresh weight (*fw*), mostly produced by Asian countries (Japan, China and the Republic of Korea) (FAO, 2020). According to the latest statistics, production of biomass has been substantially increasing in the last years, mainly by a significatively growth of seaweeds' aquaculture: while wild harvesting has stagnated around 1.1 million tones from 2006 until 2018, aquaculture has doubled (from 15.9 million to around 33.2 million tonnes) in the same period (FAO, 2018). Although globally the harvest of seaweed constitutes less than 4% versus 96,5% of biomass from aquaculture, in Europe (mainly in Norway) harvesting still constitutes the main source of biomass (98%) and only a residual portion (2%) from aquaculture (European Comission, 2020).

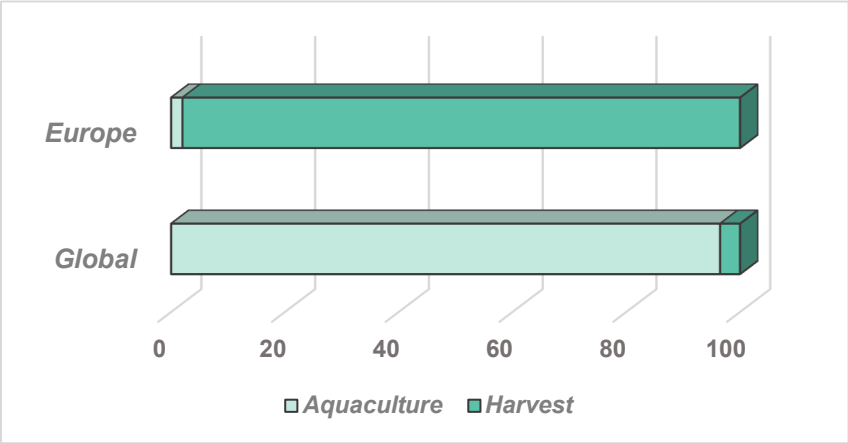


Figure 1 Percentage of Aquacultured and Harvested seaweed: comparison of global and European results (adapted from European Comission, 2020)

Seaweed's classification is based on their different combination of photosynthetic pigments, consequently attributing them different colors, respectively distributed in three different phyla, Chlorophyta, Ochrophyta, and Rhodophyta (Pereira & Correia, 2015). Different phyla also correspond to different nutritional profiles and cell wall constitutions, as storage polysaccharides (Pereira, 2018). Due to the presence of chlorophyll a and b (and additionally α-, β- and γ-carotenes, lutein and prasinoxanthin), Chlorophyta, also known as green algae, present similar pigmentation to terrestrial plants (Pereira & Correia, 2015). Primarily freshwater, only about 1500 species of Chlorophyta are marine, presenting the same typical main cell wall compound and storage products that higher terrestrial plants have: cellulose and starch, respectively (Lee, 2008).

About 2000 species of seaweed are categorized as brown algae, from phylum Ochrophyta, class *Phaeophyceae* (Pereira, 2009). Their brownish pigmentation derives from the large amounts of fucoxanthin and violaxanthin, that, along with diadinoxanthin, heteroxanthin and vaucheriaxanthin mask the green coloration of chlorophylls a, c_1, and c_2. (Pereira, 2009). Some of the species present a yellowish color due to a higher content of α-, β- and ε-carotenes (Gallardo, 2015). The cell walls of brown algae are composed of at least two layers: an inner structural one composed of cellulose, and an outer layer of mucilage, comprising alginates and fucoidans (Gallardo, 2015; Lee, 2008). As the characteristic storage product, *Phaeophyceae* accumulate laminarin (Lee, 2008).

Rhodophyta (red algae) constitute the largest and most diverse phylum of seaweeds, where *Porphyra* spp. are included (Lee, 2008; Pereira, 2009). Chlorophyll a is the responsible pigment for photosynthesis, while phycobiliproteins attribute their coloration: phycoerythrin is the main one and responsible for the red coloration; other

colors (red-blue colorations) are imparted by phycocyanin and allophycocyanin, varying the shade with the proportion of the different phycobiliproteins (Gallardo, 2015; Lee, 2008). Other pigments, like α- and β-carotenes and lutein are also present in red algae (Lee, 2008). Similar to *Phaeophyceae,* the cell wall possesses two layers, cellulose and mucilage, the source of the important hydrocolloids (agar and carrageenan) (Gallardo, 2015). Rhodophyta has a specific storage polysaccharide, the floridean starch, also found in a small group of freshwater algae (*Glaucophytes*). The following table (*Table 1*) resumes the characteristical pigments, storage products, and cell wall constituents of each phylum of seaweed.

Table 1. Characteristic pigments, storage products, and cell wall constituents of each phylum of seaweed (adapted from Pereira, 2018)

		Chlorophyta	**Ochrophyta**	**Rhodophyta**
Pigments	**Chlorophylls**	chlorophylls a and b	chlorophylls a, c_1, and c_2.	chlorophyll a
	Carotenes	α-, β-, γ-carotenes	α-, β-, ε-carotenes	α-, β-carotenes
	Xanthophylls	lutein, prasinoxanthin	fucoxanthin, violaxanthin, diadinoxanthin, heteroxanthin, vaucheriaxanthin	lutein
	Phicobiliproteins	absent	absent	phycoerythrin, phycocyanin, allophycocyanin
Storage product		starch	laminarin	floredian starch
Cell wall		cellulose	cellulose and mucilage	cellulose and mucilage

1.*Porphyra* spp.

1.1.Taxonomy and distribution

1.1.1.Taxonomy

The *Bangiales* (Nägeli, 1847) is a diverse order of the phylum Rhodophyta (Wettstein, 1901) where the genus *Porphyra* is included. Its taxonomy has been suffering several changes during times, leading to some growing confusion about the systematics of its species (Dumilag *et* al., 2017), justifying the need for studying this topic (Sutherland *et* al., 2011). Until the application of molecular techniques, the identification and taxonomic placement within the bladed *Bangiales* were highly problematic (Brodie *et* al., 2008; Gunnarsson *et* al., 2016), consisting mainly in the identification of simple morphological features of the different genera of the order *Bangiales* (blade color, size, the shape of the margin of the thalli, number of cell layers, and texture) (Abe *et* al., 2013) and variation within and between species (Brodie *et* al., 2008; Gunnarsson *et* al., 2016). Regardless, since only a reduced number of species in the order *Bangiales* were described using molecular data until now, possibly, systematics of these species will still suffer some fluctuations in the future (Yang *et* al., 2020).

The primary references to the genus *Porphyra* remote to the initial studies in plant systematics (Linnaeus, 1753); notwithstanding, the genus *Porphyra* (Agardh, 1824) was only proposed in the nineteenth century, separating the bladed purple-reddish specimens from the green ones, *Ulva* spp. (Linnaeus, 1824). At the time of the first reference to the class *Bangiphyceae* (Wettstein, 1901), represented by only one order - *Bangiales* (Nägeli, 1847) - this taxon was considered to have only two genera, basing this division on the morphology of the gametophytes: genus *Bangia* (with unbranched filaments gametophytes) (Lyngbye, 1819) and genus *Porphyra* (with bladed gametophytes) (Agardh, 1824). From this time, several different genera of bladed *Bangiales* were proposed; yet, these doubts were revised during the 70s (Hawkes, 1977; 1978) and, from then until around a decade ago, it was accepted that *Porphyra* spp. was the only genus of bladed *Bangiales* (Sutherland *et* al., 2011).

The last changes in systematics before Yang *et* al. (2020) considered nine genera of bladed Bangiales, bringing back genera *Pyropia* (Agardh, 1899) and *Wildemania* (De Toni, 1890), redefining *Porphyra* spp., and proposing *Boreophyllum, Clymene, Fuscifolium, Lysithea, Miuraea* (Sutherland *et* al., 2011) and later *Neothemis* as new genera (Sánchez *et* al., 2014). These genera share between themselves the

same kind of life cycle (biphasic type); morphologically, identification is only made by blade features and marginal reproductive structures (Kurogi, 1972; Nelson *et* al., 1999). Since these studies, it is common to find both references of Porphyra spp: "*Porphyra sensu lato*" (to mention bladed Bangiales in general) and "*Porphyra sensu stricto*" (to mention the redefined genus *Porphyra*) (Blouin *et* al., 2011). During the last decade, several studies in bladed *Bangiales* have been done, mainly on its molecular taxonomy (Meynard *et* al., 2019; Milstein *et* al., 2012; Nelson & D'Archino, 2014; Vergés *et* al., 2013) or describing new species of *Porphyra*, *Pyropia* and *Wildemania* (Meynard *et* al., 2019) since they have the majority of the most important produced *Bangiales* (Lim *et* al., 2017).

Empire *Eukaryota*
(Chatton, 1925)
Kingdom *Plantae*
(Haeckel, 1866)
Phylum *Rhodophyta*
(Wettstein, 1901)
Class *Bangiophyceae*
(Wettstein, 1901)
Order *Bangiales*
(Nägeli, 1847)
Family *Bangiaceae*
(Duby, 1830)
Genus Porphyra
(Blouin *et* al., 2011)

Figure 2. Taxonomy of *Porphyra* spp.

The most recent study in the taxonomy of this group of algae proposes the redefinition of genera *Pyropia* and *Porphyra,* readmitting *Porphyrella* spp. (Smith & Hollenberg, 1943) and with the creation of four new genera: *Neoporphyra, Neopyropia, Uedaea,* and *Calidia* (Yang *et* al., 2020), being the last one now renamed as *Phycocalidia* (Santiañez & Wynne, 2020). At the moment, the accepted taxonomy for genus *Porphyra ("Porphyra sensu stricto")* according to *Algaebase* (Guiry & Guiry, 2020) is that it belongs

to empire Eukaryota, (Chatton, 1925) kingdom Plantae (Haeckel, 1866), phylum Rhodophyta (Wettstein, 1901), class Bangiophyceae and subclass Bangiophycidae (Wettstein, 1901), order *Bangiales* (Nägeli, 1847) and family *Bangiaceae* (Duby, 1830) (*Figure 2*).

There are 57 accepted species in the genus *Porphyra* (Yang *et* al., 2020), being one of them *Porphyra umbilicalis* (Kützing, 1843). "*Porphyra sensu lato"* (*Figure 3*) includes now around one hundred and fifty-five species and fifteen genera: *Porphyra* (Agardh, 1824), *Boreophyllum, Clymene, Fuscifolium, Lysithea,* (Sutherland *et* al., 2011), *Neomiuraea* – former *Miuraea* (Kikuchi *et* al., 2018) *Neothemis* (Sánchez *et* al., 2014), *Neoporphyra, Neopyropia, Uedaea, Calidia, Porphyrella* (Smith & Hollenberg, 1943), *Phycocalidia* (Santiañez & Wynne, 2020), *Pyropia* (Agardh, 1899) and *Porphyrella* (Smith & Hollenberg, 1943), being the last seven genera mentioned in some papers as "*Pyropia sensu lato*" (Yang *et* al., 2020).

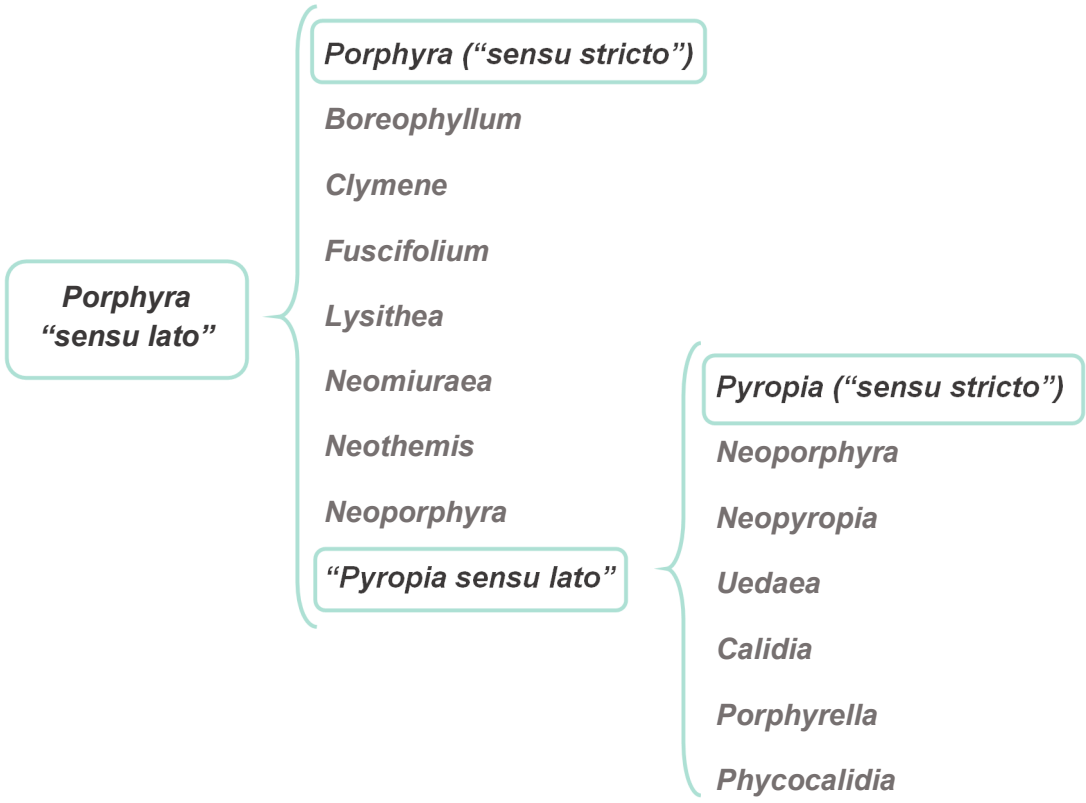


Figure 3. Elucidation of bladed *Bangiales* taxonomics (adapted from Yang *et* al., 2020)

1.1.2.Distribution

From cold to warm waters, *Porphyra* spp. is distributed around the world, substantially well demarked in the boreal and cold-temperate regions, with more evident speciation in the North Atlantic and North Pacific (Sánchez *et* al., 2014). Typically growing attached to a substrate, some species occur in the intertidal and low-intertidal zones, while others can be found in shallow subtidal zones (Sánchez *et* al., 2014; Sutherland *et* al., 2011). Typically, conchocelis, regardless of their species, grow in the subtidal zone, whereby they are more protected from dissection and salinity stress (Redmond et al., 2014b). Specimens of *Porphyra umbilicalis* are found on rocky substrates in the intertidal zones of the North Atlantic (Royer, 2017). At the Northwest Atlantic, *P. umbilicals* can be found in the Canada shores (from Labrador) to the mid-Atlantic coast of the United States of America (Virginia), while in the east of the North Atlantic there are descriptions of *Porphyra umbilicalis* in Iceland, East Europe (from Norway to Portugal) and in the Western Mediterranean (*Figure 4*), considered one of the species of "Atlantic Nori" of increasing interest (Pimentel *et* al., 2020; Royer, 2017).

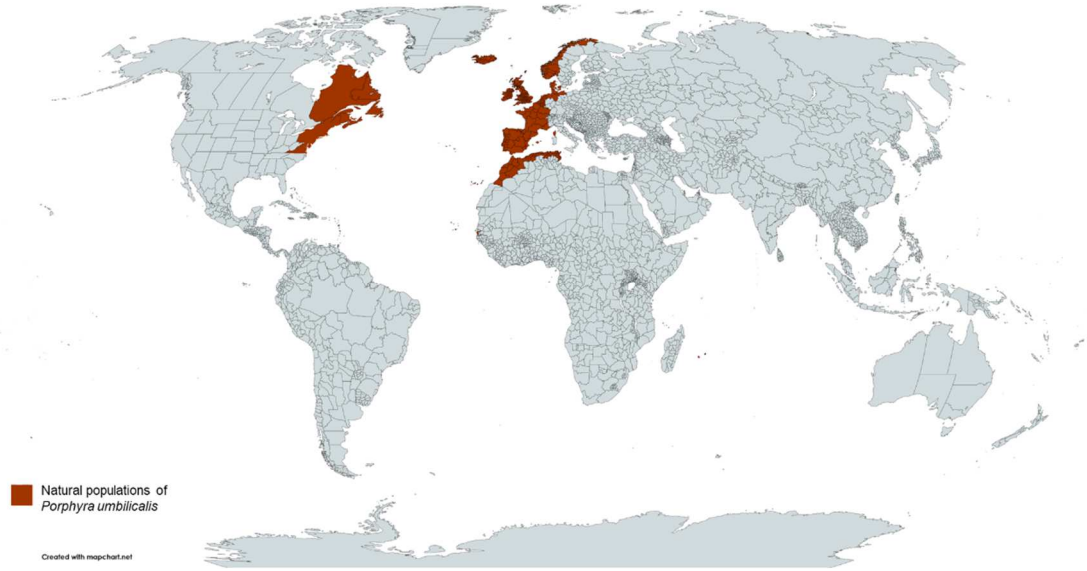


Figure 4. Distribution of *Porphyra umbilicalis* (created at *https://mapchart.net/)*

1.2.Morphological features and life cycle

Porphyra spp. is characterized by a digenetic heteromorphic life cycle, where the microscopic conchocelis (sporophyte phase) – diploid stage - intercalates with macroscopic blades (gametophytic phase) – haploid stage (Sutherland *et* al., 2011). The denomination “digenetic heteromorphic” advents from the existence of both haploid and diploid phases, yet morphologically different (Nelson *et* al., 1999). Conchocelis are characterized as thin brown-reddish filamentous structures, constituted by single cells connected by pit plugs, *(Figure 5*) initially assumed to be a separate species (*Conchocelis rosea*), until Kathleen Drew (1949) elucidated them as a phase of *Porphyra* spp. Its name comes from its usual growth attached to bivalve shells and it is not easy to observe in nature because of its size and coloration (Blouin *et* al., 2011).

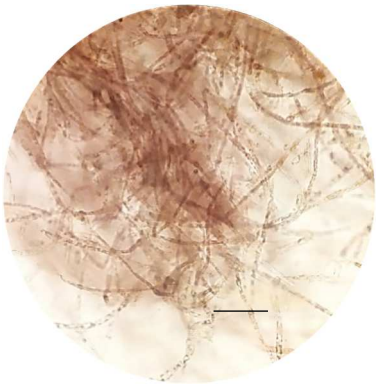


Figure 5. Conchocelis of *Porphyra* spp. ©Inês Oliveira

Thalli are foliose blades, with one or two layers of mononuclear cells (Sutherland *et* al., 2011). Oval to lance-shaped and, in some species, hook-shaped, with “margins entire, planar, dentate, undulate, or ruffled”, their colors vary from olive-green, violet, blue, red, pink, reddish-brown, brown, black according to the proportions of pigments of each species, but also influenced by dissection, light, temperature changes, enzyme treatment or bacteriosis (Lavik, 2016; Sutherland *et* al., 2011). Species of *Porphyra* spp. can be monoecious (both male and female present in the same blade) or dioecious (male and female in separated blades) which morphologically shows some differences: in monoecious specimens, in the same individual it is possible to notice darker (carpogonia) and lighter zones (spermatia) when mature, while in dioecious blades, female blades appear to be darker than the male ones (Pereira & Correia, 2015; Sutherland *et* al., 2011).

Specimens of *Porphyra umbilicalis* consist of monostromatic blades (usually dioecious) that tend to be greenish when younger, reddish-brown, brownish or olive green when adult and underwater, and brownish-purple when exposed to the sun and dissection (Guiry & Guiry, 2020). Circular to irregularly shaped and d*escribed as* “membranous, but resilient”, they expand around a singular holdfast in a cordate shape, creating overlapped spots that give it the appearance of an umbilicus, and creating sometimes freckles in the margins, resembling a rosette (Guiry & Guiry, 2020). The holdfast constitutes the attaching point to the substrate; when adults, the individuals of

P. umbilicalis are small in comparison with other species of *Porphyra* spp., sized around 5 to 10 centimeters per 20 centimeters (Guiry & Guiry, 2020) (*Figure 6*). both monoecious and dioecious blades have been reported in this species, as alternative reproduction pathways besides the typical sexual reproduction one (Royer, 2017).

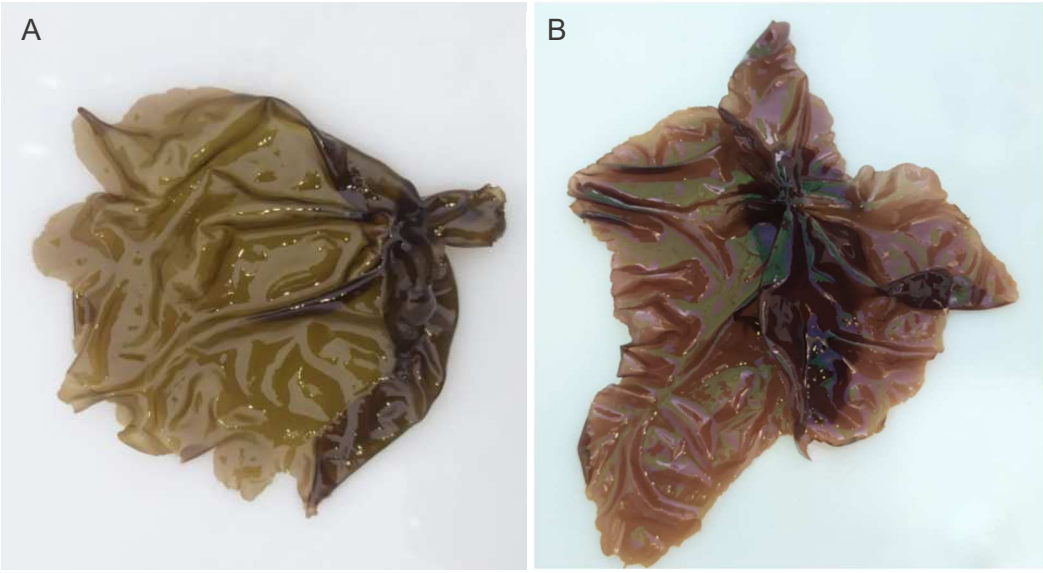


Figure 6. Specimens of *Porphyra umbilicalis* of different colorations: A (olive-green) and B (reddish brown). ©Inês Oliveira

Several pathways of reproduction of *Porphyra* spp. have been described, both sexual and asexual, the first one involving both haploid and diploid structures (corresponding to the typical heteromorphic life history), while in the second one all the structures are haploid (Kornmann, 1994; Nelson *et* al., 1999; Pereira, 2004; Pereira & Yarish, 2010). Asexual reproduction assures phenotype and genotype maintenance, which plays both advantages and disadvantages since genetic diversity declines, a breaking point in an ecological stress event. On the other hand, conchocelis play an important asset in this topic, avoiding competition, predators and salinity stress (Holmes & Brodie, 2004).

Sexual reproduction in *Porphyra* spp. (*Figure 7*) is reached when the non-flagellate spermatia, already mature, are released by the gelling of the male gametocyst and taken through water flow to the carpogonium, where it is fertilized (Pereira & Correia, 2015). A diploid structure – the zygote – will develop and result in several carpospores by mitosis, which will be released in the water when mature (Pereira & Correia, 2015). Once they settle in a suitable substrate – usually bivalves shells – they germinate,

originating the conchocelis, starting at this stage the diploid phase of the cycle (Drew, 1954).

For months, conchocelis develop, originating other filamentous structures called conchosporangia, similar to conchocelis, but with larger and more irregular branches than them, with rectangular outlines and stronger coloration of the cell walls, as its size and thickness, that will later release conchospores (Pereira & Correia, 2015). Although not consensual, it is currently accepted that meiosis is associated with the germination of the conchospores (Mitman & van der Meer, 1994). Conchospores, haploid structures, will find an appropriate substrate to adhere to and start developing new young blades, that will grow until being adult individuals and restart the cycle when matured (Pereira & Correia, 2015).

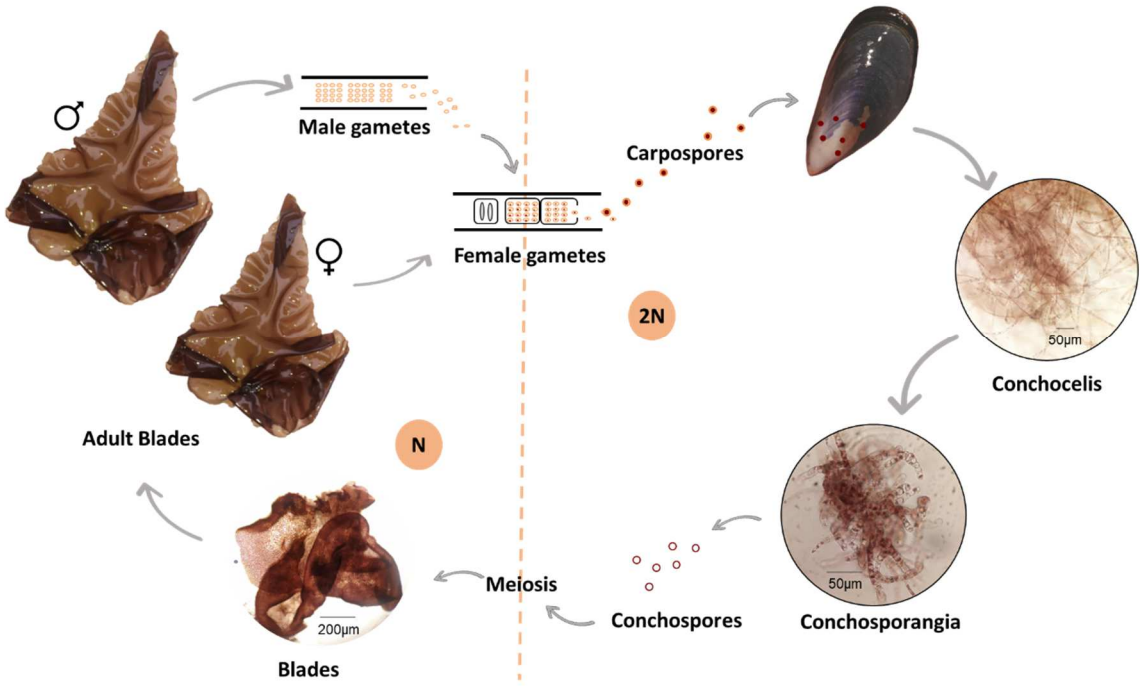


Figure 7. Life cycle of *Porphyra* spp. (sexual reproduction). Adult blades (n) realease the gametes occurring fecundation and release of carpospores (2n), that will germinate into conchocelis and mature to conchosporangia. Mature conchosporangia release conchospores that, trough meiosis, origin new blades.(Scheme by ©Inês Oliveira)

Some asexual reproduction alternatives to the typical alternation of haploid and diploid stages have been reported in species of *Porphyra* spp., through the development of agamospores, neutral spores, endospores or archeospores in the carpogonia of the blade phase (Redmond *et* al., 2014b). By mitotic cleavage of the blade cells, (without

occurring fertilization) agamospores are released from the carpogonia (referred by some authors as agamosporangia) (Nelson *et* al., 1999), which will germinate into haploid conchocelis (Redmond *et* al., 2014b). The haploid conchocelis will produce conchosporangial filaments that will also release agamospores and directly develop into new blades (Nelson *et* al., 1999).

Asexual reproduction by the development of both archeospores, endospores and neutral spores sustain the direct development of spores obtained, from the mitotic division of the carponogia, into new blades (Kornmann, 1994; Redmond et al., 2014b). Reproduction by archeospores implies the release of a unique archeospore from each carpogonium that will generate a new blade (Redmond *et* al., 2014b). Consisting both on the release of multiple spores, the major difference between endospores and neutral resumes in the equality and regularity of the neutral spores, against the disparity and irregularity of endospores (Redmond *et* al., 2014b). Populations of *Porphyra umbilicalis* have been reported with both sexual and asexual life histories: in the northeast Atlantic, they are known for both sexual and asexual reproduction by neutral spores (*Figure 8*), while in the northwest there have been only reports of asexual reproduction (Redmond *et* al., 2014b; Royer, 2017).

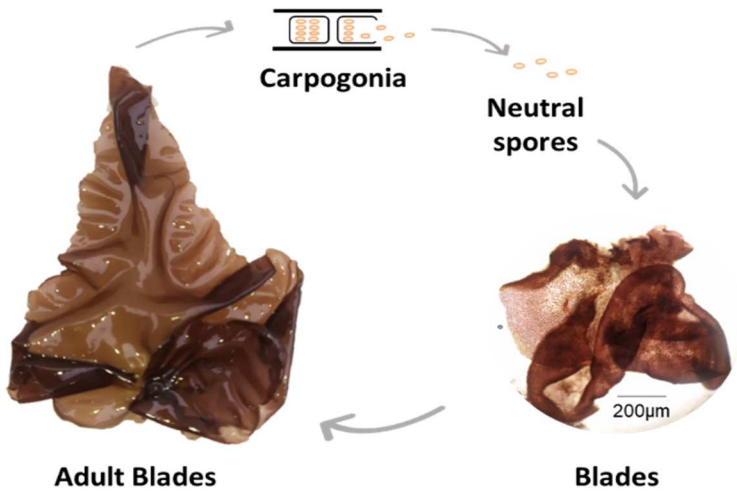


Figure 8. Asexual reproduction by neutral spores. Carpogonia from adult blades release neutral spores that will develop into new blades. (Scheme by ©Inês Oliveira)

Different phases and structures of the life history of *Porphyra* spp. (sexual and asexual pathways) are usually associated with specific times and/or seasons of the year, an indicator of the influence of the environmental factors on their development (Pereira

& Yarish, 2010). Photoperiod, temperature and light intensity are particularly important on the development of the sporophytic and gametophytic phases: withstanding wide ranges of temperature, their performances do not seem to be affected by them, although short-day conditions and specific temperatures (depending on the species) are needed for the formation of conchosporangia and release of conchospores (Pereira & Yarish, 2010). During all the phases of the life cycle, the preferred light intensity ranges between 25 and 50 $\mu mol.photon.m^{-2}s^{-1}$ (Redmond *et* al., 2014a). In species of the intertidal zone, (like *P. umbilicalis*) salinity seems to not influence the development and growth in a range of 20 to 40 ppt (Kim *et* al., 2019; Pereira & Yarish, 2010). As an economically important genus, its physiology has been substantially studied in the last decades, showing that light frequency, air exposure, nutrient availability and the material and shape of the substrate also demonstrate affect the phenotype, nutritional profile and pigment content of *Porphyra* spp. (Pereira & Yarish, 2010).

1.3.Economical value

When referring to the economical value and biomass production, it is impossible to separate *Porphyra* from other important bladed Bangiales (mainly *Pyropia*), due to the recentness of the taxonomical studies that separate them into distinct genera and the substantial occurrence of *Pyropia* spp. in the countries, mainly responsible for seaweed production and uses (Zuccarello, 2011). Hereupon, it is common to find the term "*Porphyra*(=*Pyropia*)", since the two genera are not dissociated in industry, with species of both genera commonly known as "Nori" (Lim *et* al., 2017; Zuccarello, 2011). *Porphyra*(=*Pyropia*) constitute some of the most valuable seaweeds, having the highest commercial value per unit in the seaweed industry (around 523 dollars per ton, wet weight) in a total of more than a billion dollars per year (Lim *et* al., 2017; Pereira & Yarish, 2008). Used for centuries in human food, as an ingredient in sushi, nori is one of the ten most consumed seaweeds, around 2 million tonnes annually (wet weight) (Baweja *et* al., 2016; Pereira & Yarish, 2008).

Rich in protein and fibers, with recordist values of protein registered in seaweeds, (25-50%, dry weight) and with an interesting profile of vitamins and polyunsaturated fatty-acids, blades of *Porphyra(=Pyropia*) have been pointed as vegan alternatives to finfish meal (Pereira & Yarish, 2008). Many studies have been reporting anti-tumoral, anti-allergic and immune-stimulating properties from the amino acids, fatty

acids and sulfated polysaccharides of *Porphyra(=Pyropia*), especially in the gastrointestinal tract (da Costa *et* al., 2018; Pereira, 2018).

As a source of phycoerythrin and phycocyanin, they have been used by their fluorescence in the medical diagnostic industry, and in food and cosmetics as natural dyes, with evidence of pharmaceutical applications (Varela-Álvarez *et* al., 2019); phenolic compounds (majorly mycosporine-like amino acids) have been studied by their UV protective properties, corroborating its use for more valuable meanings, like nutraceuticals, cosmetics and pharmaceuticals (Pereira, 2018; Varela-Álvarez *et* al., 2019) (*Figure 9*).

Figure 9. Economical valorization of different application of *Porphyra (=Pyropia*) (adapted from European Comission, 2020)

1.3.1.Economical potential of conchocelis

Although worldwide used and intensively studied in the last decade, scientific investigation in *Porphyra* has been focusing mostly on the blade phase, and still little is known about conchocelis and their potential as a source of bioactive compounds (da Costa *et* al., 2018; Pimentel *et* al., 2020). Studies on conchocelis have been converging on its characterization of fatty acids (da Costa *et* al., 2018) and amino acids profile (Pimentel *et* al., 2020), as in the phycobilin and mycosporine-like amino acids (MAAs) content (Lee *et* al., 2017; Pereira & Yarish, 2008; Pimentel *et* al., 2020) (*Table 2*).

Conchocelis present a higher lipid content than blades, with a similar fatty acid profile (da Costa *et* al., 2018; Patarra *et* al., 2013). With a higher content of mono and

polyunsaturated fatty acids, constituting an important source of arachidonic acid (20:4) and eicosapentaenoic acid (20:5), conchocelis are a source of excellence for n-3 polyunsaturated fatty acids, described as important regulators of the inflammatory response (da Costa *et* al., 2018). Plus, they present an n-6/n-3 PUFA ratio placed in the recommended range in the human diet for chronic disease prevention (da Costa *et* al., 2018). The protein portion of conchocelis has been highlighted for its antioxidant potential (Machado *et* al., 2020; Pimentel *et* al., 2021).

Studies on both blades and conchocelis of two species from the northeast Atlantic (*P. dioica* and *P. umbilicalis*) show higher protein percentages and better essential amino acids index (in comparison to pattern values) in conchocelis, although the nitrogen content seems to be similar in both phases (Machado *et* al., 2020). Several pros have been pointed regarding the commercial extraction of phycobilin, like their growth rate, ease production and maintenance of crops for a long period, even though the content of MAAs seems to be lower than in blades (Lin & Stekoll, 2011). When compared with the blade phase, conchocelis outstand by far in phycobilin content (30-60 $mg.g.dwt^{-1}$ and more than 100 $mg.g.dwt^{-1}$, respectively, under optimal conditions) (Lin & Stekoll, 2011). These extracts have been documented for antioxidant and UV protective properties (Pereira, 2018).

Lack of awareness, undersupply from local markers and high production prices in comparison to the Asian markets have been restraining the European nori market (European Comission, 2020). In the last decade, the interest to include sea vegetables in the European diet led to rising interest for local species, the so-called “Atlantic nori” (ie. *Porphyra umbilicalis, P. dioica* and *P. purpurea)* (European Comission, 2020; Pimentel *et* al., 2020). Until the recent closure of the production cycle of *P. umbilicalis* and *P. dioica in 2017 by ALGAplus Lda*, biomass supply in Europe was mainly imported from Asian countries, and only about one percent came from local species, predominantly from seasonal harvest in France (Pimentel *et* al., 2020). These developments bring the possibility of biomass supply on the European markets of food and cosmetics, establishing the main step for the growth of the Atlantic nori market, for both blades and conchocelis (Pimentel *et* al., 2020).

Table 2. Potential of conchocelis and different applications

	Potential	Applications
Fatty acids	Higher content than blades; Higher portions of PUFAs and MUFAs and lower of SFAs; An n-6/n-3 PUFA ratio on the recommended range (da Costa *et* al., 2018; Patarra *et* al., 2013)	Chronic disease prevention; Regulation of the inflammatory response (da Costa *et* al., 2018)
Amino acids	Higher percentage than blades; Better essential amino acids index (Machado *et* al., 2020)	Antioxidant (Pimentel *et* al., 2020
Phycobilins	Higher content than blades (phycobiliproteins) (Lin & Stekoll, 2011)	Antioxidant; UV-protection; Natural dyes (cosmetics) (L. Pereira, 2018)

1.4.Aquaculture

Nori aquaculture is responsible for almost all the biomass supply (Kim *et* al., 2017). Mainly cultivated in three countries, (China, Korea and Japan) the major species produced are *Neopyropia yezoensis* and *Neopyropia haitanensis* (Kim *et* al., 2017). Dated in the 17th century, *Porphyra(=Pyropia*) is one of the most ancient cultures of seaweed (Pereira & Yarish, 2013). At the time, cultivation consisted of placing nets on the shores at a certain time of the year, where adult blades would appear naturally and harvested from the nets or cut and harvested from the same individuals more than once (Sahoo & Yarish, 2005). After the elucidation of the life cycle by Drew (1949), modern techniques were developed, and cultivation methods were proposed in the late 1960s to the early 1980s (Pereira & Yarish, 2010).

Currently in large-scale production of Nori (Asia), cultivation settles in four major steps: culture of conchocelis, seeding nets with conchospores, nursery of the nets seeded with small blades and harvest of adult blades (Sahoo & Yarish, 2005). Similar in all countries, some variations on the methods have been described (Kim *et* al., 2017). After conchospores release, induced by stirring or by low-temperature seawater conditions , seeding nets is done differently on open water or in land-based tanks (Sahoo & Yarish, 2005). In open water, several nets are overlapped in support systems, where the cultures of conchospores are dropped over the nets and adhere to them (Sahoo & Yarish, 2005) (*Figure 10A*). In indoor conditions, similar nets are slowly rotated by a rotative wheel inside the tanks (*Figure 10B),* or the nets are placed at the water surface and the cultures are stirred with an air pump to fixate to the nets (*Figure 10C)* (Sahoo & Yarish, 2005).

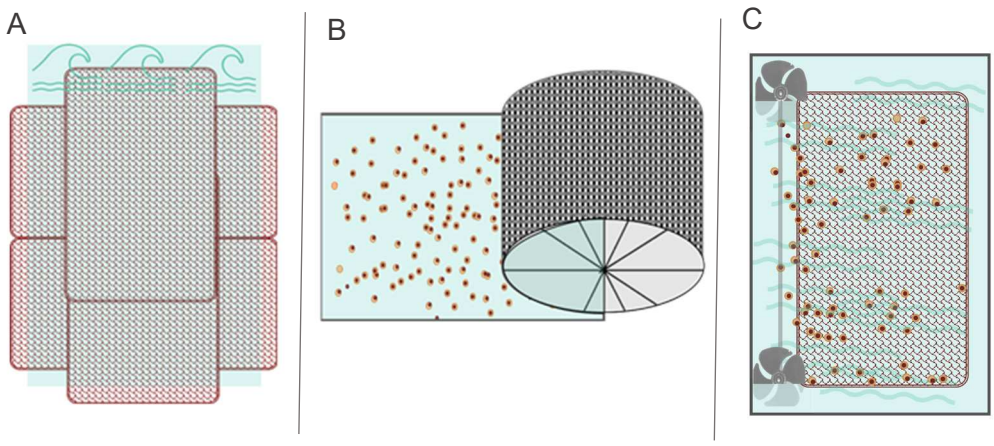


Figure 10. Seeding methods on open water (A) and in land-base tanks, by rotatery wheel (B) and by water stirring (C). (Scheme by ©Inês Oliveira)

The methods of control of epiphytes' growth may also vary: most Chinese aquacultures raise the nets daily, exposing the cultures to dissection, while Korean prefer to control the pH by applying organic acids to the nets (Kim *et* al., 2017). Three different substrate systems have been described: fixed nets (according to the tides, nets are air-exposed – low tides - or underwater – high tides), floating nets (nets mounted that are always floating at the surface of the water), or semi-floating nets (possibility to fix them at a determined level or to be floating at the surface) (Redmond *et* al., 2014).

1.4.1.Opportunities and challenges

Combining the economic value, increasing utilization on more valuable applications and their interesting growth rates (10-13% per day), nori cultivation is one of the most lucrative and promising in the seaweed industry (Kim *et* al., 2017; Redmond *et* al., 2014; Royer *et* al., 2019). One of the major advantages of cultivation is the possibility to develop a market; harvested from natural banks biomass is not enough for that (FAO, 2016). Plus, by controlling water quality on the cultivation spots, enables to reduce their heavy metals toxicity and provide products of higher quality (FAO, 2016).

Some of the current challenges consist of disease control (Ward *et* al., 2020). Still little is known about contaminants of nori, diagnosis and prophylaxis; yet, the high prevalence of insurgent diseases and contaminants increasingly limits the industry growth (Rusekwa *et* al., 2020). Nori cultures seem to be more affected by viruses than bacteria, harder to control and fight, leading to biomass and quality losses, since blades tend to be weak and discolorated (Ward *et* al., 2020). Directly associated, one of the weaknesses of nori cultivation and transversal to seaweed production is related to the absence of effective biosecurity policies and best aquaculture of seaweed practices (Bera *et* al., 2018; Rusekwa *et* al., 2020).

It is intended by biorisk the combination of occurrences of harm, and its severity, which the cause is a biological agent or toxin, making biorisk management the development of a set of strategies and protocols to minimize the likelihood of the occurrence of these occurrences (Smith, 1993). Biosafety and biosecurity are essential in successful biorisk management, biosafety englobing the protocols and practices that aim the protection and reduction of the risks of accidental infection to the operator, while biosecurity focuses on the protection of the population from microbial contamination (Rusekwa *et* al., 2020; Smith, 1993). Some of the biosafety practices also cover biosecurity aspects, as some biosecurity measures reinforce biosafety, making them interconnected concepts that should be applied together in aquaculture production (Rusekwa *et* al., 2020; Smith, 1993). Developing biorisk practices and specific legislation applied in seaweed aquaculture is urgently required to limit these impacts and increase the acceptance of nori aquaculture in Western countries, particularly in Europe (Rusekwa *et* al., 2020; Ward *et* al., 2020).

Since the advances in the early 1980s related to the elucidation of the life cycle, fewer advances were settled, besides cold storage of seeded nets with conchospores, allowing the extension of the harvest season (Blouin *et* al., 2007; Kim *et* al., 2017). The turn of the century revived the interest to develop nori cultivation in western countries focused mainly on two strands: machinery development and the establishing of more

effective production protocols, increasing cost-effectiveness and improving yield results, respectively (Blouin *et* al., 2011; Kim *et* al., 2017). Cultivation of free-living conchocelis in controlled indoor environments and establishing the most suitable combinations of nutrient availability, temperature, photon flux density (PFD), photoperiod, and salinity conditions species and phase-specifics have been the main focus on improving the efficiency of production protocols (Redmond *et* al., 2014).

Traditionally, conchocelis culture in Asia consists of a simulation of its natural conditions of development, obtained from stocked or harvested blades stress-stimulated by dissection to release zygotospores in oysters or artificial shells (Redmond *et* al., 2014). Under low photon flux density (25-50 μmol.photons.m^{-2}.s^{-1}) at long-day conditions (16L:8D) and around 23˚C, in large tanks of 25-50 cm depth, (Pereira & Yarish, 2010) conchocelis will grow vegetatively, in substrates hunged vertically (*Figure 11A*) or spread at the bottom of the tanks (*Figure 11B*) (Sahoo & Yarish, 2005). Recent work on developing protocols of mass production of free-living conchocelis of *Porphyra* spp. in indoor flasks and carboys up to 15 L (He & Yarish, 2006) and photobioreactors of 300mL (*Figure 11C-D*) allow to obtain pure cultures from specific strains and induce conchospore release several times per year, harvesting blades during all year (Pereira & Yarish, 2010; Zhang *et* al., 2006). These protocols are especially important in conchocelis cultivation, since it is possible, logistically, to produce them indoor, under controlled nutrient availability, light, temperature and PFD, difficult to control in outdoor conditions (Varela-Álvarez *et* al., 2019).

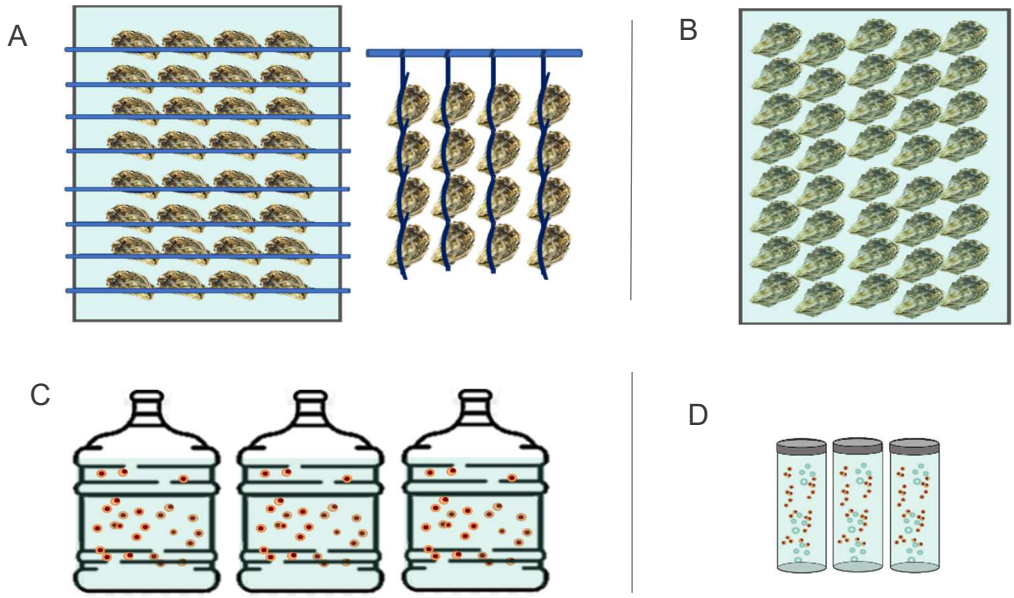


Figure 11. Conchocelis culture: in substrate (A-B) and free-living mass production in carboys (C) and in photobioreactors (D). Substrates can be placed vertically along the water column (A, top view (left) and close up of the disposition on the water column - right), or on the bottom of the tank (B). (Scheme by ©Inês Oliveira)

With the increasing interest in using nori in nutraceuticals, cosmetics and pharmaceuticals, recent studies have been also focusing on the influence of these conditions on the pigment and nitrogen content (Varela-Álvarez *et* al., 2019). The requirements for purer strains and elucidated genotypes of these industries have been leading to the increasing work on molecular verification of specimens, which may also contribute to product acceptance by European consumers (Yang *et* al., 2020). For free-living conchocelis culture, temperature, photoperiod and PFD seem to be the turning-point conditions: temperatures under 25 ºC (10-15 ºC in some species), photoperiod over 12h and low PFD (25-100 $\mu mol. photons. m^{-2}. s^{-1}$) have been described in some species of *Pyropia* as the optimal conditions (Lu & Yarish, 2011; Redmond *et* al., 2014; Zhong *et* al., 2016). Salinity has been described, similarly to the registered in the blade phase, not showing significatively differences between 20-40 ppt (Pereira & Yarish, 2010).

The light type and wavelength have been indicated by literature to influence vegetative growth, photosynthetic activity and pigment content (Osório *et* al., 2020) but studies in *Pyropia dentata* showed no differences in cultures under LED of blue, red, blue+red, and fluorescent lamps (Kim *et* al., 2019). It has been reported that higher temperatures and PFD values, on the verge of the lethal values, show higher growth rates of conchocelis cultures, but it has consequences on the development and quality

of the biomass of the blades (Waaland *et* al., 1987) that can be useful in the optimization of production of conchocelis as a final product. Waaland *et* al. (1987) described that free-living conchocelis of *Porphyra torta* in LD conditions showed the fastest growth at 300 $\mu mol.photons.m^{-2}.s^{-1}$ and 15 ºC than at 25-100 $\mu mol.photons.m^{-2}.s^{-1}$ and 10-15 ºC.

Studies on *P. umbilicalis* cultures in New England (Canada) shown that growth rates of 6.98$\pm$0.51 $\%.d^{-1}$ were reported in 10-40 $\mu mol.photons.m^{-2}.s^{-1}$ and 5-20 ºC and the upper lethal temperature was registered at 25 ºC (Chopin *et* al., 1999). According to the "New England Seaweed Culture Handbook" (Redmond *et* al., 2014), the optimal conditions of free-living conchocelis in laboratory conditions (flasks or gallons) presuppose temperatures of 10-15 ºC, LD or neutral (12L:12D) photoperiod and 25-50 $\mu mol.photons.m^{-2}.s^{-1}$. Pereira *et* al. (2001) in a study in the east Atlantic (Portugal) described that the optimal conditions for these species were obtained in LD photoperiod, at 15 ºC and 25 $\mu mol.photons.m^{-2}.s^{-1}$ in conchocelis culture.

1.4.2.Nutritional requirements

Macronutrient requirements are transverse to all seaweed: nitrogen (N), phosphorus (P) and carbon (C), but the major limiting nutrient in seaweed production is the first one (Redmond *et* al., 2014). *Rhodophyta* and pigment proteic levels are closely related to the N content (Redmond *et* al., 2014). Leading to a decrease in protein content, the use up of the N content leads to a consequent increase on the polysaccharides and lipid content in nori thalli; insufficient nitrogen causes depigmentation and bleaching of the biomass. (Redmond *et* al., 2014). Bleaching and lower protein content are responsible for lower market acceptance and, consequent decrease economical interest (Redmond *et* al., 2014; Zhong *et* al., 2016) Some work has been developed to create simpler methods of evaluation of the N content in seaweed (*Ulva* sp. and *Gracilaria* sp.), associating Pantone® colors and N content, allowing the producers to rapidly diagnose the quality of the biomass (Robertson-Andersson *et* al., 2009); yet, there is no published work applicable in nori specimens. Nitrogen is available in the form of nitrate (NO_3^-), ammonium (NH_4^+) and urea. Usually, NO_3^- is added by artificial supplementation, but it has lower N utptake than NH_4^+ and urea, result products of invertebrates and fish metabolism (Roleda & Hurd, 2019).

Carbon requirements are directly linked to photosynthesis. It is usually obtained from bicarbonate (HCO_3^-) in seawater, or from the residual production of carbon dioxide

(CO_2) from fish metabolism; yet, utilization of C from HCO_3^- is more limited than from CO_2 (Roleda & Hurd, 2019). As it is used, seawater pH increases, until the depletion point, when photosynthesis stops; bubbling or stirring CO_2 have been used to incorporate CO_2 in the water, balancing pH under optimal values (7.80–8.01) (Blouin *et* al., 2007; Redmond *et* al., 2014). Water motion also increases nutrient uptake of N and P (Redmond *et* al., 2014; Roleda & Hurd, 2019). Phosphorus is involved in protein synthesis since it is a major constituent of ribonucleic acid, whereby P is crucial to tissue growth (Douglas et al., 2014; Louren et al., 2007). It can be obtained from ammonium phosphate monobasic ($(NH_4)_2HPO_4$) or from phosphates (PO_4^{3-}) resulting from fish farming (Douglas *et* al., 2014; Redmond *et* al., 2014). When phosphorus supplementation is used, lag periods after the artificial supply have been reported (Wells *et* al., 2017).

Specific micronutrients required for nori cultures include metallic elements (iron, copper, manganese, calcium, potassium, zinc, cobalt, magnesium, molybdenum), chlorine, sulfur, oxygen and hydrogen, usually found in seawater (Redmond *et* al., 2014). These elements usually do not need to be artificially supplied (especially metals) if high flow rates from natural seawater are used (Redmond *et* al., 2014). Artificial supplementation of macronutrients must be considered the optimal C: N: P ratio for the specific culture (around 132: 26: 1 for nori) (Kawaguchi *et* al., 2005). Better results were registered in a three-times per week supplementation comparing to one weekly and keeping cultures in batch during a period after adding nutrients to decrease nutrient losses (Redmond et al., 2014). Yet, artificial supplementation in outdoor tanks or open water cultures turns the methodology ineffective and costly (Roleda & Hurd, 2019). The ability of these species to use C, N and P from chemical compounds resulting from finfish aquaculture feed bleaching and fish effluents lead to the increasing use of integrated multitrophic aquaculture (IMTA) systems in seaweed culture (Roleda & Hurd, 2019).

1.4.3.*Porphyra* spp. in IMTA: bioremediation potential

Finfish and seaweed aquaculture may be the response in the future to the exponential population growth and consequent food needs, but finfish production has been currently limited due to water scarcity, limited availability and increasing costs of fish meal, whereby blue economy initiatives have been promoted (FAO, 2016). Blue economy defends production growth under sustainable management of water resources, assuring human welfare and environmental stability, defending the need to become less dependent on natural resources, studying the viability of other species, and investing in

best aquaculture practices, minimizing the environmental impact of the aquaculture industry (FAO, 2016; Martinez-Porchas & Martinez-Cordova, 2012).

Treatment of finfish aquaculture effluents depends on one of two biological techniques: bacterial nitrification or plant biofiltration; yet, systems using bacterial nitrification require high technology and usually accumulate nitrates and residuals that need to be eliminated, which is expensive (Pereira & Yarish, 2010). Seaweed biofiltration is settled on the assimilation of nutrients and production of biomass that can be removed or used by its economical value (Martinez-Porchas & Martinez-Cordova, 2012; Pereira & Yarish, 2010). Values from the last decade indicate that seaweed production in China (around 7.5 million tonnes) was responsible for the removal of more than 40 thousand tonnes of nitrogen from coastal waters, sustaining its potential on the mitigation of the environmental impact of finfish aquaculture (Cofrades *et* al., 2010; Pereira & Yarish, 2010).

IMTA systems have been proposed for their environmental sustainability and social acceptance, bringing economic and ecological diversity (Chopin *et* al., 2008). Proven to be the most effective on C, N and P utilization, they achieve a 50% uptake rate against a 25-35% of monocultures (Martinez-Porchas & Martinez-Cordova, 2012). The term “multitrophic” implies the incorporation of species of different trophic levels on the same system (Pereira & Correia, 2015). The principle of these systems consists of combining, in a balanced ecosystem, one culture of fed species (usually finfish), another culture of organic extractive species (shellfish), and/or an inorganic assimilative culture (seaweed) (Chopin & Sawhney, 2009; Pereira & Yarish, 2010). Effluents rich in organic and inorganic matter (ammonium, carbon dioxide and phosphate) of finfish are used as a nutrient source by the other species, converting waste into valuable resources for other cultures of commercial value and minimizing waste production by bioremediation (Pereira & Correia, 2015) (*Figure 12*).

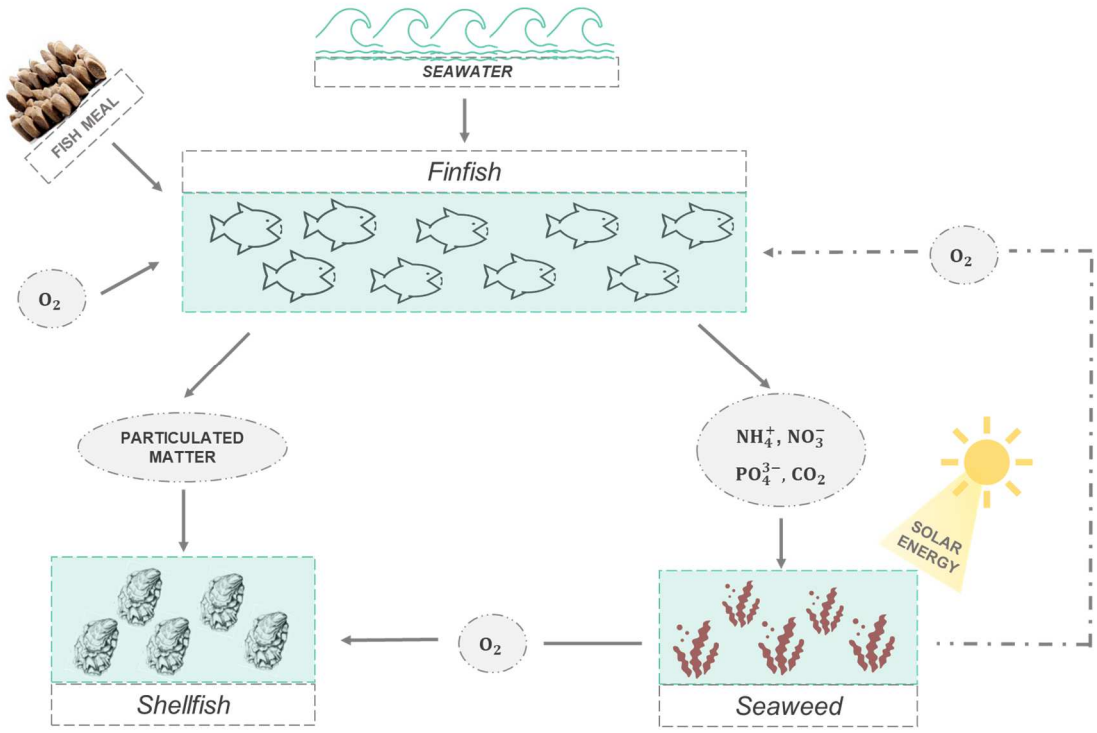


Figure 12 Example of an IMTA system. Effluents rich in organic and inorganic matter of finfish are used as a nutrient source by the other species, converting waste into valuable resources for other cultures of commercial value and minimizing waste production. Water from seaweed production can return to finfish production, oxigenated by seaweed. (Scheme by ©Inês Oliveira)

Integrated aquaculture of nori has been practiced in Asia since its primordials, but not intentionally: due to the extension of fish and shellfish aquaculture in the continent, most seaweed farms happened to be placed near these cultures and assimilate nutrients from their residuals (Pereira *et* al., 2013). The first integrated fish and Nori systems (established for that purpose) were reported to be associated with the implementation of nori cultivation in western countries (Kim *et* al., 2017). At the turn of the century, after a failed try on the cultivation of *Neopyropia yezoensis* in open water in the state of Maine by lack of nutrients, *PhycoGen, Inc*. was successfully able to cultivate *Neopyropia yezoensis* near an Atlantic salmon (*Salmo salar)* farm, leading to the development of an IMTA (Chopin *et* al., 1999).

Since then, western farms (mostly on the American East coast) have been converging their efforts on cultivating species with asexual reproduction, mainly by neutral spores, like *Porphyra umbilicalis* (Blouin *et* al., 2007; Kim *et* al., 2007; Korbee *et* al., 2005; Royer *et* al., 2019; Sampath-Wiley *et* al., 2008). *P. umbilicalis* seems to be the only species described to exclusively reproduce asexually on the American East coast,

corroborating the lack of studies on the optimal conditions of conchocelis culture for this species (Blouin *et* al., 2007). Cultivation of neutral spores species constitutes an advantage in the production of blades biomass, skipping steps of structural development, monetizing the production (Blouin *et* al., 2007). Plus, a market study has shown no consumer acceptance differences between the Asian species *Neopyropia yezoensis* and *Porphyra umbilicalis,* supporting its potential (Blouin *et* al., 2006).

In Europe, only ALGAplus, Ltd. farms *Porphyra umbilicalis* and *P. dioica* on a commercial scale year-round, along with several other seaweed species in an integrated land-based system of fish (sea bream and seabass) (ALGAplus, 2021). In this system, sexual and asexual (by neutral spores) specimens of *Porphyra umbilicalis* and sexual of *P. dioica* are produced in free-floating, recurring to an initial hatchery phase to produce conchocelis and obtain new blades from conchospores, and outdoor tanks to upscale blades (ALGAplus, 2021; da Costa *et* al., 2018; Pimentel *et* al., 2020).

Regarding its performance in IMTA, *Porphyra umbilicalis* presented, in lab-scale experiments, high photosynthetic rates and nutrient uptake efficiency, reaching maximum levels of growth rate (10-13%) and N content (up to 5% dry weight) at 150-300 µM N and 720-1440 µM P concentrations (Carmona *et* al., 2006). It responds better to N in NH_4^+ than NO_3^-, with N removal rates up to 77-100% and 72-87%, respectively; P removal rates rounded 81-91% and 71-79% at 120-1440 µM P from PO_4^{3-} (Carmona *et* al., 2006). Photosynthetic rates were shown to be higher than most species (23 $mmol\ O_2\ g^{-1}.DW.min^{-1}$). While several species can only use CO_2, *P. umbilicalis* has been shown to metabolize C from both CO_2 and HCO_3^- (Maberly, 1990).

II.Internship at ALGAplus, Ltd.

1.ALGAplus, Produção e comercialização de algas e seus derivados Lda.

ALGAplus is a biotechnological Portuguese small and medium-size enterprise (SME) founded in 2011, dedicating its work to produce Atlantic native seaweed species, settling on sustainable and innovative techniques in its process, and promoting the implementation of IMTA systems. Considered one of the European pioneers in seaweed farming, it is a forerunner in its work controlling all the phases of the life cycle of *Porphyra dioica* and *Porphyra umbilicalis*, the company highlights itself for its fish and seaweed integrated land-based system unique in Europe: water rich in nutrients and carbon dioxide (CO_2) from the fishponds feeding seaweed cultures developed in proprietary tank system; seaweed yield is increased and they act as bioremediation agents, oxygenating and freeing water of effluents before it is released back to Ria de Aveiro lagoon. Supporting its work on research, development, and innovation, ALGAplus sums a series of scientific projects on its curriculum and more ongoing, along with a series of partnerships with other companies and entities.

Located in Ílhavo (*Figure 13)* and inserted in a 14ha area of former sea salt pans of the channels of the Aveiro Lagoon (Boco river), ALGAplus started committing its work only to seaweed production, but always working in an integrated system with the fish earthen ponds of the formerly *Materáqua*, pisciculture of sea bream (*Sparus aurata*) and sea bass *(Dicentrarchus labrax).* Nowadays, the company manages both the seaweed and the fish production, having achieved organic certification in both production lines.

Figure 13 Localization of ALGAplus, Ltd. (Google Earth, 2021 (adapted))

With over 30 tonnes of seaweed produced per year (*fw*), the company farms and trades a panoply of red (*Chondrus crispus, P. dioica*, *P. umbilicalis*, *Palmaria palmata* and *Gracilaria gracilis*), brown (*Fucus vesiculosus*) and green (*Ulva rigida* and *Codium tomentosum*) species mainly for food and cosmetics (INE, 2020; ALGAplus, 2021) (*Table 3*), exporting since 2014 over 75%. It possesses seven commercial brands: ALGAplus®, for the trade of services and products in the B2B segment, *Tok de Mar*®, for seaweed-based food products sold in specialized retail and HoReCa channels, Pão d'Algas® for a bread-ready mix developed and co-branded with IPL, *SeaOriginals*, with an offer of well-being products, *Algaessence*®, a combination of macro and microalgae food and feed products (co-branded with the company Allmicroalgae S.A.) and more recently the brands for products sold in wholesalers (e.g. Auchan, Pingo Doce,…) *Companhia das Algas*® for seaweed products and AquiLusa®, for organic certified fish.

Table 3. Production, in tonnes (wet weight), of seaweed and fish in ALGAplus, Ltd. in 2019 (INE, 2019, adapted) (* values not available)

	Species	***Production (ton)***
Seaweed	*Ochrophyta*	*0.24*
	Rhodophyta	*1.7*
	Chlorophyta	*43.3*
	Total	***35***
Fish	*Sparus aurata*	*X**
	Dicentrarchus labrax	*X**
	Total	***x****

1.1.Work Facilities

The production area (*Figure 14*, marked at yellow) consists of an integrated system of a polyculture fish production (sea bream and seabass), which takes place in two earthen ponds, along with a series of polyethylene and cement tanks of different volumes, where, sideways with a hatchery, the seaweed cultivation is done. At the moment, the remaining area (*Figure 14*, marked at red) belonging to the company and mainly constituted of earthen ponds is still unused, foreseeing the possible upscale of fish and seaweed production in raceways.

Figure 14. Company area (in red), including the production area (in yellow), where Water capture (A), Fish production (B), Sedimentation tank (C) and Seaweed production (D) are inserted. (Google Earth, 2021 (adapted))

The fish earthen ponds are naturally water-supplied by the channel of the Aveiro Lagoon and renewed by the tides' cycle. From the earthen ponds, water with dissolved nutrients flows to a sedimentation tank and is pumped into the seaweed system. Before entering the seaweed cultivation units, the water follows to a rotofilter and a sand filter, a skimmer and sterilized with UV and ozone to ensure that cultures are free from microalgae and other biological contaminations. Water that supplies the seaweed production also supplies the deposit, from where it is mechanically filtered through different sieves – filtered seawater (FSW) and supply maternity and seaweed production. In Maternity, FSW can be sterilized in the autoclave (autoclaved seawater - ASW) to be used in more sensitive cultures.

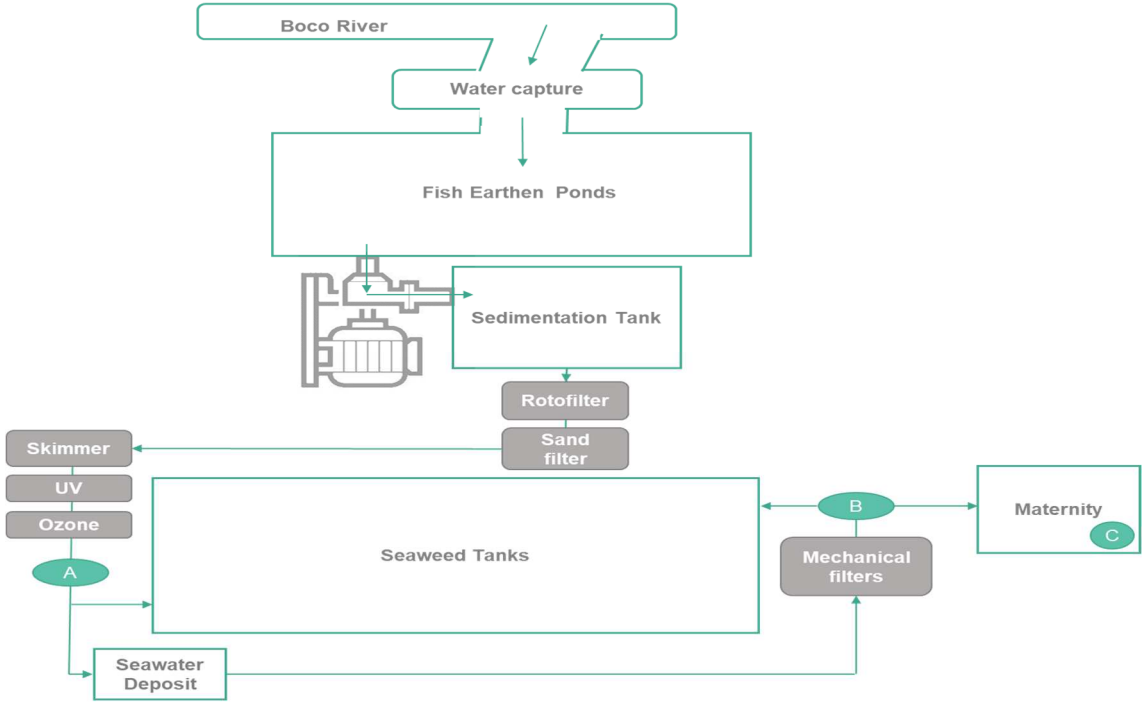


Figure 15. Draft of seawater pahways in the aquaculture. From Rio Boco, water feeds the fish earthen ponds. A pump supplies the sedimentation tank, following from here to the rotofilter and the sand filter, then to the skimmer and UV radiation, before being used in the seaweed system. Water that supplies the seaweed production also supplies the deposit (A) from where it is filtered by mechanical filters (B) and supply maternity and seaweed production. In Maternity, FSW (B) can be sterilized in autoclave (ASW) to be used in more sensitive cultures (C).(Scheme by ©Inês Oliveira)

1.2.Work Departments

1.2.1.RDI Department

The RDI Department is composed of a technical team of Biologists created to provide support to the Operations team and lead the innovation activities of the company. It has two main working areas: a indoor lab and hatchery, and an outdoor experimental-scale tank system (15 to 1000 L capacity), the Experimental Outdoor Area (EOA) (*Figure 16*). As the name indicates, the goals of this department include research projects applied to algae biotechnology and cultivation, optimization, including specific biochemical features, the maintenance of a biobank with multiple species and strains, and the assurance of the quality of the cultures. Therefore, the RDI department studies the optimal culture conditions of different species (stock density, availability of nutrients, photoperiod, temperature, salinity and light intensity) and tries to domesticate economically important seaweed species. The maintenance of these conditions on the current cultures, both in and outdoors, are assured daily.

Figure 16. Tanks from EOA of different volumes (up to 1000 L) ©Inês Oliveira

The maternity/nursery is composed of a laboratory room, equipped with all the necessary material, equipment and conditions to assure all the procedures needed, along with two environmental chambers (*Figure 17*) with controlled temperature, light intensity and photoperiod, producing cultures in units of different sized (from 250 ml flasks to 80 L column PBRs). These two chambers are specially programmed for the optimal conditions (confidential) of cultivation of *P. dioica* and *P. umbilicalis,* allowing the manipulation of the life cycle of these species all year. In the maternity, the following activities take place: start of biomass cultivation, the maintenance of the biobank, as of some cultures of ongoing research projects, requiring a more controlled and clean work environment using FSW or ASW. Cultures are continuously upscaled to the outdoors area according to biomass evolution.

Figure 17. Maternity chambers @Inês Oliveira

When new cultures arrive in the maternity, they are attributed a batch number, stored in the biobank and/or used to initiate a new production. Firstly, they pass a standard cleaning internal protocol (confidential information) intending the elimination of epiphytes and other contaminating agents (i.e. microalgae and cyanobacteria), sand and small predators, improving their performance of growth and keeping all the environment

in the lab cleaner and safer for seaweed production. After cleaning, new cultures initiate acclimatization in the maternity and are upscaled gradually until transferred to the EOA, when already more resistant and better adjusted to grow in outside conditions.

For the majority of species in production, (except *Porphyra dioica* and *P. umbilicalis,* which the company targeted to manipulate the life cycle) biomass cultivation consists of vegetative propagation. For more sensitive species, starting the production in the maternity leads to better results when transferred to EOA, enabling the control of contaminations and the presence of epiphytes. For *Porphyra dioica* and *P. umbilicalis, cultivation* differs a little bit: the early stages of the life cycle (conchocelis, conchosporangia to juvenile blades) are manipulated via photoperiod and temperature, in the maternity, following the "Atlantic Nori hatchery/nursery (*Porphyra dioica* and *Porphyra umbilicalis*)" protocols, established by ALGAplus. The maturation of conchocelis into conchosporangia and release of conchospores is induced by photoperiod and temperature changes. Excess conchocelis biomass is discharged or stored, while juvenile blades pass through an upscaling procedure from maternity to the outdoors system until being ready to harvest, all year round. (*Figure 18).*

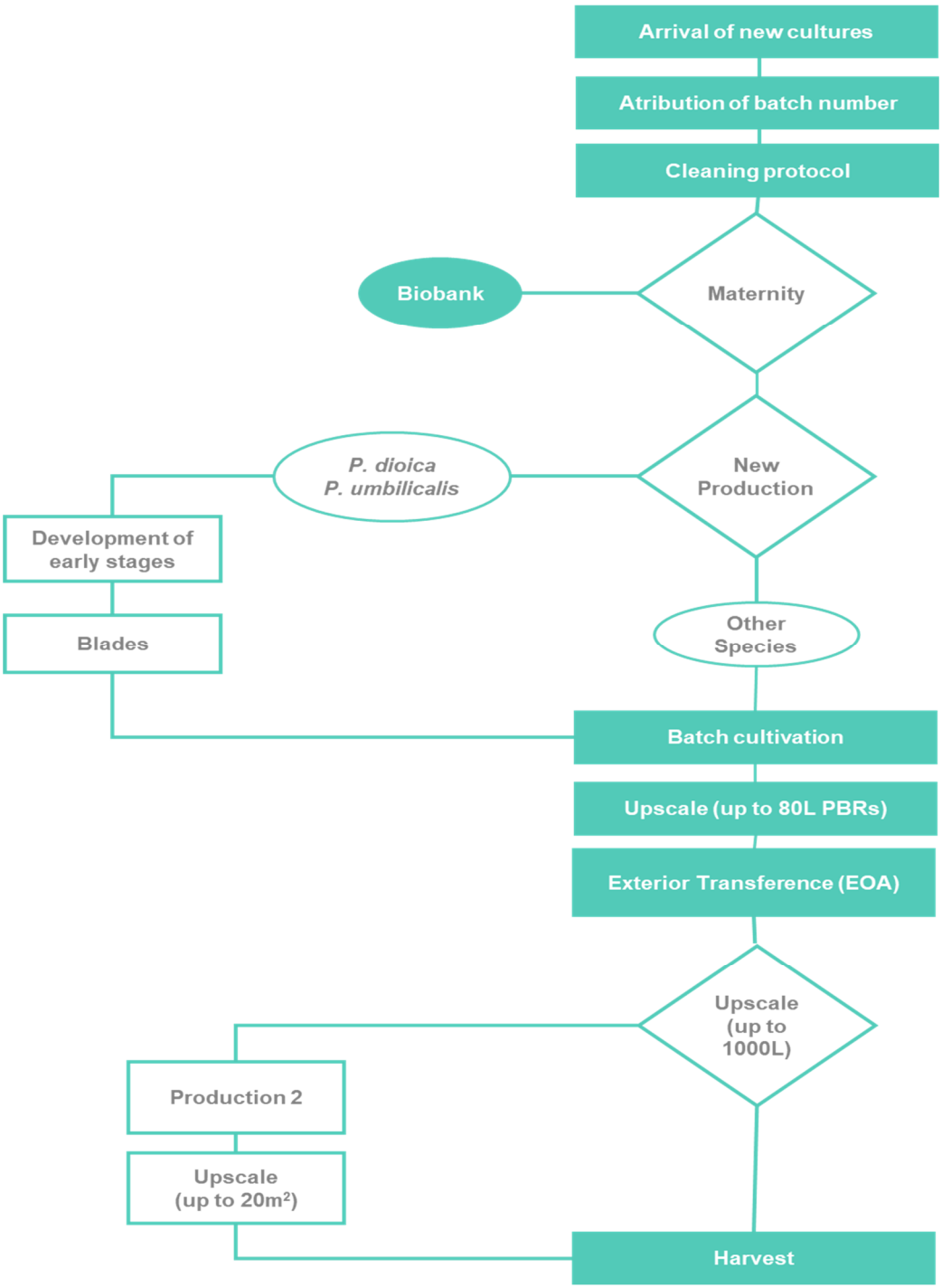


Figure 18. Cultures pathway in RDI department (Scheme by @Inês Oliveira)

All the maternity cultures' are connected to the aeration system of the chambers, keeping them in constant movement; these cultures, unlike in the majority of the outdoor ones, are cultivated in batch (*Figure 19*). Routine management of both maternity and EOA cultures implies stocking density adjustment/upscale, and medium

changes. The outdoors cultivation area is also connected to an aeration system and these cultures are maintained in a constant water flow system from the seaweed seawater system, with species customized daily renovations. The first quality control in the production process is assured by the technical team, by evaluating different parameters in cultivation. Weekly, macro and microscopic observations are performed in all production tanks to assess: color, texture, size, reproductive status (vegetative or reproductive), the presence of strange objects, contaminations, epiphytes, biofouling and other predatory organisms.

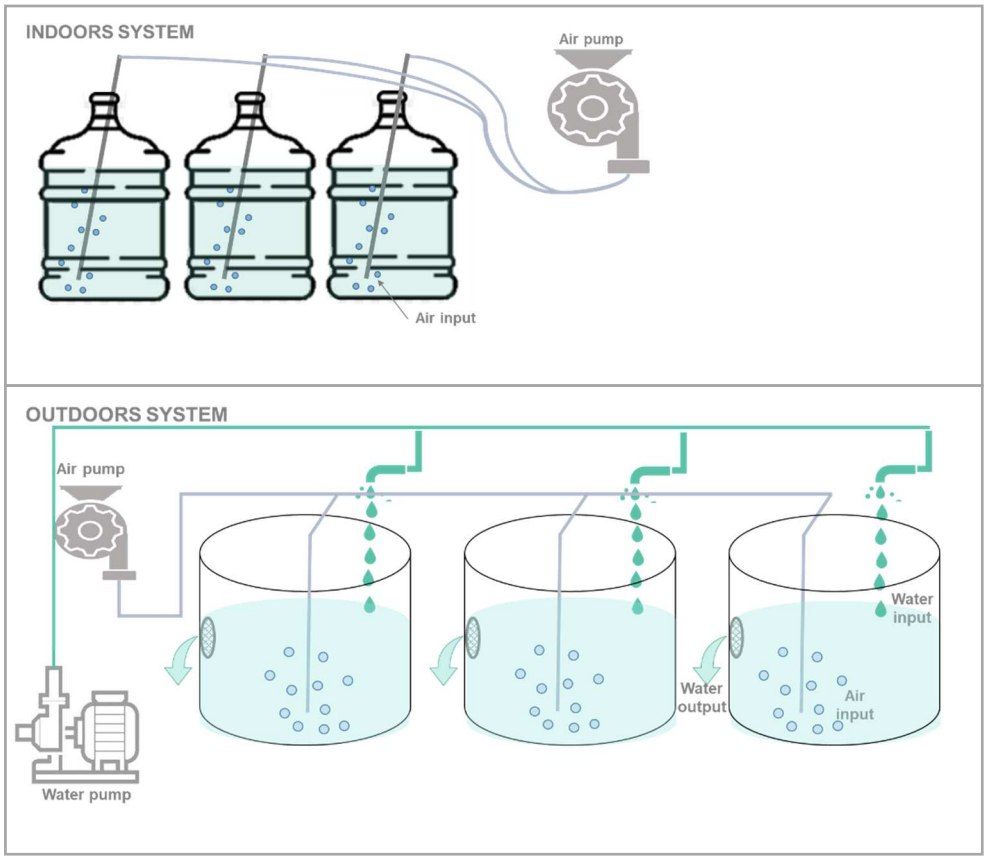


Figure 19. Draft of the indoor and outdoor system. In indoors, cultures are maintained in batch, while outdoors, they are cultured in continuous flow (although some cultues are also cultured in batch) (Scheme by @Inês Oliveira)

1.2.2.Operations Department

The Operations Department manages the fish and seaweed commercial-scale activities and includes different workspaces corresponding to the maintenance of the larger tanks in production (7 m^2 to 20 m^2) and all the product transformation chain, from harvest to final product packaging and storage until they are sold. The production department is also responsible for water temperature, salinity and light intensity daily measurements in seaweed production in random tanks of each typology. Weekly, water temperature, salinity and pH from the seawater deposit are measured. Another important action passes from inspection of abnormal situations or behaviors, along with notice of mortality.

Figure 20. Production tanks (up to 20 m^2) @Inês Oliveira

The first step in seaweed processing (harvest from the tanks) is done manually, with fishnets, from the Production tanks, based on biomass weight evolution and quality control report. In the Processing facility, *fw* and centrifuged weight are registered. Seaweed are then washed with seawater and afterward manually inspected to remove biomass of small crustaceans, shells, sand and rocks not cleaned in the washing process. After weighing and washing, seaweed can be processed fresh or dried. The algae meant for fresh selling are packed for direct consumption, or preserved in salt and packed in plastic buckets or bags. Seaweed for dry selling is subjected to a low-temperature drying process, until a constant (and proved safe) moisture content is

reached, minimizing the enzymatic activity, preserving the properties of the bioactive compounds. Dried algae can be packed either whole or milled in flakes of different sizes or powders.

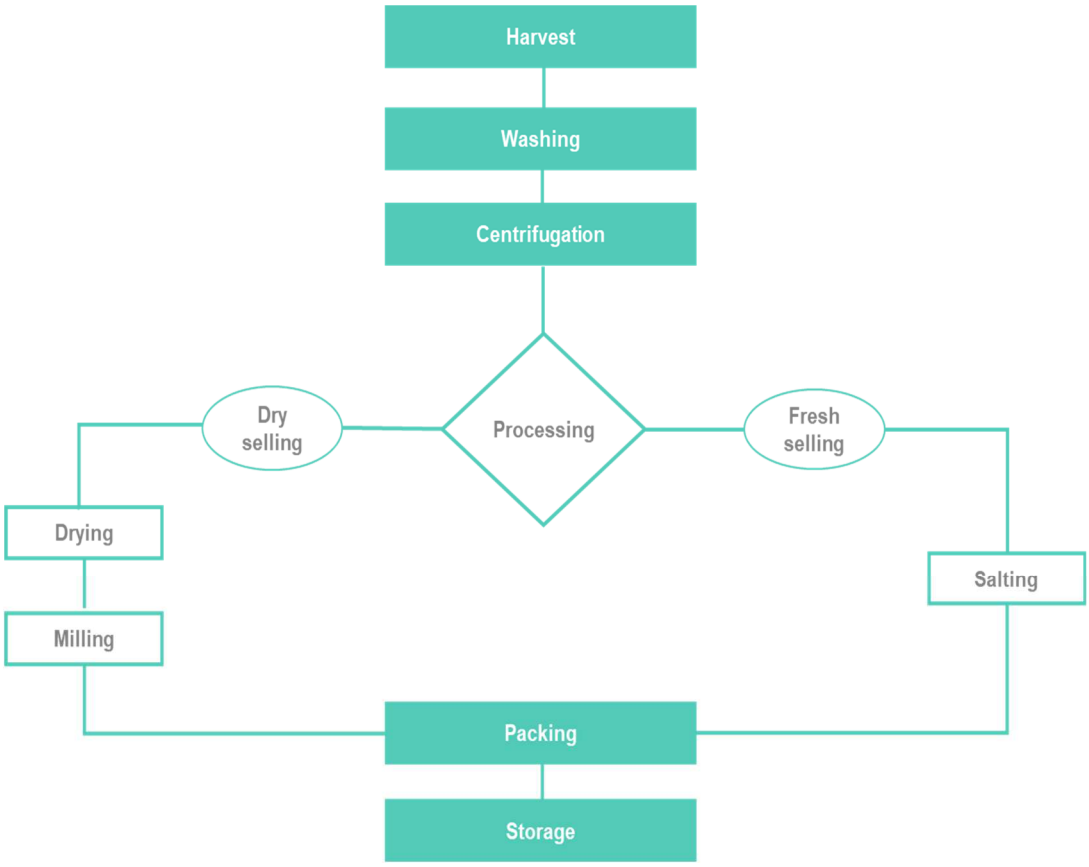


Figure 21. Fluxogram of the cultures pathway in the Operations Department (Scheme by @Inês Oliveira)

1.3.Recording and adjustment of abiotic factors

Abiotic factors like water temperature, salinity, pH and light intensity are known to affect the performance of seaweed and fish cultures, therefore measuring and keeping a database of these parameters is crucial. The seasonality of abiotic factors in the Aveiro lagoon is well marked: with hot and dry summers, and cold winters with heavy rains more incident in December and January in this region, factors like water temperature, salinity, pH and dissolved oxygen derived from the channel that supplies the production experiment some variation during all year, as light intensity in the production tanks

(Rebelo, 1992). Water temperature and salinity variations differ between the various volumes and shapes found in the system; smaller masses have bigger temperature variations when receiving the same heat energy, whereby water temperatures in cultures in smaller tanks is more significant, and the quantity of freshwater from raining affects salinity differently conforming the proportion volume/surface area (Hemminger & Höhne, 1894).

The schedule of these measurements has a purpose: dissolved oxygen in the fish earthen ponds is affected by photosynthetic activity that drops, during the night, whereby dissolved oxygen at the beginning of the daylight is at its lowest and therefore it must be checked out during the day. Parameters are measured in the middle of the afternoon in the seaweed system because high-temperature water constitutes an alarming factor in seaweed production of the species in culture, and at this time of the day, the water temperature reaches its highest (Harrison & Hurd, 2001).

Even though there is some resistance of the cultures to temperature, salinity and light intensity variations, some adjustments in the production can be done when the parameters reach limiting values. When salinity drops under critical values, (usually in the winter, because of the heavy rains and values depending on the species) sodium chloride is added to the cultures, to adjust salinity at 35 ppt (standard seawater salinity) (Harrison & Hurd, 2001). In these cases, if precipitation lasts heavily for several days, the salinity is also measured in the morning (at 8:30h) since during these times salinity variations are higher and require more supervision. During summer/months of higher temperatures and light intensity, shading nets are placed to lower water temperature and to adjust light intensity above 55 $\mu mol.photons.m^{-2}.s^{-1}$ just under the seawater surface of the tanks) (Harrison & Hurd, 2001). For the fish, the alarming parameter is dissolved oxygen: when under 3 $mg.L^{-1}$ (value considerable critical to the conditions on the fish tanks of the company), the oxygenation paddles are turned on and start to agitate the surface of the water, rising the levels of dissolved oxygen.

1.4.Biorisk Management

Different biorisk management policies are applied in ALGAplus facilities, some of them transversal to all the company, others specific for some workspaces; to assure the worker's understanding of these practices, periodically there are training actions about this subject. Due to the COVID-19 pandemic, along with all the biosafety protocols already implemented at the company, a contingency plan was created on the 4th of May of 2020 to minimize the risk of contagion of the collaborators, including the mandatory

use of mask, alcohol gel disinfection of hands, disinfection of surfaces and fomites with a 70% ethanol solution, telework whenever it is possible and rotative schedules (including breaks and lunchtime) to limit the number of people in each division according to the government measures.

Some of the biosafety strategies implemented at the company include the restricted access of authorized people in the production area, biohazard labeling of dangerous matter and their containment in inert containers, and mandatory use of personal protective equipment specific, adapted to the different workspaces: gloves, at any workspace, when manipulating potentially hazardous materials; lab coat, autoclave-resistant shoes in the laboratory and heat-protective gloves when manipulating hot material from the autoclave; rainboots in the outside production, and lab coat, rain boots and mob cap for the processing section. Each element of the personal equipment must be exclusive to each workspace. Other biosafety practices pass by hand washing before and after handling biomass or potentially hazardous materials, avoid hand-to-face contact in the same circumstances, protect open wounds with gloves and periodically disinfection during the day of work surfaces and hands with an ethanol solution, along with daily disinfection of the laboratory and processing section with 5% sodium hypochlorite solution.

Biosecurity in aquaculture settles in three main cornerstones: population management, pathogen management and people management. Biosecurity protocols at ALGAplus follow the same base and line of thought, although with some adjustments, since the concepts established for “biosecurity in aquaculture” refer to animal productions, with very little work done in the development of biosecurity in seaweed production (Rusekwa *et* al., 2020).

Population management starts with choosing native local species, and whenever possible, more resistant to contaminations and abiotic stress strains, and the application of an effective cleaning protocol that eliminates microorganisms and other contamination agents before introducing new cultures (described in the description of the RDI department). When introduced, each culture, after attributing its batch number, is maintained in separated balloons with the opening protected by parafilm. Identification and physical isolation of the cultures are practices that consent with the “all-in-all-out stocking” policy implemented in animal productions, preventing the exposure to contaminations or its spread, and balancing the development in the same container. Routine management action and observations, along with quality control protocols (also already described) and routine observations are another set of practices that enforces biosecurity in populations, by allowing the detection of losses of biomass or weight gain

under the expected, discoloration of the cultures, abnormality of the individuals or an unusual presence of contaminations, indicators of distemper.

Biosecurity associated with pathogen management consists of preventing, reducing or eliminate pathogens. Being able to recognize and diagnose a pathogen infection is crucial in pathogen management; therefore, one of the practices includes constant revision work in pathogens affecting seaweed cultures. Pathogens can be living in reservoirs, including equipment, seawater, system components, work surfaces, pavements and walls. Good sanitation and disinfection practices are severely important in this goal, whereby all equipment, work surfaces and pavements, and all vehicles pass through a cleaning and disinfection protocol: these must be removed of dirt and/or organic matter, rinsed with freshwater, disinfected with a 5% solution of sodium hypochlorite, rinsed again and left to dry naturally.

The material used in the maternity must be sterilized in the autoclave after its cleaning and disinfection, covering it when necessary (in materials with an opening and without proper cover) with aluminum foil and stored in closed cabinets. For the material not resistant to autoclave, it must be disinfected with a 70% ethanol solution. Work surfaces in this workspace, similarly to what happens with the material, must be complementarily disinfected with a 70% ethanol solution before each culture manipulation. Since in this closed workspace different cultures of different species are maintained and can be manipulated at the same time, all the beakers' and gallons' tops are covered by parafilm and the cultures are disposed at the chambers so different species are distributed in different aeration lines, and when using the same line, cleaner and more sensitive cultures must be placed first, so if there is accidentally crossed contaminations, it is not as much severe. When manipulating different cultures at the same time, it is especially important to create barriers of disinfection or even physical barriers: in these cases, cleaner and more sensitive cultures must be manipulated as far as possible from the most contaminated ones. For more sensitive cultures, it must be done in the laminar-flow chamber, equipped with ultra-violet sterilization, which provides a more sterilized work environment. Independently of the workspace, the cleaning and disinfection protocol must follow a unidirectional line of dirty/clean materials, avoiding that already clean and disinfected material crosses with dirty material.

Notwithstanding, seawater and system components require biosecurity practices, such as daily purging the tubes of each seawater supply in the exterior production tanks (to unblock them and free the seawater of dirt), changing their exit filters, changing or cleaning and disinfecting the deposit of FSW filters, and cleaning and disinfection of the aeration and water supply tubes weekly, when weighing and stock

density adjustment of each culture, and according to the sanitation and disinfection protocol applied in the equipment, work surfaces and pavements.

As an important living reservoir, people require specific biosafety management, risking the increase of biorisk when not followed. The majority of the biosafety practices referred above reinforce and/or converge with biosecurity associated with people management, like the restriction of access for authorized people, handwashing, avoid hand-to-face contact, protect open wounds with gloves, mandatory use of personal protective equipment of exclusive utilization to each workspace, and the complementary measures associated to COVID-19 pandemic. To help prevent the introduction of diseases, there are several material disinfection stations distributed at the outside production and disinfectant footbaths in the entrance of the Processing section. At the laboratory, the use of autoclave-resistant shoes of exclusive use at this workspace makes the existence of disinfectant footbaths unnecessary. Along with the regular biosafety practices communications, communications with these biosecurity practices are also given at the company, to raise awareness of the collaborators for the importance of following these practices.

2.Daily Activities

The internships provided by ALGAplus have the main goal of giving the experience and knowledge to work in an integrated seaweed and fish production, in a business context. Specific work plans of each internship are attributed to each intern according to the subject of the experiment they develop, whereby my internship consisted of a work experience focusing on the work developed in the RDI department, participating in almost all the activities carried out to gain experience of keeping seaweed cultures and all the activities associated to the methodology of the experimental project.

This internship began on the 7th October of 2019 and ended on the 21st December 2020, with a break of six weeks (from the 19th March of 2020 to the 3rd May of 2020) corresponding to the national lockdown, due to the COVID-19 pandemic. Like the collaborators, my work plan consisted of eight hours per day, (8:00h to 17:00h, with a lunchtime break of an hour at noon) from Monday to Friday (excepting holidays). Due to the COVID-19 pandemic, from the 4th May of 2020, my schedule was reduced to Monday, Tuesday and Friday afternoon (13:00h to 17:00h), on the weeks with ongoing trials of my project. Work schedule varied during the internship, according to my

experience and autonomy, the readiness to initiate the activities of the experimental project, as the need to follow the contingency plan.

From the 27th April of 2021 to the 27th May of 2021, biochemical analysis (ashes determination, lipid content and fatty acid profile) was carried out as part of the experiment. Yet, these laboratory procedures took place at the Chemistry Department of University and Aveiro facilities, whereby not included in this chapter.

2.1.Bibliographic review, integration to the company and training

During the first eight weeks of the internship, (from October 2019 to December of 2019) it was intended my incorporation on the company team, to meet the facilities and understand how the company works, gradually starting to integrate several tasks, mainly on the RDI department, as part of my training, and doing some research about the experimental project to develop. Training activities included both indoors and outdoors daily routines:

- **Indoors (maternity),** performing activities of cleaning, disinfection and preparation for the autoclave of the lab material; weekly routines of *Porphyra* spp. (cleaning, weighing, stocking density adjustment, medium changes, upscaling of blade cultures) and the same for other species like *Codium tomentossum*, *Palmaria palmata* and *Ulva rigida*

- **Outdoors**, daily measuring the abiotic factors in the fish and seaweed tanks; assuring water purge of the tanks; cleaning and disinfection/ changing water filters; readjusting the water flow in the tanks; weekly routines of *Porphyra* spp., *Codium tomentossum*, *Palmaria palmata* and *Ulva rigida* (weighing the cultures, stocking density adjustment, cleaning and disinfection of the tanks and material used in the process like fishnets and boxes).

My first contact with cultures of conchocelis of *Porphyra umbilicalis* took place during this period, starting to follow a culture of conchocelis growing in one of the photobioreactors. During this time, I was in charge of all of the routine activities associated with its maintenance (cleaning and disinfection, biomass weighing, stocking density adjustment and medium changes). This first contact with a culture of conchocelis allowed me to get used to the cleaning and disinfection protocols for the

photobioreactors, their system assembly and functioning, as starting to understand the adjustments needed to viable the use of the photobioreactors in the production of conchocelis, like using ASW or FSW, the credibility of using the biomass present in 1L of culture to estimate the total biomass on the culture, how to proceed at the stock density (withdrawing part of the biomass or increasing the volume of seawater) and how often. During this time, the preliminary experiments in outside conditions, considering previous work developed in the company were also planned.

2.2.Preliminary experiments on the production of conchocelis

After a period of integration in the company and learning the skills needed to incorporate its activities and to be able to perform the methodology of the experiments, the preliminary experiments started. Starting in outside conditions on the 4th of December of 2019 and on inside conditions on the 19th of February of 2020, and ending on the 18th of March of 2020, these preliminary studies were crucial to define the methodology of the experimental design:

- Duration of each trial;
- Frequency of the nutrient supplementation
- Abiotic parameters to measure and frequency
- Procedures of the stock density adjustment
- Important factors influencing the production of conchocelis to study.

Along with the preliminary studies, I continued with my training activities, namely participating in some quality control routines for *Ulva rigida.*

2.3.Production of conchocelis in indoor and outdoor conditions

After the preliminary studies, several indoor and outdoor trials were carried out at the company, from 4th May 2021 to 21st December 2021. As described more extensively below, ten trials were performerd, four indoor and six outdoor. During this period, testing different starter cultures, the influence of an internal protocolar treatment

(Treatment x) and the influence of abiotic factors like salinity, temperature, pH, PFD and photoperiod.

2.4. Other Activities

Along with the training activities and trials, some other relevant activities took place, like field trips and training actions/workshops. During this internship, I had the opportunity to participate in a field trip at Aguda beach, to harvest some biomass of *Porphyra dioica* and *Porphyra umbilicalis*, and two more in the Buarcos beach to harvest some *Grateloupia tururu* biomass for RDI trials.

Two training actions in best practices were carried out at the company during my internship, one specific to best practices to adopt in the maternity, and another to common spaces of the company (seaweed production, kitchen and office). Due to the COVID-19 pandemic and after a six-week national lockdown, the company implemented a contingency plan to minimize the risk of infection in a work context, requiring a training action about the COVID-19. This training action instructed us about the symptoms, period of inoculation, transmission and infection, and the new biosafety measures adopted by the company. Besides these best practices/biosafety actions, I also did a five-hour workshop about hydroponic cultivation systems ("*Introdução à Hidroponia*"), guided by GroHo (GroHo, 2020).

III. Production of Conchocelis

1.Aim of the study

Although research projects focused on *Porphyra* spp. production, compounds and applications have been increasing in the last decades, the sporophyte stage of the life cycle of *Porphyra* spp. – the conchocelis phase – has received little attention, and bibliographic work on its potential as a source of bioactive compounds is still scarce (da Costa *et* al., 2018; Pimentel *et* al., 2020). Only seen as a life stage in *Porphyra* spp., the protocols of production of conchocelis assure its propagation for the generation of new blades, and not for the utilization of the conchocelis as a final product; when possible, asexual strains are used, skipping the time of the development of conchocelis and conchosporangia, making the production more efficient neglecting the study of the optimal conditions and viability of production of conchocelis (He & Yarish, 2006).

At the moment, the main purpose of vegetative growth of conchocelis at ALGAplus facilities has been blade production, using the life cycle of *P. umbilicalis*: conchocelis are cultivated in lab-scale in indoor conditions, in the LD chamber (P3 LD), where temperature, light intensity and photoperiod are controlled. After some time in vegetative growth, the conchocelis are transferred to the SD chamber to induce their maturation into conchosporangia and the conchospores' release.

Excess conchocelis (P3 SD) are a by-product at the end of the blade production (Figure 23) and typically present mix of differentiated conchocelis, (primordial conchosporangia, conchosporangia, and sometimes microscopic free-floating blades, that did not attach to a substrate). Conchocelis excesses are currently not used and therefore ALGAplus sees it as an opportunity to be marketed for use as an ingredient in food, feed, cosmetics or other applications.

This study was done within a confidential service provided to an ALGAplus customer and intended to optimize and upscale the vegetative growth of conchocelis of *Porphyra umbilicalis* in aquaculture production, establishing viable protocols of cultivation in indoor PBR and outdoor tanks. The results will be helpful to use the conchocelis as a promising final product, using the resources and production conditions of the company. Producing conchocelis indoors without the need for autoclave seawater will decrease sterilization and storage costs, as for the possibility of producing outdoors uses sunlight as a natural resource, and resorts to closed systems of bigger volumes than in inside conditions that require less manpower and are more efficient in the

energy/volume produced ratio. In these trials, the industrial upscale of conchocelis is intended to decrease the production costs.

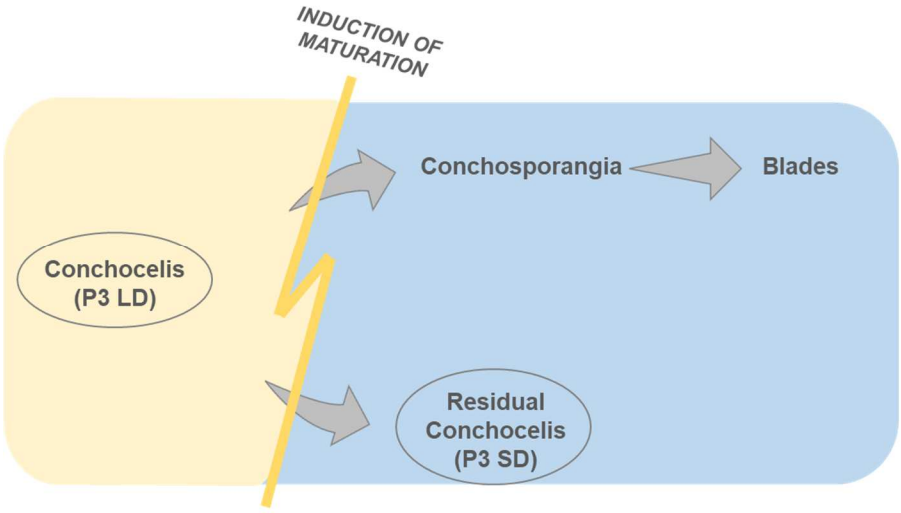


Figure 22. P3 LD and P3 SD elucidation (Scheme by ©Inês Oliveira)

2.Experimental design

From the 4th of December of 2019 until the 18th of March of 2020, preliminary experiments were developed in indoors/maternity and outdoors conditions to establish the methodology to be used in the posterior trials, like stock density, duration of each trial, rate of seawater renewal, nutritional supplementation, stock density adjustment, and volumes to test. The trials started on the 14th of May of 2020 until the 21st of December of 2020, for four weeks each, taking place on the ALGAplus' production facilities: for the indoor condition trials, three PBR (80L capacity) located in the LD chamber of the maternity were used; for the outdoor condition trials, three tanks (15L capacity), placed in EOA, were used. During the trials, three main factors were tested and evaluated:

- Productivity of the biomass, yield and relative growth rate (RGR) % $d^{-1,}$ in indoor and outdoor conditions;
- Ashes and lipid content;
- Two different conchocelis starter cultures: LD stock cultures (P3 LD) versus SD by-products (P3 SD) from the blade production cycle;
- Influence of Treatment x on the conchocelis starter cultures;
- The differences between seasonality (for P3 SD, outdoors).

2.1.Trials

During this internship, two preliminary studies in outside conditions (named 1T and 2T) and one in inside conditions (named 1PBR) were conducted to set some details and procedures. After that, in a total of 30 samples, six trials were developed in the outside tanks (3T, 4T, 5T, 6T, 7T and 8T), and four in the inside photobioreactors (3PBR, 4PBR, 5PBR and 6PBR). The design of these protocols was planned to use the available resources at the company to their fullest, minimizing waste and increasing the sustainability of the company.

In indoor conditions, 3PBR and 4PBR tested the results of production of conchocelis of P3 SD with and without treatment, respectively, while 5PBR and 6PBR tested the performance of conchocelis of P3 LD with and without Treatment x, respectively. The 3T and 7T, as 4T and 8T trials constitute similar trials (testing the production of conchocelis of P3 SD in outdoor conditions, with and without Treatment x, respectively). 5T and 6T tested the results of production for conchocelis of P3 LD under outdoor conditions, with and without treatment x, respectively. These repetitions were performed to test the influence of seasonality on the performance of the biomass. Stock density (medium) was the same for each trial. The following tables (*Table 4* and *Table 5*) resume the number of trials and replicas per condition and the description of the conditions of each trial.

Table 4. Number of cases per Biomass used, Environment and Treatment (Yes/No)

		Treatment x (Yes/No)	Environment		Total (n)
			Outdoors (n)	Indoors (n)	
Biomass used	P3 SD (n)	No	6	3	9
		Yes	6	3	9
		Total	**12**	**6**	**18**
	P3 LD (n)	No	3	3	6
		Yes	3	3	6
		Total	**6**	**6**	**12**
Total			**18**	**12**	**30**

Table 5. Biomass used, Environment and Treatment x (Yes/No) and number of cases of each trial

Environment	Trial	Stock density	Biomass (P3 SD/P3LD)	Treatment x (Yes/No)	Cases (n)
Outdoors	3T	medium	P3 SD	No	3
	4T	medium	P3 SD	Yes	3
	5T	medium	P3 LD	No	3
	6T	medium	P3 LD	Yes	3
	7T	medium	P3 SD	No	3
	8T	medium	P3 SD	Yes	3
	Total Outdoors				**18**
Indoors	3PBR	medium	P3 SD	No	3
	4PBR	medium	P3 SD	Yes	3
	5PBR	medium	P3 LD	No	3
	6PBR	medium	P3 LD	Yes	3
	Total Indoors				12
	Total				**30**

2.1.1. Indoor condition trials

The inside conditions trials intended to test the influence of two variables using the same protocol: the origin of the biomass (P3LD or P3SD) and the influence of treatment x, summarized in *Table 6*. From the 14th of May of 2020 (T_0) to the 8th of June of 2020 (T_{4weeks}), the third trial in inside conditions (3PBR) tested the performance of P3 SD without Treatment x in the beginning. From the 13th of July of 2020 to the 10th of August of 2020, the fourth trial in inside conditions (4PBR) tested the performance of P3 SD, using treated conchocelis in the beginning. The other trials tested the performance of P3LD: from the 19th of October of 2020 to the 16th of November of 2020, the fifth trial in inside conditions (5PBR) tested P3LD without treatment x in the beginning, while the sixth trial (6PBR) tested P3LD with treated conchocelis in a trial began in the 23rd of November to the 21st of December of 2020. Stock density adjustment took place in every trial two weeks after T_0 (T_{2weeks}).

Table 6. Indoor trials

Biomass used (P3 SD/P3 LD)	Trial	Treatment X (Yes/No)	Time of the trial		
			Beginning (T_0)	Stocking density adjustment (T_{2weeks})	End (T_{4weeks})
P3 SD	3PBR	No	14/05/2020	01/06/2020	08/06/2020
	4PBR	Yes	13/07/2020	27/07/2020	10/08/2020
P3 LD	5PBR	No	19/10/2020	03/11/2020	16/11/2020
	6PBR	Yes	23/11/2020	09/12/2020	21/12/2020

2.1.2.Outdoor condition trials

The outside conditions trials intended to test the influence of three variables using the same protocol: the origin of the biomass, (P3SD or P3LD) the influence of treatment x on the conchocelis and the influence of different abiotic conditions, summarized in *Table 7.* The third and fourth trials tested the performance of the residuals of conchocelis of the short day chamber (P3 SD) on the warm and dry season in outside conditions: from the 18th of May of 2020 to the 15th of June of 2020, the third trial in outside conditions (3T) tested the performance of residuals of conchocelis of the short day chamber (P3 SD) without treatment x the conchocelis in the beginning.

From the 20th of July of 2020 to the 17th of August of 2020, the fourth trial in outside conditions (4T) tested the performance of residuals of conchocelis of the short day chamber (P3 SD), using treated conchocelis in the beginning. The seventh and eighth trials in outside conditions (7T and 8T) were similar to the third and fourth, respectively, testing the performance of the short day chamber (P3 SD) on the dry and cold season in outside conditions. Ran simultaneously from the 23rd of November of 2020

to the 21st of December of 2020, the seventh trial in outside conditions (7T) tested the performance of residuals of conchocelis of the short day chamber (P3 SD) without treating the conchocelis in the beginning, while the eighth trial in outside conditions (8T) tested the performance of residuals of conchocelis of the short day chamber (P3 SD) using treated the conchocelis in the beginning.

Although it was foreseen to test both origins of the cultures of conchocelis, exceptionally P3 LD biomass was only tested during one time of the year. Developed simultaneously, from the 19th of October of 2020 to the 16th of November of 2020, the fifth trial in outside conditions (5T) tested P3LD culture without treating the conchocelis in the beginning, while the sixth trial in outside conditions (6T) tested P3 LD cultures with treated conchocelis. Stock density adjustment took place in every trial two weeks after T_0 (T_{2weeks}).

Table 7. Outdoor trials

Biomass used (P3 SD/P3 LD)	Trial	Treatment X (Yes/No)	Time of the trial			
			Time of the year	Beginning (T_0)	Stocking density adjustment (T_{2weeks})	End (T_{4weeks})
P3 SD	3T	no	May to August	18/05/2020	01/06/2020	15/06/2020
	4T	yes		20/07/2020	03/08/2020	17/08/2020
	7T	no	November to December	23/11/2020	09/12/2020	21/12/2020
	8T	yes		23/11/2020	09/12/2020	21/12/2020
P3 LD	5T	no	October to November	19/10/2020	03/11/2020	16/11/2020
	6T	yes		19/10/2020	03/11/2020	16/11/2020

2.2.Conchocelis

The conchocelis of *Porphyra umbilicalis* used for the trials originated from a combination of mixed strains isolated in ALGAplus biobank: (P3.02.1.1.140204; P3.02.3.1.180323) from both stock cultures (P3 LD) and by-products of the blade production process (P3 SD) (*Figure 23*). P3 SD of different batches no longer being used to produce blades (from April to July 2020 for 3PBR, 4PBR, 3T and 4T, and September to October 2020 to 7T and 8T), were collected and stored in 20 L carboys in LD chamber until being used for trials.

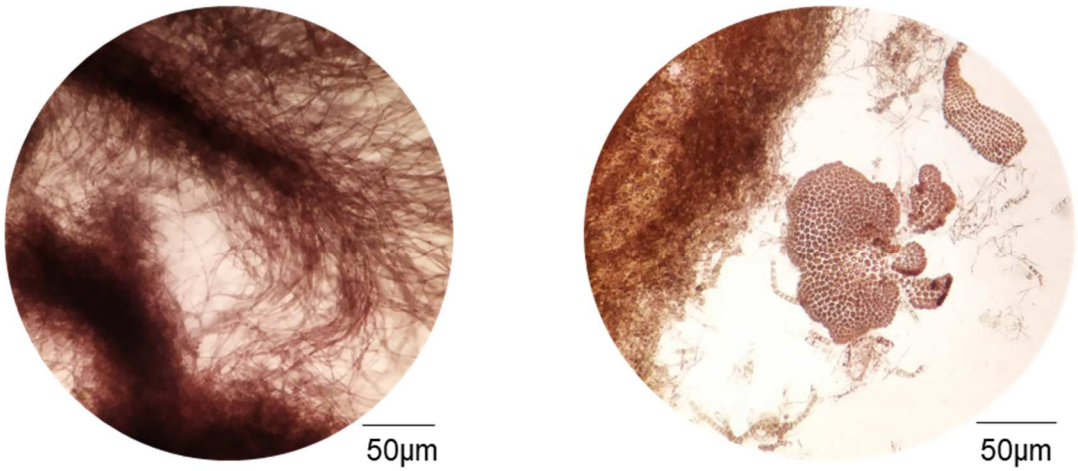


Figure 23. P3 LD (on the left) in comparison with P3 SD (on the right) (10x ampliation). P3 SD is constituted by a mix of conchocelis, and differentiated primordial conchosporangia, conchospores, and microscopic free-floating blades. ©Inês Oliveira

2.3.System assembly

Cultures from both indoor and outdoor systems were maintained in FSW batch, with aeration from air pumps/aeration system from the company facilities, with periodical seawater renews/adding and nutritional supplementation. The assembly of these systems is described above: indoors, consisting of three PBRs of 80L placed in the LD chamber, and outdoors, constituted by three tanks of 15L from Production 1 (although not assembled in continuous flow as the majority of the tanks).

2.3.1.Indoor systems

The PBRs *(Figure 24)* consisted of three vertical water columns (80L capacity) of transparent acrylic,with a storz plug cover in the superior base, a tap at the 37L level (for sampling during the trials), and two hose-adjustable openings: one on the side (just under the top) for water supply and the other in the middle of the bottom, provided with a tap to control water output and air input, where the air provided by an aquarium pump is introduced in each photobioreactor. The output tap has four positions: two of them neutral (no output of water and no input of air) one position of functioning (no output of water with the input of air) and another of discharging (output of water and no input of air*).* The PBRs are placed vertically in the LD chamber, where the temperature and photoperiod are programmed; the illumination by three cool-white fluorescent tubes (actinic light) assures constant light intensity (*Figure 25).*

Figure 24. PBRs used in the trials, placed in the LD chamber. ©Inês Oliveira

Before all trials, the PBRs were cleaned and disinfected with tap water and a 5% concentration of sodium hypochlorite overnight or until the walls were cleaned and the water colorless. After discharging the solution, the photobioreactors are filled with clean seawater (at least three times), until the sample collected from the tap in the middle of the column presents no coloration or odor. The covers and top openings of the photobioreactors must be pulverized with a 70% ethanol solution whenever manipulated, to minimize contaminations.

Not well-adjusted aeration in each culture can compromise the results, whereby it is crucial to regulate it to assure the homogeneity of movement of the culture and supervise it during the trial. Given the results of the preliminary studies and to minimize the manipulation of the biomass during the trial, a few adjustments were established: stocking density was adjusted by increasing the volume of water; use of FSW instead of ASW; use of an organic nutritional medium; starting volume was settled at 40 L (half of the total volume) and increased up to its full capacity (80 L) in the middle of the trial (T_{2weeks}) as stocking density procedure.

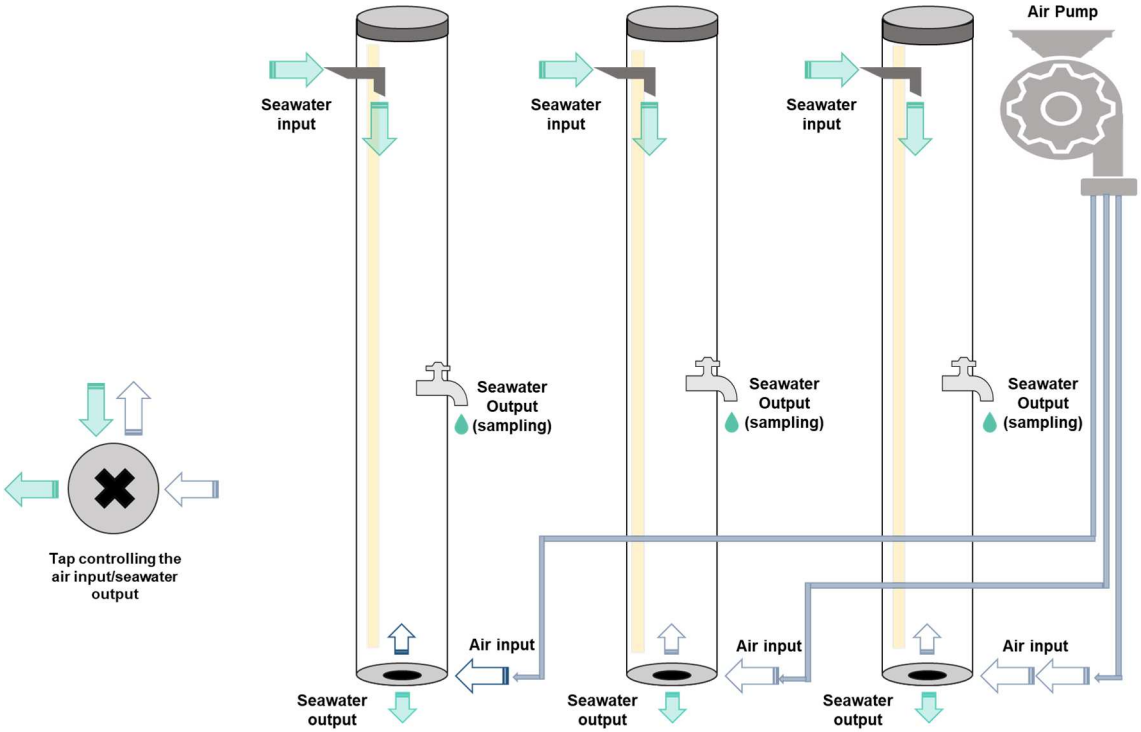


Figure 25. Draft of the indoor system assembly. PBRs are air supplied from the bottom, with an air pump. The bottom tap (on the right) controls the input of air and the output of seawater. *(Scheme by ©Inês Oliveira)*

2.3.2.Outdoor systems

The tanks used for the outside trials (15L capacity) are equal to the ones used in Production 1. They consist of opaque white polymer tanks, approximately cylinder-shaped, with a top opening of 0,13m^2 (radius around 0,20m) exposed to air and light. For each trial, three tanks were placed in EOA. The air supply was connected to the aeration system of the outside production (*Figure 26*). At some time, light exposure was reduced (confidential internal protocol).

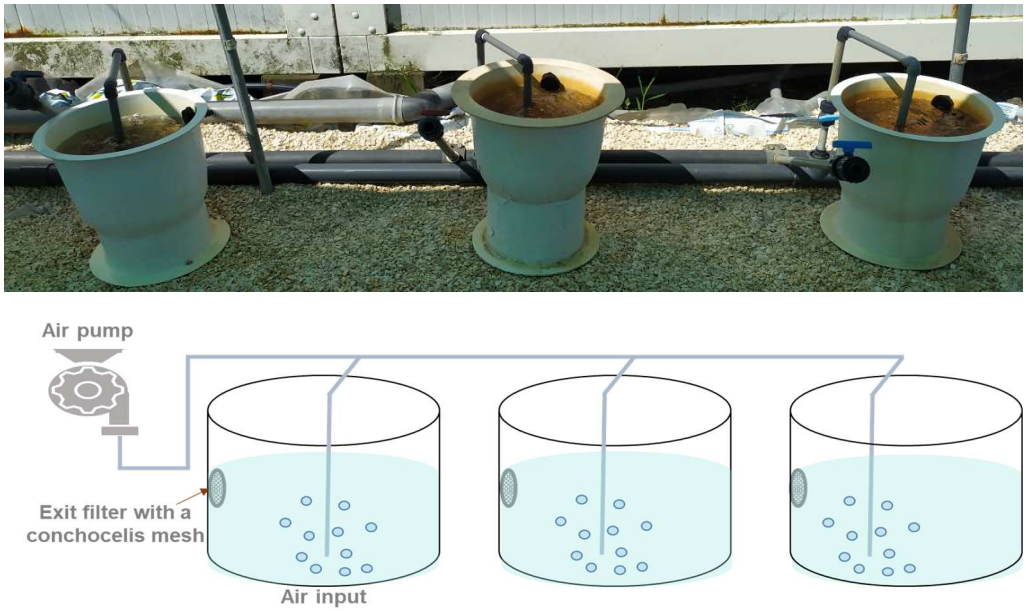


Figure 26. Tanks display for outdoor trials (above), with a draft showing system assembly (under) (Note: the tanks are opaque; they are only translucid in the draft for a better understanding) ©Inês Oliveira.

Before each trial, the tanks and aeration tubes were cleaned and disinfected with a 5% hypochlorite sodium solution and their walls were scrubbed from microalgae and cyanobacteria. Since these tanks are designed for cultures with continuous seawater renewal, they possess an exit filter at the 15L level, covered with a conchocelis mesh to avoid biomass losses for the trials. The aeration tubes were adjusted to the length of the tank, with capacity enough that allow circulation but avoid risking overflow, which could cause biomass loss or sedimentation and heterogeneity of the cultures.

2.4.Biomass starter cultures

According to the defined stocking density for the protocols (medium), the total biomass for the initial volume was obtained by filtering the conchocelis in culture with an appropriate sieve for conchocelis, previously cleaned, disinfected, and pulverized with a 70% ethanol solution. Biomass (fw, g) was weighed in autoclaved beakers after no more water drained from the conchocelis sieves (*Figure 27*). After weighing, the biomass was kept covered with aluminum and rehydrated with FSW when necessary, preventing dry distress to the cultures. From the initial starter biomass of each trial, three samples of 50 g (fw) were collected for microscopic observations, photographic registers were taken from macro and microscopic observations and frozen at -20ºC for posterior biochemical analysis.

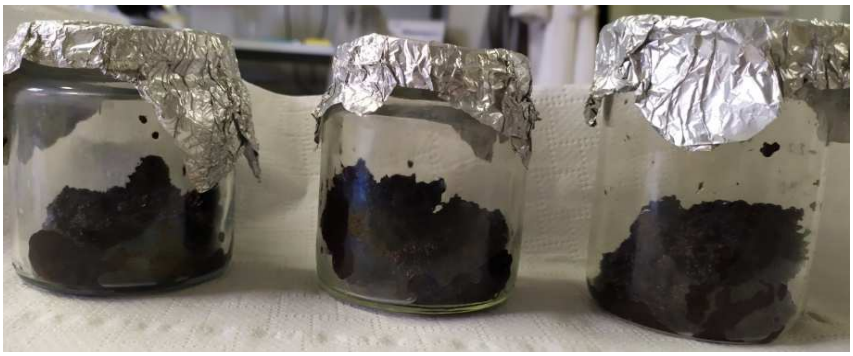


Figure 27. Biomass starter cultures after previously weighted @Inês Oliveira

2.5.Treatment x (confidential internal protocol)

One of the parameters evaluated was the influence of Treatment x on the growth performance and quality of the cultures (*Figure 28*). After weighing all replicates, each beaker was subjected to treatment x at the beginning of the trial, confidential internal protocol. The procedure was not repeated at any time during the trials.

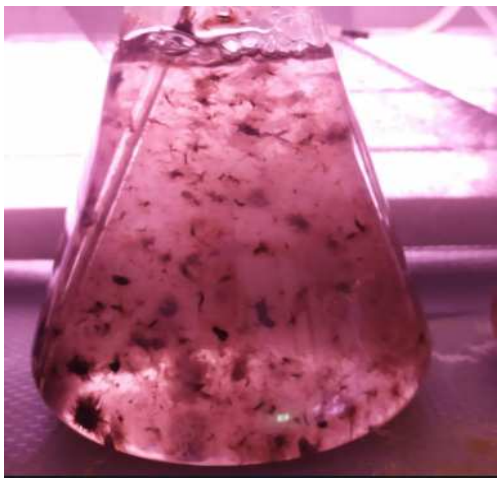


Figure 28. *Culture subjected to treatment x @Inês Oliveira*

2.6.Abiotic factors

The following abiotic factors were monitored and registered for all trials, photoperiod (hours), temperature (ºC), PFD ($\mu\text{mol. photons. m}^{-2}.s^{-1}$), salinity (ppt) and pH. PFD was measured with a PFD sensor; the other parameters were measured with a multiparameter analyzer with two probes: one for salinity and temperature, and other for pH. Photoperiod was registered from "*Sunrise and Sunset"* (*www.sunrise-and-sunset.com)* (*Figure 29).*

Figure 29. PFD sensor (left) and multiparameter analyzer with probes for salinity and temperature and pH (right). (*www.apogeeinstruments.com; www.hach.com*)

On the indoor trials, photoperiod, temperature, and light intensity are programmed in the chambers; however, values were measured once a week, as salinity and pH whenever the estimative of total biomass weight was done. Salinity, temperature, and pH were measured from a sample of the 1 L taken for estimative of total weight, while PFD was measured in four defined spots from the acrylic surface of each PBR. On the indoor trials, the parameters were measured three times per week whenever the weekly weighing (after the medium was changed) plus the twice-weekly partial medium changes (before them). In outdoor trials, the abiotic parameters are measured directly in the cultures; light intensity is measured in two spots of each tank: just above the water surface and just under it (*Figure 30*).

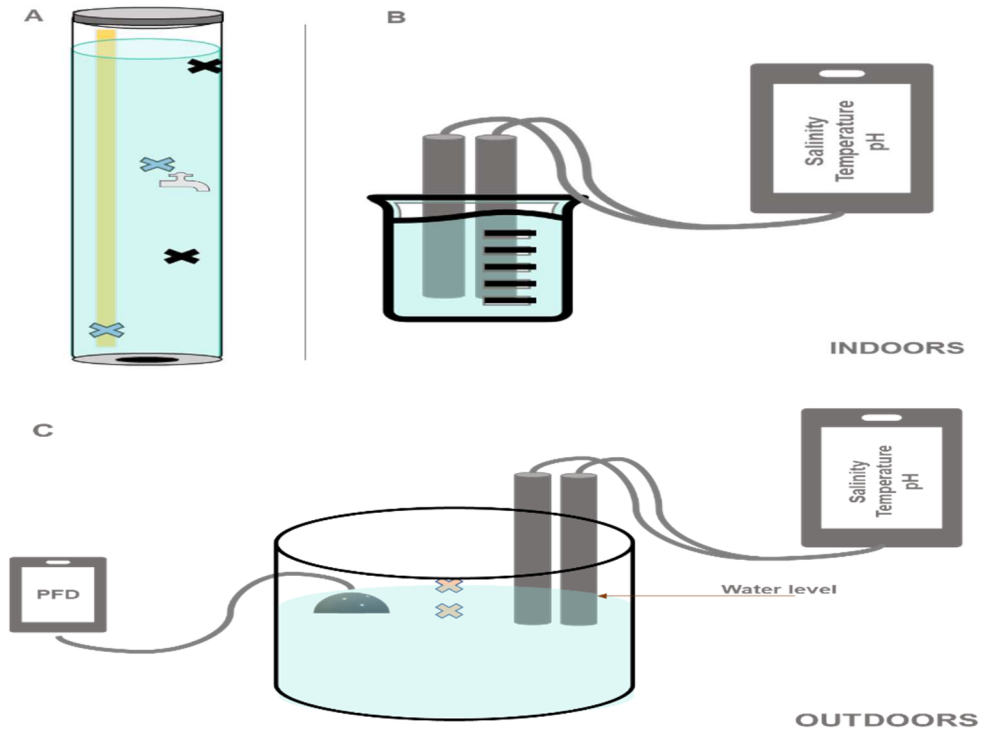


Figure 30. Abiotic factors measurements. Indoors, PFD was measured on the surface of the PBRs in four points of each (A), while salinity, temperature and pH were measured in a water sample from the 1 L weight estimate (B); outdoors, salinity, temperature, pH, and PFD were measured directly in the cultures tanks, the last one just above the water surface and just under it (C). **Note**: in (A), black crosses indicate spots in the front of the PBR, while blue ones in the back. (Scheme by ©Inês Oliveira)

2.7.Medium renewals and nutritional supplementation

In indoor trials, although the initial 40L was not renewed, after two weeks (whenever stocking density is adjusted), the volume was increased to 80L with FSW. For nutritional supplementation, a nutritional medium was added weekly, (at the moment of the estimate of total weight) in a concentration of x $g.L^{-1}$ (confidential). Since the outside trials were more exposed to contaminations and kept in batch, the cultures required more than once a week seawater renewal, when they are weighed; to fulfill that need, two partial seawater renewals per week were done: turning off the aeration system and letting the biomass settle, 10L of water (two-thirds of the content) is discharged by the exit filter (preventing biomass losses) and the tanks fulfilled with FSW. In these partial seawater renewals, as in the weekly weightings, an organic nutritional supplement was also added in a concentration of x $g.L^{-1}$ (confidential), counting it for the 15L.

2.8.Conchocelis Filters/Weight assessment

In outdoors trials, fresh weight (g) was weighed weekly by filtering the content of each tank separately. The biomass was drained until the sieve no longer drained water permanently from the conchocelis. At T_{2weeks}, stock density was readjusted by collecting the excess biomass. Samples from the excess and the end of each trial were collected for further analysis and stored at -20ºC. The samples were weighed as previously described and microscopically observed; afterward, the conchocelis biomass was placed back into the respective cultures, to not skew the results.

Figure 31. *Sieves used in the trials. In most of the weighings, a smaller sieve was used (above); yet, for the final weighings of PBRs, it was used a bigger one (under). ©Inês Oliveira*

In indoor trials, the total biomass was only weighed at the beginning (T_0) and end of the trials (T_{4weeks}). Weekly, weight assessment was done by collecting a 1 L sample from the culture: with the tap opened at maximum flow and the system in function (to promote movement and homogeneity of the culture along the water column). The sample was collected to a beaker, filtering the culture (similarly to outdoor trials) and estimating the total weight by extrapolation of those results. For the final weighing, the sieve used was different from the one used in outdoor trials or the beginning/weekly estimates, due to the bigger volume of culture in the PBRs (*Figure 31*).

2.9.Cleaning, disinfection and contamination control

All the equipment used in the manipulation of the cultures was sterilized by autoclave or cleaned and disinfected accordingly to the protocol of clean and disinfection described in Biorisk Management. Dirt and/or organic matter were emoved, the material was rinsed with freshwater, disinfected with a 5% solution of sodium hypochlorite, and a 70% ethanol solution for materials not resistant to sterilization. After each weighing in the exterior, the tanks were also cleaned and disinfected before restocking them. To keep the cultures healthier and cleaner of contaminations, the outdoor cultures were submitted to an internal cleaning procedure.

2.10.Observations and sampling

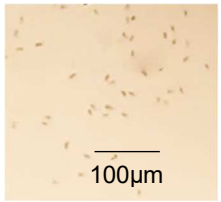
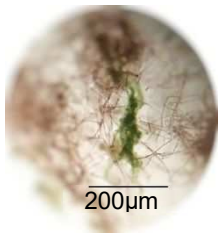
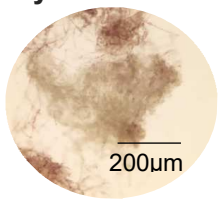
Along with weighing procedures, the cultures were observed macroscopically. Three samples from each tank/PBR were collected and analyzed at the microscope, intending to observe the coloration of the cultures, structures observed (conchocelis, conchosporangia and/or blades) and contaminations (microalgae, ciliates and other contaminators). Photographic registers were taken from macro and microscopic observations.

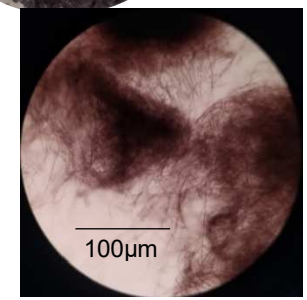
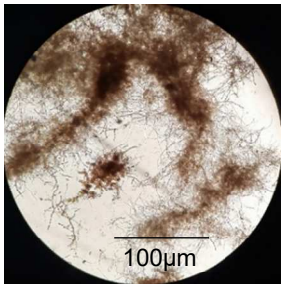
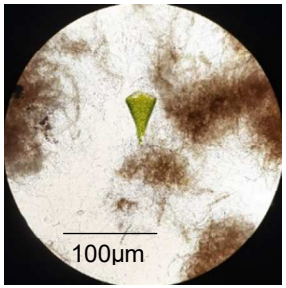
To ease the analysis of the quality of the biomass, the following qualitative "Quality and Contamination scale" was created, where "Very Good" corresponded to the best biomass and "Satisfactory" the worst (*Table 8*). For each trial, the quality of the biomass was considered at the beginning (T_0), after two weeks (at the moment of stock density adjustment) (T_{2weeks}) and at the end of the trial (T_{4weeks}) It was also created the variable "Changes in Quality" with three degrees (*Changes in quality = Quality at the ending–Quality in the beginning), where:*

"No change" corresponds to Changes in quality = 0
"Small change" to Changes in quality = ±1
"Big change" to Changes in quality = ±2

For indoor trials, since stocking density adjustment did not include collecting biomass and not bias the growth results, sampling for biochemical analysis was only collected from the starter cultures and the final biomass (a sample from each PBR and trial in the last case). In outdoors, since stocking density adjustment implied collecting biomass, a sample from each tank and trial was collected whenever the stocking density adjustment was performed and at the end of the trials. Samples of 50 g (*fw*) were stored at -20 ºC for further biochemical analysis. Tables following in "ANNEX 1 and ANNEX 2" summarize the adjacent tasks in trials.

Table 8. Quality and Contamination scale

	Parameters/Level	Very Good	Good	Satisfactory
MICROscopic observations	**Conchocelis filaments**	Fine filaments well outlined and discernible	Fine filaments, generally well outlined and discernible	Ruptured cells or damaged filaments, general difficulty identifying the margins
		Not detected or no significative presence	Presence of some (up to 10 per 10x field)	Presence of many (more than 10 per field)
	Microalgae 	Not detected or no significative presence	The occasional presence of microalgae	Many blots
	Cyanobacteria 	Not detected or no significative presence	Few blots (less than 40% coverage)	Many blots (more than 40% coverage)

	Parameters/Level	Very Good	Good	Satisfactory
MACROscopic observations	Coloration	Dark brown-reddish 	Mocha to Pecan 	Mocha to pecan, with red (unpigmented) and/or green blotches 
	Consistency	Firm	Firm	Soft and mushy
	Examples	 	 	 

2.11.Biochemical analysis

For biochemical analysis, samples were freeze-dried and stored at -80 ºC until the analysis. Samples were also taken for fatty acid analysis and elementary analysis (determination of N, P and C content), but it was not possible to accomplish these results during the time of the internship. The analysis took place from 27th April of 2021 to 27th May of 2021, at Mass Spectrometry Center, Chemistry Department, University of Aveiro.

2.11.1.Ash content determination

Before the ashes determination, porcelain crucibles, (one per each sample) already identified, were incinerated at 575 ºC for 6 h and let for cool overnight before opening. The porcelain crucibles were weighted and 250 mg of sample were weighed and placed in the muffle at 105ºC for 20h. After the muffle dry period, the crucibles were let in a dissector for at least 30min to cool and then weighed the dried biomass and calculate the moisture content (value not considered for the final results of this work):

$$\text{Moisture (\%)} = \frac{dry\ sample\ weight}{initial\ sample\ weight}\ x\ 100$$

For ashes determination, the crucibles were put in the muffle and submitted to the following furnace temperature ramp program: ramp from room temperature to 105 ºC and hold it for 12 min; ramp to 250 ºC at 10 ºC.min^{-1} and hold it for 30 min; ramp to 575 ºC at 20 ºC.min^{-1} and hold it for 360min; allow the temperature to drop to 105 ºC and hold it until the samples were removed. After opening the muffle, the crucibles were let in a dissector for at least 30min to cool and then weighted to calculate the ashes:

$$\text{Ash (\%)} = \frac{Final\ weight}{Dry\ sample\ weight}\ x\ 100$$

2.11.2.Lipid extraction procedure

Using a modified Bligh & Dyer (1959) method, 3.75 ml of dichloromethane/methanol (1:2) were added to 100 mg of conchocelis in a PIREX tube, vortexed for 2 min and incubated by an hour in the orbital shaker, vortexing three times during the incubation. The mixture was centrifuged at 2000 rotations per minute (rpm) for 10 min, and the organic phase (supernatant) was collected to another PIREX tube. The solid phase was re-extracted with 3.75 ml of dichloromethane/methanol (1:2), homogenized by vortexing for 2 min, centrifuged at 2000 rpm for 10 min and the supernatant was collected to the same PIREX tube, which was dried under a stream of nitrogen gas.

To remove the non-lipid contaminants from the dried organic phase, it was added 2ml of dichloromethane and 2 ml of methanol, homogenized by a 1 min vortex, and 1.8 ml of Mili-Q water, followed by vortexing for 2 min. The mixture is then centrifuged at 2000 rpm for 10 min and the organic (lower) phase is collected to a new PIREX tube, re-extracting the aqueous phase with 2ml dichloromethane, vortexing for 2 min, and centrifuging at 2000 rpm for 10 min. The organic phase from this step was collected to the same tube as before and the combined organic phases were dried under a stream of nitrogen gas.

Afterward, the organic phase, total lipid extract, was transferred to dark vials, previously pre-heated at 100 ºC for two hours and cooled in the desiccator for at least 30 min. 500 µl of dichloromethane were added to the organic phase tube, vortex and transferred to dark vials. This procedure was repeated to recover all lipid extract. The lipid extract content of the vials was dried under a stream of nitrogen gas, and total lipid content was calculated by the difference of the weighted vials with the lipid extract and the pre-weighed vials.

2.12.Data Analysis

The results analyzed corresponded to vegetative growth, abiotic conditions, environment (indoor/outdoor), the origin of the biomass, apply of treatment x, quality of the biomass/changes in quality and biochemical analysis (ashes and lipid content). All samples were analyzed in triplicates (corresponding to the tanks/PBR replicas). Vegetative growth was analyzed according to two parameters – Yield and Relative growth rate (RGR) - calculated by the following formulas:

$$\textit{Yield}\ (g.l.d^{-1}) = \frac{Wf - Wi}{Volume * Time}$$

$$\textit{RGR}\ (\%.d^{-1}) = \frac{ln(Wf) - ln\,(Wi)}{Time} * 100$$

Where Wf refers to the final *fw* (g), Wi refers to the initial *fw* (g), volume to the volume of the cultures (L) and Time to the days from the initial weight up to the final weight. Yield ($g.l.d^{-1}$) refers to fresh weight biomass produced per litter per day; RGR ($\%.d^{-1}$) refers to the relative increase of fresh weight per day. Yield and RGR were calculated by trials and by halves. By halves, it is understood the moments from the beginning of the trial to stock density adjustment and from the moment to the end of the trial, where:

Yield_0-14 ($g.l.d^{-1}$) refers to biomass produced per litter, per day, from the beginning of the trial up to the moment of stock density adjustment;

Yield_15-28 ($g.l.d^{-1}$) refers to biomass produced per litter, per day, from the moment of stock density adjustment to the final of the trial;

RGR_0-14 ($\%.d^{-1}$) refers to the relative increase of fresh weight per day from the beginning of the trial up to the moment of stock density adjustment;

RGR_15-28 ($\%.d^{-1}$) refers to the relative increase of fresh weight per day from the moment of stock density adjustment to the final of the trial.

For the parameters by trials (RGR_trial and Yield_trial), Yield_trial is calculated:

$$\text{Yield\_trial}\ (g.l.d^{-1}) = \frac{(Yield\_0{-}14 * \text{Time\_0}{-}14) + (Yield\_15{-}28 * \text{Time\_15}{-}28)}{\text{Time}}$$

Quality of the biomass and Changes in Quality were described by absolute and relative frequency tables. Inferences of dependency of these variables with Environment, origin of the biomass and Treatment were verified with Qui-square tests, considering T_0, T_{2weeks}, and T_{4weeks} for quality of the biomass and the Changes in quality between T_{4weeks} and T_0. Growth parameters (*Yield_0-14, Yield_15-28, Yield_trial,* RGR*_0-14, RGR_15-28* and *RGR_trial)* and abiotic conditions (salinity, temperature, pH, PFD and photoperiod) were described by mean±std dev by trial and per environment, and inferences were verified separating the results by environment (indoors and outdoors).

Differences between trials for each variable were studied by One-way-ANOVA after verifying the assumptions, as differences between *Yield_0-14 and Yield_15-28, and between* RGR_*0-14 and RGR_15-28,* to understand if the performance of growth tends to differentiate with time. The influence of treatment x and origin of the biomass on the growth parameters and on the differences between times were studied by Two-way ANOVA tests after verifying the assumptions.

The correlation between the vegetative growth and abiotic conditions was analyzed for the parameters showing significant differences, to conclude if there was an influence of abiotic factors on the growth. The relationship between vegetative growth and changes in quality/quality of the biomass at T_{4weeks}, and abiotic conditions (with significant differences between trials) and "Changes in quality" and "Quality of the biomass" at T_{4weeks} was studied by Qui-square tests, to observe if trials with higher yield and/or growth rate had worse quality/more contaminations or, on the contrary, the quality was better when the cultures had better growth performances. For PFD in outdoor conditions, it was studied an additional correlation between the measured values (mean per trial) on the tanks and the values from HIDROMOD (mean of eight measures per day from the days corresponding at each trial) and settled by the light exposure reduce or not.

In outdoor conditions, it was hard to adjust the abiotic conditions, since they are majorly dependent on the environment and connected: in warmer and drier months in Ria de Aveiro, temperature, PFD and photoperiod are usually higher, as salinity, due to evaporation from the heat (Rebelo, 1992); in colder and wet months, temperature, PFD and photoperiod are usually lower, as salinity, due to the rain (Rebelo, 1992). Towards, the relationship between and other variables were analyzed in two ways: analyzing each factor singularly, and the abiotic conditions (AC) as a block:

*AC = Salinity * Temperature * pH * PFD * Photoperiod*

Differences in ashes and lipid content during the three moments of measuring were studied by One-way-ANOVA tests (assumptions assured). Differences in T_0 content according to the biomass used (P3 SD or P3 LD) were analyzed by One-way-ANOVA tests, and the influence of environment, use of the treatment x and biomass used on the increase/decrease during each measurement was analyzed by three-way-ANOVA tests (assumptions assured). A 5% level of significance was considered. Statistic analysis was performed recurring to IBM SPSS Statistics 25 (Corporation, 2017). All graphics were created using Microsoft Office®.

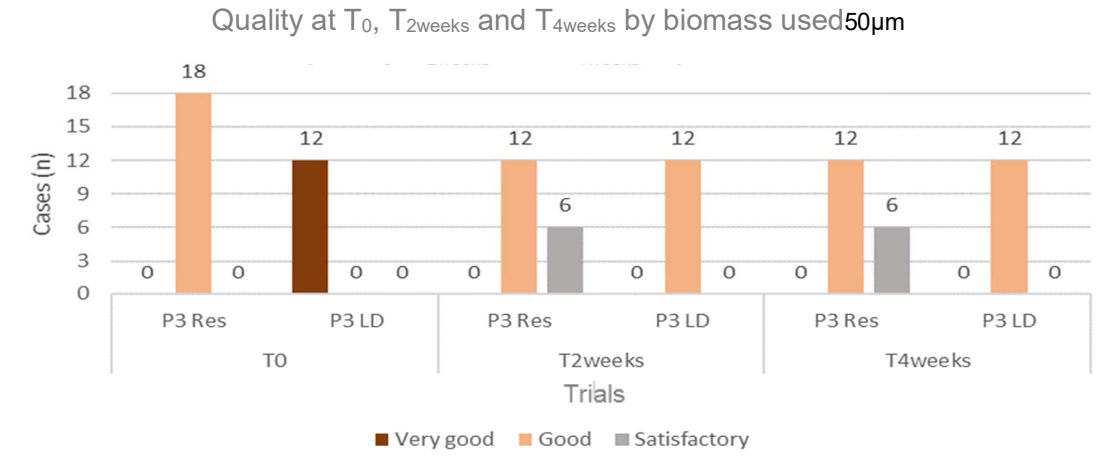
IV.Results

This chapter includes all the results corresponding to the post-preliminary trials (indoors 3PBR, 4PBR, 5PBR and 6PBR and outdoors 3T, 4T, 5T, 6T, 7T and 8T). the results for quality of the biomass, abiotic conditions, growth parameters and biochemical analysis odf the preliminary trials (1T, 2T and 1PBR) were not considered in this chapter since the importance of them was the decision of the methodology used (number of water renews/nutritional supplementation; periodicity of abiotic conditions measuring; weight assessment; stock density adjustment periodicity and procedure.

1.Quality of the Biomass (based on the Quality and Contamination scale)

Figure 32 and Table 9 show the results for quality. In *Figure 32 A*, it is possible to observe that the quality at T_0 depended on the origin of the biomass (Pvalue < 0.001), since quality in all of the P3 LD trials were “Very good” and “Good” in P3 SD ones (T_0); also, the results at T_{2weeks} and T_{4weeks} were similar (the replicas “Good” and “Satisfactory” at T_{2weeks} are the same as these classifications at $T_{4\ weeks}$).

The quality of the biomass at T_{2weeks} and T_{4weeks} was influenced by the environment (Pvalue = 0.025) and biomass used (Pvalue = 0.025), but not for treatment x (Pvalue = 1.000). Crossing the results at T_{4weeks} with the environment (Figure 32 B) it is possible to see that, independently of the origin of the biomass, the quality in indoor trials was “Good” at T_{4weeks}; yet, in outdoor trials, it was “Good” in all the trials and replicas with P3 LD, “Satisfactory” in six cases of P3 SD and Good on the other six, corresponding to the trials from 18th May to 17th August (3T and 4T) and 23rd November to 21st December (7T and 8T), respectively (Figure 32 C).



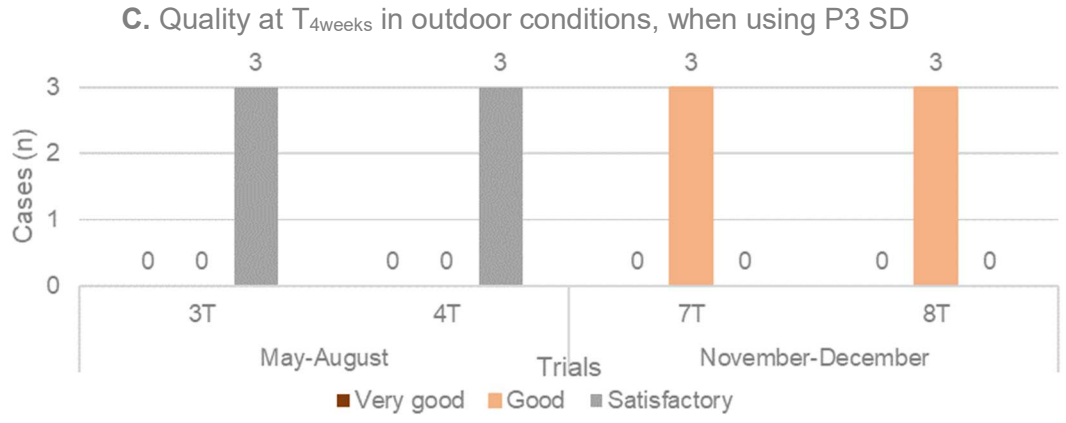


Figure 32. Results of the Quality of the biomass at differentes times of trial and by trial conditions (biomass used and environment)

Table 9. Quality of the biomass at different times of trial by trial conditions (Biomass used, Treatment x and Environment). *Categories influenced the quality of the biomass (Pvalue < 0.005).

	Categories		Quality						Total	
			Very Good		Good		Satisfactory			
			n	%	n	%	n	%	n	%
T_0	Biomass*	P3 SD	0	0%	18	100%	0	0%	**18**	**67%**
		P3 LD	12	100%	0	0%	0	0%	**12**	**33%**
	Treatment x	Yes	6	33%	9	67%	0	0%	**15**	**50%**
		No	6	33%	9	67%	0	0%	**15**	**50%**
	Environment	Outdoor	6	33%	12	67%	0	0%	**18**	**67%**
		Indoor	6	50%	6	50%	0	0%	**12**	**33%**
	Total		**12**	**40%**	**18**	**60%**	**0**	**0%**	**30**	**100%**
T_{2weeks}	Biomass*	P3 SD	0	0%	12	67%	6	33%	**18**	**67%**
		P3 LD	0	0%	12	100%	0	0%	**12**	**33%**
	Treatment x	Yes	0	0%	12	80%	3	20%	**15**	**50%**
		No	0	0%	12	80%	3	20%	**15**	**50%**
	Environment*	Outdoor	0	0%	12	67%	6	33%	**18**	**67%**
		Indoor	0	0%	12	100%	0	0%	**12**	**33%**
	Total		**0**	**0%**	**24**	**80%**	**6**	**20%**	**30**	**100%**
T_{4weeks}	Biomass*	P3 SD	0	0%	12	67%	6	33%	**18**	**67%**
		P3 LD	0	0%	12	100%	0	0%	**12**	**33%**
	Treatment x	Yes	0	0%	12	80%	3	20%	**15**	**50%**
		No	0	0%	12	80%	3	20%	**15**	**50%**
	Environment*	Outdoor	0	0%	12	67%	6	33%	**18**	**67%**
		Indoor	0	0%	12	100%	0	0%	**12**	**33%**
	Total		**0**	**0%**	**24**	**80%**	**6**	**20%**	**30**	**100%**

Regarding the changes in the quality (Table 10) during the trials, the results showed that 12 samples (40%) suffered “No change” (Changes in quality = 0) and the remaining 18 (60%) suffered a “Small change” (Changes in quality = 1). There were not any samples presenting a “Big change”. Inference about proportions showed significant differences between the changes in quality and the origin of biomass: P3 SD suffered No change in 67% of the cases and a Small change in 33% of the cases, while all of the samples of P3 LD suffered a Small change (Pvalue < 0.001). There were no significant differences between the percentage of cases with no changes (40% suffered “No change” and 60% suffered “Small change”) when biomass was treated or not; 33% of the cases in outdoor conditions suffered “No change” and the remaining 67% suffered a “Small change”, with no significant differences to the 50% in indoor conditions for each change.

Table 10. Changes in Quality by trial conditions (Biomass used, Treatment x and Environment). *Categories influenced the quality of the biomass (Pvalue < 0.005).

		Changes in Quality							
Trial Conditions		No change (=0)		Small change (±1)		Big change (±2)		TOTAL	
		n	%	n	%	n	%	n	%
Biomass*	P3 SD	12	67%	6	33%	0	0%	**18**	**67%**
	P3 LD	0	0%	12	100%	0	0%	12	33%
Treatment x	Yes	6	40%	9	60%	0	0%	**15**	**50%**
	No	6	40%	9	60%	0	0%	**15**	**50%**
Environment	Outdoor	6	33%	12	67%	0	0%	**18**	**60%**
	Indoor	6	50%	6	50%	0	0%	**12**	**40%**
Total		**12**	**40%**	**18**	**60%**	**0**	**0%**	**30**	**100%**

2.Abiotic Conditions and Growth Parameters

Mean and standard deviation (mean±sd) of the abiotic factors are discriminated in *Table 11*, with the respective results of the inference of significative differences in salinity (F = 70.477, with 12 df), temperature (F = 3.578, with 12 df), pH (F = 13.153, with 12 df), PFD (F = 26.263, with 12 df), and photoperiod (F = 4843.013, with 12 df), between indoor and outdoor conditions and within trials. Outdoor (salinity = 32.57 ± 3.31 ppt; temperature = 18.53 ± 10.53 ºC; pH = 7.90 ± 0.31; PFD = 76.33 ± 39.90 $\mu\text{mol.photons.m}^{-2}s^{-1}$; photoperiod = 11.37 ± 2.38 hours of light) and indoor abiotic conditions (salinity = 33.68 ± 2.20 ppt; temperature = 15.75 ± 1.61 ºC; pH = 7.43 ± 0.47; PFD = 24.02 ± 19.57 µmol.photons.$m^{-2}s^{-1}$; photoperiod = 16.00 ± 0.00hours of light) showed relevant differences only in photoperiod (Pvalue < 0.001); yet, within trials of the same environment, there were differences in all of the measured abiotic parameters in outdoor conditions, whilst in indoor conditions, differences were only significant for salinity (Pvalue < 0.05). PFD showed significant differences within replicas in 3T and all of the indoor trials (sd overloads half of the mean).

The results of mean, maximum and minimum per trial for each abiotic factor (salinity, temperature, pH, photoperiod and PFD) are shown in *Figure 33* and *Figure 34,* simplifying the understanding of the variations of the parameters during trials. Ranges were higher in outdoor trials for all the parameters, except for pH. Salinity was higher at 4T (41.6 ppt); the lowest salinity and temperature values were registered in 7T and 8T (27 ppt and 8.3 ºC, respectively), while the highest water temperature (26.1 ºC) was registered in 3T. pH varied from 6.4 (5PBR) to 8.26 (7T and 8T). PFD registered the highest values in 3T (302 µmol.photons.photons.$m^{-2}.s^{-1}$) - vertiginously higher than the second-highest (93.5 in 4T) – and the lowest in 3PBR (302 µmol.photons.$m^{-2}.s^{-1}$) – vertiginously lower than the lowest in outdoor trials (29.5 µmol.photons.$m^{-2}.s^{-1}$ in 7T and 8T). The highest photoperiod was registered (and constant at this value) in indoor trials (16 hours of daylight); in outdoor trials, hours of light varied from 8.3 hours (7T and 8T) to 15 hours (3T).

Table 11. Abiotic conditions per trial (mean ± sd) (significative differences were measured per column (Pvalue < 0.005)). *µmol photons.$m^{-2}.s^{-1}$; **hours of daylight

Trial	Schedule (starting – ending)	n	Abiotic Conditions (mean±sd)				
			Salinity (ppt)	T(ºC)	pH	PFD*	Photoperiod**
3T	*18/05/20 – 15/06/20*	*36*	33.79 ± 2.04^{ace}	23.43 ± 2.03^{a}	8.09 ± 0.31^{a}	128.86 ± 95.41^{a}	14.79 ± 0.17^{a}
4T	*20/07/20 - 17/08/20*	*36*	38.00 ± 1.11^{b}	21.64 ± 1.09^{ab}	7.74 ± 0.26^{b}	71.10 ± 25.12^{b}	14.23 ± 0.28^{b}
5T	*19/10/20 - 16/11/20*	*36*	32.44 ± 1.54^{c}	17.12 ± 1.94^{ab}	8.07 ± 0.16^{a}	67.53 ± 22.35^{b}	10.38 ± 0.35^{c}
6T	*19/10/20 - 16/11/20*	*36*	32.01 ± 1.08^{c}	16.83 ± 1.75^{abc}	7.99 ± 0.21^{ac}	67.26 ± 21.89^{b}	10.38 ± 0.35^{c}
7T	*23/11/20-21/12/20*	*36*	29.53 ± 1.99^{d}	14.35 ± 2.66^{c}	7.81 ± 0.31^{bc}	62.28 ± 25.96^{b}	9.37 ± 0.14^{d}
8T	*23/11/20-21/12/20*	*36*	29.63 ± 2.00^{d}	14.40 ± 2.66^{c}	7.74 ± 0.33^{b}	61.61 ± 26.56^{b}	9.37 ± 0.14^{d}
Total Outdoors		***216***	32.57 ± 3.31^{acde}	18.53 ± 10.53^{abc}	7.90 ± 0.31^{abd}	76.33 ± 39.90^{abc}	11.37 ± 2.38^{e}
3PBR	*14/05/20 – 08/06/20*	*15*	30.91 ± 0.49^{cd}	17.08 ± 0.97^{ac}	7.47 ± 0.50^{bd}		
4PBR	*13/07/20 - 10/08/20*	*15*	35.03 ± 1.74^{a}	17.23 ± 0.85^{ac}	7.29 ± 0.46^{d}		
5PBR	*19/10/20 - 16/11/20*	*15*	35.82 ± 0.97^{a}	14.42 ± 0.92^{bc}	7.81 ± 0.52^{bd}	24.02 ± 19.57^{c}	16.00 ± 0.00^{f}
6PBR	*23/11/20-21/12/20*	*15*	32.97 ± 0.71^{e}	14.28 ± 0.15^{bc}	7.80 ± 0.38^{bd}		
Total Indoors		***60***	33.68 ± 2.20^{ace}	15.75 ± 1.61^{abc}	7.43 ± 0.47^{abd}		
Total		***276***	32.81 ± 3.13^{ace}	18.01 ± 9.40^{abc}	7.80 ± 0.40^{abd}	64.97 ± 41.35^{bc}	12.37 ± 2.84^{g}

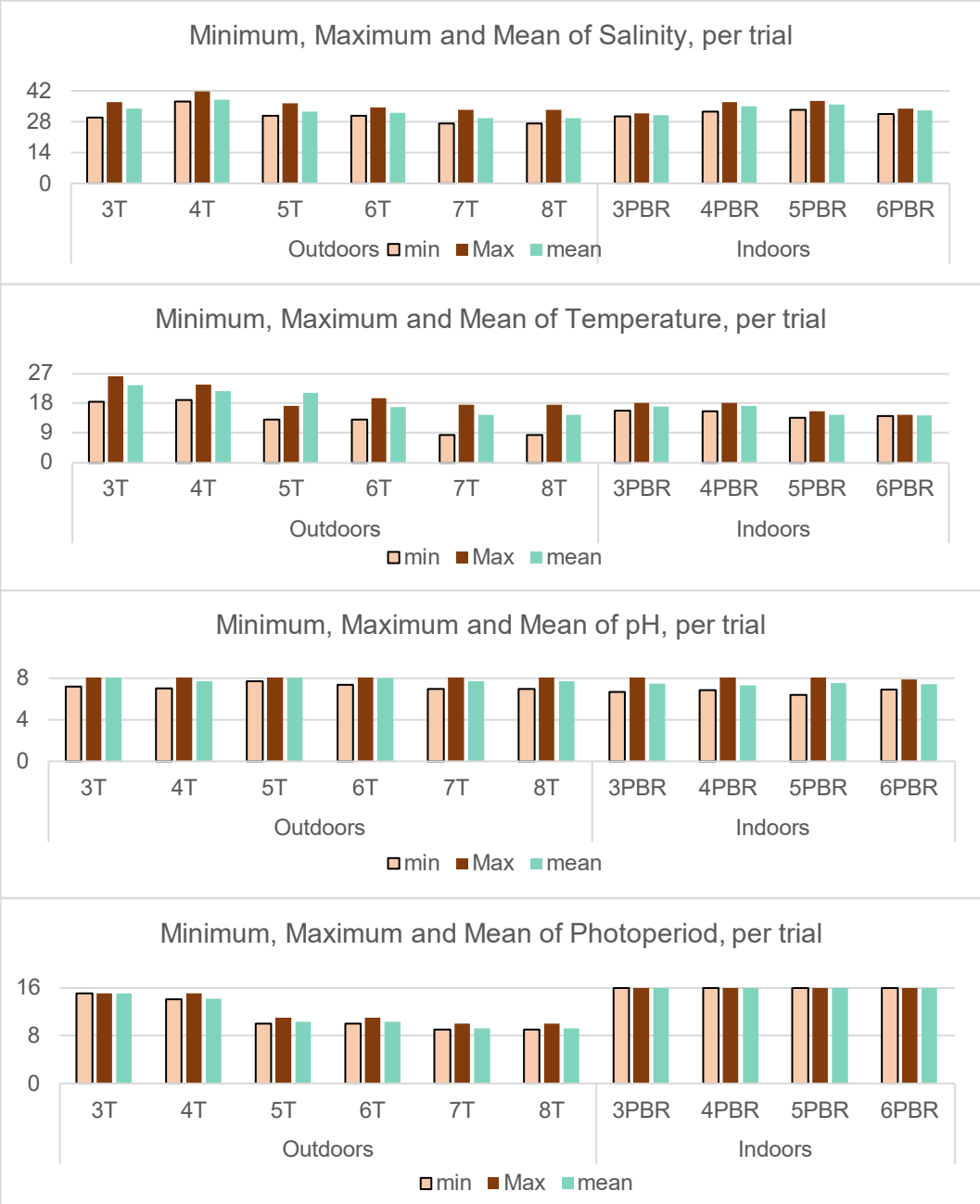


Figure 33. Minimum, Maximum and Mean of Salinity (ppt), Temperature (ºC), pH and Photoperiod (hours of daylight) per trial

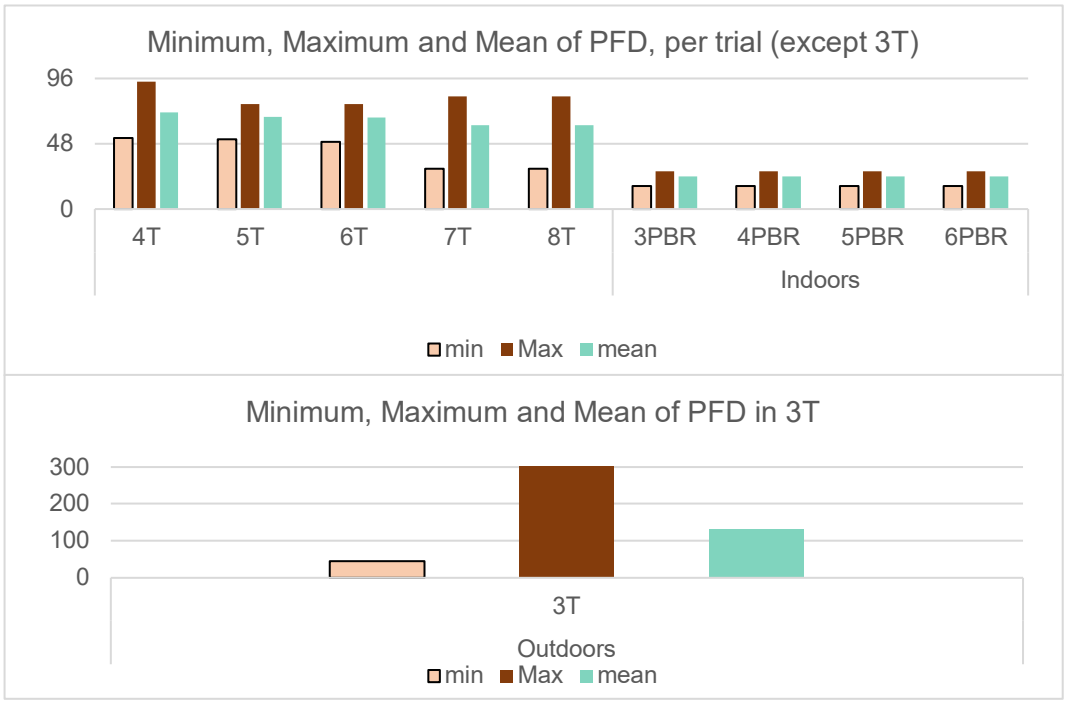


Figure 34. Minimum, Maximum and Mean of PFD (µmol.photons.m^{-2}.s^{-1}) per trial (3T was separated from the other trials since the scale of the results was very different from the other ones).

Figure 35 displays mean±sd for Yield_0-14, Yield_15-28, Yield_trial RGR_0-14, RGR_15-28 and RGR_trial in indoor and outdoor. Indoor growth parameters (Yield_0-14 = 0.84 ± 0.27 $gL^{-1}d^{-1}$; Yield_15-28 = 0.28 ± 0.17 $gL^{-1}d^{-1}$; Yield_trial=0.55 ± 0.09 $gL^{-1}d^{-1}$; RGR_0-14 = 9.94 ± 2.58 $\%d^{-1}$; RGR_15-28 = 3.06 ± 1.93 $\%d^{-1}$; and RGR_trial = 6.36 ± 0.69 $\%d^{-1}$) were significantly higher (Pvalue < 0.005) than the outdoor ones (Yield_0-14 = 0.25 ± 0.09 $gL^{-1}d^{-1}$; Yield_15-28 = 0.32 ± 0.09 $gL^{-1}d^{-1}$; Yield_trial = 0.28 ± 0.05 $gL^{-1}d^{-1}$; RGR_0-14 = 4.37 ± 1.3 $\%d^{-1}$; RGR_15-28 = 5.47 ± 0.86 $\%d^{-1}$ and RGR_trial = 4.92 ± 0.78 $\%d^{-1}$), except Yield_15-28 that showed no significant differences. *Table 12* shows mean±sd for the growth parameters per trial, with the results of the inference of significative differences in Yield_0-14, Yield_15-28, Yield_trial RGR_0-14, RGR_15-28 and RGR_trial per trial. Within indoors and outdoors trials, there were also significant differences in all parameters, except in outdoors Yield_15-28 (Pvalue < 0.05).

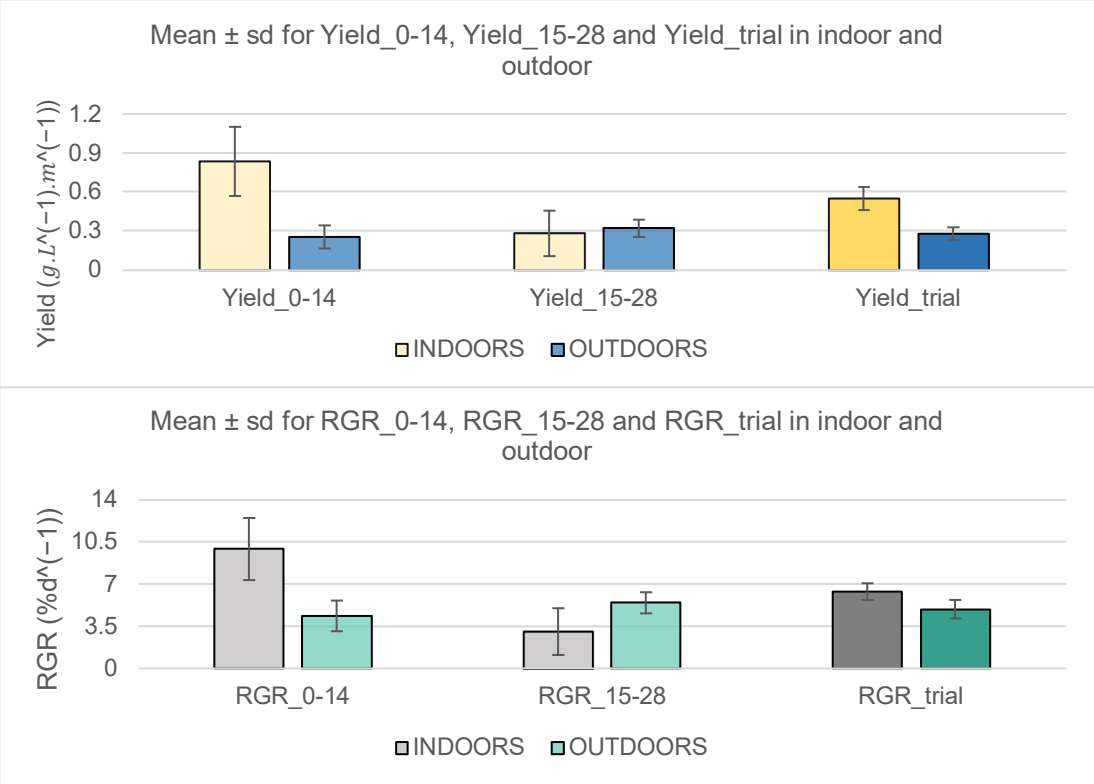


Figure 35. Mean ± sd of growth parameters (RGR and Yield) in indoor and outdoor trials

Table 12. Yield and RGR (mean ± sd) per halves and trial (significative differences were measured per column (Pavlue < 0.005)).

Trial	n	Yield ($gL^{-1}d^{-1}$)			RGR by halves ($\%d^{-1}$)		
		Yield_0-14	Yield_15-28	Yield_trial	RGR_0-14	RGR_15-28	RGR trial
3T	3	0.35 ± 0.03^{ab}	0.32 ± 0.08^{abc}	$\mathbf{0.33 \pm 0.04^{ac}}$	5.70 ± 0.30^{ab}	5.30 ± 0.90^{abcd}	$\mathbf{5.50 \pm 0.48^{ac}}$
4T	3	0.32 ± 0.02^{ac}	0.30 ± 0.04^{abc}	$\mathbf{0.30 \pm 0.02^{abc}}$	5.28 ± 0.29^{bc}	5.06 ± 0.47^{abcd}	$\mathbf{5.17 \pm 0.25^{abe}}$
5T	3	0.09 ± 0.04^{d}	0.33 ± 0.03^{abc}	$\mathbf{0.20 \pm 0.04^{b}}$	1.97 ± 0.90^{d}	5.56 ± 0.32^{abcd}	$\mathbf{3.77 \pm 0.58^{d}}$
6T	3	0.26 ± 0.02^{a}	0.35 ± 0.07^{abc}	$\mathbf{0.30 \pm 0.02^{abc}}$	4.53 ± 0.22^{bce}	5.81 ± 0.80^{abdi}	$\mathbf{5.17 \pm 0.29^{abe}}$
7T	3	0.29 ± 0.02^{a}	0.39 ± 0.03^{abc}	$\mathbf{0.33 \pm 0.03^{ac}}$	4.81 ± 0.27^{bce}	6.44 ± 0.32^{d}	$\mathbf{5.63 \pm 0.29^{ac}}$
8T	3	0.23 ± 0.03^{cd}	0.25 ± 0.08^{bc}	$\mathbf{0.23 \pm 0.03^{bc}}$	3.89 ± 0.34^{e}	4.63 ± 1.23^{abce}	$\mathbf{4.26 \pm 0.51^{de}}$
Total Outdoors	**18**	$\mathbf{0.25 \pm 0.09^{ac}}$	$\mathbf{0.32 \pm 0.09^{abc}}$	$\mathbf{0.28 \pm 0.05^{abc}}$	$\mathbf{4.37 \pm 1.30^{ce}}$	$\mathbf{5.47 \pm 0.86^{adi}}$	$\mathbf{4.92 \pm 0.78^{ae}}$
3PBR	3	1.18 ± 0.01^{e}	0.22 ± 0.06^{c}	$\mathbf{0.62 \pm 0.04^{de}}$	13.74 ± 0.08^{f}	2.29 ± 1.04^{fh}	$\mathbf{6.87 \pm 0.64^{f}}$
4PBR	3	0.48 ± 0.04^{bf}	0.43 ± 0.08^{a}	$\mathbf{0.45 \pm 0.06^{df}}$	7.00 ± 0.39^{g}	5.38 ± 0.57^{bdg}	$\mathbf{6.19 \pm 0.48^{bcf}}$
5PBR	3	0.86 ± 0.12^{g}	0.42 ± 0.09^{ab}	$\mathbf{0.64 \pm 0.07^{e}}$	9.87 ± 0.80^{h}	3.94 ± 0.77^{cghi}	$\mathbf{6.91 \pm 0.36^{f}}$
6PBR	3	0.83 ± 0.04^{g}	0.05 ± 0.02^{d}	$\mathbf{0.49 \pm 0.02^{d}}$	9.13 ± 0.24^{h}	0.57 ± 0.32^{f}	$\mathbf{5.46 \pm 0.26^{abg}}$
Total Indoors	***12***	$\mathbf{0.84 \pm 0.27^{g}}$	$\mathbf{0.28 \pm 0.17^{abc}}$	$\mathbf{0.55 \pm 0.09^{d}}$	$\mathbf{9.94 \pm 2.58^{h}}$	$\mathbf{3.06 \pm 1.93^{eh}}$	$\mathbf{6.36 \pm 0.69^{cfg}}$
Total	***30***	$\mathbf{0.49 \pm 0.34^{f}}$	$\mathbf{0.31 \pm 0.12^{abc}}$	$\mathbf{0.39 \pm 0.15^{af}}$	$\mathbf{6.60 \pm 3.35^{ag}}$	$\mathbf{4.50 \pm 1.81^{abi}}$	$\mathbf{5.50 \pm 1.03^{ac}}$

2.1.Indoor conditions

The results of the parameters of vegetative growth showed a higher yield (F = 7.213, with 3 df) in trial 5PBR compared to trials 4PBR (Pvalue = 0.010) and 6PBR (Pvalue = 0.036), without relevant differences between the other trial, as illustrated in *Figure 36*. RGR was significantly lower in 6PBR when compared with 3PBR (Pvalue = 0.004) and 5PBR (Pvalue = 0.004), without relevant differences between the other trials.

There were significant differences in Yield_trial between P3 SD and P3 LD (Pvalue = 0.047), as between treated and not treated biomass (Pvalue = 0.005), without interaction between the two factors (Pvalue = 0.365), showing higher values in P3 LD (Yield_trial = 0.57 ± 0.09 $\mathrm{gL^{-1}d^{-1}}$) than in P3 SD (Yield_trial = 0.53 ± 0.10 $\mathrm{gL^{-1}d^{-1}}$). Treated biomass had lower values (Yield_trial = 0.47 $\pm$ 0.05 $\mathrm{gL^{-1}d^{-1}}$) than not treated (Yield_trial = 0.62 ± 0.09 $\mathrm{gL^{-1}d^{-1}}$). For RGR_trial (F = 11.957, with 3 df), there were only significant differences for treatment x (Pvalue = 0.001), corroborating with the inference for Yield_trial: better performance when biomass was not treated (RGR_trial = 6.90 ± 0.12 $\%\mathrm{d^{-1}}$) than when treated (RGR_trial = 5.83 ± 0.21 $\%\mathrm{d^{-1}}$).

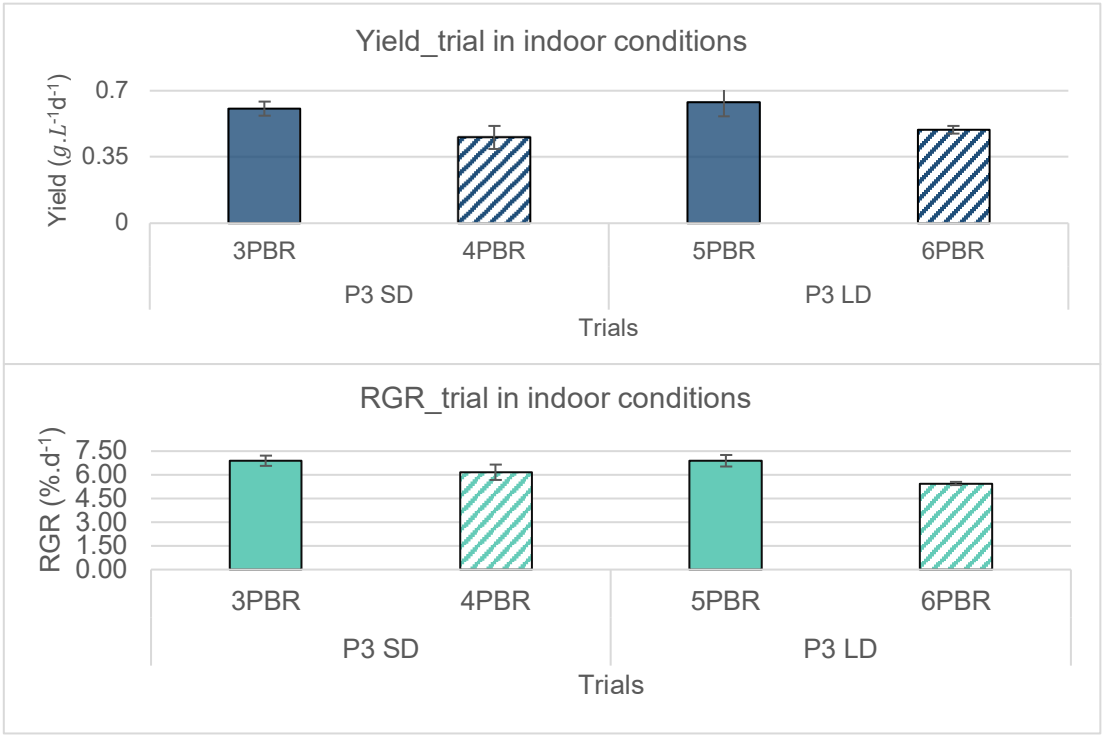


Figure 36. Mean ± sd of the growth parameters (Yield_trial and RGR_trial) in outdoor trials (stripped bars match trials with treated biomass)

2.1.1.Relationship between Quality, Abiotic Conditions and Growth

As previously indicated, differences in abiotic conditions in indoor conditions were just registered in salinity: 3PBR showed the lowest values of salinity (30.9 ± 10.49 ppt) and 6PBR the second-lowest (32.97 ± 0.71 ppt). 4PBR and 5PBR showed no significant difference between themselves, presenting with the highest salinity levels (35.03 ± 1.74 ppt and 35.82 ± 0.97 ppt, respectively) (*Figure 37*).

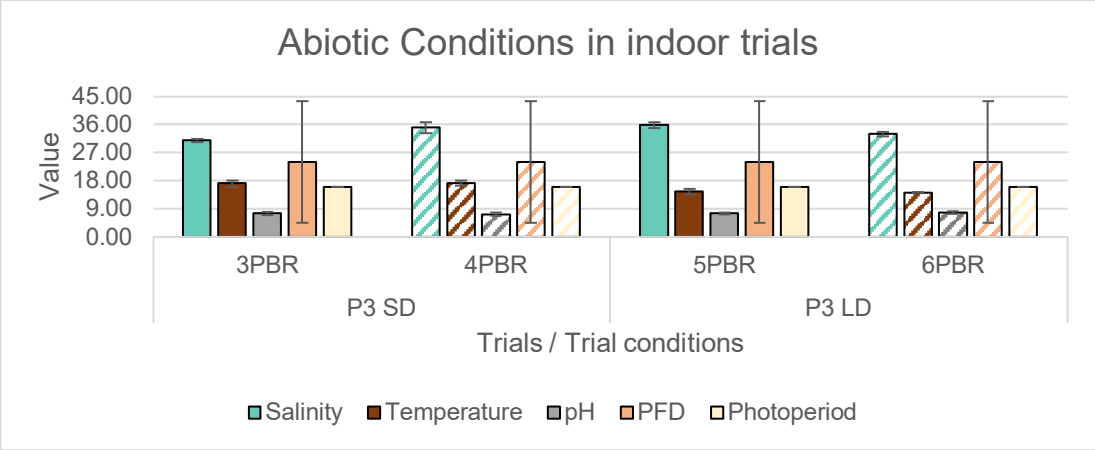


Figure 37. Mean ± sd of the abiotic conditions (Salinity in ppt, Temperature in ºC, pH, PFD in µmol.photons,m^{-2}.s^{-1}, and Photoperiod in hours of daylight) in indoor trials (stripped bars match trials with treated biomass)

In PFD, it was verified relevant variations within replicas, as shown for the values of standard deviation, due to the differences of light intensity in the different spots of measurement (Figure 38); yet, PFD was constant within replicas and trials in each spot. Photoperiod was also constant within trials and replicas (16 ± 0.00 hours of daylight. Although temperature and pH did not show significant differences within trials, it was studied the relationship between them (along with salinity), quality and growth, since the variations in these values were registered during the trials and not in the initial value.

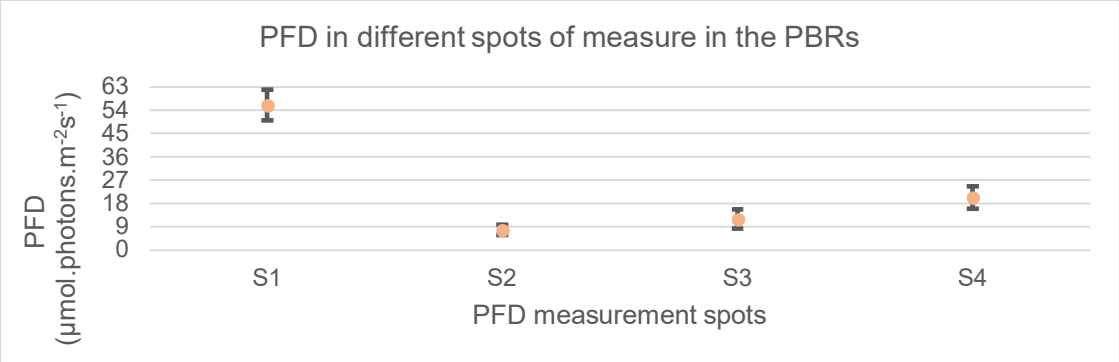


Figure 38. PFD (mean ± sd) in the different spots of measurement. Standard deviation refers to the variation within replicas. These values were constant during the trials and between trials.

It was not possible to establish a relationship between salinity and temperature and Yield_trial or RGR_trial (Pvalue>0.096) or pH and Yield_trial (Pvalue=0.056), but there was a good positive relationship between pH and RGR_trial ($r^2 = 0.695, \text{Pvalue} = 0.002$). The initial pH in these trials was similar in all the trials (Pvalue=0.819) and lower at the end *(Figure 39*), suggesting that the decrease over the weeks has a negative influence on RGR_trial.

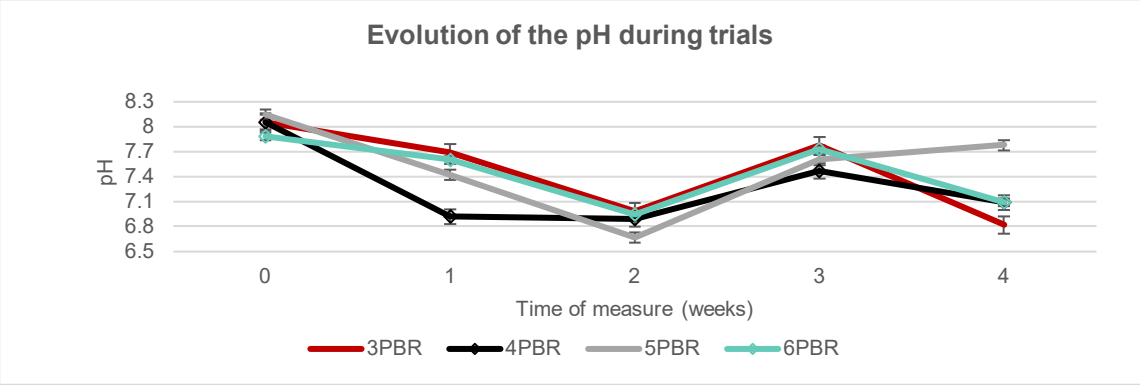


Figure 39. Evolution of pH by trials. 0=starting of the trial, 1= 1st weighing, 2=2nd weighning, 3=3rd weighing, 4=final of the trial

Since the final Quality in indoor was similar in all trials, the relationship between Quality and RGR_trial and Yield_trial was verified by the change in quality, showing that both parameters were lower when there was a “Small change” and higher when occurred “No change”; yet these results of change in quality coincide with the trials with biomass of P3 LD, where growth parameters were lower. “No change” was verified in 100% of the

cases when using P3 SD, while “Small change was noticed in 100% of the samples of P3 LD, showing dependency between Change in Quality (Qui-square = 12,00, with 1 df, Pvalue = 0.001). The influence of pH in the Change of quality (F = 0.005, with 1 df) showed no significant differences (Pvalue = 0.543).

2.1.2. Differences between halves on RGR and Yield

Looking at the results of Yield and RGR on the first and second halves in indoor conditions (*Figure 40*), it is possible to see there were differences between halves for both growth parameters, showing higher performances in the first half. Within trials, the first half on YIeld showed better results than the second half (F = 71.319, with 5 df), with significant differences in all of the indoor trials (Pavlue > 0.001), except for 4PBR (Pvalue = 0.975). Analyzing the influence of treatment x and origin of the biomass in these factors, P3 SD and P3 LD showed no differences between themselves; yet, there was an influence of the interaction of both factors: while P3 SD trials showed more homogeneity with treated biomass (corresponding this trial to the one without significant differences between halves), trials with P3 LD showed higher differences when the biomass was treated.

For RGR, the first half also showed better results than the second half (F = 131.169, with 5 df), with significant differences in all of the indoor trials (Pavlue > 0.001), except for 4PBR (Pvalue = 0.080). Analyzing the influence of treatment x and origin of the biomass in these factors, and similarly to what was observed in Yield, P3 SD and P3 LD showed no differences between themselves; but, while P3 SD trials showed more homogeneity with treated biomass (corresponding this trials to the one without significant differences between halves), trials with P3 LD showed higher differences when the biomass was treated. The relationship between parameter growth and Quality of the biomass/Changes in Quality was not analyzed for T_{2weeks} and T_{4weeks} since they obtained similar results.

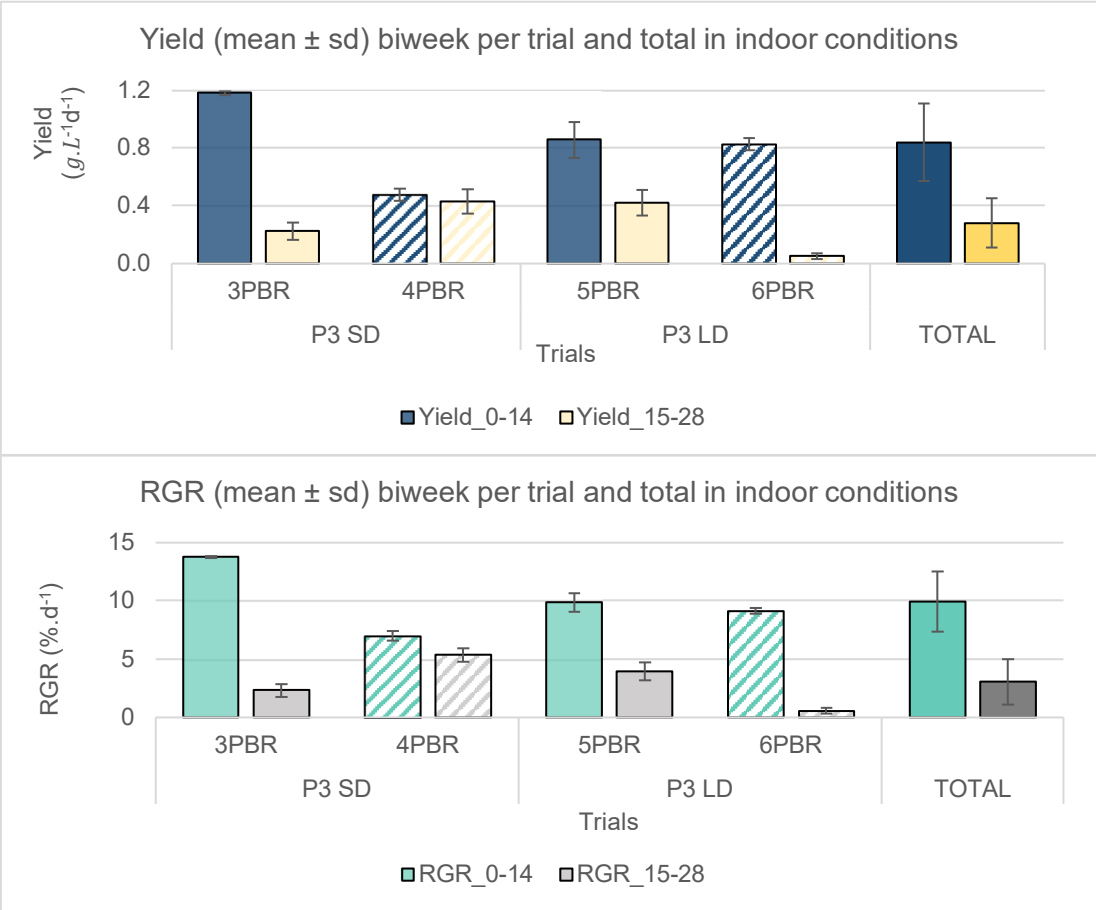


Figure 40. Comparison between Yield_0-14 and Yield_15-28 and RGR_0-14 and RGR_15-28 in indoor conditions (stripped bars match trials with treated biomass)

Figure 41 compares the daily growth in indoor trials considering the RGR of the first and second halves and the RGR of the trials (calculated by the total weight at the end and the beginning). In *Figure 41 A*, RGR is presented in all of the trials (except in 4PBR) with a major break after two weeks, indicating almost a stagnancy on growth in the second half; *Figure 41 B* shows the prediction of weight evolution if RGR was similar during all trial.

Figure 41. Weight evolution connsidering the RGR per halves (A) and RGR_trial (continuous lines correspond to P3SD and dashed lines to trials with P3LD; trials without markers correspond to not treated biomass and with square markers to treated one).

2.2.Outdoor conditions

Regarding yield, results showed similar values (F = 10.112, with 7 df) in trials 3T (Yield_trial = 0.33 ± 0.04 $gL^{-1}d^{-1}$), 4T (Yield_trial = 0.30 ± 0.02 $gL^{-1}d^{-1}$), 6T (Yield_trial = 0.30 ± 0.02 $gL^{-1}d^{-1}$), and 7T (Yield_trial = 0.33 ± 0.03 $gL^{-1}d^{-1}$), (Pvalue>0.768). 5T (Yield_trial = 0.20 ± 0.04 $gl^{-1}d^{-1}$) and 8T (Yield_trial = 0.23 ± 0.03 $gL^{-1}d^{-1}$) were significantly lower (Pvalue = 0.706), although 4T and 6T did not show significant differences from 8T (Pvalue > 0.114). 5T and 8T did not show significant differences between them (Pvalue = 0.820).

Regarding RGR and corroborating the Yield results, trials 3T (RGR_trial = 5.50 ± 0.48 $\%d^{-1}$), 4T (RGR_trial = 5.17 ± 0.25 $\%d^{-1}$), 6T (RGR_trial = 5.17 ± 0.29 $\%d^{-1}$ and 7T (RGR_trial = 5.63 ± 0.29 $\%d^{-1}$) (Pvalue > 0.919). 5T (RGR_trial = 3.77 ± 0.58 $\%d^{-1}$)

and 8T (RGR_trial = 4.26 ± 0.51 $\%d^{-1}$) were significantly lower (Pvalue = 0.692), although 4T and 6Tdid not show significant differences from 8T (Pvalue > 0.163) (*Figure 42*).

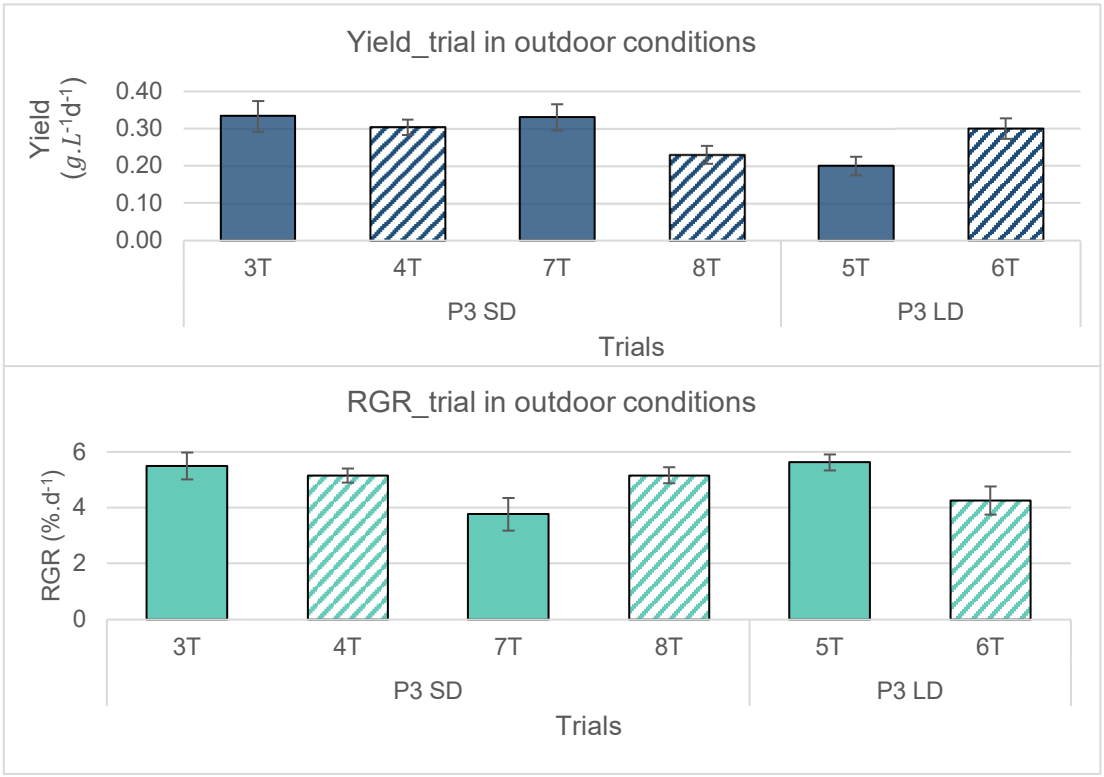


Figure 42. Mean ± sd of growth parameters (Yield_trial and RGR_trial) in outdoor trials (stripped bars match trials with treated biomass).

The inference on the influence of the origin of the biomass and treatment on the vegetative growth (F = 9.343, 7 df) showed significant differences in Yield_trial between P3 SD and P3 LD (Pvalue = 0.018), with better results in P3 SD (Yield_trial = 0.30 ± 0.05 $gL^{-1}d^{-1}$) than P3 LD (Yield_trial = 0.25 ± 0.06 $gL^{-1}d^{-1}$). There was no influence of treatment x on the Yield_trial results (Pvalue = 0.364), but there was an influence of the interaction between the two factors (Pvalue < 0.001): there were better results in P3 SD when the biomass was not treated (3T and 7T), while there were better results in P3 LD when it was treated (6T).

The results for RGR_trial were similar to the other parameter: there were significant differences in RGR_trial between P3 SD and P3 LD (Pvalue = 0.016) showing better results with P3 SD (RGR_trial = 5.140 ± 0.14 $\%d^{-1}$) than with P3 LD (RGR_trial = 4.468 ± 0.20 $\%d^{-1}$). Treatment x did not influence RGR_trial results, but the inference

showed an interaction between the two factors: there were better growth results in P3 SD when the biomass was not treated (3T and 7T), while there were better results in P3 LD when it was treated (6T), corroborating with the Yield_trial results.

2.2.1.Relationship between Quality, Abiotic Conditions and Vegetative Growth

Outdoor trials showed significant differences in all abiotic conditions, without differences in any parameter between 5T and 6T and 7T and 8T (*Figure 43)*. Salinity was lower in 7T and 8T (29.53 ± 1.99 ppt and 29.63 ± 2.00 ppt, respectively) and higher in 4T (38 ± 1.11 ppt), with similar values for 3T, 5T and 6T (33.79 ± 2.04 ppt, 32.44 ± 1.54 ppt and 32.01 ± 1.08 ppt, respectively). 7T and 8T registered the lowest temperatures (14.35 ± 2.66ºC and 14.40 ± 2.66ºC); the rest of the trials registered similar temperatures (3T = 23.43 ± 2.03 ºC, 4T = 21.64 ± 0.09 ºC, 5T = 17.12 ± 1.94 ºC and 6T = 16.83 ± 1.75 ºC), 6T being similar also to 7T and 8T. pH was higher in 3T, 5T and 6T (3T = 8.09 ± 0.31, 5T = 8.07 ± 0.16 and 6T = 7.99 ± 0.21) and lower in the rest of the trials (4T = 7.74 ± 0.26, 7T = 7.81 ± 0.31 and 8T = 7.74 ± 0.33), although 6T and 7T showed no significant differences between themselves. Photoperiod was higher in 3T (14.79 ± 0.17 hours of daylight) followed by 4T (14.23 ± 0.28 hours of daylight), 5T and 6T (10.38 ± 0.35 hours of daylight in both trials), with the lowest values in 7T and 8T (9.37 ± 0.14 hours of daylight).

Similar to what was noticed in indoor trials, PFD was the only parameter with significant variations within replicas, although in outdoor trials only registered in 3T. although standard variations are high in every trial (also due to the differences between the measurement spots – above the water and immediately under), in 3T, PFD measurements above the water varied from 61 to 430 µmol.photons.$m^{-2}s^{-1}$ above the water, and 25 to 192 µmol.photons.$m^{-2}s^{-1}$ immediately under. PFD was higher in 3T (128.86 ± 95.41 µmol.photons.$m^{-2}s^{-1}$) and similar in all the other trials (4T = 71.10 ± 25.12 µmol.photons.$m^{-2}s^{-1}$, 5T = 67.53 ± 22.35 µmol.$m^{-2}s^{-1}$, 6T = 67.26 ± 21.89 µmol.photons.$m^{-2}s^{-1}$, 7T = 62.28 ± 25.96 µmol.$m^{-2}s^{-1}$ and 8T = 61.61 ± 26.56 µmol.photons.$m^{-2}s^{-1}$).

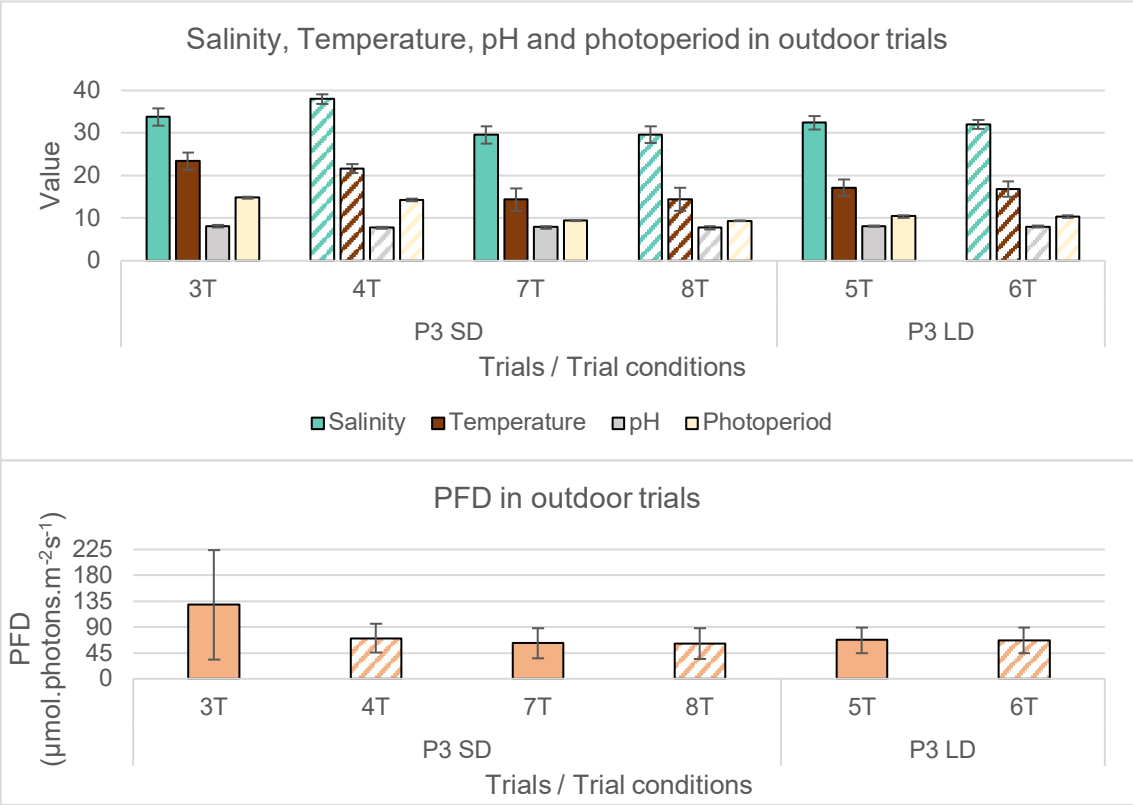


Figure 43. Mean ± sd for abiotic conditions (Salinity in ppt, Temperature in ºC, pH, PFD in µmol.photons.m^{-2}.s^{-1}, and Photoperiod in hours of daylight) in outdoor trials (stripped bars match trials with treated biomass)

Comparing the results of PFD measured in the tanks during the trials with the mean values of PFD registered by Hidromod in the days corresponding to each trial, it is possible to establish a perfect relationship (r^2=1) between the values registered in the tanks when reducing light intensity (3T and 4T, corresponding to the time from 18th May to 15th June and 20th July to 17th August 2020) and a very good relationship (r^2=0.99) when not reducing it (5T, 6T, 7T and 8T, corresponding to the time from 19th October to 16th June and 23rd November to 21st December 2020). The models predict *PFD in tanks = 0.51 (PFD in Hidromol) - 544.75* µmol.photons.$m^{-2}s^{-1}$, for the trials reducing light intensity and PFD in tanks = 0.04 (PFD in Hidromol) + 52.39 µmol.photons.$m^{-2}s^{-1}$ for the trials without reducing it, showing a more accentuated cleavage when using the protocol (*Figure 44*).

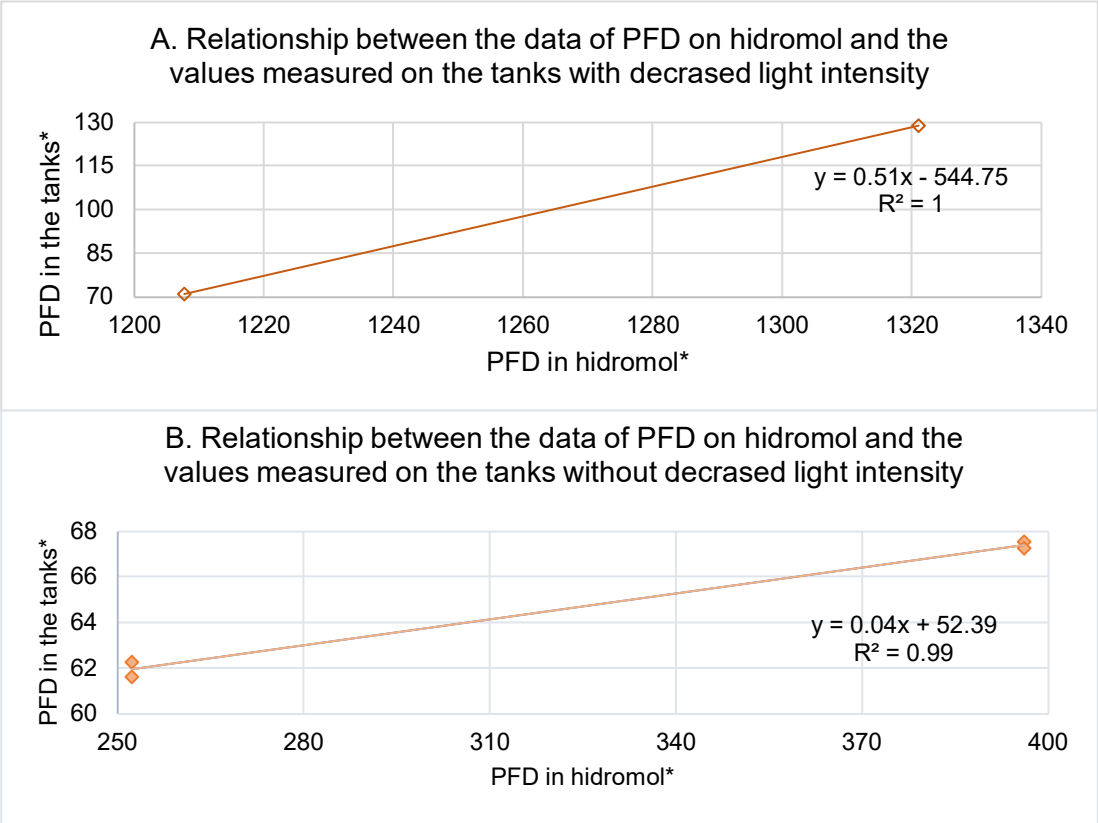


Figure 44. Relationship between the data of PFD on hidromol and the values measured on the tanks with (A) and without decrased light intensity (B). (*PFD in *µmol.photons.m^{-2}s^{-1})*

It was not possible to establish a relationship between vegetative growth parameters (Yield_trial and RGR_trial) and any abiotic factor in outdoor conditions (F = 4.237, 5 df, Pvalue > 0.128 for Yield_trial, and F = 3.427, 5 df, Pvalue > 0.128), nor between vegetative growth parameters and AC (F = 3.813, 1 df, Pvalue > 0.069 for Yield_trial, and F = 2.723, 1 df, Pvalue > 0.118). Analyzing the results of the inference of Quality at the ending of the trials, as the Change in Quality, with Yield_trial and RGR_trial, it was also not possible to establish any relationship between quality and vegetative growth (Pvale > 0.068).

Since the initial results of quality were similar when using biomass with the same origin (Good in P3 SD and Very Good in P3 LD), when analyzing the results separated by origin, the results of inference are similar for “Quality at the ending” or “Change in quality”. Contrary to indoor conditions, there were different results of final quality/change in quality in trials using biomass with the same origin, for P3 SD trials. As previously shown in *Figure 32 C*, two of the trials with P3 SD (3T and 4T) presented as “Satisfactory" at the end of the trial, while the other two (7T and 8T) presented as “Good", when both started as “Good”. Considering these, the results showed that AC, the origin of the

biomass and quality at the ending of the trials are related (F = 9.374, 2 df, Pvalue = 0.002): while P3 LD trials had the same quality at the end ("Good", presenting "No Change" in both trials), "Satisfactory" (Small Change) and "Good" (No Change) P3 SD trials at the end correspond to statistically different means of AC (AC = 10426087.36 and AC = 3538940.40, respectively).

2.2.2.Differences between halves on RGR and Yield

Contrarily to what was noticed in indoor conditions, there were no differences between halves in growth (both for Yield and RGR), except for 5T. Unlike in indoor trials, 5T shows a higher Yield and RGR in the second half than in the first one (*Figure 45*).

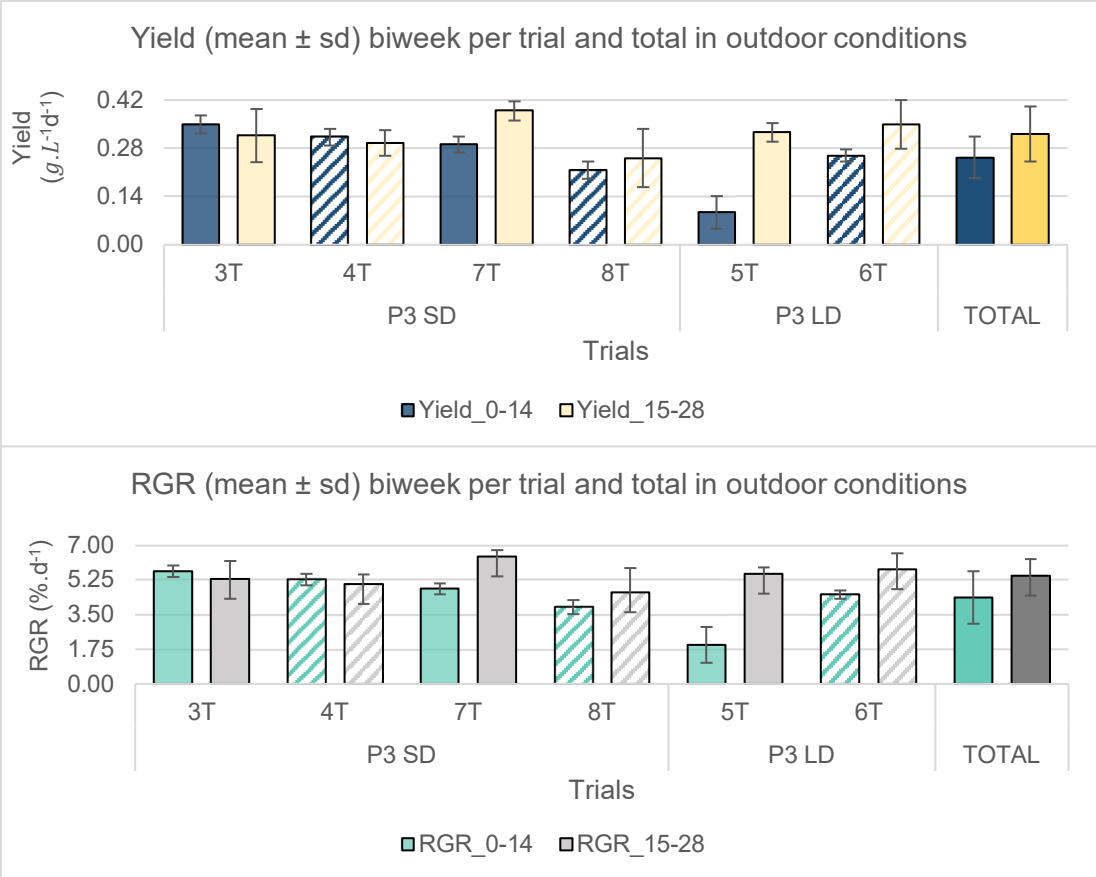


Figure 45. Comparison between Yield_0-14 and Yield_15-28 and RGR_0-14 and RGR_15-28 in indoor conditions (stripped bars match trials with treated biomass)

3.Biochemical analysis

3.1.Ashes Content

The following table *(Table 13*) shows the results of ash content in percentage of dry matter (%DM), per trial at T_0, T_{2weeks} (only for outdoor trials) and T_{4weeks}. There were differences between T_0, T_{2weeks} and T_{4weeks}: ashes content, at T_{2weeks}, is significantly higher than in the start (Pvalue < 0.001); ash content, at T_{4weeks}, is significantly lower than after two weeks (Pvalue = 0.009), but significantly higher than at the beginning (Pvalue < 0.001).

Table 13. Mean ± sd of ashes content (%DM) per trial at T_0, T_{2weeks} and T_{4weeks}. (significative differences were found between ash content at T_0, T_{2weeks} and T_{4weeks}. (Pvalue < 0.005)

		Ashes content (% DM)		
	Trials	T_0	T_{2weeks}	T_{4weeks}
Outdoors	**3T**	62.37±1.19	63.52±2.08	63.00±0.07
	4T	54.87±1.15	59.56±1.88	51.93±0.18
	5T	59.94±0.67	65.62±0.28	60.55±1.07
	6T	59.94±0.67	64.04±3.82	63.10±2.61
	7T	52.50±3.12	63.57±1.54	64.78±1.54
	8T	52.50±3.12	68.25±1.92	65.18±1.07
	total outdoors	56.78 ± 4.34	64.09±3.25	61.42±4.77
Indoors	**3PBR**	55.82±1.75	-	61.84±0.89
	4PBR	54.87±1.15		55.62±2.27
	5PBR	59.94±0.67		60.29±4,15
	6PBR	55.03±0.88		66.99±6.25
	total indoors	56.41±2.38	-	61.18±5.41
	Total	56.78 ± 3.64[a]	64.09 ± 3.25[b]	61.33 ± 4.95[c]

At T_0, ash content was different according to the origin of the biomass: P3 SD (ashes = 55.49 ± 3.84%) showed a lower percentage than P3 LD (58.71 ± 2.13%). Since the initial content is different according to the origin of the biomass, the influence of treatment, origin, environment and abiotic conditions was studied on the difference between the three moments of analysis: T_0 and T_{2weeks}, T_{2weeks} and T_{4weeks}, and T_0 and T_{4weeks}. The inference on the increase of ashes content from T_0 to T_{4weeks} showed that the increase in ash content was influenced by treatment and origin of biomass: while P3 SD showed a higher increase when not treated (trials 3T, 7T and 3PBR), P3 LD showed a higher increase when treated (trials 6T and 6PBR) (Pvalue=0.035) (*Figure 46*).

Comparing the decrease from T_{2weeks} to T_{4weeks} (outdoor trials only) P3 SD showed a higher decrease when treated (trials 4T and 8T), while P3 LD showed a higher decrease when not treated (6T) (Pvalue = 0.005) (*Figure 46*). It was not possible to establish a relationship between these factors and the increase of ashes content from T_0 and T_{2weeks} (Pvalue>0.267).

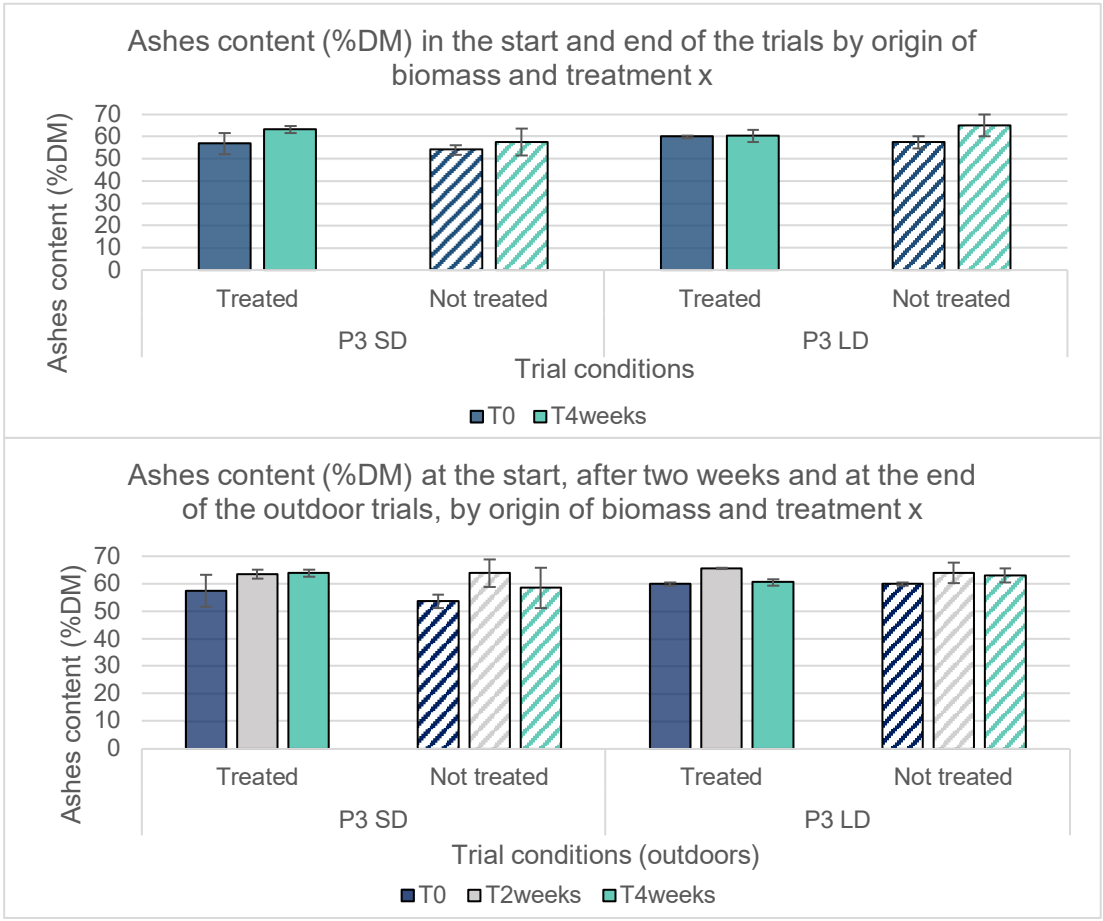


Figure 46. Influence of treatment x and origin of the biomass in the increase of ashes content (%DM). P3 SD showed a higher increase in the end when not tretaed, while P3 LD's increase was higher when treated.

3.2.Lipid content

The results of lipid content (%DM) per trial are shown in *Table 14*. There were no differences between the lipid content (%DM) in the T_0, T_{2weks} and T_{4weeks} (Pvalue > 0.381). Results showed that the lipid content in the start was not influenced by the origin of the biomass, showing no differences between P3 LD and P3 SD (Pvalue = 0.228) (*Figure 47*). Considering all measurements, since none of the factors showed to influence it, the biomass showed 4.09 ± 0.88% of lipid content.

Table 14. Mean ± sd of lipid content (%DM) per trial at T_0, T_{2weeks} and T_{4weeks}

	Trials	T_0	T_{2weeks}	T_{4weeks}
INDOORS	**3PBR**	3.38±0.11	–	3.94±0.52
	4PBR	6.16±0.86		4.50±0.28
	5PBR	3.71±0.86		4.92±0.38
	6PBR	3.82±0.37		4.27±0.14
	total indoors	4.27±1.28		4.41±0.48
OUTDOORS	**3T**	4.38±0.69	3.26±0.31	3.70±0.37
	4T	6.16±086	4.21±0.44	3.87±0.76
	5T	3.71±0.86	4.05±0.06	3.28±0.11
	6T	3.71±0.86	3.87±0.09	3.80±0.46
	7T	2.90±022	4.32±0.38	4.35±0.55
	8T	2.90±0.22	4.57±0.89	4.57±0.39
	total outdoors	3.96±1.28	4.04±0.50	3.93±0.53
	Total	4.08±1.26	4.04±0.50	4.12±0.56

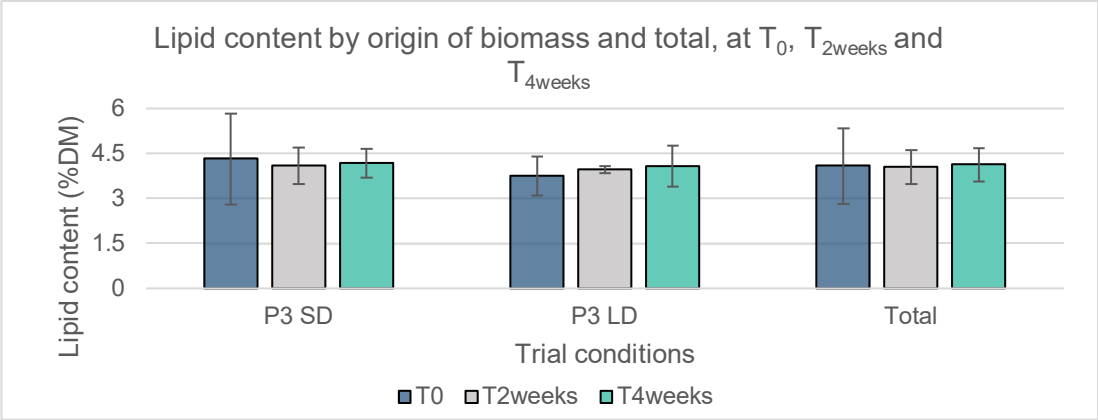


Figure 47. Lipid content (mean ± sd) by origin of biomass and total, at T_0, T_{2weeks} and T_{4weeks}

V.DISCUSSION

This study presents the first long-term, seasonal and pilot-scale study on the optimized cultivation of *Porphyra umbilicalis* conchocelis, a commercially important seaweed species in Europe, in a land-based IMTA system. The results showed significant differences in the quality of the biomass at T_0, according to the origin of culture starter biomass (P3 SD or P3 LD) which was expected since P3 SD cultures suffered some photoperiod distress before the trials (passing from LD to SD). Photoperiods over 12h have been described as preferable in several free-living conchocelis cultures of Porphyra/Pyropia (Lu & Yarish, 2011; Redmond *et* al., 2014; Zhong *et* al., 2016), corroborating that a decrease in photoperiod has a negative impact in the cultures, inhibiting its growth and, consequently, increasing the possibilities for contaminations to grow.

The results at the end of the trials were also influenced by the origin of the biomass and by the environment (indoor/outdoor conditions). Besides, the abiotic conditions in the outdoor tanks suffer more variations than in indoor PBRs, tanks are more exposed to crossed contaminations due to the top opening, sustaining the results obtained. The decrease in quality in P3 LD in indoor trials might be explained by the use of FSW (instead of ASW, used in the company protocols), making the culture medium less sterile.

Abiotic conditions in indoor trials showed significant differences only in salinity. Photoperiod, PFD and temperature were controlled by the LD chamber conditions; temperature, even though, suffered some variations, due to high air temperatures registered in June, July and August that made the temperatures in the chambers rise. Salinity variations within trials are explained by the salinity variations in nature, while the variations during the trials are due to some evaporation over the four weeks and the 40L added after two weeks. Although pH did not show significant differences within trials, it suffered some variations during each trial, which can be related to a higher/lower photosynthetic activity over the weeks.

In outdoor conditions, differences in the abiotic conditions were noticed in all of the parameters, with more accentuated variations along the trials than in the indoor ones. Temperature, PFD and photoperiod are dependent on the environment and not controlled like in the LD chamber; salinity is influenced by variations in salinity from the capture and more accentuated by precipitation and evaporation, amplified by the small volumes of the tanks (Hemminger & Höhne, 1894; Rebelo, 1992). pH variations might

be from variations in seawater pH from the supply, as from differences in photosynthetic activity during the trials.

Yield_trial and RGR_trial were significantly higher in indoor trials as expected since the outdoor environment is more subjected to contaminations, abiotic distress (contrarily to indoors, it is not possible to control temperature, PFD and photoperiod) and more variations during the trials. RGR in indoors (RGR_trial= 6.36 ± 0.69 $\%.d^{-1}$) was similar to what was described in the bibliography (6.98 ± 0.51 $\%.d^{-1}$) (Chopin *et* al., 1999), where it was described the use of smaller volumes (up to 10 L) in ASW and weekly medium changes, indicating that the use of FSW was not responsible for a decrease in growth. Differences within trials in the same environment were an indicator of the influence of other factors (treatment, origin of the biomass, or abiotic conditions), confirmed in the posterior inference.

Indoor inference showed the best results in RGR and Yield for not treated P3 LD biomass (5PBR trial), showing no significant differences between P3 SD and P3 LD, but worse results in treated biomass, corroborating that treatment x constitutes distress for the cultures, having a negative impact in growth during four weeks of trial. When comparing the results of growth with changes in quality and abiotic conditions, it was only possible to establish a good positive relationship between pH and RGR. Since the initial pH was similar in all trials, it suggests that the decrease in pH affects negatively growth. Higher pH values are usually associated with high photosynthetic activity (Blouin *et* al., 2007; Redmond *et* al., 2014), suggesting that photosynthetic activity tended to decrease during the trials, and, consequently, jeopardizing growth results. Photosynthesis depends on two variables – CO_2 and light. Insufficient CO_2 leads to an increase in pH, whereby pH decrease might be caused by low PFD. Although the PFD results suited the PFD optimal range described in the bibliography, it is not clear if these values were obtained on the glass flasks or under the cultures; plus, PBRs walls are made of polyethylene, which might lead to lower light intensity inside the containers than in glass flasks.

All the indoor trials had better performances in the first two weeks (except for 4PBR with no significant differences), P3 SD trials showing a less steep drop with treated biomass (corresponding to 4PBR), while trials with P3 LD showed a higher drop when the biomass was treated. These results do not agree with what was expected: treatment x might constitute physiological distress for the cultures at the moment, but it also contributes to the increase in the SA/V ratio of the cultures, described as an indicator of better results in nutrient assimilation and consequently better growth performances (Pereira & Yarish, 2010). Therefore, it was expected that in the first half, trials in which

biomass was treated might have lower yield and RGR, but showed better performances in the second half or a less evident decrease.

The results of RGR and Yield between halves in indoor conditions and the decrease verified in the second half do not corroborate with the results obtained in the same analysis in outdoor trials. In outdoor trials, (where weight assessment was always calculated by weighing the total biomass), it showed no significant differences between the first and second halves, except for 5T, which, contrarily to indoor trials, showed an improvement in the second half. These results (along with the tendency lines in *Figure 41*) suggest that indoor RGR_0-14 and Yield_0-14 might have been overestimated, biased by the adopted method of weighting: the 1 L estimative might over or underestimate the total weight after two weeks since this estimative assumes a homogeneity of the culture along the water column that might not be accurate. This might indicate the adopted methodology on weight assessment not to be the most suitable.

Trial 5T might have suffered the influence of other factors (not enough aeration, biomass losses by the exit sieves, or overflow from too strong aeration or precipitation); plus, the value of standard deviation in the first half of both parameters corroborates that other factors might have been the cause for these results, with one of the replicas performing as an outlier by default.

The differences in the quality at the end of the trials found between the outdoor P3 SD trials (3T and 4T versus 7T and 8T) and their relation with the abiotic conditions can be justified by the different abiotic conditions: salinity, temperature and photoperiod were higher in 3T and 4T and pH and PFD were higher in 3T than on the other trials. Results showed that salinity did not influence quality or growth in the range of 20-40 ppt; yet, conchocelis cultures react better to low PFD (under 50 μmol.photons.m^{-2}.s^{-1}) and 5-20 ºC temperatures, registering lethal values at 25 ºC (Waaland *et* al., 1987). PFD was in every trial in outdoor conditions over 50 μmol.photons.m^{-2}.s^{-1}, but the bibliography is not clear about the method/depth of the measure. Temperature, on the other hand, was over 20 ºC in 3T and 4T, which might be the reason why the final quality was worse than in 7T and 8T, where temperatures were under 20 ºC, suggesting that 18-20 ºC constitute a “danger range” for conchocelis cultures quality, resulting in discolored cultures and an increase in contaminations.

Conchocelis showed high ashes content (over 50%), with higher values for P3 LD than P3 SD. According to what was noticed in the micro and macroscopic observations, P3 SD presents as a mix of conchocelis and differentiated primordial conchosporangia, conchospores, and microscopic free-floating blades, while P3 LD is usually more “pure” in conchocelis, suggesting that conchocelis might present a higher

mineral content than blades and other structures of the life cycle. High amounts of minerals are usually associated with a higher capacity of inorganic matter (Rupérez, 2002), whereby conchocelis might be excellent for bioremediation.

Indoor and outdoor trials showed similar results, although the ash content was higher at the end of the trials, suggesting that FSW is richer in minerals, as the biological nutritional supplementation when confronted with the nutritional option used on the company protocols. Treatment x seemed to influence ash content: although the results showed that the increase was lower with treated biomass of P3 SD, in P3 LD the treatment improved the final results. Considering that P3 SD contains a mix of conchocelis and other structures, with more heterogeneity of results and that treatment x improves SA/V ratio, it might increase conchocelis ash content and, consequently, improve their bioremediation potential. In outdoor trials, where the mineral content after two weeks was also analyzed, showed that the ash content decreased in the last two weeks, although higher at the end than at the beginning, suggesting retain capacity/bioremediation potential decreases with time.

Lipid content found in conchocelis suited the range described in the bibliography for *Porphyra* spp. and red macroalgae (0.5-5%) (Sanina *et* al., 2004). According to the results, there were no differences between P3 SD and P3 LD, nor did the trial conditions (treatment x, environment) influence the lipid content of the conchocelis. It was not possible to find any references to corroborate/refute these results since nutritional studies in conchocelis are still scarce.

VI. Final considerations

This work intended to optimize the protocols of biomass production of conchocelis of *Porphyra umbilicalis*, in indoor and outdoor conditions, using the company resources, and to study the viability of producing conchocelis as a final product. The results showed that conchocelis produced in indoor PBRs had better RGR and yield than outdoor tanks. The best results were achieved in indoors PBRs with P3 LD not treated (5PBR, Yield_trial = 0.64 ± 0.07 gLd^{-1} and RGR_trial = 6.91 ± 0.36%d^{-1}); assuming 52 weeks as a year and that each trial has a duration of four weeks (28 days), yearly, production rounds 157.08±30.59 gL^{-1} (results for the studied stock density).

Although indoor trials had better growth performances (RGR and yield) than outdoor trials, outdoor results are encouraging; abiotic conditions did not show to affect growth, allowing to produce conchocelis during all year with fewer energy costs (PBR accommodation requires energy for photoperiod simulation and temperature control). These results corroborate that outdoor production may be an added value; in the future, it was interesting to do an economic balance on indoors/outdoors production. P3 SD conchocelis production could be an important asset for the company in the future, valuing a by-product of blades production and reducing waste.

One of the weaknesses of this experiment was the difference in abiotic conditions during the trials when testing different factors (treatment x, biomass used); in the future, for validation of these results, all the intended factors to test should be tested at the same time, and during 12 months, so the results about the influence of abiotic conditions were more reliable; also, it would give a real perspective about the yearly production. More experiments that would include abiotic conditions of the entire year could also create a stronger correlation between PFD measurement in the tanks/HIDROMOL values, and estimate these values only from the HIDROMOL tables (and possible extrapolation for other tanks at the company).

Upscaling outdoor trials for bigger volumes would also be interesting: working hours/volume might decrease and it would allow more interesting values of yearly production. Another factor that could be tested in the future is the time of maintenance of the cultures in production: testing the influence of time in a longer period allows the same culture to stay and produce more biomass and might have promising results since in four weeks there were no significant differences between the first and the second ones in these trials.

Relying on the possibility of the estimation of total biomass in 1L of culture biased the results of growth at T_{2weeks}, alternative protocols of weight assessment in

PBRs should be studied, like image processing and conchocelis area (mm^2) as described in other studies (Lu & Yarish, 2011; Varela-Alvarez *et* al., 2004). A more trusting weight assessment method would enable the study of the increase or decrease of the time of cultures' maintenance in production. This type of weight assessment could be also useful in outdoor tanks in bigger volumes, reducing working time and cultures' manipulation.

Concerning the quality of the biomass, P3 LD presented with better quality than P3 SD at T_0; at T_{4weeks}, P3 SD indoor and had better results in indoor trials than in outdoor trials in general, but similar during 7T and 8T, and P3 LD had similar results independently of the environment. The previous suggestion of 12 months would also give stronger results about the final quality of the biomass.

In terms of biochemical analysis, N, P and C determination (as planned) would have given important results about nutrient assimilation, nutritional profile and bioremediation potential, which could corroborate conchocelis potential and expand the range of possible applications (food, cosmetics, biofilters, nutraceuticals, pharmaceuticals). Plus, the correlation between N content and the colors of quality and contamination scale, similar to what was done in a previous study with *Ulva latuca* and *Gracilia gracilis* (Robertson-Andersson *et* al., 2009), allows the assessment of N content by rapid classification of cultures coloration and validating the created scale.

Both indoor and outdoor, other variables were interesting to be tested: the performance of isolated conchocelis strains (instead of a mix) and different light wavelengths, recurring to colored vinyl. Indoors, it would be interesting to test LEDs instead of fluorescent lights, since it showed to improve growth in conchocelis (Kim *et* al., 2019). Since conchocelis had interesting results in PBRs, which typically are used for microalgae, cultivation in green wall panels or horizontal PBRs. These structures, along with the tested PBRs, may also be tested in outdoor conditions, reducing electricity costs and easing cleaning and maintenance actions.

The opportunity to be an intern at a leading land-based IMTA European company was very rewarding, fulfilling a personal desire of working both with seaweed and to know more about IMTA systems. To be on the vanguard in conchocelis production was a unique experience that enriched my academic route, providing me with work tools and knowledge about seaweed biology and cultivation protocols that only working directly in the field could give. As my first experience in a company environment, it allowed me to have a different perspective than academic research work; in a company, development needs to be particularly applied in the company work and reality, forcing the team to be more flexible and to think more outside of the box sometimes. Cost-

effectiveness is a primary concept in a company and a goal in every work and developed protocol in its context, which, sometimes, is forgotten in the academic field.

Continuing the good work in innovation and development, work with mathematic models of growth prediction for both fish and seaweed might be an important future step for the company, rising cost-effectiveness and reducing losses, easing work planning and management, sustaining decisions for new practices, and perpetuating the blue economy concept of the project. The work invested in seaweed production (not only *Porphyra* spp.) in artificial substrates might be a key step for the company growth and it would allow focusing work in conchocelis as a good. Conchocelis potential for more valuable markets sustains a rising focus on its production as a final product.

Ongoing work in biorisk management policies (and possible legislation and certification) and waste management might be an extremely important step in customer acceptance - seaweed is still a low-known resource outside the Asian market. As a workspace with some associated hazards to the workers, first aid training to the team might be a good biosafety strategy to minimize prolonged work injuries and health consequences to the workers. Biorisk certificated measures, along with marketing work (increasing show cookings and partnerships with restaurants and local businesses) might lead to higher costume familiarity and acceptance/product buy, by creating safety standards of these products, teaching the general public about how to incorporate seaweed in their diet and dishes and increasing people awareness for seaweed potential and wide range of applications.

Carrying out a study during a pandemic was very challenging, leading to facing unique problems arising from national lockdown and posterior contingency measures several adaptations to the initial project and leading to the impossibility of some analysis and factors tested. Yet, this study provided important results, allowing taking important conclusions in conchocelis production that will be very useful for further and more extended studies.

The increasing pursuit of nori for food, nutraceuticals and cosmetics in Europe suits space for the interest of Atlantic nori species to grow and, consequently, Atlantic nori farms and applied research. There is still some fear and unacceptance of seaweed products in the European general public since there is not a tradition of its use or, when there was, it was lost with industrialization and economic growth. The intensive work developed in the last decade on taxonomy review (Yang *et* al., 2020) and increasing interest in molecular validation species (Varela-Álvarez *et* al., 2018) along with the implementation of biorisk management policies and legislation specific to seaweed may

bring more safety and acceptance of seaweed products, which might be the last piece for nori industry development in Europe.

Both biochemistry and production conditions of conchocelis (*P. umbilicalis* or other *Porphyra/Pyropia* species) are still very few described (da Costa *et* al., 2018; Pimentel *et* al., 2020). Yet, the little available data about the subject sustains the interest and research: conchocelis overloads blades in phycobiliproteins, fatty acids and amino acid content, with higher portions of PUFAs and MUFAs and lower of SFAs and better essential amino acids index (Lin & Stekoll, 2011; Pereira, 2018; Pimentel *et* al., 2020), with interesting daily growth rates (Chopin *et* al., 1999). However, a lot of work (especially cultivation issues, like production protocols and growth rates) may not be published: as important data for nori aquacultures, it is possible that some companies keep their work unpublished and not patented for confidentiality, mostly in Asian countries, where nori cultivation has centuries of history.

Working with seaweed cultivation is very challenging and research-requiring: as one of the most studied fields in the last decades, new data about changes in taxonomy, molecular validation, cultivation protocols, or life cycle revisions/alternative life cycle pathways are constantly emerging. Notwithstanding, it is working in a valuable natural resource, full of potential and new applications, in the current context of overpopulation and overexploitation of resources.

VII. References

Abe, M., Kobayashi, M., Fujiyoshi, E., Tamaki, M., Kikuchi, N., & Murase, N. (2013). Use of PCR-RFLP for the discrimination of Japanese *Porphyra* and *Pyropia* species (Bangiales, Rhodophyta). *Journal of Applied Phycology*, *25*(1), 225–232. doi: 10.1007/s10811-012-9856-7.

Agardh, C. A. (1824). *Systema algarum* (1st edition). Lundu: Gleerups.

Agardh, J. G. (1899). *Analecta algologica* (1st edition). Lundu: Acta Universal.

ALGAplus. (2021). *ALGAplus, Ltd.* https://www.algaplus.pt/ (accessed January 20, 2021).

Baweja, P., Kumar, S., Sahoo, D., & Levine, I. (2016). Biology of seaweeds. *Seaweed in Health and Disease Prevention*. Elsevier. doi: 10.1016/B978-0-12-802772-1.00003-8.

Bera, K. K., Karmakar, S., Sciences, F., Das, S. K., Purkait, S., & Sciences, F. (2018). *Biosecurity in aquaculture : an overview in shrimp feed production in India.*

Biris-Dorhoi, E. S., Michiu, D., Pop, C. R., Rotar, A. M., Tofana, M., Pop, O. L., Socaci, S. A., & Farcas, A. C. (2020). Macroalgae - a sustainable source of chemical compounds with biological activities. *Nutrients*, *12*(10), 1–23. doi: 10.3390/nu12103085.

Blouin, N. A., Brodie, J. A., Grossman, A. C., Xu, P., & Brawley, S. H. (2011). *Porphyra*: a marine crop shaped by stress. *Trends in Plant Science*, *16*(1), 29–37. doi: 10.1016/j.tplants.2010.10.004.

Blouin, N., L., & Brawley S.H. (2006). Sensory and fatty acid analyses of two Atlantic species of *Porphyra* (Rhodophyta). *Applied Phycology*, *18*, 79–85.

Blouin, N., Xiugeng, F., Peng, J., Yarish, C., & Brawley, S. H. (2007). Seeding nets with neutral spores of the red alga *Porphyra umbilicalis (L.) Kützing* for use in integrated multi-trophic aquaculture (IMTA). *Aquaculture*, *270*(1–4), 77–91. doi: 10.1016/j.aquaculture.2007.03.002.

Brodie, J., Irvine, L., Neefus, C. D., & Russell, S. (2008). *Ulva umbilicalis L.* and *Porphyra umbilicalis Kütz.* (Rhodophyta, *Bangiaceae*): a molecular and morphological redescription of the species, with a typification update. *Taxon*, *57*(4), 1328–1331. doi: 10.1002/tax.574022.

Carmona, R., Kraemer, G. P., & Yarish, C. (2006). Exploring Northeast American and Asian species of *Porphyra* for use in an integrated finfish-algal aquaculture system. *Aquaculture*, *252*(1), 54–65. doi: 10.1016/j.aquaculture.2005.11.049.

Chatton, É. (1925). *Pansporella perplexa*, amoebiens à spores protégées parasite de

daphnies. *Annales des Sciences Naturelles* (pp. 5–84).

Chopin, T., Robinson, S. M. C., Troell, M., Neori, A., Buschmann, A. H., & Fang, J. (2008). Multitrophic integration for sustainable marine aquaculture. *Encyclopedia of Ecology,* 2463–2475. doi: 10.1016/B978-008045405-4.00065-3.

Chopin, T., & Sawhney, M. (2009). Seaweeds and their mariculture. *Encyclopedia of Ocean Sciences* (2nd edition), 317–326. doi: 10.1016/B978-012374473-9.00757-8.

Chopin, T, Yarish, C., Wilkes, R., Belyea, E., Lu, S., & Mathieson, A. (1999). Developing *Porphyra*/salmon integrated aquaculture for bioremediation and diversification of the aquaculture industry. *Journal of Applied Phycology*, *11*(5), 463–472. doi: 10.1023/A:1008114112852

Cofrades, S., López-Lopez, I., Bravo, L., Ruiz-Capillas, C., Bastida, S., Larrea, M. T., & Jiménez-Colmenero, F. (2010). Nutritional and antioxidant properties of different brown and red Spanish edible seaweeds. *Food Science and Technology International*, *16*(5), 361–370. doi: 10.1177/1082013210367049

da Costa, E., Azevedo, V., Melo, T., Rego, A. M., Evtuguin, D. V., Domingues, R., Calado, R., Pereira, R., Abreu, M. H., & Domingues, M. R. (2018). High-resolution lipidomics of the early life stages of the red seaweed *Porphyra dioica*. *Molecules*, *23*(1). doi: 10.3390/molecules23010187

De Toni, G. B. (1890). Frammenti algologici. *Nuova Notarisia*, *1*, 141–144.

Dhargalkar, V. K., & Kavlekar, D. (2004). Seaweeds – A field manual. *National Institute of Oceanography, Dona Paula, Goa. 403 004*, 36.

Douglas, E. J., Haggitt, T. R., & Rees, T. A. V. (2014). Supply- and demand-driven phosphate uptake and tissue phosphorus in temperate seaweeds. *Aquatic Biology*, *23*(1), 49–60. doi: 10.3354/ab00601

Drew, K M. (1954). Studies in the *Bangioideae*. *Annals of Botany*, *18*(2), 183–216.

Drew, K. M. (1949). Conchocelis-phase in the life-history of *Porphyra umbilicalis (L.) Kütz*. *Nature*, *164*(4174), 748–749. doi: 10.1038/164748a0.

Duby, J. É. (1830). Pyrami de candolle botanicon gallicum sen synopsis plantarum in flora gallica descriptarum. *Ex herbariis et schedis Candollianis propriisque digestum a J. É. Duby V.D.M.* (2nd edition).

Dumilag, R. V., Aguinaldo, Z. A., Mintu, C. B., Quinto, M. P., Ame, E. C., Andres, R. C., Monotilla, W. D., Yap, S. L., Cao, E. P., Vital, P. G., & Fontanilla, I. K. C. (2017). A review of the current taxonomic status of foliose *Bangiales* (Rhodophyta) in the Philippines. *Phytotaxa*, *312*(1), 47–59. doi: 10.11646/phytotaxa.312.1.3.

European Comission. (2020). *The EU blue economy report 2020*.

FAO. (2016). *The State of World Fisheries and Aquaculture 2016*.

FAO. (2018). The global status of seaweed production, trade and utilization. *Globefish Research Programme* (124).

Gallardo, T. (2015). Marine algae: general aspects (biology, systematics, field and laboratory techniques). *Marine Algae: Biodiversity, Taxonomy, Environmental Assessment, and Biotechnology* (1st edition, pp. 1–67). CRC Press.

Gómez-Zorita, S., González-Arceo, M., Trepiana, J., Eseberri, I., Fernández-Quintela, A., Milton-Laskibar, I., Aguirre, L., González, M., & Portillo, M. P. (2020). Anti-obesity effects of macroalgae. *Nutrients*, *12*(8), 1–29. doi: 10.3390/nu12082378.

Guiry, M. D., & Guiry, G. M. (2020). *Algaebase*. http://www.algaebase.org (accessed November 4, 2020).

Gunnarsson, K., Nielsen, R., Egilsdóttir, E., & Brodie, J. (2016). A collections-based approach to the species and their distribution based on the bladed *Bangiales* (Rhodophyta) of Iceland. *Botanica Marina*, *59*, 223–229.

Haeckel, G. (1866). *Generale morphologie der organismen* (Verlag von).

Hawkes, M. W. (1977). A field, culture and cytological study of *Porphyra gardneri* (Smith & Hollenberg) comb. nov., (=*Porphyrella gardneri* Smith & Hollenberg), (Bangiales, Rhodophyta). *Phycologia*, *16*, 456–469.

Hawkes, M. W. (1978). Sexual reproduction in *Porphyra gardneri* (Smith et Hollenberg) Hawkes *(Bangiales,* Rhodophyta*). Phycologia*. *17*, 329–353.

He, P., & Yarish, C. (2006). The developmental regulation of mass cultures of free-living conchocelis for commercial net seeding of *Porphyra leucosticta* from Northeast America. *Aquaculture*, *257*(1–4), 373–381. doi: 10.1016/j.aquaculture.2006.03.017.

Holmes, M. J., & Brodie, J. (2004). Morphology, seasonal phenology and observations on some aspects of the life history in culture of *Porphyra dioica* (*Bangiales*, Rhodophyta) from Devon, UK. *Phycologia*, *43*(2), 176–188. doi: 10.2216/i0031-8884-43-2-176.1.

Kawaguchi, O., Yamamoto, T., & Hashimoto, T. (2005). Carbon, nitrogen and phosphorus contents of cultured nori (*Porphyra yezoensis)*. *Journal of the Graduate School of Biosphere Science*, *42*, 7–9.

Kikuchi, N., Arai, S., Yoshida, G., Shin, J. A., & Miyata, M. (2018). *Neomiuraea* nom. nov., a replacement name for *Miuraea* N. Kikuchi, S.Arai, G.Yoshida, J. A.Shin & Miyata (*Bangiales*, Rhodophyta), nom. illeg. *Notulae Algarum*, *62*, 1.

Kim, J. H., Choi, S. J., & Lee, S. (2019). Effects of temperature and light on photosynthesis and growth of red alga *Pyropia dentata (Bangiales*, Rhodophyta) in a conchocelis phase. *Aquaculture*, *505*, 167–172. doi: 10.1016/j.aquaculture.2019.02.058.

Kim, J. K., Kraemer, G. P., Neefus, C. D., Chung, I. K., & Yarish, C. (2007). Effects of temperature and ammonium on growth, pigment production and nitrogen uptake by four species of *Porphyra* (Bangiales, Rhodophyta) native to the New England coast. *Journal of Applied Phycology*, *19*(5), 431–440. doi: 10.1007/s10811-006-9150-7.

Kim, Jang K., Yarish, C., Hwang, E. K., Park, M., & Kim, Y. (2017). Seaweed aquaculture: cultivation technologies, challenges and its ecosystem services. *Algae*, *32*(1), 1–13. doi: 10.4490/algae.2017.32.3.3.

Kim, J. K., Stekoll, M., & Yarish, C. (2019). Opportunities, challenges and future directions of open-water seaweed aquaculture in the United States. *Phycologia*, *58*(5), 446–461. doi: 10.1080/00318884.2019.1625611.

Korbee, N., Figueroa, F. L., & Aguilera, J. (2005). Effect of light quality on the accumulation of photosynthetic pigments, proteins and mycosporine-like amino acids in the red alga *Porphyra leucosticta* (*Bangiales*, Rhodophyta). *Journal of Photochemistry and Photobiology B: Biology*, *80*(2), 71–78. doi: 10.1016/j.jphotobiol.2005.03.002.

Kornmann, P. (1994). Life histories of monostromatic *Porphyra* species as a basis for taxonomy and classification. *European Journal of Phycology*, *29*(2), 69–71. doi: 10.1080/09670269400650511.

Kurogi, M. (1972). Systematics of *Porphyra* in Japan. *Japanese Society of Phycology*, *Contributions to the Systematics of the Benthic Marine Algae of the North Pacific*, 167–91.

Kützing, F. T. (1843). Phycologia generalis oder anatomie, physiologie und systemkunde der Tange. *F. A. Brockhaus*,143-458.

Lavik, A. Q. (2016). Developing a laboratory cultivation protocol for local species of *Porphyra* spp.

Lee, D., Nishizawa, M., Shimizu, Y., & Saeki, H. (2017). Anti-inflammatory effects of dulse (*Palmaria palmata*) resulting from the simultaneous water-extraction of phycobiliproteins and chlorophyll a. *Food Research International*, *100*, 514–521. doi: 10.1016/j.foodres.2017.06.040.

Lee, R. E. (2008). *Phycology* (4th edition). Cambridge University Press.

Lim, P. E., Yang, L. E., Tan, J., Maggs, C. A., & Brodie, J. (2017). Advancing the taxonomy of economically important red seaweeds (Rhodophyta). *European Journal of Phycology*, *52*(4), 438–451. doi: 10.1080/09670262.2017.1365174.

Lin, R., & Stekoll, M. S. (2011). Phycobilin content of the conchocelis phase of alaskan *Porphyra* (Bangiales, Rhodophyta) species: responses to environmental variables. *Journal of Phycology*, *47*(1), 208–214. doi: 10.1111/j.1529-8817.2010.00933.x.

Linnaeus, C. (1753). *Species plantarum* (Laurentius).

Linnaeus, C. (1824). *Philosophia Botanica*. Kessinger Publishing.

Louren, S. O., Barbarino, E., Nascimento, A., Freitas, J. N. P., & Diniz, G. S. (2007). *Eighteenth International Seaweed Symposium*, *January 2007*. doi: 10.1007/978-1-4020-5670-3.

Lu, S., & Yarish, C. (2011). Interaction of photoperiod and temperature in the development of conchocelis of *Porphyra purpurea* (Rhodophyta: Bangiales). *Journal of Applied Phycology*, *23*(1), 89–96. doi: 10.1007/s10811-010-9541-7.

Lyngbye, H. C. (1819). *Tentamen hydrophytologiae danicae continens omnia*.

Maberly, S. C. (1990). Exogenous sources of inorganic carbon for photosynthesis by marine macroalgae. *Journal of Phycology,* 26, 439–449. doi: 10.1111/j.0022-3646.1990.00439.x.

Machado, M., Machado, S., Pimentel, F. B., Freitas, V., Alves, R. C., & Oliveira, M. B. P. P. (2020). Amino acid profile and protein quality assessment of macroalgae produced in an integrated multi-trophic aquaculture system. *Foods*, *9*(10). doi: 10.3390/foods9101382.

Martinez-Porchas, M., & Martinez-Cordova, L. R. (2012). World aquaculture: environmental impacts and troubleshooting alternatives. *The Scientific World Journal*.

Meynard, A., Zapata, J., Salas, N., Betancourtt, C., Pérez-Lara, G., Castañeda, F., Ramírez, M. E., Bulboa Contador, C., Guillemin, M. L., & Contreras-Porcia, L. (2019). Genetic and morphological differentiation of *Porphyra* and *Pyropia* species (*Bangiales*, Rhodophyta) coexisting in a rocky intertidal in Central Chile. *Journal of Phycology*, *55*(2), 297–313. doi: 10.1111/jpy.12829.

Milstein, D., Medeiros, A. S., Oliveira, E. C., & Oliveira, M. C. (2012). Will a DNA barcoding approach be useful to identify *Porphyra* species (*Bangiales*, Rhodophyta)? *Journal of Applied Phycology*, *24*, 837–845.

Mitman, G. G., & van der Meer, J. P. (1994). Meiosis, blade development, and sex determination in *Porphyra purpurea* (Rhodophyta). *Journal of Phycology*, *30*, 147–159.

Nägeli, C. (1847). Die neuern algensysteme und versuch zur Begründung eines eigenen systems der algen und florideen. *Neue Denkschriften Der Allgemeinen Schweizerischen Gesellschaft Für Die Gesammten*, *9*(2), 1–275.

Nelson, W. A., Brodie, J., & Guiry, M. D. (1999). Terminology used to describe reproduction and life history stages in the genus *Porphyra* (*Bangiales*, Rhodophyta). *Journal of Applied Phycology*, *11*(5), 407–410. doi: 10.1023/A:1008174307352.

Nelson, W. A., & D'Archino, R. (2014). Three new macroalgae from the three kings islands New Zealand including the first southern Pacific Ocean record of the *Furcellariaceae* (Rhodophyta). *Phycologia*, *53*, 602–613. doi: 10.2216/14-60R1.1.

Osório, C., Machado, S., Peixoto, J., Bessada, S., Pimentel, F. B., Alves, R. C., & Oliveira, M. B. P. (2020). Pigments content (chlorophylls, fucoxanthin and phycobiliproteins) of different commercial dried algae. *Separations*, *7*(2), 1–14. doi: 10.3390/separations7020033.

Pereira, L. (2009). *Guia ilustrado das macroalgas: conhecer e reconhecer algumas espécies da flora portuguesa*. doi: 10.14195/978-989-26-0397-1

Pereira, L. (2018). *Therapeutic and nutritional uses of algae*. Taylor & Francis Group.

Pereira, L., & Correia, F. (2015). *Macroalgas marinhas da costa portuguesa : biodiversidade, ecologia e utilizações* (1st edition). Nota de Rodapé Edições.

Pereira, R., Sousa-Pinto, I., & Yarish, C. (2001). Studies on the life cycle of *Porphyra dioica* and *Porphyra umbilicalis* from Portugal. *Journal of Phycology*, *33*(3), 41. doi: 10.1111/j.1529-8817.2001.jpy37303-106.x.

Pereira, R., & Yarish, C. (2008). Mass production of marine macroalgae. *Encyclopedia of Ecology,* 2236–2247. doi: 10.1016/B978-008045405-4.00066-5.

Pereira, R., & Yarish, C. (2010). The role of Porphyra in sustainable culture systems: physiology and applications. doi: 10.1007/978-90-481-8569-6.

Pereira, R. (2004). Studies on the Ecophysiology and Biochemistry of Porphyra dioica Brodie et Irvine in culture.

Pereira, R., & Yarish, C. (2013). S*ustainable food production.* doi: 10.1007/978-1-4614-5797-8.

Pereira, R., Yarish, C., & Critchley, A. T. (2013). Seaweed aquaculture for human foods in land-based and IMTA systems. *Sustainable Food Production*, 1405–1424. doi: 10.1007/978-1-4614-5797-8_189.

Pimentel, F. B., Cermeño, M., Kleekayai, T., Machado, S., Rego, A., Fernandes, E., Alves, R. C., Oliveira, M. B. P. P., & FitzGerald, R. J. (2020). Contribution of in vitro simulated gastrointestinal digestion to the antioxidant activity of *Porphyra dioica* conchocelis. *Algal Research*, *51*(228). doi: 10.1016/j.algal.2020.102085.

Pimentel, F. B., Machado, M., Cermeño, M., Kleekayai, T., Machado, S., Rego, A. M., Abreu, M. H., Alves, R. C., Oliveira, M. B. P. P., & Fitzgerald, R. J. (2021). Enzyme-assisted release of antioxidant peptides from *Porphyra dioica* conchocelis. *Antioxidants*, *10*(2), 1–13. doi: 10.3390/antiox10020249.

Redmond, S., Green, L., Yarish, C., Kim, J., & Neefus, C. (2014a). New England nursery systems. *Connecticut Sea Grant*.

Redmond, S., Green, L., Yarish, C., Kim, J., & Neefus, C. (2014b). *New England Seaweed Culture Handbook. Connecticut Sea Grant.*

Robertson-Andersson, D. V., Wilson, D. T., Bolton, J. J., Anderson, R. J., & Maneveldt, G. W. (2009). Rapid assessment of tissue nitrogen in cultivated *Gracilaria gracilis* (Rhodophyta) and *Ulva lactuca* (Chlorophyta). *African Journal of Aquatic Science*, *34*(2), 169–172. doi: 10.2989/AJAS.2009.34.2.7.894.

Roleda, M. Y., & Hurd, C. L. (2019). Seaweed nutrient physiology: application of concepts to aquaculture and bioremediation. *Phycologia*, *58*(5), 552–562. doi: 10.1080/00318884.2019.1622920.

Royer, C. J. (2017). Advancing development of *Porphyra umbilicalis* as a red algal model system and aquaculture crop. *Dissertation*.

Royer, C. J., Redmond, S., Lai, C. S., & Brawley, S. H. (2019). *Porphyra umbilicalis* in applied and basic research: reproductive phenology, development, seed stock culture, and a field trial for aquaculture. *Journal of Applied Phycology*, *31*(1), 547–560. doi: 10.1007/s10811-018-1538-7.

Rusekwa, S. B., Campbell, I., Msuya, F. E., Buriyo, A. S., & Cottier-Cook, E. J. (2020). Biosecurity policy and legislation of the seaweed aquaculture industry in Tanzania. *Journal of Applied Phycology*, *32*(6), 4411–22. doi: 10.1007/s10811-020-02194-1.

Sahoo, D., & Yarish, C. (2005). Mariculture of seaweeds. *Algal culturing techniques*, 219–230. Elsevier.

Sampath-Wiley, P., Neefus, C. D., & Jahnke, L. S. (2008). Seasonal effects of sun exposure and emersion on intertidal seaweed physiology: Fluctuations in antioxidant contents, photosynthetic pigments and photosynthetic efficiency in the red alga *Porphyra umbilicalis Kützing* (Rhodophyta, *Bangiales*). *Journal of Experimental Marine Biology and Ecology*, *361*(2), 83–91. doi: 10.1016/j.jembe.2008.05.001.

Sánchez, N., Vergés, A., Peteiro, C., Sutherland, J. E., & Brodie, J. (2014). Diversity of bladed *Bangiales* (Rhodophyta) in Western Mediterranean: recognition of the genus themis and descriptions of *T. ballesterosii sp. nov., T. iberica sp. nov*., and *Pyropia parva sp. nov. Journal of Phycology*, *50*(5), 908–929. doi: 10.1111/jpy.12223.

Santiañez, W. J. E., & Wynne, M. J. (2020). Proposal of *Phycocalidia* Santiañez & M.J.Wynne nom. nov. to replace *Calidia* L.-E.Yang & J.Brodie nom. illeg. (*Bangiales*, Rhodophyta). *Notulae Algarum*, *140*, 1–3.

Silva, A., Carpena, M., Garcia-Oliveira, P., Gullón, P., Barroso, M. F., Prieto, M. A., & Simal-Gandara, J. (2020). Macroalgae as a source of valuable antimicrobial compounds: extraction and applications. *Antibiotics*, *9*(10), 1–41. doi:

10.3390/antibiotics9100642.

Smith, G. M., & Hollenberg, G. J. (1943). On some *Rhodophyceae* from the Monterey Peninsula, California. *American Journal of Botany*, *30*, 211–22.

Sutherland, J. E., et al. (2011). A new look at an ancient order: generic revision of the *Bangiales* (Rhodophyta). *Journal of Phycology*, *47*(5), 1131–1151. doi: 10.1111/j.1529-8817.2011.01052.x.

Varela-Álvarez, E., Tobin, P. R., Guihéneuf, F., FitzGerald, R. J., & Stengel, D. B. (2019). Phycobiliproteins, nitrogenous compounds and fatty acid contents in field-collected and cultured gametophytes of Porphyra dioica, a red sea vegetable. *Journal of Applied Phycology*, *31*(6), 3849–3860. doi: 10.1007/s10811-019-01841-6.

Vergés, A., Sánchez, N., Peteiro, C., Polo, L., & Brodie, J. (2013). *Pyropia suborbiculata* (*Bangiales*, Rhodophyta): first records from the Northeastern Atlantic and Mediterranean of this North Pacific species. *Phycologia*, *52*, 121–129.

Waaland, J. R., Dickson, L. G., & Carrier, J. E. (1987). Conchocelis growth and photoperiodic control of conchospore release in *Porphyra Torta* (Rhodophyta). *Journal of Phycology*, *23*(3), 399–406. doi: 10.1111/j.1529-8817.1987.tb02525.x.

Wang, X., He, L., Ma, Y., Huan, L., Wang, Y., Xia, B., & Wang, G. (2020). Economically important red algae resources along the Chinese coast: history, status, and prospects for their utilization. *Algal Research*, *46*(January), 101817. doi: 10.1016/j.algal.2020.101817.

Ward, G. M., Faisan, J. P., Cottier-Cook, E. J., Gachon, C., Hurtado, A. Q., Lim, P. E., Matoju, I., Msuya, F. E., Bass, D., & Brodie, J. (2020). A review of reported seaweed diseases and pests in aquaculture in Asia. *Journal of the World Aquaculture Society*, *51*(4), 815–828. doi: 10.1111/jwas.12649.

Wells, M. L., Potin, P., Craigie, J. S., Raven, J. A., Merchant, S. S., Helliwell, K. E., Smith, A. G., Camire, M. E., & Brawley, S. H. (2017). Algae as nutritional and functional food sources: revisiting our understanding. *Journal of Applied Phycology,* 29 (2) 949–982. Springer Netherlands. doi: 10.1007/s10811-016-0974-5.

Wettstein, R. von D. (1901). *Handbuch der systematischen Botanik.* Franz Deut.

Yang, L. E., Deng, Y. Y., Xu, G. P., Russell, S., Lu, Q. Q., & Brodie, J. (2020). Redefining *Pyropia* (*Bangiales*, Rhodophyta): four new genera, resurrection of *Porphyrella* and description of *Calidia pseudolobata* sp. nov. from China. *Journal of Phycology*, *56*(4), 862–879. doi: 10.1111/jpy.12992.

Zhang, W., Gao, J. T., Zhang, Y. C., & Qin, S. (2006). Optimization of conditions for cell cultivation of *Porphyra haitanensis* conchocelis in a bubble-column bioreactor. *World Journal of Microbiology and Biotechnology*, *22*(7), 655–660. doi:

10.1007/s11274-005-9050-z.

Zhong, Z. hai, Wang, W. jun, Sun, X. tao, Liu, F. li, Liang, Z. rui, Wang, F. jiu, & Chen, W. zhou. (2016). Developmental and physiological properties of *Pyropia dentata* (*Bangiales*, Rhodophyta) conchocelis in culture. *Journal of Applied Phycology*, *28*(6), 3435–3445. doi: 10.1007/s10811-016-0877-5.

Zuccarello, J. (2011). What are you eating? It may be nori, but it is probably not *Porphyra* anymore! *Journal of Phycology*, *47*(5), 967–968. doi: 10.1111/j.1529-8817.2011.01064.x.

Annex A

	Weighing day
Start (T_0)	PBRs cleaning and disinfection Weighing of starter cultures and treatment x** Sampling and Macro and micro observations Freezing samples (50g*3) Abiotic factors measuring Settling the cultures in 40L Nutritional supplementation
T_{1week}	Weighing estimative in 1L sampling Macro and micro observations Abiotic factors measuring Nutritional supplementation
$T_{2weeks*}$	Weighing estimative in 1L sampling Abiotic factors measuring Macro and microbservations Rising the water volume to 80L Nutritional supplementation
T_{3weeks}	Weighing estimative in 1L sampling Macro and microbservations Abiotic factors measuring Nutritional supplementation
End ($T_{4\ weeks}$)	Emptying of the PBRs Abiotic factors measuring Weighing total biomass Macro and micro observations Freezing samples (50g per PBR)

Annex B

	Tasks	Start (T_0)	$T_{week\ 1}$	$T_{week\ 2}$*	$T_{week\ 3}$	End ($T_{week\ 4}$)
Weighing day	Tank cleaning and disinfection	✓	✓	✓	✓	✓
	Weighing of starter cultures	✓				
	Weighing biomass		✓	✓	✓	✓
	Treatment x**	✓				
	Sampling	✓		✓		✓
	Macro and microscopic observations	✓	✓	✓		✓
	Freezing samples (50g*3)	✓		✓		✓
	Abiotic factors measuring	✓	✓	✓		✓
	Restock		✓		✓	
	Restock with stocking density adjustment			✓		
	Settling the cultures	✓				
	Nutritional supplementation	✓	✓	✓	✓	
1st + 2nd Partial medium changes	Partial medium changing	✓	✓	✓	✓	✓
	Nutritional supplementation	✓	✓	✓	✓	✓
	Abiotic factors measuring	✓	✓	✓	✓	✓