Optimization of seedling production using vegetative gametophytes of *Alaria esculenta*

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Abstract

Alaria esculenta is a brown seaweed with a great potential for biomass production due to its high productivity and high content of carbohydrates, proteins, vitamins, minerals and bioactive compounds like phlorotannins and pigments. *Alaria* cultivation is performed either by collection and settlement of zoospores on ropes to develop seedlings, or by production of the seedlings from vegetative gametophyte cultures. Contrary to the zoospore technique, the use of gametophyte cultures has the potential to provide a constant, year-round supply of seedlings. There is a need for optimizing the production methods such as the maintenance of the gametophyte cultures, gametogenesis, seeding process and deployment time.

The purpose of this dissertation is to optimize several parameters involved in *Alaria* cultivation using vegetative gametophytes and provide qualitative and quantitative information related to kelp phlorotannins.

Optical density and *in vivo* fluorescence were evaluated as an alternative method to estimate gametophyte biomass. Both methods showed linearity with the dry weight. The cultures were also supplemented with a possible growth enhancer and the effect evaluated on the cultures, however no growth improvements were noticed on these cultures.

When the seedlings production is initiated, the fertility of vegetative gametophytes needs to be switched on. The fertility induction was evaluated with three different photoperiod regimes under white light, where the best performance was accomplished by 23 hours light over 8 days. The seeding density is the next step to optimize seaweed industry. Higher and lower densities bring several disadvantages to the development of the sporophytes. Thus, four densities of fertile gametophyte cultures were tested, where a density per dry weight of 0.8 mg/mL produced the most acceptable number of sporophytes on the twines.

For the estimation of sporophyte growth, manual measurements, such as sporophytes counting and length measurement, are extremely time-consuming methods. An alternative method based on image analysis was tested to estimate the percentage of growth of the seedlings and compared with the manual method. The image analysis method was shown to have a good relationship with the sporophyte measurements, bringing a faster and easier way to estimate the seedlings growth.

A. esculenta and *S. latissima* could represent a viable source of phlorotannins (PHL) due to the fast and efficient grow of these species. The juvenile stages of these species had an average of 4.11 and 3.08 mg PHL/g algae, respectively. It was also observed that the phlorotannin content in the *Alaria* gametophytes increased during fertilization. Two forms of phloroglucinol were documented in both species but due to the lack of studies related with the phlorotannin characterization of these species no more compounds were identified.

Resumo

Alaria esculenta é uma alga castanha com um grande potencial para a produção de biomassa devido à sua alta produtividade e aos elevados conteúdos de hidratos de carbono, proteinas, minerais e compostos activos como florotaninos e pigmentos. O cultivo desta espécie é realizado pela apanha de esporófitos maduros e pela fixação de zoosporos em cordas para produzir algas juvenis ou pela produção de algas juvenis a partir de culturas de gametófitos vegetativos. Contrariamente à primeira técnica, o uso de culturas de gametófitos têm o potencial de iniciar esta produção durante todo o ano. Para utilizar este método é ainda necessário otimizar todos os processos envolvidos nesta produção, desde a manutenção das culturas de gametófitos à determinação do período de plantação dos esporófitos no mar.

O presente trabalho procura otimizar diferentes parâmetros envolvidos no cultivo de *A. esculenta* utilizando gametófitos vegetativos. Procura também fornecer informações qualitativas e quantitativas dos florotaninos presentes nestas espécies.

A densidade óptica e a fluorescência *in vivo* foram avaliadas como um método alternativo para estimar a biomassa de gametófitos. Estes dois métodos apresentaram linearidade com o peso seco. As culturas foram também suplementadas com um possível indutor de crescimento e o seu efeito foi avaliado, não sendo observadas melhorias no seu crescimento.

Para a produção de algas juvenis a partir de gametófitos a crescer de forma vegetativa, a fertilidade destes gametófitos têm de ser induzida. Esta indução foi avaliada usando três regimes de fotoperíodo, onde o melhor desempenho foi obtido nas culturas de gametófitos expostas a 23 horas de luz e uma de obscuridade durante 8 dias.

A densidade da cultura que inicia a sementeira é outro passo a ser optimizado. Elevadas e reduzidas densidades trazem desvantagens ao desenvolvimento dos esporófitos. Desta forma, quatro densidades de culturas de gametófitos foram testadas, onde uma densidade de 0.8 mg/mL por peso seco obteve os melhores resultados.

Para avaliar o desenvolvimento dos esporófitos juvenis, a contagem e a medição do comprimento de esporófitos são métodos extremamente morosos. Um método baseado na análise de imagem foi testado para estimar a percentagem de crescimento dos juvenis. Este método mostrou uma boa relação com as medições manuais dos esporófitos, permitindo estimar rapidamente o crescimento destes.

A. esculenta e *S. latissima* podem representar uma fonte viável de florotaninos (PHL) devido ao rápido e eficiente crescimento destas espécies. Estas algas apresentaram, respectivamente, um conteúdo médio de florotaninos de 4.11 e 3.08 mg PHL/g alga. Os gametófitos de *A. esculenta* apresentaram um aumento dos teores de florotaninos após a indução da fertilidade. Apenas dois tipos de floroglucinol foram identificados nestas espécies, porque devido à falta de estudos relacionados com a caracterização destes compostos, não foi possível identificar outros florotaninos.

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List of abbreviations

AcUW	Acetone:Ultrawater
CLP	Counting and length product
DHPG	Dihydrophloroglucinol
DW	Dry weight
FC	Folin Ciocalteu
GeO2	Germanium dioxide
ivF	*in vivo* fluorescence
LD	long-day
mbar	Millibar
MS	Mass spectrometry
OD	Optical density
PC	Percentage covered
PES	Provisoli Enriched Seawater
PG	Phloroglucinol
PHL	Phlorotannins
rpm	rotations per minute
SD	short-day
SR	Sedgewick Rafter
SW	Seawater
TOF	Time of Flight
UV	Ultraviolet

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1. Introduction

The seaweeds are a diverse group of marine algae. They are critical primary producers using photosynthesis to convert CO_2 and nutrients into living biomass. These organisms support the remaining marine life through the oxygen production, their contribution to marine food webs, and by providing structure and habitat for other species. Seaweeds are also an important resource for humans. Historically, seaweeds have been used around the world for human consumption, as a fertilizer, as medicine and as animal food additive (Arbona and Molla, 2006; Seth and Shanmugam, 2016).

1.1 Worldwide aquaculture

Seaweeds are one of the largest unexploited global biomass resources. For centuries, macroalgae has been farmed in China, Japan, and Korea, but now this cultivation has expanded across the world. The main species harvested include *Laminaria sp*, *Undaria sp*, *Eucheuma sp*, *Gracilaria sp*, and *Porphyra sp.* As seaweed consumption has increased in the last several decades, seaweed mariculture had filled the gap between wild stock harvest and the present demand. ”Aquatic plants” represent 27.6 percent of global aquaculture production, where seaweeds are by far the dominant group (FAO, 2016). In 2015, aquaculture produced 29.3 million tonnes (live weight equivalent) of “aquatic plants”, with a total estimated value of US$4.84 billion. Countries in East and Southeast Asia dominate the global seaweed production by volume and value. There are innumerous applications involving these organisms (table1).

Table 1- Applications and their principal components

Application	Components	References
Food	Seaweed blade, proteins, amino acids, minerals, vitamin C and A, iodine, lipids, alginate, agar, carrageenan, mannitol, laminarin	(Holdt and Kraan, 2011; Kim and Pangestuti, 2011; Thomas and Kim, 2011; Vijayaraghavan et al., 2012; Balboa et al., 2013; Evans and Critchley, 2014; Silva et al., 2015; Seth and Shanmugam, 2016)
Fertilizer	Minerals and phytohormones	
Biofuel	Alginate, laminarin	
Feed additives	Protein, lipid, dietary fibers, alginate, iodine, fucoidan	
Medicine and Pharmaceutical	Phlorotannins, fucoidan, agar, fucoxanthine, carrageenan, alginate, enzymes, tocoferol, β-carotene, phospholipids,	
IMTA system	Whole seaweed	
Bioremediation	Whole seaweed, capsules from alginate	

Seaweeds are industrially processed to extract thickening substances such as alginate, agar and carrageenan. A growing attention is focused on seaweed nutritional value due to their natural abundance of vitamins, minerals, and plant-based proteins. The use of seaweed as an alternative to salt is also being explored. Procedures are being developed for the industrial

preparation of biofuel from fish waste and seaweeds (Teresa Fernandes and McWhinnie, 2011; Hurd et al., 2014; Skjermo et al., 2014; FAO, 2015, 2016).

Before 2050, the global population will reach 9 billion people and will require millions of tons of new biomass resources. The cultivated seaweed biomass is a new entry into the growing global bioeconomy. The uncontaminated and relatively sheltered coast allied to a strong competence in aquaculture and off-shore constructions create many opportunities for seaweed cultivation and processing in Norway. Whereas *Laminaria hyperborea* and *Ascophyllum nodosum* are the main seaweeds harvested and exploited in Norway today, the kelp species *Saccharina latissima* and *Alaria esculenta* have the biggest potential for industrial scale farming, being already cultivated by several companies. The number of research projects on macroalgae cultivation has also increased due the potential to increase the volumes of renewable biomass for third generation biofuel production and to supply the global market with food, feed ingredients and other products (Meland and Rebours, 2012; Skjermo et al., 2014).

1.2 Experimental organism

Alaria esculenta (Linnaeus) Greville is an abundant brown algal species of the order Laminariales populating sublittoral zones of Arctic and cold temperate coastal ecosystems (Fig.1). Low tide level mainly determines its upper vertical distribution limit. Also called winged kelp, this seaweed is among the highest biomass producers and grows naturally down to at least 8 meters at moderately to highly exposed areas (Bischof et al., 1999).

Figure 1. *A. esculenta* distribution in European coastal waters (from http://www.ukmarinesac.org.uk/communities/infralittoral/ ik1_2_2.htm)

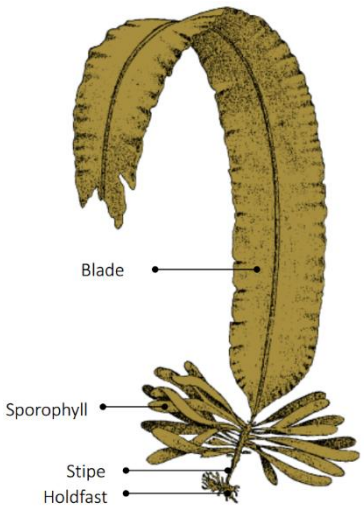

Figure 2. *Alaria esculenta* morphology

A. esculenta is composed by a long non-digitate monostromatic blade, a stipe arranged with sporophylls and a root-like organ called holdfast (Fig.2). The stipe is short, continuing as a prominent midrib in the blade, which is smooth with deep ridges. These ridges extend from the periphery towards the central midrib on the adult plant. The plant attaches to the substratum with the narrow but resistant holdfast. The reproductive cells are produced in small blades with few centimeters up from the holdfast called sporophylls (Druehl, 1988; Arbona and Molla, 2006).

1.3 Life history

The life history of a species is a continuous interaction between the organism and its biotic and abiotic environments. Kelp species, including *Alaria esculenta*, have a heteromorphic diplohaplontic reproductive cycle (Fig.3). After maturation, the adult sporophyte releases zoospores (sporulation), which develop into male or female microscopic gametophytes. The sexual fertilization of the gametes results in a zygote, which develops into a sporophyte.

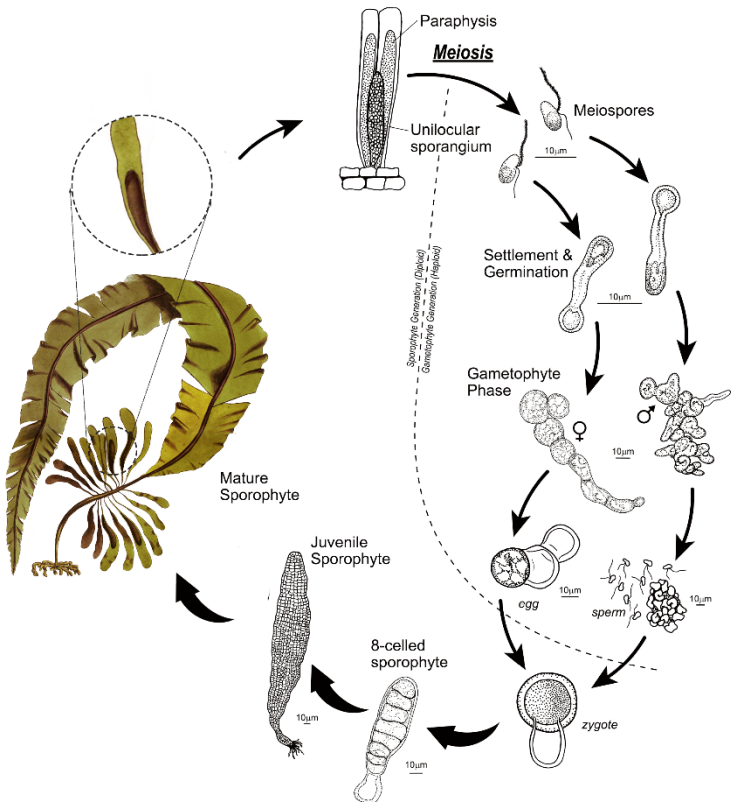

Figure 3. The life cycle of *Alaria esculenta* (Adapted from Redmond et al. (2014)).

The sporulation season lasts for about 2-3 months with a peak in October-November. Zoospores or meiospores are produced in a unilocular sporangium called sorus, which is present in the mature sporophylls (Kain, 1979). These biflagellate zoospores become haploid through meiosis when released. Before settling in a suitable substratum, zoospores are able

to move actively for more than 48 h under experimental conditions. The flagella are then resorbed and germination occurs immediately. The initial germling stage involves the elongation of the spore and after 11-13 days, male and female individuals can be distinguished.

The development of the gametophytes involves three stages: embryospore germination phase, vegetative growth phase and reproductive phase. The first stage leads to the primary cell of the gametophyte. The second is characterized by the primary cell increase in the female gametophyte or by the production of a few cells in the male gametophyte. The last stage only occurs if there are favorable conditions for the gametophyte fertilization, otherwise they may grow vegetatively, forming filamentous structure (Cuijuan et al., 2005). This type of fertilization involving a mobile and a non-mobile gamete is termed oogamy. Female gametophytes can be distinguished from males by having less branching and being thicker. Males develop antheridia where spermatozoids are produced and females develop oocysts or oogonia producing oospheres. After the oosphere is produced, female gametophytes secrete pheromones such as lamoxirene that induce the spermatozoids release and attract towards the oosphere, where the fusion occurs to form a zygote (Maier et al., 2001). The sexual maturation of gametophytes occurs within 15-20 days after sporulation, but large variations can be observed from 8 days to 70 days. Finally, the zygote germinates forming diploid plantlets, termed seedlings. Unfertilized egg cells may develop into haploid parthenosporophytes. (Kraan and Guiry, 2000; Cuijuan et al., 2005; Arbona and Molla, 2006; Lüning, 2008; Steinhoff et al., 2011)

1.4 Biomass composition

Seaweeds are known for their richness in carbohydrates, minerals and certain vitamins, but they also contain bioactive substances like polysaccharides, proteins, lipids and polyphenols. Antibacterial, antiviral and antifungal properties are just a few applications used by macroalgae. These give seaweed great potential as a supplement in functional food or for the extraction of compounds. However, these organisms are characterized by a highly variable composition, depending on species, collection time and habitat. These seasonal and environmental variations in the composition of seaweed make generalizations impossible (Holdt and Kraan, 2011; Schiener et al., 2015).

The moisture content of fresh marine algae is very high and can account for up to 94% of the biomass. The ash content of *Alaria sp* blades is lowest during September, October and November and highest during February to June. The dry weight is lowest from January to March and highest from July to September for these species (Holdt and Kraan, 2011).

Marine algae contain large amounts of polysaccharides. The cell wall and storage polysaccharides most common in brown algae are the alginic acid, mannitol, fucoidan and laminarin (β-1,3 glucan). The storage carbohydrates mannitol and laminarin in Laminariales tend to accumulate during summer and autumn and then utilized during winter as an energy source for new tissue growth (Holdt and Kraan, 2011; Schiener et al., 2015).

The structure and biological properties of seaweed proteins are still poorly documented. The protein fraction of seaweed varies with the species, but is generally small in brown seaweed. Most seaweed species contain all the essential amino acids and are a rich source of the acidic amino acids, aspartic acid and glutamic acid. Contrary to carbohydrate profiles, protein contents were found to be highest from February to May, where it has been suggested that this build-up of nitrogen reserves is to sustain the rapid growth rates during the summer (Holdt and Kraan, 2011; Schiener et al., 2015).

Lipids represent up to 4.5% of the seaweed on a dry weight basis, and this content is lower than other marine organisms. The lack of studies of the bioavailability of algal lipids currently limits their nutritional evaluation. Like other biochemical components, the fatty acid content varies with the season and other environmental factors. The maximum content of lipids in the fronds of *A. esculenta* was generally found in winter (Holdt and Kraan, 2011).

Seaweed pigmentation is composed by chlorophylls, carotenoids and other pigments. Chlorophylls are green lipid-soluble pigments which carry out photosynthesis. Chlorophyll a is essential in the reaction center of the thylakoid, light-harvesting structures in which photosynthesis is carried out. The carotenoids such as β-carotene, violaxanthin and fucoxanthin are photosynthetic pigments that absorb into the blue-green region. The latter is one of the most abundant carotenoids in nature where its content varies during the season and life cycle (Holdt and Kraan, 2011; Hurd et al., 2014).

Marine algae are also known for the high mineral content, being used as feed and food supplements to supply minerals. The brown seaweeds are an excellent source of iodine (Holdt and Kraan, 2011).

1.5 Phlorotannins

Polyphenols have been emerging as one major category of natural product important to human health. Beside polyphenols from terrestrial plants, seaweeds are another source of polyphenols with unique structural properties (Zhang et al., 2006).

Phlorotannin is a group of phenolic compounds widely distributed in brown macroalgae (Fig. 4). It consists of phloroglucinol (1,3,5-trihydroxybenzene) units with different degrees of polymerization and a group of heterogeneous polymeric compounds. Phlorotannins are

subdivided in four subclasses based on their type of linkage, i.e., phlorotannins with phenyl linkage (fucols), with both phenyl and ether linkages (fucophlorethols), with dibenzodioxin linkages (eckols) and with ether linkages (fuhalols and phlorethols). The molecular weight (MW) of these compounds can range from 126 Da to 650kDa.

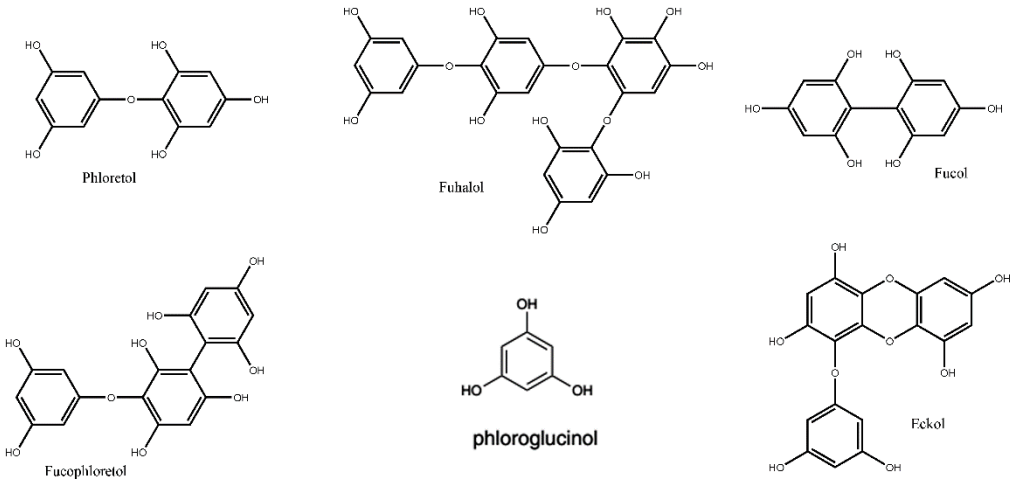

Figure 4. Different classes of phlorotannins from brown seaweeds (Adapted from Thomas and Kim (2011))

Phlorotannins are biosynthesized through the acetate-malonate pathway in the Golgi apparatus, in the perinuclear area of the cell. Soluble phlorotannins are stored in physodes which are membrane-bound cytoplasmic vesicles. These organelles fuse with the cell membrane and the phlorotannins are secreted into the cell wall, forming complexes with different components of the cell. Phlorotannins are essential to the physiological integrity of the seaweeds. These compounds act as a defense mechanism against herbivores and other organisms by releasing an insoluble agar matrix containing phlorotannins into the surrounding water directly via exudation. Phlorotannins have an allelopathic activity against epibionts and they are responsible for the absorption of ultraviolet radiation. Phlorotannins can be found in brown seaweeds, depending on the species, age and tissue type, with concentrations between 0.5 to 20% of dry weight. These concentrations show a phenotypic plasticity in response to environmental parameters such seasonal variations, nutrient availability, intensity of herbivory, light intensity exposure, water temperature and others (Koivikko et al., 2005; Holdt and Kraan, 2011; Lopes et al., 2012; Steevensz et al., 2012; Agregán et al., 2017; Li et al., 2017).

Recently, phlorotannins showed a wide variety of bioactivities and potential beneficial health effects, including antioxidant properties (Balboa et al., 2013), anti-carcinogenic activity (Yuan and Walsh, 2006) , anti-allergic effects (Shim et al., 2009) , anti-HIV-1 activity (Ahn et al., 2004), acting as radioprotective effect (Yuan and Walsh, 2006), as bactericides (Nagayama et al., 2002) and antidiabetic activity (Okada et al., 2004). Phlorotannins are analyzed as total phenolics, where the total contents of phenolic compounds can be measured by colorimetric assays such as the Folin-Ciocalteu, the Folin–Denis reagent and 2,4- dimethoxybenzaldehyde

(DMBA). These methods are simple to use but they provide little information on the chemical composition of phenolic extracts. Chromatographic techniques have been shown to be a suitable option for this kind of analysis, providing qualitative and quantitative analysis of phenolic extracts (Steevensz et al., 2012).

1.6 From the sea to the lab

1.6.1. Current process

The *Alaria esculenta* cultivation is mainly performed by the collection of wild sporophytes, release and settlement of zoospores into strings (sporelings), and development of the seaweed on the twine (seedlings). Finally, there is a deployment, outgrowing and eventually harvesting of the plants. Sporelings can be made using zoospores released seasonally from mature sporophytes and by vegetative filamentous gametophytes cultures (Xu et al., 2009; Forbord et al., 2012). The cultivation protocol for *A. esculenta* developed by Arbona and Molla (2006) is currently used by both scientists and seaweed farmers, but possibly with some adaptions.

1.6.2. Zoospore *versus* gametophyte method

The technique using zoospores is practiced for a long time and for that reason is well studied, being crucial to the seaweed industry. However, this method is dependent on the availability of mature algae, which is limited by seasonal and local variations. Furthermore, the process involving the collection and transporting of the mature sporophytes and the release of the zoospores from the sporophylls could be quite time consuming, and the number of zoospores obtained not predictable. Gametophytes can be kept in cultures under laboratory conditions and enable access independent of the season. The technique using gametophytes also has challenges. The gametophytes tend to cluster due to the filamentous structure of these organisms, which could affect the development of the algae by overlapping during growth, where nutrients, light and the proper space are limited. The attachment of the gametophytes to the twine could be also a bottleneck, which depends on the polysaccharides quality released by the gametophytes. However, since the gametophyte method is not dependent of the seasonality, it could provide seedlings through the whole year, increasing the availability of demanded biomass of macroalgae (Xu et al., 2009; Forbord et al., 2012; Hurd et al., 2014).

1.7 Growth conditions for gametophyte cultures

Gametophyte cultures are kept under manipulated conditions, such as light and temperature, to maintain these organisms in a vegetative state. This state allows the cultures to be

maintained for years and there is a constant increase of biomass since the vegetative growth of the gametophytes is favored. The fertilization can be triggered at any moment by modifying the previous conditions for seedling production (Arbona and Molla, 2006).

1.7.1. Light and photoperiod

In their natural environments, seaweeds grow in exceptionally diverse and dynamic light climates. The season, tides, water turbidity and other factors have a marked effect on the quantity and quality of light that reaches seaweeds at their growth sites. Thus, the quality and the intensity of light influences the growth, development and reproduction of the Laminariales species (Lüning and Dring, 1972, 1975; Lüning, 1981; Cuijuan et al., 2005).

Blue light alone or as part of white light is required for the gametogenesis of *Alaria esculenta*. In red light, gametophytes grow only vegetatively. The ability to grow vegetatively in extremely dim light and reproduce only when irradiance increases provides a mechanism for populations to retain space after the canopy of parent sporophytes is lost (Hurd et al., 2014). The chlorophylls and other light harvesting pigments have different absorption peaks, and together they absorb across a broad region (400-700nm) of what is called photosynthetic active radiation (PAR). Three kinds of pigments are directly involved in algal photosynthesis: chlorophylls, phycobiliproteins and carotenoids. Chlorophyll a is present in all algae. The chlorophyll c1 and c2 occurs in brown seaweed, which they absorb blue light more strongly and red light less strongly than chlorophyll a. Fucoxanthin, b-carotene and siphonaxanthin are carotenoids that absorb into the blue-green region. The blue light affects specifically the reproductive development of the gametophytes as a photomorphogenetic response (Lüning and Dring, 1972; Hurd et al., 2014). The importance of carotenoids as light harvesting pigments is much more pronounced in macroalgae than in terrestrial plants because the spectral composition underwater is particular rich in blue and green light. Therefore, species from sublittoral zones such as *A. esculenta* are exposed to these conditions and it's expected to have a better performance under blue light. Indeed, light acts as an important ambient factor to regulate plant growth and it also has a strong effect on algae in morphological and reproductive development (Hurd et al., 2014).

Photoperiodism is the ability of an organism to detect day length, which is a key factor to determine the timing of reproduction and growth activity in macroalgae species. The two-main photoperiodic responses are short-day plants (SD) and long-day plants (LD). Notwithstanding these names, plants actually measure the length of uninterrupted night, not day length. For terrestrial plants, the biochemistry and molecular biology of photoperiodic responses are well understood, which cannot be said about seaweeds. Most macroalgae species that show

photoperiodism are short-day. Higher plants have a family of photoreceptors, the phytochromes, which detect light in the red region of the spectrum and are involved in their systems for measuring and responding to light/dark cycles (Pr/Pfr). The presence of phytochromes has not been confirmed for brown seaweeds. The microstages of the life cycle are not under photoperiod control. The meiospore germination is dependent on light dose, whereas gametogenesis is triggered by a specific dose of blue light, which that means when the sporophyte canopy is removed, the increased blue light triggers gametogenesis (Hurd et al., 2014; Taiz et al., 2015).

The ability of the seaweed to modify its reproductive status in response to an environmental stimulus can be categorized in “anticipators” and “responders”. Season responders sense and respond directly when environmental conditions are favorable. Yet, season anticipators grow and reproduce in a strategic annual rhythm suitable for the species. The growth is not a response to suitable environmental conditions but a response to a trigger (eg. low light). Contrary to a responder organism, an anticipator would show a slower growth rate during the summer, when irradiance is maximal. The organic matter produced in excess is stored and then used for growth during the winter, when the light is low and the nutrients are high. The species from the order Laminariales are mainly season anticipators, since their reproduction and sporophyte development occur during the winter. The biochemical and molecular mechanisms underlying circannual endogenous clocks are barely known for seaweeds (Kain, 1989).

1.7.2. Temperature

The temperature primarily controls the biogeography of seaweed specie. Seawater temperature has been increasing annually due to the global warming, which is directly related to the amount of light reaching the sea. These climate changes have caused measurable effects on kelp near their thermal limit, whereas blades may decay or even drop away from floating twines. The temperature can also affect reproduction through its effects on metabolism rates. The reproduction and sorus induction time, and the enhancement of the reproductive traits are dependent from temperature, being a few examples within the brown seaweed (Zhang et al., 2013; Hurd et al., 2014).

1.7.3. Growth medium

Seaweeds require inorganic carbon, water, light and various mineral ions for photosynthesis and growth. In 1960, the development of defined culture media for growing algae allowed the

testing of a variety of elements to determine which are essential and required for growth. All the major constituents of seawater, except Sr and F, are required by macroalgae.

The main sources of nitrogen for seaweeds are nitrate, nitrite and ammonium (inorganic nitrogen) and urea (organic nitrogen). Nitrogen has a major metabolic importance in compounds, being assimilated into amino acids and proteins. Phosphorus is mainly available as the inorganic ions PO_4^{3-} and H_2PO_4. Phosphorus plays key roles in nucleic acids, proteins and phospholipids. The most important role is in energy transfer through ATP and other high-energy compounds in photosynthesis and respiration. Nitrogen is the most frequently limiting nutrient followed by phosphorus. Iron is an important trace element for macroalgae growth, involved in the photosynthesis, chlorophyll synthesis, respiration, mitochondria electron transport, nitrogen reduction and gametogenesis induction. Elements such as magnesium, cooper, zinc, selenium, nickel and molybdenum are mainly involved as enzyme cofactors. Iodine is mainly stored as iodide, which scavenges a variety of reactive oxygen species.

The use of natural seawater in macroalgae cultures usually involves the addition of an enrichment solution to produce an enhanced growth of the organisms. Among the many media, one dominates citations when working with seaweeds - the Provasoli's Enriched Seawater medium (PES). Essential macronutrient elements (carbon, nitrogen and phosphorous), ions elements (Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, and SO_4^{2-}) and micronutrient metals (iron, zinc, cobalt, and manganese) are added to these cultures for the reasons mentioned before. Contrary to most of the higher plants, macroalgae don't synthesize their own vitamins and depend on environmental sources. The three vitamins are added to culture media are B12 (cyanocobalamin), thiamine and biotin. Vitamin B12 is the most required by seaweeds because it's present in lower amounts than the remaining in seawater (Harrison and Berges, 2005; Hurd et al., 2014).

1.7.4. Germanium dioxide

The cultivation of the microscopic stage has the potential to be contaminated with diatoms, microalgae and other. The use of the germanium dioxide (GeO_2) interferes with the formation of the diatom's silica frustule inhibiting their growth. Ge blocks Si uptake and blocks the metabolism of Si already taken up, which affects protein and especially chlorophyll synthesis. Silica is not a major or essential element for kelp species, however the effects of GeO_2 on micro- and macroscopic stages of Laminariales is still not entirely clear. Either studies pointed out inhibitory effect of GeO_2 or no serious damaged for given GeO_2 doses in brown macroalgae cultures. Currently, the concentration of GeO_2 usually applied on gametophyte cultures is between 0.1 and 0.5 mL per liter (Lewin, 1966; Markham and Hagmeier, 1982; Merrill and Gillingham, 1991; Shea and Chopin, 2007; Kerrison et al., 2016).

1.7.5. Growth enhancers

Seaweeds have been used in agriculture for decades due to the presence of several plant growth-stimulating compounds in crop production systems. The phytohormones present in higher plants such as auxin, cytokinin gibberellins, abscisic acid and polyamines are also present in brown macroalgae extracts, playing a physiological role in the regulation of growth and development these organisms. Numerous studies have revealed a wide range of beneficial effects of seaweed extract applications on plants such as enhancement of growth and development and establishment, and elevated resistance to biotic and abiotic stress, for example. Seaweed extracts might also produce these effects in macroalgae as in higher plants. Studies where macroalgae cultures were treated with seaweed extracts demonstrated higher growth rates and effects in growth and development comparing with the controls. The growth enhancer Algeafert was used in *S. latissima* gametophytes with positive effects on these cultures (Stirk et al., 2003; Robertson-Andersson et al., 2007; Tarakhovskaya et al., 2007; Khan et al., 2009; Hurtado et al., 2012; Panda et al., 2012; Matsson, 2013).

2. Experimental design

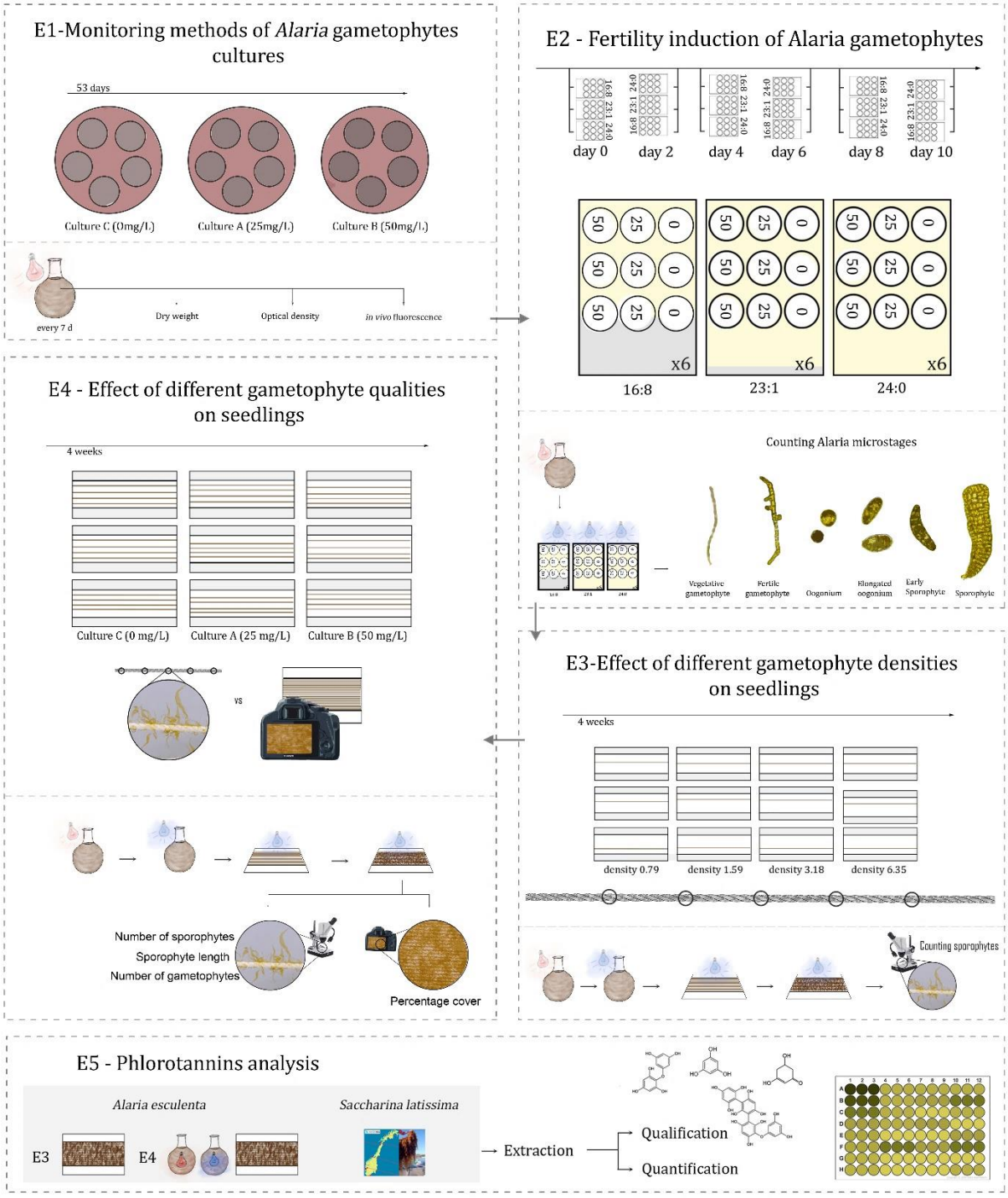

Figure 5. Experiment design of the dissertation.

Experiment 1 (E1) evaluates the effect of a possible growth enhancer on gametophyte cultures of *Alaria esculenta*. Experiment 2 (E2) evaluates the fertility induction of the previous cultures under three different photoperiod regimes. Experiment 3 (E3) intends to optimize the density of gametophyte cultures as a starting point of the seedlings of *A. esculenta*. The control culture from E1 was used and the fertility of the gametophytes was induced based on E2. Experiment 4 (E4) evaluates the seedlings quality initiated by different gametophyte cultures of *A. esculenta*. One culture from each condition was selected from E1. The induction of the gametophytes fertility was performed based on E2 and the density of the cultures was adjusted based on E3. Phlorotannin analysis (E5) was performed using samples from E3 (*Alaria* sporophytes), E4 (*Alaria* vegetative and fertile gametophytes and sporophytes) and Saccharina sporophytes from another experiment group from the MACROSEA project.

3. Monitoring methods of *A. esculenta* gametophytes cultures (E1)

3.1. Scientific questions

The use of gametophyte cultures as source for seedling production is an increasingly common experimental alternative to the zoospore release method, but in the upscaling of the seaweed cultivation there is a need for optimizing of the production methods and different techniques that are used during different steps. The measurements of growth allows to understand the performance of a gametophyte culture. These measurements have been archived by wet weight (Zhang et al., 2008; Liu et al., 2016), dry weight (Ratcliff et al., 2017) and counting in microscope. Wet weight requires large amounts of samples and counting in microscope is very time-consuming. The dry weight method is the most accurate for filamentous cultures, but it takes a considerable time to obtain the biomass value in gametophyte cultures. It is necessary to establish an alternative method, less time-consuming, to follow these gametophyte cultures. *Question 1: Is it feasible to measure the biomass of Alaria gametophyte cultures with optical density or in vivo fluorescence alternatively to the dry weight method?*

The addiction of fertilizers has been used in higher plants for a long time. Not only the plant growth and development are stimulated but also their resistance against biotic and abiotic factors. These growth enhancers might show similar effects in the seaweed production. *Question 2: Could the addition of different concentrations of a possible growth enhancer affect the growth rate and/or the quality of Alaria gametophyte cultures?*

3.2. Materials and methods

Cultures initiation and maintenance: A gametophyte culture of *A. esculenta* was provided by the company Hortimare. This culture was kept in a culture room at 10°C under a 24h photoperiod with a red light at 640 nm and intensity of 30 µmol m^{-2} s^{-1}. The culture was equally distributed into three groups of five flasks. The cultures were renewed each 10-12 day with Provasoli Enriched Seawater (PES) medium supplemented with germanium dioxide (GeO_2). Each group was supplemented with a different concentration of a growth enhancer (AlgeaFert Solid K+) – 0 mg/L (control, culture C), 25mg/L (culture A) and 50mg/L (culture B).

Material and reagents: AlgeaFert Solid K+ (*Ascophyllum nodosum* extract) was purchased from Algea (Kristiansund, Norway). Sodium beta-glycerophosphate pentahydrate and germanium (IV) oxide were purchased from Alfa Aesar. Tris(hydroxymethyl) aminomethane, ammonium iron (II) sulfate hexahydrate and sodium nitrate were purchased from Merck.

Culture monitoring: Every 10-12 days, the gametophytes cultures of *A. esculenta* were measured by dry weight, optical density and *in vivo* fluorescence over 53 days. Calibration curves were established for each method. The dry weight method was performed by weighting pre-dried filters (24h at 80°C), filtrating the sample using a vacuum filtration system, rinsing with distilled water, drying the filters for 24h at 80°C and weighting them. The optical density method was measured in a visible spectrometer (UviLine 9100 from SCHOTT Instruments) using a plastic cuvette at 750nm. The *in vivo* fluorescence method was performed in a fluorescence spectrophotometer (Cary Eclipse from Agilent Technologies) with an excitation wavelength of 436.00nm and an emission wavelength of 685nm. A plastic cuvette was used. The medium of the culture was used as a blank in both methods.

Statistics: All data are expressed as means ± SD. Two-way analysis of variance (ANOVA; GraphPad PRISM 6.01 for windows) was used to test for differences between sample treatments, using the Tukey's multiple comparison test. A level of statistical significance at $p<0.05$ was used. The plots presented were made on the program R v3.3.3 with the package ggplot2.

3.3. Results

An alternative method to dry weight is mandatory. Although this method is the most accurate for filamentous cultures, it takes a long time to obtain the number of biomass existing in gametophyte cultures. Thus, the gametophyte cultures were followed through dry weight (DW), optical density (OD) and *in vivo* fluorescence (ivF). Standard curves were made to calculate the dry weight (mg/mL) from the measurements of optical density and *in vivo* fluorescence (Appendix I: Supplemented figure 1 and 2).

Figure 6 represents the biomass of *A. esculenta* gametophytes, treated with 3 concentrations of a putative growth enhancer, obtained by the three methods over 53 days. For DW, the cultures C and B had similar profiles with an exponential phase ending on day 32. For OD and ivF measurements, these same cultures were still in exponential phase on day 53, contrary to the DW. For all methods, the culture A entered in stationary phase always on day 43. Beside the controversial profiles, the estimated biomass revealed different values at the end of the experiment.

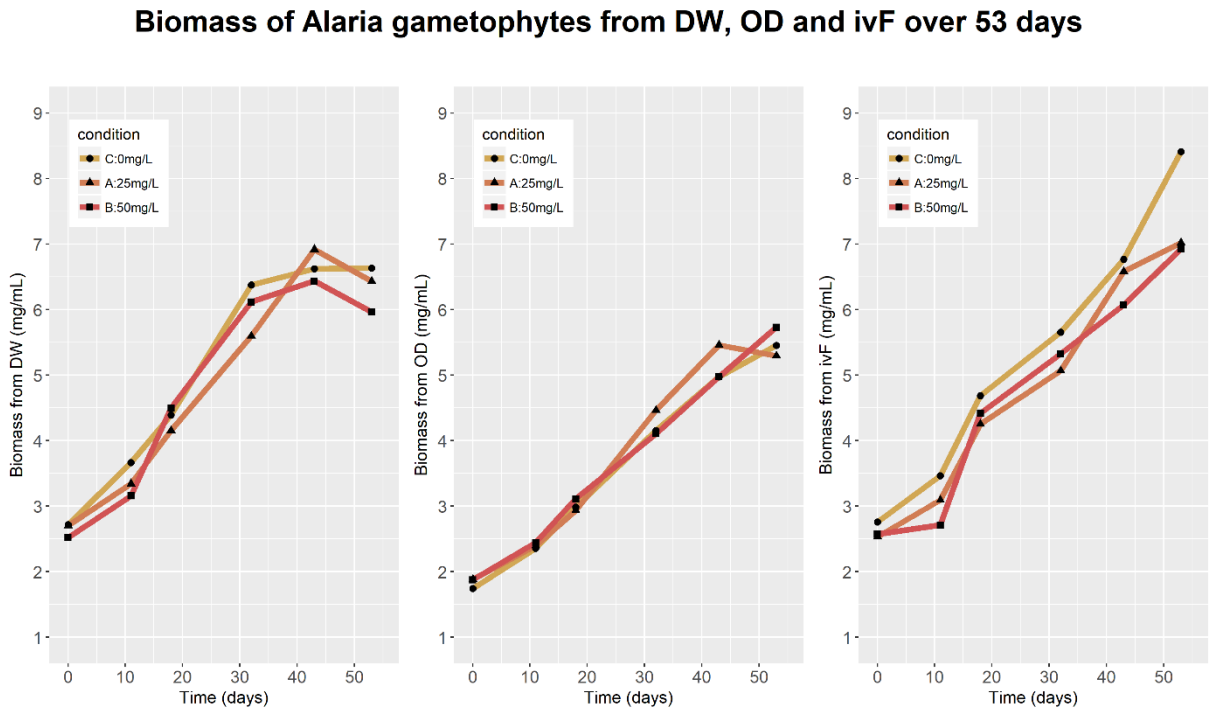

Figure 6. Representation of the biomass of *Alaria* gametophytes obtained by dry weight (left), optical density (center) and *in vivo* fluorescence (right) over 53 days. The yellow line represents the cultures non-treated (C), the orange line represents the culture treated with 25mg/L (A) and the red line the culture treated with 50mg/L (B) of growth enhancer. Values are means (n=5).

With the interest to perceive the differences of growth in each gametophyte culture treatment, two comparisons by two-way ANOVA were performed at the end of the experiment (Fig. 7). The first evaluate the effect of the putative growth enhancer comparing the biomass obtained

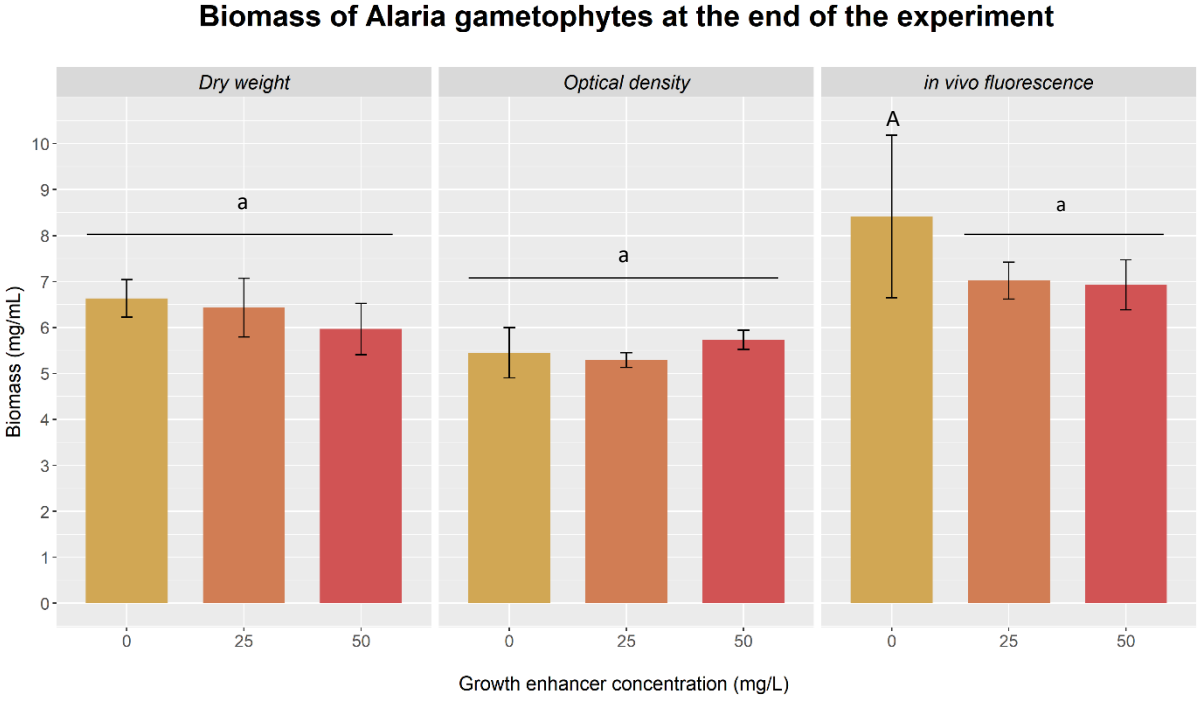

Figure 7. Biomass of *Alaria* gametophyte cultures after 53 days under three different concentrations of growth enhancer measured by dry weight, optical density and *in vivo* fluorescence. Values are means ± SD (n=5). Similar letters denote no statistical significant differences between treatments and measure techniques.

by the different measurement techniques. The cultures C, A and B had no significant differences (p>0.05) on the DW method. The same happened on the OD measurements. The biomass of the culture C, measured by ivF, was significant higher (p≤0.05) than the culture A and B, which no significant differences (p>0.05) were found between them.

The second comparison evaluate the differences of the alternative methods with the dry weight, which was considered as a control method in this statistic analysis. The comparison of the DW with the OD method showed no significant difference (p>0.05) in any treatments. The same comparison was made with ivF method, which showed a significant increase (p≤0.05) of the biomass in cultures C, but no significant difference (p>0.05) was found on the treated cultures obtained by these two methods.

A regression was made between the dry weight and the remaining methods to better understand how the first method varies with the other methods (Fig. 8). During the experiment, the biomass obtained by DW was positively correlated to the biomass from OD (p<0.0001) and to the biomass of ivF (p<0.0001). The linearity between dry weight and optical density was confirmed with 74% of variability of the dependent variable (biomass from OD) being explained by the independent variable (biomass from DW). The linearity between dry weight and *in vivo* fluorescence is also confirmed with 65% of variability of the dependent variable (biomass from ivF) being explained by the independent variable (biomass from DW).

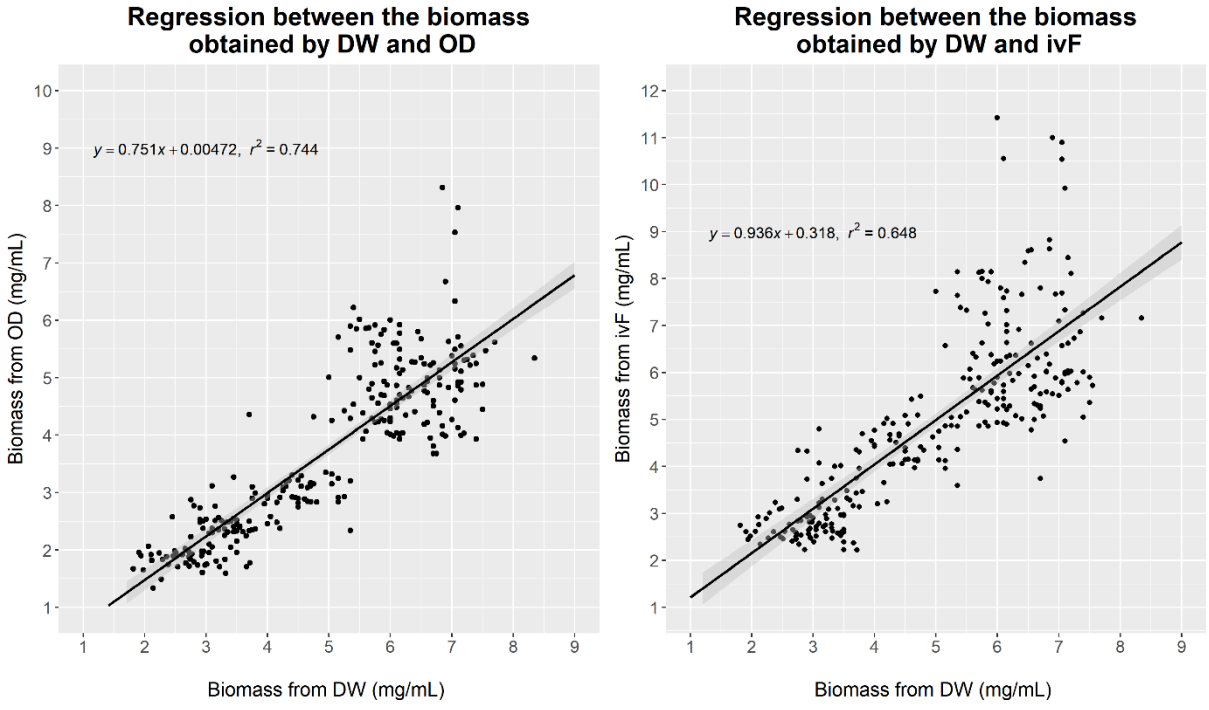

Figure 8. Regression between the biomass (mg/mL) obtained by dry weight and optical density and between the biomass (mg/mL) obtained by dry weight and *in vivo* fluorescence (p<0.0001)

The calculation of the ratio OD/DW and ivF/DW allowed to perceive how is the data behaving over the population, i.e., if the number obtained by the alternative methods is close to the true biomass obtained by the DW method. The figure 9 represents the biomass ratio OD/DW and the biomass ratio ivF/DW over the biomass obtained by the dry weight. The optical density method is underestimating the value of biomass in 25%, independently from the population size. The *in vivo* fluorescence method is under- and overestimating the value of biomass in 7%, which seems be dependent of the population size.

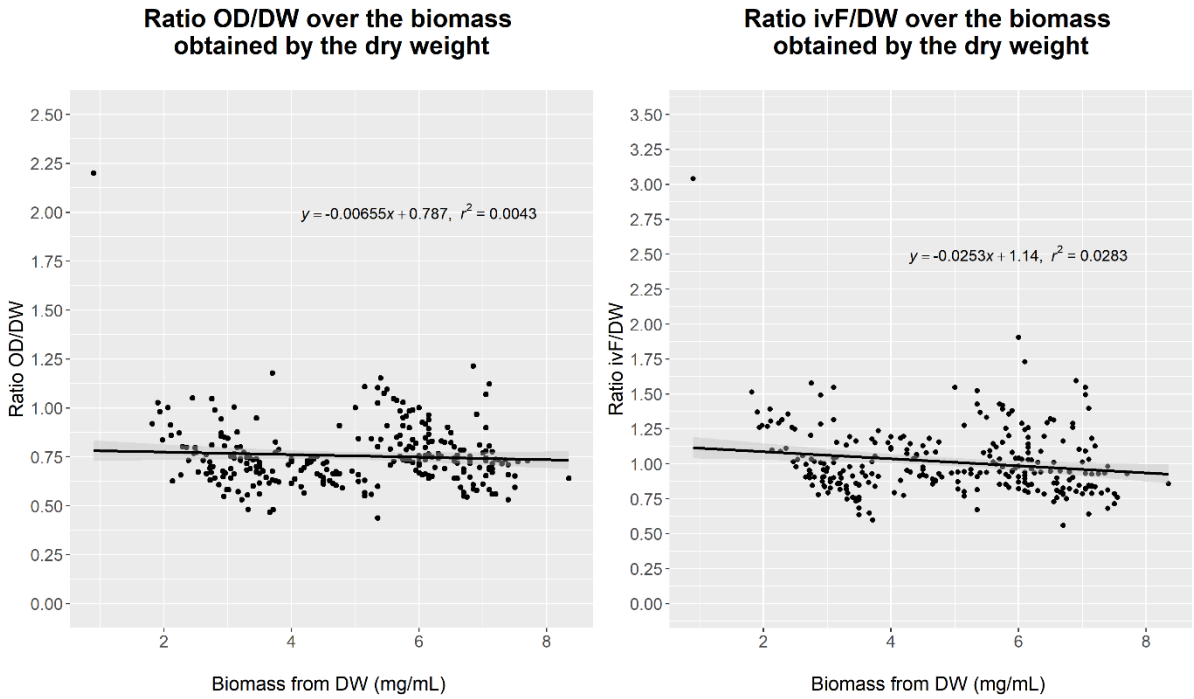

Figure 9 . Representation of the ratio OD/DW and ivF/DW over the biomass obtained by the dry weight. The ratio 1 corresponds to the true value of the biomass, where an underestimation is represented by the line being below 1 and the opposite represents an overestimation.

3.4. Discussion

The seedling production using gametophytes cultures has a great potential to increase seaweed biomass. It's important to find an easy and fast method to monitoring growth of these cultures. Dry weight is an accurate method to estimate biomass when a culture has the tendency to aggregate such as the gametophyte culture of *A. esculenta*. This method is very time consuming, where it's necessary at least two days to know the exact biomass present on the cultures. Therefore, for industrial production, it is mandatory an alternative method to dry weight. Optical density and *in vivo* fluorescence are two methods which need a small volume of sample and the biomass can be estimated quickly. In this experiment, the estimation of the culture biomass by these methods allowed to know how the relationship between optical density and *in vivo* fluorescence differ from the dry weight.

At the end of the experiment, the dry weight and the optical density method had no statistically differences contrarily to the *in vivo* fluorescence. The addiction of the growth enhancer on the treated cultures gave a brownish tone to the media. Therefore, these cultures had a darker medium than the control cultures, increasing with the concentration of growth enhancer. The *in vivo* fluorescence measured the intensity of reflected light by chlorophyll a present in the cultures. The higher the reflected light, the higher is the chlorophyll content. The higher the chlorophyll content, higher is the biomass on the culture. If there was a decrease of chlorophyll content on the cultures treated with the growth enhancer, it means the medium is, somehow affecting the production/reflection of the chlorophyll.

The regression between the three methods was performed to estimate the strength of the relationship (R^2) between variables. There was a positive relationship in DW /OD and DW/ivF, however the relationship of the first was higher than the second. Contrarily to the relationship strength, the slop of the linear equation of DW/OD was lower than DW/ivF. In a perfect model, the slop would be one (y=1x) but, for both regressions, this perfection wasn't achieved. The optical density method is underestimation the biomass for 25%, yet the *in vivo* fluorescence method is under- and overestimation 6,4%. It seems there was a regression between the overestimation with the decrease of population and the underestimation with the increase of the population. Such thing doesn't happen with the optical density method, where the underestimation is constant over the population.

Both methods can measure the biomass of a gametophyte cultures of *A. esculenta*, however there will be always an error associated to these measurements. The choice between these three methods depends on time limitations and investment of the laboratory.

4. Fertility induction of *Alaria* gametophytes (E2)

4.1. Scientific questions

The seedlings production using gametophytes cultures needs the right conditions to maintain these filamentous organisms under vegetative growth (eg. red light). However, when the seedlings production is initiated, the fertility of these vegetative gametophytes needs to be switch on. The fertility induction should be performed under blue or white light and an appropriate photoperiod regime. The nutrients concentration is also considered as a factor that influence the reproduction of the kelp species (Lüning and Dring, 1972). *Question 3: Could the rate of developmental stage of Alaria esculenta be optimized by applying different photoperiod regimes? Question 4: Could also the addition of the growth enhancer benefit the fertility induction of Alaria gametophytes?*

4.2. Materials and methods

Seaweed material: The gametophyte cultures of *Alaria esculenta*, from the previous experiment, were maintained in PES supplemented with GeO_2 in a culture room at 10°C under a 24h photoperiod with a red light intensity of 30 µmol m^{-2} s^{-1}. The medium of the cultures was renewed to remove the GeO_2. From each condition, one flask was chosen randomly to make part of the fertility induction experiment.

Material and reagents: AlgeaFert Solid K+ was purchased from Algea. Sodium beta-glycerophosphate pentahydrate was purchased from Alfa Aesar. Tris(hydroxymethyl) aminomethane, ammonium iron (II) sulfate hexahydrate and sodium nitrate were purchased from Merck.

Fertility induction: The experiment was performed in a climate controlled room at 10°C under a white light with an intensity range of 30-60 µmol m^{-2} s^{-1}. Three photoperiod regimes were selected - 16:8, 24:0 and 23:1 (light:dark) and the duration of this experiment was 10 days. For each photoperiod regime six 6 well-plates were used, where each one of those correspond to a monitoring day: 0, 2nd, 4th, 6th, 8th, 10th day. Inside of these plates three replicates from each condition was added. A total of 18 plates was used in this experiment.

Monitoring: The monitoring was made every 2 days by removing one of the plates from each treatment. Lugol was used to preserve the cells. From each well, 50 organisms were counted with a Sedgewick-Rafter chamber under an inverted microscope (Nikon Eclipse TS100). A counting pattern was used covering whole chamber. All organisms were categorized according their development phase: Vegetative gametophyte, fertile gametophyte, mature

oogonia, elongated oogonia, early sporophyte and sporophyte. In fertile gametophytes, the number of early, mature and elongated oogonia, and early and late sporophytes were counted.

Statistics: All data are expressed as means ± SD. Two-way analysis of variance (ANOVA; GraphPad PRISM 6.01 for windows) was used to test for differences between photoperiod regimes on the culture C (Tukey's multiple comparison test) and on cultures with growth enhancer (Dunnett's multiple comparisons test). A level of statistical significance at $p<0.05$ was used. The plots presented were made on the program R v3.3.3 with the package ggplot2.

4.3. Results

The experiment 2 attempt to reduce the time of fertility induction of *Alaria* gametophytes by testing three different photoperiods regimes over 10 days. Along the counting chamber, the different life stages of *A. esculenta* were counted and categorized. The figure 10 represents the percentage of these microstages under 16:8, 23:1 and 24:0 (light:dark regime) over 10 days on a gametophyte culture C.

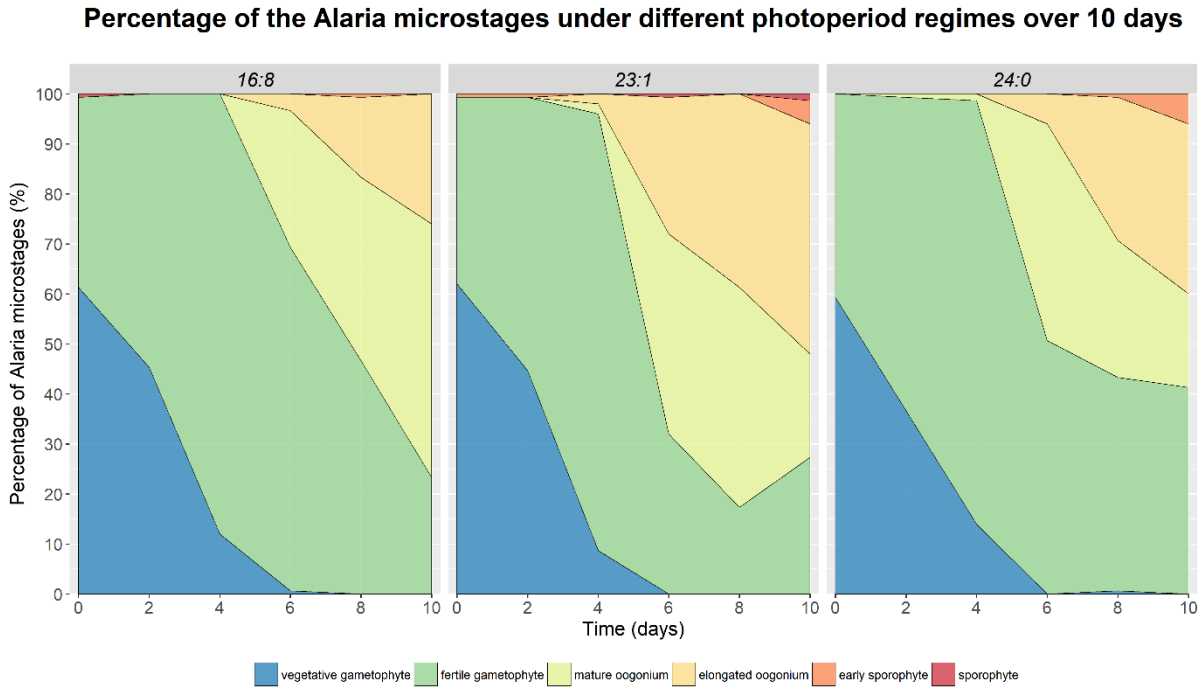

Figure 10. Percentage of the microstages of *Alaria esculenta* under photoperiod 16:8 (left), 23:1 (center) and 24:0 (right) over 10 days. The microstages represented are vegetative gametophytes (blue), fertile gametophytes (green), mature oogonium (yellow), elongated oogonium (light orange), early sporophyte (orange) and sporophyte (red). Values are means (n=3).

After 4 days of white light exposure, the percentage of vegetative gametophyte decreased from ~61% to ~12% and the fertile gametophytes increased from ~39% to ~87% in the cultures from the different photoperiod regimes. In the 6th day, the vegetative gametophytes

percentage was close to 0% in all photoperiod treatments. The fertile gametophytes percentage decreased 20%, 55% and 34% under 16:8, 23:1 and 24:0 regime, respectively. The mature oogonia percentage was 27%, 40% and 43% and the elongated oogonia percentage was 3%, 27%, 6% under 16:8, 23:1 and 24:0 regime, respectively. In the 8th day, the fertile gametophytes percentage was 47%, 17% and 43% under 16:8, 23:1 and 24:0 regime, respectively. The mature oogonia percentage was 37, 44 and 27 % and the elongated oogonia percentage was 16%, 39%, 29% under 16:8, 23:1 and 24:0 regime, respectively. On the last day, the fertile gametophytes percentage was around 30% under 16:8 and 23:1 regime, respectively, and 40% under 24:0 regime. The mature oogonia percentage was 51%, 21%, 19% and the elongated oogonia percentage was 26%, 46%, 34% under 16:8, 23:1 and 24:0 regime. The early stage of sporophyte started to appear with a percentage around 5% only on 23:1 and 24:0 photoperiod regimes.

The developmental rate was calculated for each photoperiod regime. A value was attributed to each microstage. The number 1 to 6 was assign to vegetative gametophyte, fertile gametophyte, mature oogonia, elongated oogonia, early sporophyte and sporophyte, respectively. From all organisms counted, the numbers assigned were summed and divided by the total of organisms counted. The final value represents the developmental rate of the microstages of *Alaria esculenta*.

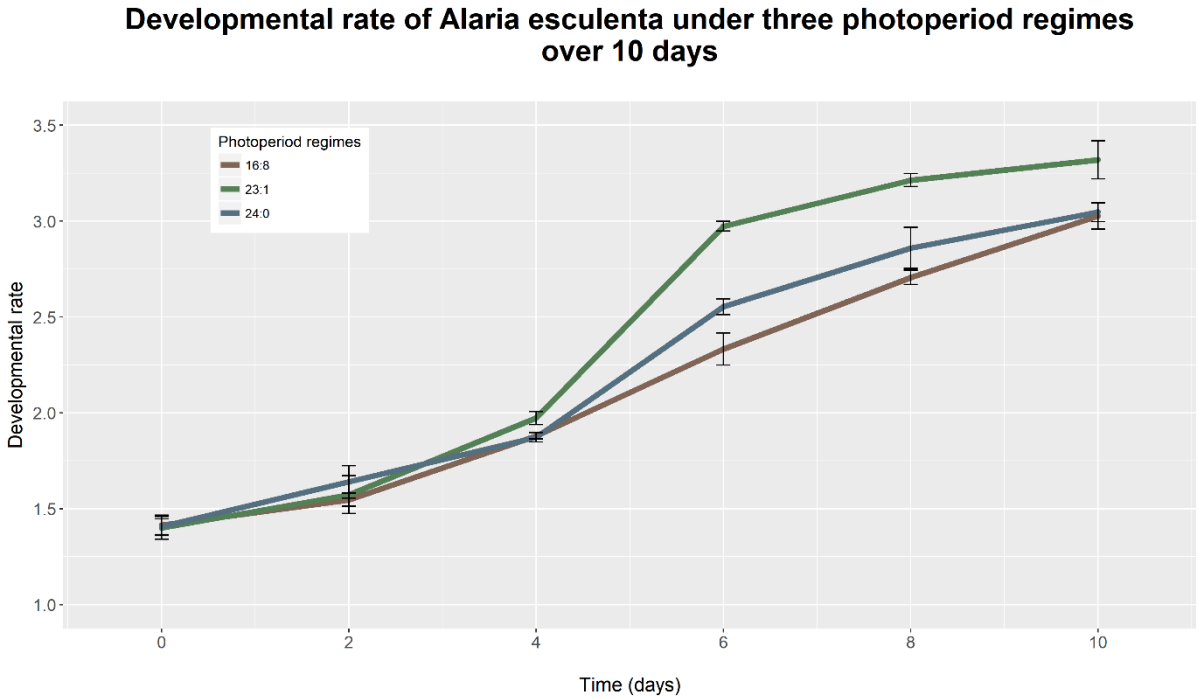

Figure 11. Developmental rate of *A. esculenta* under the photoperiod 16:8 (brown), 23:1 (green) and 24:0 (blue) over 10 days Values are means ± SD (n=3).

The figure 11 represents the developmental rate of *A. esculenta* under different photoperiod regime over 10 days. From day 4 to 6, the photoperiod 23:1 and 24:0 were at exponential phase, however the photoperiod 16:8 was still in exponential phase until day 10.

Figure 12 represents the developmental rate of *A. esculenta* under the three photoperiod regimes on the day 6, 8 and 10. The effect of the photoperiod and the time on the developmental rate was compared statistically (two-way ANOVA).

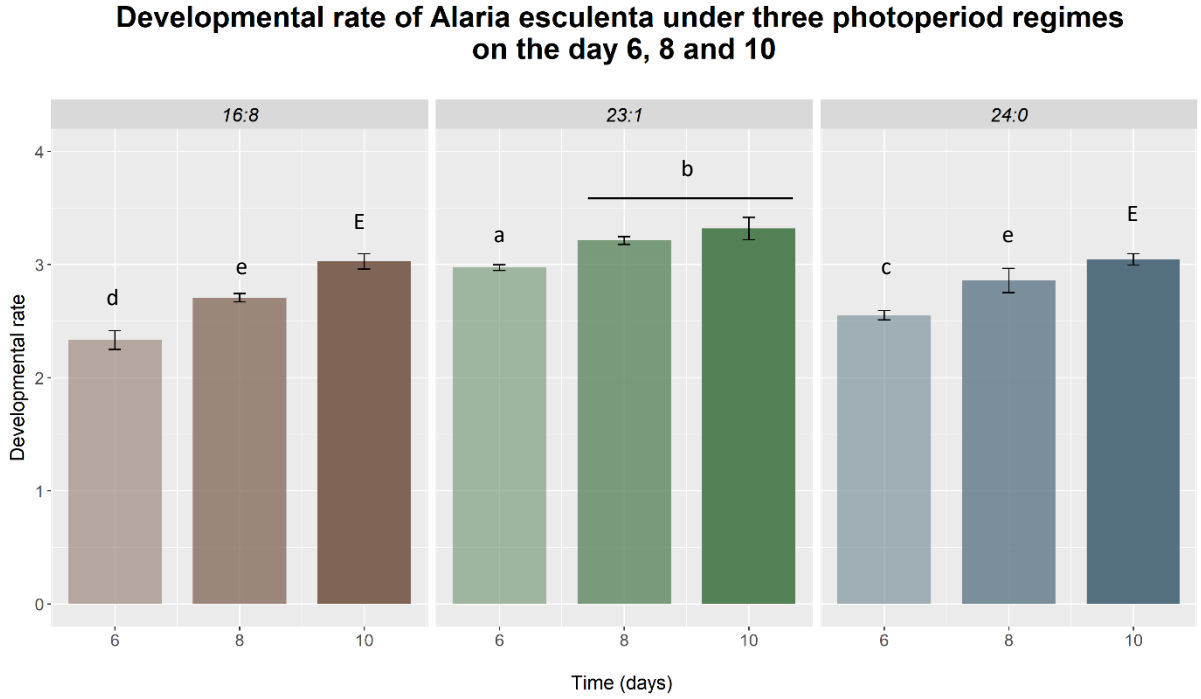

Figure 12. Developmental rate of *A. esculenta* under the three different photoperiod regimes on day 6 (light color), 8 (medium color) and 10 (dark color). Values are means ± SD (n=3). Similar letters denote no statistical significant differences between photoperiod conditions and days.

In the 6th day, the developmental rate of the photoperiod 16:8 was statistically different from the 23:1 ($p \leq 0.0001$) and 24:0 ($p \leq 0.05$). In the days 8 and 10, the developmental rate of the photoperiod 23:1 was significant higher than the photoperiods 16:8 ($p \leq 0.0001$; $p \leq 0.01$) and 24:0 ($p \leq 0.001$; $p \leq 0.01$). By comparing the days within each condition, the photoperiod 16:8 and 24:0 had a significant increase during these 3 days. However, for the photoperiod 23:1 there was no longer a significant difference ($p > 0.05$) after the 8th day.

After the understanding of the control cultures under the previous experimental conditions, the effect of the fertility induction in gametophytes cultures supplemented with 25 and 50mg/L of growth enhancer was evaluated and compared with the control culture.
The figure 13 represents the developmental rate of the *Alaria* gametophytes C, A and B under three photoperiod regimes on the day 6, 8 and 10.

Figure 13. Developmental rate of the microstages of *Alaria esculenta*, treated with different concentrations of a growth enhancer, under three different photoperiod regimes (16:8, 23:1 and 24:0) on the day 6 (light color), day 8 (medium color) and day 10 (dark color). Values are means ± SD (n=3). The symbol (**) denote statistical significant differences against the control.

The data from the treated cultures were compared statically (two-way ANOVA) with the culture C: 0mg/L. Within the photoperiod groups there was no significant difference ($p>0.05$) between the treated cultures and the control culture, excepting for the photoperiod 24:0 on the day 8 where a significant decrease ($p\leq 0.01$) of the developmental rate was found in the culture A.

4.4. Discussion

The fertility induction of vegetative gametophytes from *A. esculenta* is one of the crucial parameters to be optimized, since could take from 8 days to 70 days depending on the culture conditions (Arbona and Molla, 2006). The switch from vegetative growth to reproductive development depends on environmental factors such as light, photoperiod, nutrient concentration and others (Lüning and Dring, 1972). A faster transition to fertility is desirable in the seaweed industry, thus different photoperiod regimes were tested, on this experiment, to evaluate the percentage of *Alaria* microstages over time. During these days, female gametophytes became fertile, where each cell of this filamentous organism had the potential to turn into a sporophyte. These cells developed into an elongated neck, then most of the contents emerged in the tip of the neck forming a spherical egg (mature oogonium). After fertilization, the spherical zygote swelled, elongated (elongated oogonium) and a transverse

division occurred resulting in a single cell row or a cruciate form (early sporophyte) (Kain, 1979).

Between the different photoperiod regimes, it seems the induction of fertility of the culture C had a better response to the photoperiod 23 hours light. On day 6 the percentage of vegetative gametophytes rounded 0%, meaning almost all gametophytes were already fertile. Two days later, the percentage of fertile gametophytes was 20% lower in the 23:1 photoperiod than the remaining conditions. The percentage of mature oogonia and elongated oogonia together was 30% higher in the 23:1 photoperiod than the remaining conditions. These different life stages are indicative the fertility induction is engaging, being expected a decrease of the fertile gametophytes and an increase of oogonia mature, elongated and sporophytes. And so, the fertility induction of the culture C exposed to 23h light had a better performance than the remaining photoperiod regimes. Not only because, these cultures showed a faster developmental rate but also because 8 days were enough to produce a good number of mature and elongated oogonia which could be seeded on the next phase of the *Alaria* cultivation. The explanation to this better performance is difficult to compare with other articles, since no studies have been explored on *A. esculenta* fertility. Even being a short-day seaweed, kelp microstages are not under photoperiod control, where gametogenesis is triggered by a specific dose of blue light (Bartsch et al., 2008). Thus, *Alaria* gametophytes exposed to a long exposure of light with at least one hour of darkness seems to be necessary for an optimized induction of the fertility.

The percentage of the *Alaria* microstages were also evaluated on the cultures A and B (Fig.13), verifying the effect of nutrients on the fertility induction. Few authors mentioned the concentration of nutrients can influence, positively and negatively, the fertility induction of gametophyte cultures (Hsiao and Druehl, 1973; Hoffmann and Santelices, 1982). Ratcliff et al. (2017) tested the gametogenesis of *L. digitata* under 12h light and a better performance was found with a f/2 medium, which is less rich in nutrients than PES. No differences were found between these conditions, except the culture A in one of the days. In this experiment, it seems the growth enhancer, which provides extra nutrients to the culture, didn't affect the fertility induction of the gametophytes cultures.

5. Effect of different gametophyte densities on seedlings (E3)

5.1. Scientific questions

The seeding density is one of the most important traits to be optimized in the seaweed industry. A too high seeding density affects the development of the seaweed seedlings by nutrient and light competition and is also a waste of material, leading to sporophytes growing on the tank walls. However, a lower density allows the growth of diatoms and other organisms in the free spaces on the twine. *Question 5: What is the optimum seeding density for A. esculenta gametophytes?*

5.2. Materials and methods

Seaweed material: The gametophyte culture of *Alaria esculenta* from the first experiment was used in the seedling experiment. The effect of the culture density was evaluated with a random flask from the culture C (0mg/L).

Material and reagents: Sodium beta-glycerophosphate pentahydrate and germanium (IV) oxide were purchased from Alfa Aesar. Tris(hydroxymethyl) aminomethane, ammonium iron (II) sulfate hexahydrate and sodium nitrate were purchased from Merck.

Fertility induction: The culture was transferred to a climate controlled room at 10°C under a 23:1 photoperiod (light: dark regime) with a white light intensity range of 30-60 µmol m^{-2} s^{-1} over 8 days. Germanium dioxide was used. Samples were taken before and after the fertilization period and organisms were counted in the same way as in E2.

Tank treatment: Flat tanks with a water volume of 27 liter, were cleaned with warm water, rubbed with sodium hypochlorite, and filled with water. A period of three days in stagnant water with this compound was applied on tanks. The water flow was turned on for three days, removing the hypochlorite from the water. The water flow at 1.5L min^{-1} and the water level was equally adjusted in each tank.

System design: A plate of polycarbonate glass was used to produce the seedlings. These seedlings had a dimension of 35x40cm. The plats extremities were cut 30cm from the center to the corners. This action confines the twines and avoids them to get loose from the platform. The twine used was made of polyester with a width of 1.35mm, which was spooled around the plates. Each seedling had one “sample” twine not connected to the main twine, allowing to remove it without compromising the seedling.

Seedling initiation: Four different densities were used (0.79, 1.59, 3.18 and 6.35 mg/mL). The seedling was initiated with 200mL. A container was developed to pour the gametophyte culture on the twines, in a distributed manner. After the pouring, spools were placed in air for 20min at 10°C, allowing the gametophytes to settle onto the strings. The spools were immersed and left in stagnant water. Three days later, the waterflow was opened at 1.5L min^{-1}. The seedlings were cultivated at 10°C under a 16:8 photoperiod (light: dark regime) and white light (fluorescent light bulb) with a mean intensity of 44.9 µmol m^{-2} s^{-1} over four weeks.

Monitoring: The sporophyte number was counted on 1,4mm of five different points in the "sample" twine on the fourth week. A device was made to hold this twine, keeping it under water and in the same position. The counting was made under a stereomicroscope (Nikon SMZ1000). All sporophytes were collected five weeks later. Sporophytes were frozen at -20°C for chemical analysis.

Statistics: All data are expressed as means ± SD. Two-way analysis of variance (ANOVA; GraphPad PRISM 6.01 for windows) was used to test for differences between sample treatments, using the Tukey's multiple comparison test. A level of statistical significance at $p<0.05$ was used. The plots presented were made on the program R v3.3.3 with the package ggplot2.

5.3. Results

The optimized parameters from E2 and the density of the seedlings, which will be developed in this experiment, permit to accelerate the hatchery phase of the seaweed industry. And so, the experiment 3 evaluated four different seedings densities of *Alaria* gametophyte cultures. The fertility of a gametophyte culture was induced under photoperiod 23:1 over 8 days and four densities with 0.79, 1.59, 3.18 and 6.35 mg/mL of biomass (DW) were made. The linear model from E1 was used to estimate the biomass of the gametophyte culture.

The figure 14 shows the percentage of *Alaria* microstages under 23:1 regime over 8 days on the gametophyte culture. After 8 days, the vegetative gametophytes percentage decreased from 48% to 28% and the fertile gametophytes percentage increased from 29% to 68%. The percentage of the reproductive structures were around 1%. The developmental rate was calculated the same way as E2. From day 0 until the end of the fertility induction there was an increase of 0.27 of developmental rate (Appendix III: Supplemental figure 3).

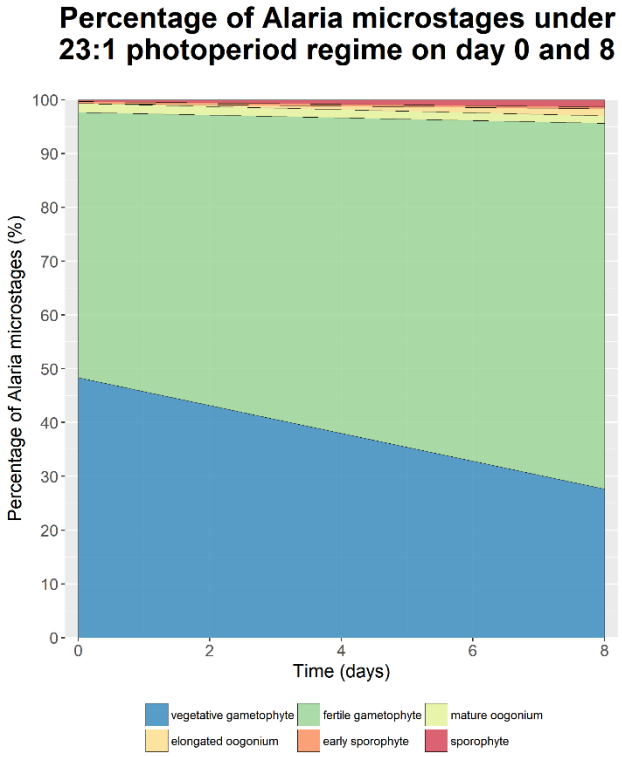

Figure 14. Percentage of the microstages of *Alaria esculenta* under photoperiod 23:1 on day 0 and 8. The microstages represented are vegetative gametophytes (blue), fertile gametophytes (green), mature oogonium (yellow), elongated oogonium (light orange), early sporophyte (orange) and sporophyte (red). Values are means (n=3).

Figure 15 represents the number of sporophytes per millimeter twine, in each seedling, after four weeks of growth. The grouped columns represent the culture densities 0.79, 1.59, 3.18 and 6.35 mg/mL applied on the seedlings, where each column illustrate the replicates within the densities group.

While, the lowest density presented a low variance between replicates with no significant differences (two-way ANOVA; $p>0.05$), the remaining densities had a high variance between replicates with a few significant differences. The average of sporophytes per mm of twine was 7, 15, 21 and 27 from the lowest to the highest density.

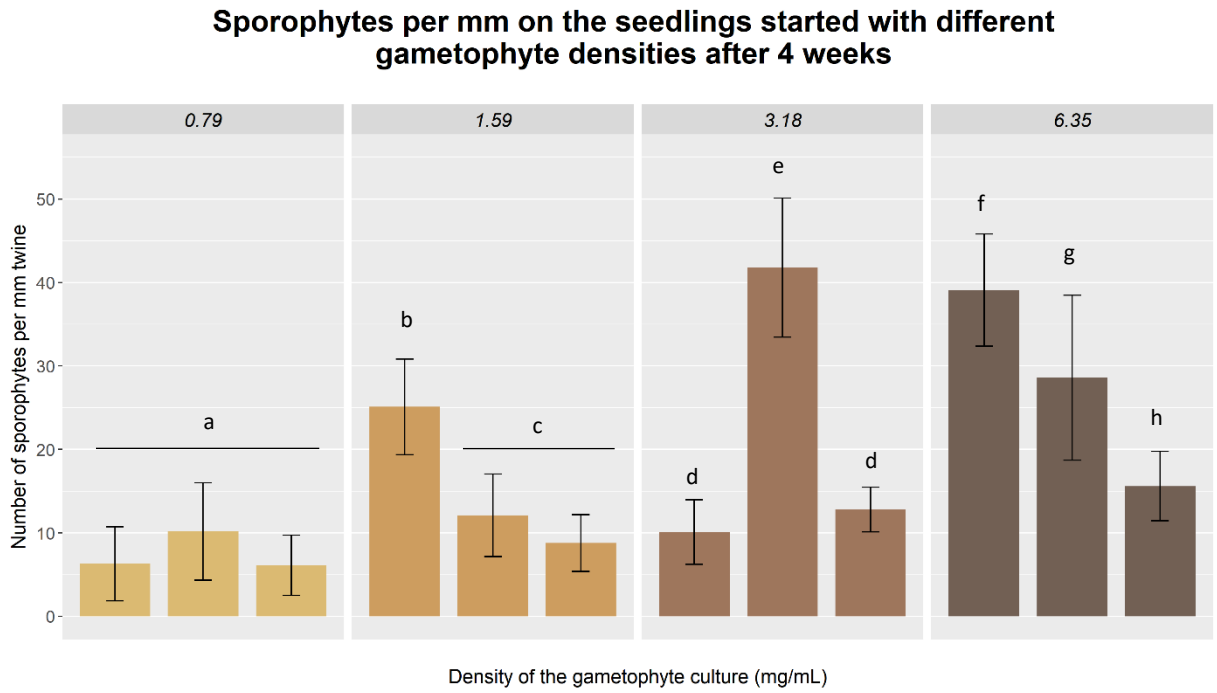

Figure 15. Sporophytes per mm twine on the seedlings started with different gametophyte densities after 4 weeks. The densities applied were 0.79, 1.59, 3.18 and 6,35 mg/mL. Values are means ± SD (n=3). Similar letters denote no statistical significant differences between replicates.

5.4. Discussion

The seeding density is another crucial parameter needed to optimize in a seaweed industry. The importance of optimizing the seeding density is because a higher and a lower density brings limitations to the development of the seedlings. On the one hand, a higher density affects the development of the seaweeds by nutrient competition and produces gametophyte waste, leading to sporophytes growing in the tank walls. On the other hand, the lower density will benefit the grow of diatoms, epiphytes and other organisms on the free spaces of the twine. Thus, a culture C was induced and different densities were applied to evaluate the optimum density of *Alaria* gametophytes.

The fertility induction performed in this experiment was clear that didn't work similarly as in E2. Eight days after transferring the culture C into white light, the number of the two oogonia type was very low and the vegetative and fertile gametophytes should be close to 0% and lower than 30%, respectively. Since all conditions, as the light and temperature, were standardized, the volume and the culture density were the only different parameters used in this experiment. While, the experiment E2 was performed in microplates with 5 mL of a diluted culture, the E3 used 1 liter of non-diluted culture. Having a culture with a higher density and a lower surface area, probably part of the gametophytes didn't receive the amount of light needed to induce their fertility, even under shaking conditions (Lüning and Dring, 1975).

After the gametogenesis, the densities were performed and the cultures were seeded on the twines. The lowest density had a low variance and the remaining densities had a high variance between replicates. The high variance on the seedlings could be explained by the high density present in the gametophyte cultures. Whereas, probably some gametophytes had no space to attach to the twine, growing freely in the tank, and/or some sporophytes had developed above other sporophytes.

The average of sporophytes on the lower density had 7 sporophytes per mm twine and the highest had 27. Since *Alaria*'s holdfast can occupy at least 10mm of twine in the adult phase, the lowest density will in theory develop 70 sporophytes in 10 mm of twine, which of course is impossible in a cultivation situation at sea. Even the lowest density seems to be preeminent in this experiment. A deployment at the sea could be useful to evaluate the survival rate of the plants. Nonetheless, this experiment is a starting point to find the ideal seedling density avoiding gametophyte waste and ensure the viability of the sporophytes after deployment.

6. Effect of different gametophyte qualities on seedlings (E4)

6.1. Scientific questions

The manual measurements of sporophytes on the twine are an accurate method, providing a real and precise number of the growing state on seedlings but these are methods extremely time-consuming. The monitoring of the seedling cultures based on digital images is an automatic method, already been used to follow the biofouling on cage nets in the fishery industry (Braithwaite et al., 2007; Guenther et al., 2010). *Question 6: When gametophyte cultures are seeded on twines for seedlings production, will gametophytes treated with growth enhancer develop differently from non-treated gametophytes? Question 7: Could the image analysis method be used to estimate the growth of A esculenta during the hatchery phase for the seaweed industry?*

6.2. Materials and methods

Seaweed material: The gametophyte cultures of *Alaria esculenta* from the first experiment were used in the seedling experiment. The effect of the quality of gametophyte culture was evaluated with three random flasks from each condition (0, 25 and 50 mg/L).

Material and reagents: AlgeaFert Solid K+ was purchased from Algea. Sodium beta-glycerophosphate pentahydrate and germanium (IV) oxide were purchased from Alfa Aesar. Tris(hydroxymethyl) aminomethane, ammonium iron (II) sulfate hexahydrate and sodium nitrate were purchased from Merck.

Fertility induction: The culture was transferred to a climate controlled room at 10°C under a 23:1 photoperiod (light: dark regime) with a white light intensity range of 30-60 µmol m^{-2} s^{-1} for 8 days. Germanium dioxide was not used. Samples were taken before and after the fertilization. Microscopic observations were performed likewise as in E2.

Tank treatment and system design: The tanks were treated and seedlings were produced the same way as the previous experiment. The number of “sample” twines present on the seedling was four.

Seedling initiation: To start the seedling a volume of 200 mL of culture with a density of 0.9mg/mL (DW) was used. The pouring and the settlement of the culture was performed similarly as the later experiment. The seedlings were cultivated at 10°C under a 16:8 photoperiod (light: dark regime) and white light (fluorescent light bulb) with a mean intensity of 58.7 µmol m^{-2} s^{-1} over four weeks

Manual monitoring: The monitoring was performed every week. The seaweeds were collected and frozen at -20°C at the end of the experiment. Number of sporophytes and gametophytes, sporophytes length, image analysis and chemical analysis were evaluated in this experiment. The sporophyte and gametophyte number were counted on 1,4mm of five different points in the “sample” twine under a stereomicroscope (Nikon SMZ1000). A device was made to hold the sample twine, keeping the twine under water and in the same position. The sporophytes from each twine point were detached and collected with a tweezer and a needle. These sporophytes were transferred to a petri dish and pictures were taken under the stereomicroscope camera (Nikon SMZ1000). The length measurements were performed using the software Lumenera INFINITY ANALYZE.

Automatic monitoring: Parallelly to the manual monitoring pictures were taken to the seedlings. The photo shoot was taken, with a Nikon D800E and a Sigma 105mm f/2.8 EX DG OS HSM Macro Lens, in a standard environment (light intensity, distance between camera and seedling, focal length and other). The pictures were analyzed with a program developed by Torfinn Solvang-Garten in the software ViewLab. This program does a thresholding on the images based on hue/value histogram, resulting in a binary image (red and black, or mathematically, 0 and 1's). The 1's are counted, and divided on the sum of both numbers, giving the percentage cover (PC), which represent the grow percentage.

Statistics: All data are expressed as means ± SD. Two-way analysis of variance (ANOVA; GraphPad PRISM 6.01 for windows) was used to test for differences between developmental rates (Sidak's multiple comparisons test) and between seedling qualities (Tukey’s multiple comparison test). A level of statistical significance at $p<0.05$ was used. The plots presented were made on the program R v3.3.3 with the package ggplot2.

6.3. Results

The previous experiments allowed to reduce the fertility induction period and to approximate the optimal density of the gametophyte cultures. These parameters are crucial to accelerate the hatchery phase and to reduce the gametophyte waste, i.e., the number of gametophytes floating freely within the seedlings. Based on these optimized parameters, experiment 4 evaluated the effect of a possible growth enhancer, supplemented to the gametophyte cultures, on the sporophytes growth.

The gametophyte cultures fertility was induced under photoperiod 23:1 (light:dark regime) over 8 days and densities performed. The figure 16 represents the percentage of *Alaria* microstages under 23:1 photoperiod regime over 8 days on the gametophyte cultures C, A and B. The control culture showed a decrease of the vegetative gametophytes percentage

from 60% to 53.3%. The fertile gametophytes, mature oogonia and elongated oogonia increased 4%, 2% and 0.6%, respectively.

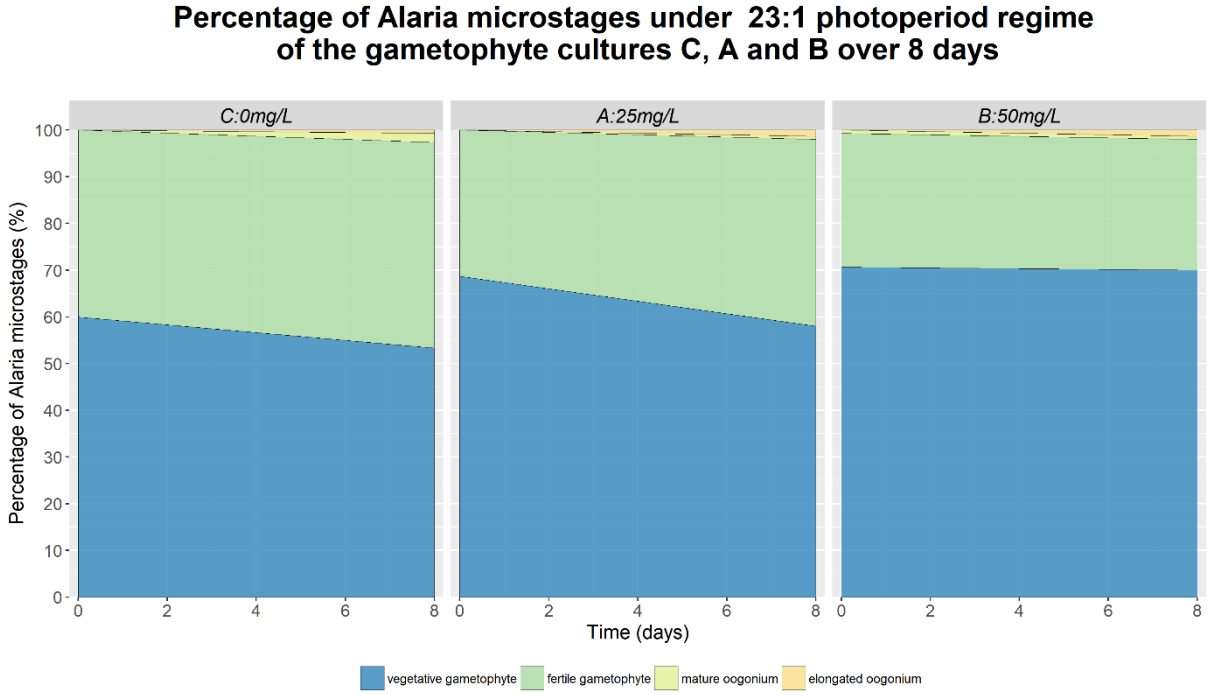

Figure 16. Percentage of the microstages of *A. esculenta*, treated with different growth enhancer concentrations, under photoperiod 23:1 over 8 days. The microstages represented are vegetative gametophytes (blue), fertile gametophytes (green), mature oogonium (yellow) and elongated oogonium (light orange). Values are means (n=3).

The culture A presented a decrease of the vegetative gametophytes percentage from 68.6% to 58%, respectively. The fertile gametophytes, mature oogonia and elongated oogonia increased 8.7%, 0.6% and 1.3%, respectively. The percentage of vegetative and fertile gametophytes decreased 0.6% after 8 days on the culture B. The number of mature oogonia remained the same and the elongated oogonia increased 1.3%.

The developmental rate was calculated the same way as E2. The figure 17 represents the developmental rate of gametophyte cultures supplemented with 0, 25, 50 mg/L of growth enhancer under the 23:1 photoperiod regime over 8 days.

From day 0 to 8 in the fertility induction there was only a significant increase (two-way ANOVA; $p \leq 0.05$) of developmental rate in the culture A but no significant differences were found between the three cultures in each day. Moreover, culture C from this experiment showed a developmental rate significant lower than culture C in E3.

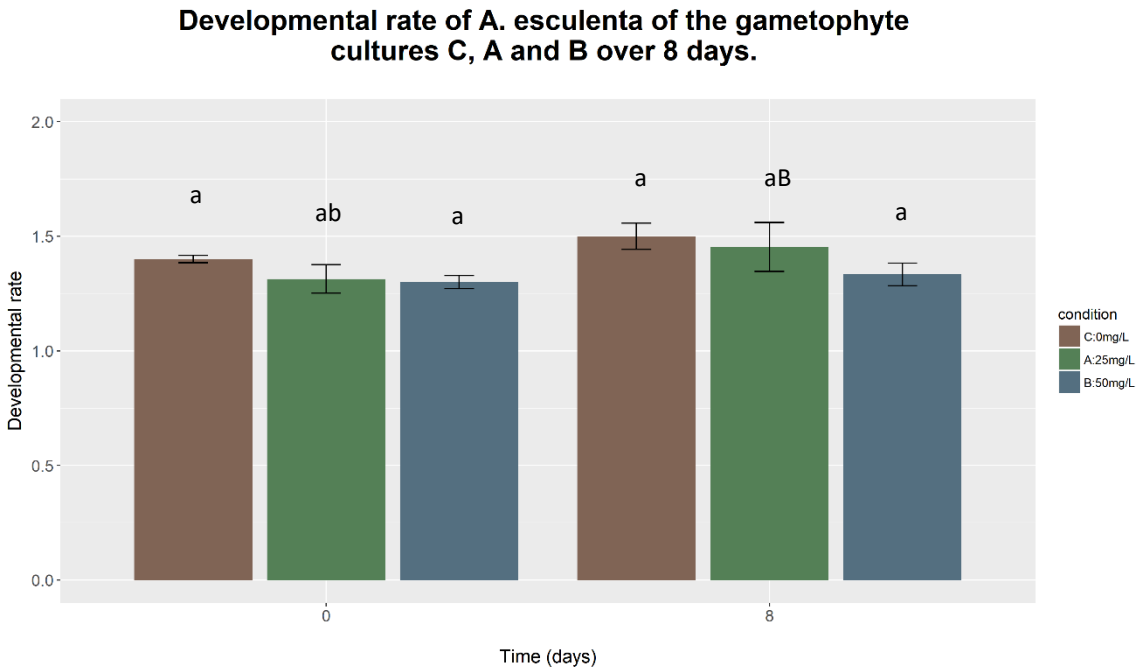

Figure 17. Developmental rate of *A. esculenta* of the gametophyte cultures C:0mg/L (brown), A:25mg/L (green) and B:50mg/L (blue) under the 23:1 photoperiod regime over 8 days. Values are means ± SD (n=3). Similar letters denote no statistical significant differences between treatments and days.

Later, the density of the gametophyte cultures C, A and B was adjusted to be similar and these cultures were seeded on the twines producing the seedlings under the names seedling C, A and B. The table 2 represents the density used in each condition of the experiment, which was near to the lowest density used on the last experiment. The linear model from E1 was used to estimate the biomass of each gametophyte culture.

Table 2. Densities applied in each seedling from the different treated cultures

Treatement used	Density applied (mg/mL)
C: 0 mg/mL	0.91
A: 25 mg/mL	0.93
B: 50 mg/mL	0.90

Figure 18 represents the number of gametophytes and sporophytes per millimeter twine, over 30 days, within the seedlings started with gametophyte cultures C, A and B.

The number of gametophytes and sporophytes were compared statistically (two-way ANOVA) against time and the different treatments. The number of gametophytes had no significant difference ($p>0.05$) between the four days and between the three treatments. No sporophytes were found on day 7 in any treatment. The seedling C had a significant increase of the number of sporophytes over the experiment days. For each day, the sporophytes number of the seedling C showed to be statistically higher than the treated seedlings. From the day 14 to 23,

the seedling A and B showed a significant increase of the number of sporophytes but after day 23 no differences were found. The treated seedlings showed to be statistically similar between them.

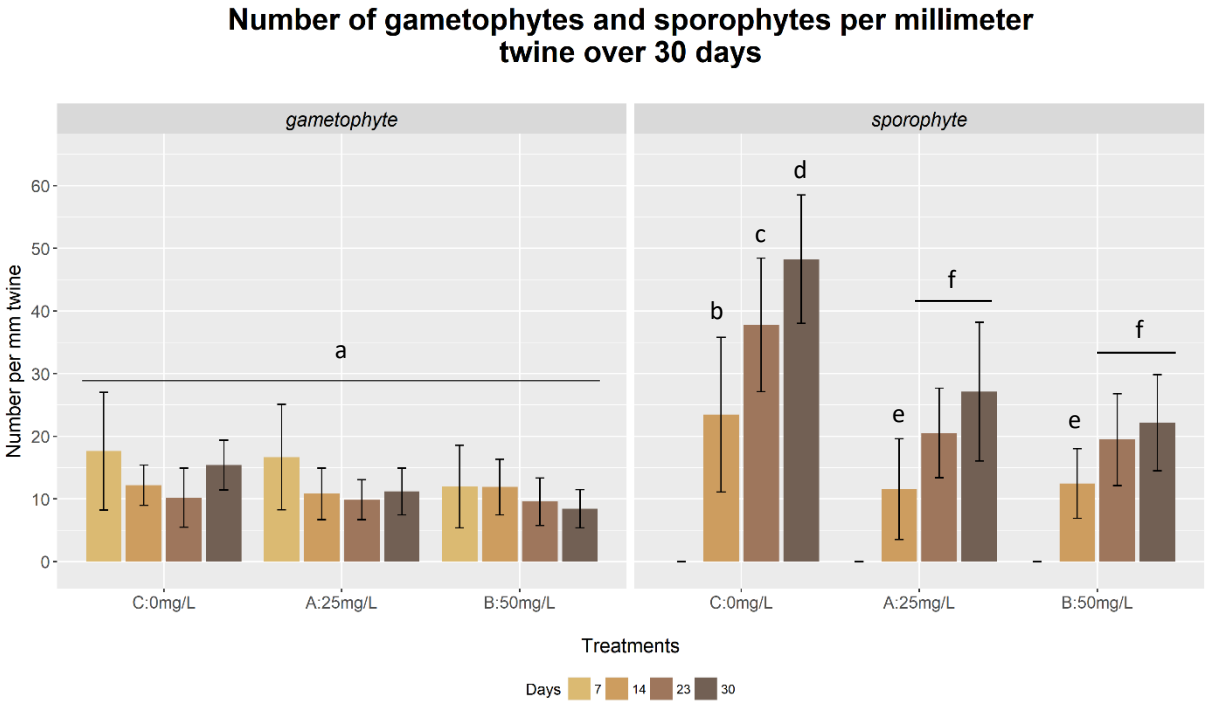

Figure 18. Number of gametophytes and sporophytes per mm twine within the seedlings, started with the gametophyte cultures C, A and B over 30 days. The days represented are day 7 (yellow), day 14 (dark yellow), day 23 (light brown) and day 30 (brown). Values are means ± SD (n=3). Similar letters denote no statistical significant differences between treatments and days.

The figure 19 represents the sporophytes length from the seedling C, A and B over 30 days. This data was not normal distributed due to the high variance of sporophyte length found within the twine. For that reason, several ranges of length were performed to clarify this data.

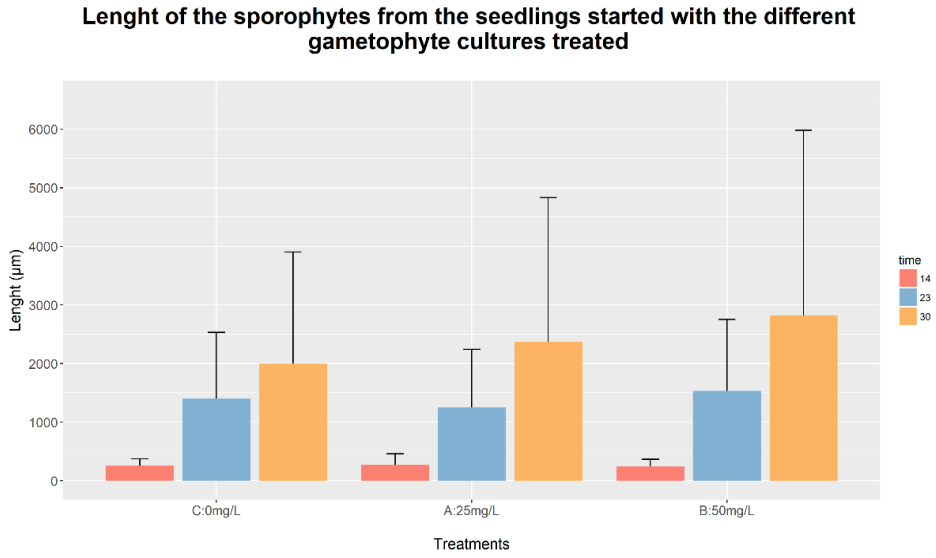

Figure 19. Length of the sporophytes found within the different seedlings. Values are means ± SD (n=3).

The table 3 shows the number of sporophytes within the range of length from the different seedlings on the day 30.

Table 3. Number of sporophytes within the several ranges of length (µm) from different gametophyte cultures at day 30. Values are means (n=3).

	Length range (µm)	Seedling C	Seedling A	Seedling B
Number of sporophytes	<300	4	4	3
	300-1000	**42**	**52**	**40**
	1000-2000	**52**	**34**	**37**
	2000-3000	**25**	**17**	**23**
	3000-4000	10	**17**	18
	4000-5000	9	10	6
	>5000	8	16	**23**

In each seedling treatment, the ranges with the highest number of sporophytes were selected to compare them. The majority of the sporophytes present in the seedling C and A had sizes between 300-3000 µm and 300-4000 µm, respectively. The seedling B showed a high number of sporophytes with sizes between 300-3000 µm and up 5000 µm.

The counting and length measurement of sporophytes are an accurate method which provide a real and precise number of the growing state on seedlings, but these are methods associated to time-consume. New ways of monitoring seedlings cultures should be explored. The use of digital images could help to estimate the product of these two methods. All parameters, such light intensity, seedling depth and camera range, were standardized to have the same settings in every day of the experiment.

The Percentage Cover (PC) was calculated as previously described. The pictures 20 illustrate the segmentation of the seedlings pictures into binary images, with black and red pixels. The first image has 1 PC with almost absence of black dots, where the background is the twine represented by the red points. Contrarily, the last picture has 97 PC being almost covered by black pixels. After day 30, the PC can only reach a percentage of 100, meaning there will be no more red points, or visible twine, on the image and so the system enters in saturation.

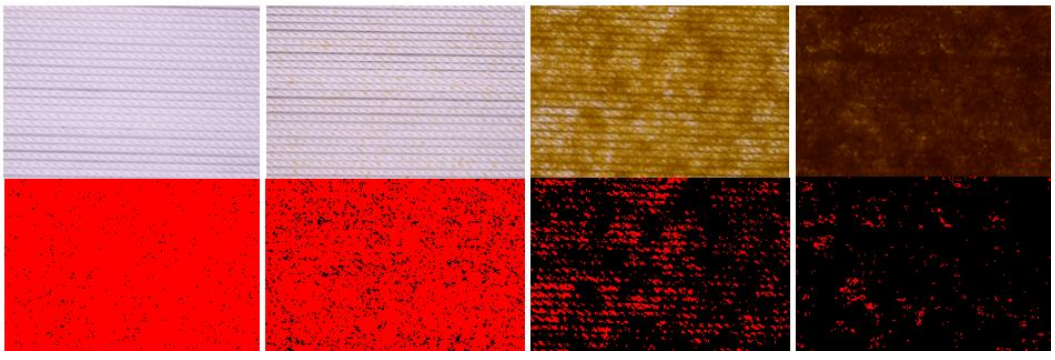

Figure 20. Representation of the segmentation process of the pictures into binary images. From the left to the right are represented the seedlings on the 7th, 14th, 23rd and 30th day.

The figure 21 (left) represents the percentage of PC obtained by the image analysis over 30 days. The percentage cover for each treatment showed a similar trend throughout the whole experiment with no significant differences between the treatments. The data showed an exponential growth right after the day 14 and a decreasing after day 23.

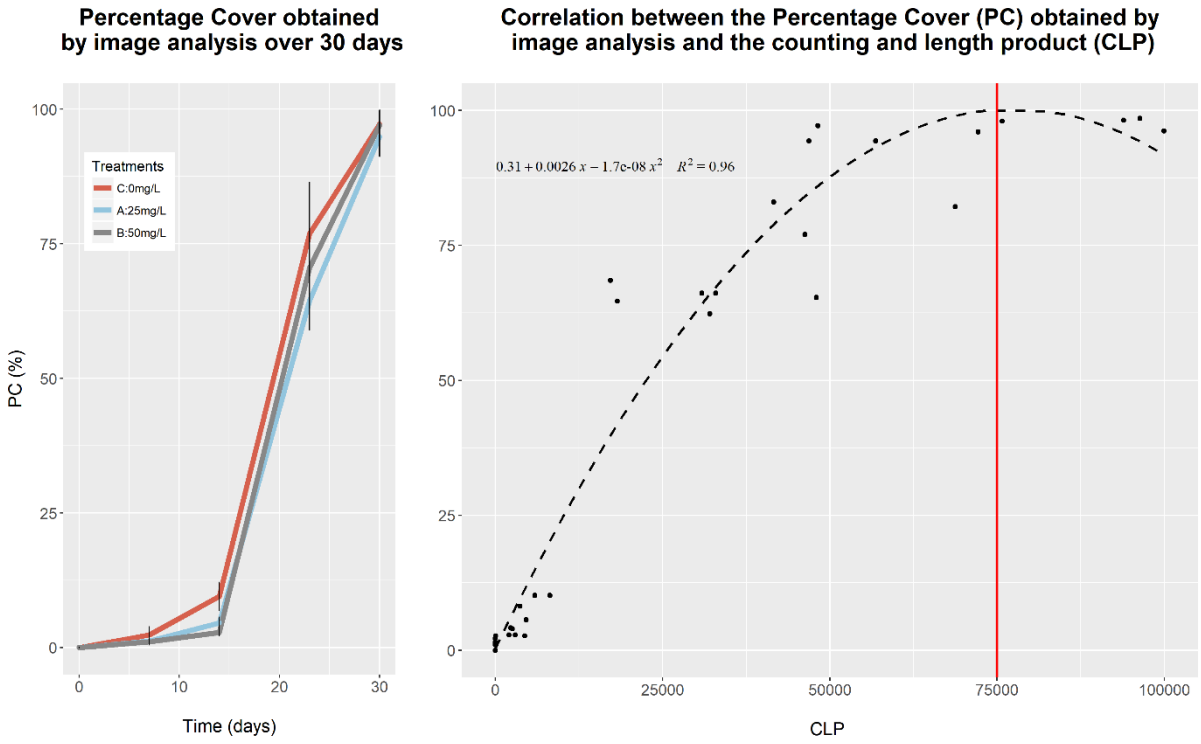

Figure 21. Left graph: Percentage Cover (PC) obtained by the image analysis over 30 days for the different seedlings quality. The seedlings were initiated with the culture C (red), A (blue) and B (gray). Values are means ± SD (n=3).
Right graph: Correlation between the PC (obtained by image analysis) and CLP (product of length and counting measurements). The red line represents the saturation of the program.

The values obtained by counting and length of the sporophytes were multiplied and then the values of the gametophyte counting was added (CLP), representing the occupied space by the gametophytes and sporophytes on the seedlings. The three parameters: number of gametophytes, number of sporophytes and length had to be grouped as a variable because each one of this is contributing to the percentage cover on the seedling. While the number of gametophytes contributes mostly during the first 14 days, due to the lack of sporophytes conversion, the length of the blades of each sporophyte is contributing from day 23 to 30.

The figure 21 (right) represents the correlation between CLP and PC to estimate the strength of the relationship between these two variables. The data was not normal distributed. A trendline with a polynomial function of 2nd degree fitted rather good between 0 and ~75 000 CLP. Could had be interesting to follow the culture during the exponential growth to fill the gap with more points between these days.

6.4. Discussion

After finding the optimum photoperiod regime for the fertility induction and the optimum seeding density, it was time to evaluate the effect of the gametophyte cultures treated with putative growth enhancer on the sporophytes development.

The fertility induction performed to initiate the experiment had a similar behavior as the previous experiment (E3). Eight days after transferring the culture C, A and B into white light, the number of the two oogonia types was very low and the percentage of vegetative and fertile gametophytes had not decreased significantly. As already mentioned, the volume and culture density were different from the E2. The experiment E2 was performed in microplates with 5 mL of a diluted culture, contrary to 1 liter of non-diluted culture used this experiment. Having a culture with a higher density and a lower surface area, part of the gametophytes probably didn't receive the amount of light needed to induce their fertility, even under shaking conditions (Lüning and Dring, 1975).

Moreover, the E3 had an addition of germanium dioxide, contrarily to this experiment. Since a few authors (Markham and Hagmeier, 1982; Shea and Chopin, 2007; Mizuta and Yasui, 2012) pointed out positive and negative effects on kelp sporophyte development, the E4 had no GeO_2 addition. Since the developmental rate of the culture C in E3 was statistically higher than the culture C in E4, it seems as addition of GeO_2 could have a benefit for fertility induction.

In the seeding experiment the lowest density seemed to be sufficient or even too high for the seedlings production, and this experiment (E4) confirms this statement. The estimated densities of gametophytes on the twines were similar, indicating that the seeding densities had been similar for the different tanks. The number of sporophytes increased over the weeks but at the same time the number of gametophytes were stable. This was not expected, as the conversion of gametophytes into sporophytes should have induced a decrease in the number of gametophytes. This means that only a part of the gametophytes on the twine had the ability to grow into sporophytes whereas the excess of the gametophytes endures in the vegetative form due to the lack of space or/and the competition for light and nutrient.

No sporophytes present at day 7 was probably caused by a fertility induction that didn’t work properly, as discussed above. In addition to the 8-day of fertility induction time, the immature gametophytes were thus still being induced 7 days after seeding. With an optimized fertility induction, the maximum number of sporophytes, as observed on day 30, could possibly have been obtained one week earlier. Since there were no significant differences in the number of the gametophytes, the difference between the control and the treated sporophytes is valid. The difference could be explained by a better quality of the gametophytes cultivated without the growth enhancer, as these gametophytes produced a higher number of sporophytes.

Since there was a high variance on the sporophyte length, also described by Xu et al. (2009), different ranges of size were performed to show how different the sporophyte length are within treatments. Despite the number of sporophytes being higher in the control seedlings, it seems as by having less sporophytes per mm twine, the treated seedlings from the cultures A and B had two and three times, respectively, more seaweeds with a length over 5000 µm than the control group. By having a lower density, these sporophytes had probably less competition for nutrients and light in the tank, resulting in a faster grow. However, this feature was not directly related with the initial application of the growth enhancer.

The percentage cover (PC) showed an exponential growth after day 14, starting to slow down one week later. The delay in the fertility induction is probably the reason why the exponential growth started after day 14 and not from the start. On day 23, the PC of the twines was around 75% and probably sufficient for deployment of the seeded twines in the sea, but it is important to study this further to find the optimum PC and the seeding density and incubation time to reach this.

The correlation between CLP and PC had a good relationship for these two variables. However, for values above 75 000 CLP, the system is in saturation and can't give a value more than 100 PC.

The goal of the PC method is to accelerate the seedling monitoring, alternatively to the manual counting and measuring the sporophytes. This method is interesting to the seaweed industry because the growth percentage can be estimated in a few minutes by the image analysis, contrary to the manual counting and length measurement that can take hours, giving a green-light to deploy the seedlings on the sea.

7. Phlorotannins analysis (E5)

7.1. Scientific questions

Seaweed phlorotannins' importance has been growing the past decades because of the potential to benefit human health. These properties come from the defense mechanism against grazing, protection against UV radiation and other. Phlorotannins are present in the brown seaweeds *Alaria esculenta* and *Saccharina latissima* and due to their fast and efficient growth, these species could represent a viable source of these compounds. *Question 8: What are the quantitative and qualitative differences between the phlorotannin contents of A. esculenta and S. latissima species? Question 9: After the fertility induction of the Alaria gametophytes, what changes in the phlorotannin content? Question 10: Can a growth enhancer supplement increase the concentration of phlorotannins in gametophyte cultures or even later in the sporophyte form?*

7.2. Materials and methods

Seaweed material: The phlorotannins analysis was performed with the seaweed material from the previous experiments and from experiments performed by others in work package (WP2) from the MACROSEA project: *Alaria* sporophytes cultivated with different densities (E3); *Alaria* gametophytes, before and after fertilization, and sporophytes with different qualities (E4); *Saccharina latissima* from different locations from Norway. The seaweed material was freeze-dried and turned to dust using a grinder (KRUPS F203).

Materials and reagents: Sodium carbonate solution was purchased from VWR. Folin-Ciocalteu's reagent, methanol, n-hexane and acetone were purchased from Merck. Phloroglucinol anhydrous was purchased from Alfa Aesar. Ultrapure water was obtained by the equipment PURELAB® Ultra from Elga.

Extraction: A quantity of 500mg was weighted in glass tubes. The lipids were removed three times by adding 1 mL n-hexane and centrifuged for 5 minutes at 4000 rpm. These extracts were saved for lipid analysis. The first part of the phlorotannin extraction was made by adding 10 mL of acetone/ultrapure water (7:3) (AcUW), mixing at 100 rpm one overnight and centrifuging 10 minutes at 4000 rpm. The second part of the extraction was made 3 times. Then,10 mL of AcUW was added, two cycles of 30 seconds in vortex and 30 seconds of rest was performed and they were centrifuged for 10 minutes at 4000rpm. In the end of all steps, the extract was transferred into a new flask. The samples were submitted to the low pressure (~50 mbar) of a rotary evaporator (Heidolph, Laborota 4000) at 30°C for 5 min. These samples, now without acetone, were transferred into glass vials and they were freeze-dried. The final powder was weighted and stored in methanol (100%) with a final concentration of 10 mg/mL.

Quantification of phlorotannins: The total phlorotannins content was performed by the Folin-ciocalteu method performed by Zhang et al. (2006). Three 96 well-plate were prepared. The structure of these plates was: standard curve samples/blank and their controls, samples/blank and their controls. The microplate was loaded with 20 µL of sample, 100 µL Folin-Ciocalteu's reagent, mixed and waited for 5 min. Then 80 µL 7.5% sodium carbonate solution was added and well mixed. The microplate was incubated at room temperature in the dark for 2 h and then mixed in a microplate mixer (Eppendorf thermomixer comfort). The absorbance was read at 750 nm using a spectrophotometer (Varian Cary 50MPR Microplate Reader).

Qualification of phlorotannins: The TOF-MS analyses were performed on an Agilent 6220 Accurate Mass Time-of- flight mass spectrometer. Samples were introduced to the ion source through flow-injection using an Agilent 1100 HPLC system. One µl of sample was injected into a mobile phase consisting of acetonitrile:water (95:5) with 4 mM ammonium-acetate. This resulted in a single peak which was integrated, and the mass spectrum was extracted. Subsequently exported to a mzXML file for import into R using the mzR library (Chambers et al., 2012).

Statistics: All data are expressed as means ± SD. One-way analysis of variance (ANOVA; GraphPad PRISM 6.01 for windows) was used to test for differences between sample treatments on E3 and E4 sporophytes, using the Tukey's multiple comparison test. Two-way analysis of variance (ANOVA; GraphPad PRISM 6.01 for windows) was used to test for differences between sample treatments on E4 gametophytes, using the Tukey's multiple comparison test. A level of statistical significance at $p<0.05$ was used. The plots presented were made on the program R v3.3.3 with the package ggplot2.

7.3. Results

The importance of phlorotannins has grown the past two decades. Phlorotannins are restricted to brown macroalgae and have been demonstrated to have a potential beneficial for human health. These compounds have been reported in *A. esculenta* and *S. latissima (Zhang et al., 2006; Lopes et al., 2012; Kim et al., 2013)*.

The qualification and quantification of phlorotannins was performed with freeze-dried samples from the previous experiments. The seaweed material used were sporophytes with 9 weeks of growth (E3), gametophyte cultures collected before and after the fertility induction from E4, sporophytes with 4 weeks of growth (E4) and Saccharina sporophytes grown in laboratory from eleven locations in Norway. From all the experiments, the content of phlorotannins of *A. esculenta* and *S. latissima* showed an average of 4.11 and 3.08 mg PHL/g algae, respectively.

The figure 22 represents the content of phlorotannins in milligrams per grams of dry algae present in sporophytes from different seedlings densities. The figure shows there was no significant difference between densities 0.79, 1.59 and 6.35 mg/mL, however a significant difference was found between the density 3.18 and the densities 1.59 and 6.35 mg/mL (One-way ANOVA; F=5.073; p=0.0055).

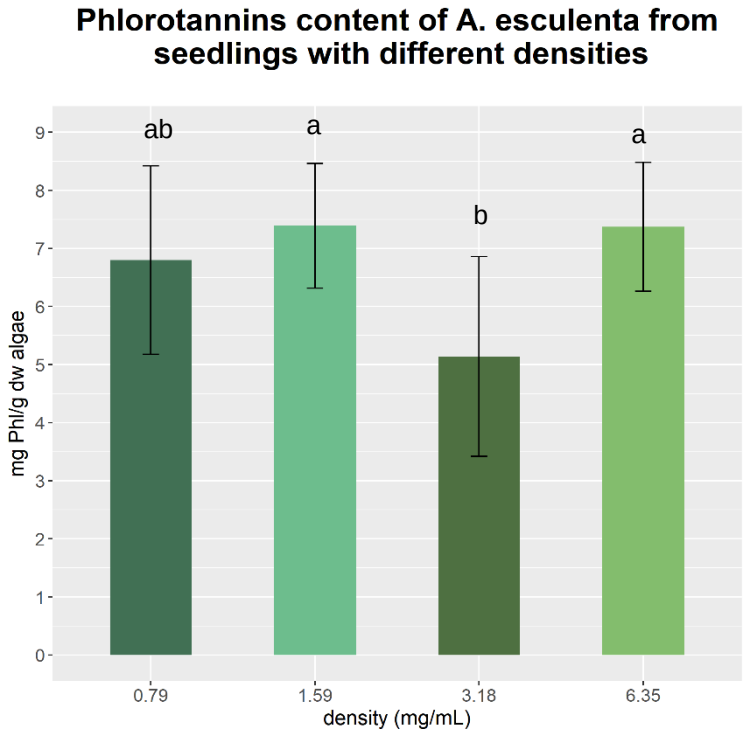

Figure 22. Phlorotannin content present in sporophytes from seedlings with different densities. Values are means ± SD (n=3). Similar letters denote no statistical significant differences between treatments.

Figure 23 represents the content of phlorotannins in milligrams per grams of dry algae present in the gametophyte cultures of *A. esculenta* treated with three growth enhancer concentrations, before and after fertilization. There was no significant difference between treatments on the non-fertile gametophytes. After the induction of the fertilization there was an increase of the phlorotannin content in all cultures, where the cultures A and B had an increase more accentuated than the control culture. The fertile gametophytes showed a significant difference of the phlorotannin content on the treated cultures comparing with the control culture (One-way ANOVA; F=49.7; p<0.0001).

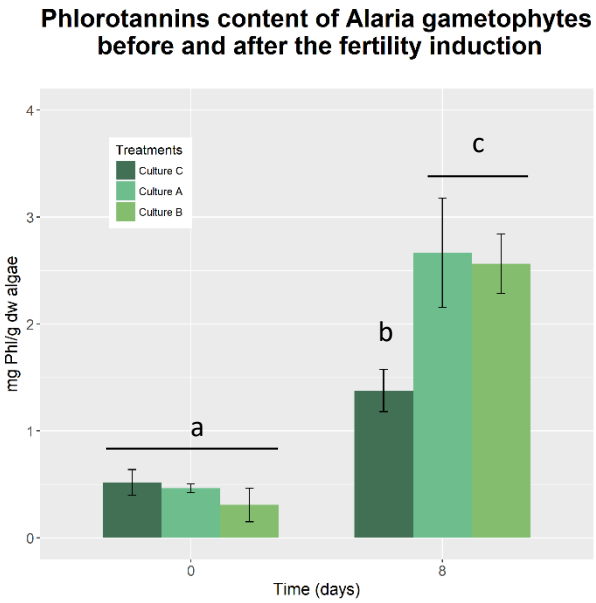

Figure 23. Phlorotannin content of *Alaria* gametophytes before and after the fertility induction. Values are means ± SD (n=3). Similar letters denote no statistical significant differences between treatments and days.

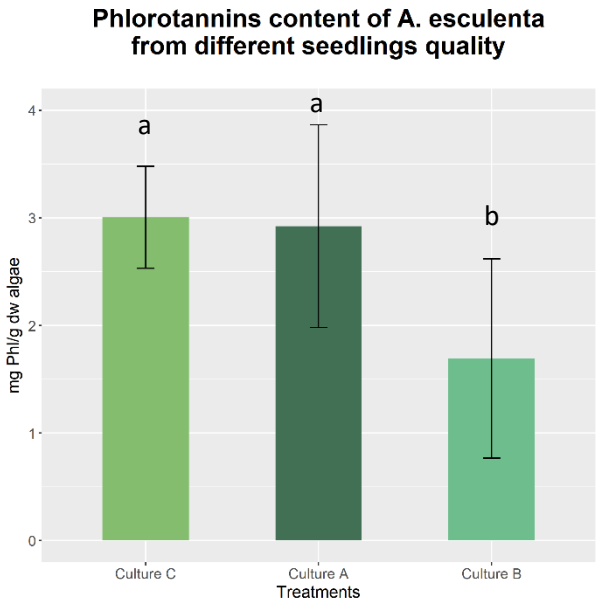

Figure 24. Phlorotannins content of *Alaria* sporophytes from seedlings initiated with three gametophyte cultures treated with growth enhancer. Values are means ± SD (n=3). Similar letters denote no statistical significant differences between treatments.

The figure 24 represents the content of phlorotannins in milligrams per grams of dry algae present in sporophytes of *A. esculenta*, where their gametophyte source was treated with three concentrations of a growth enhancer. The sporophytes showed significant differences between the culture B and the culture C and A, with no significant differences between the later (one-way ANOVA; F=7.414; p=0.0031).

The figure 25 represents the content of phlorotannins in milligrams per grams of dry algae present in sporophytes of *Saccharina latissima* from eleven locations of Norway over three months. Mature plants from these locations were collected and zoospores were released in the laboratory. All seedlings from the different locations grown under similar conditions over three months. A high variance was noticed on the phlorotannin concentration of Saccharina sporophytes from the eleven locations. The sporophytes from location 9 had a clear increase of phlorotannins concentration in April.

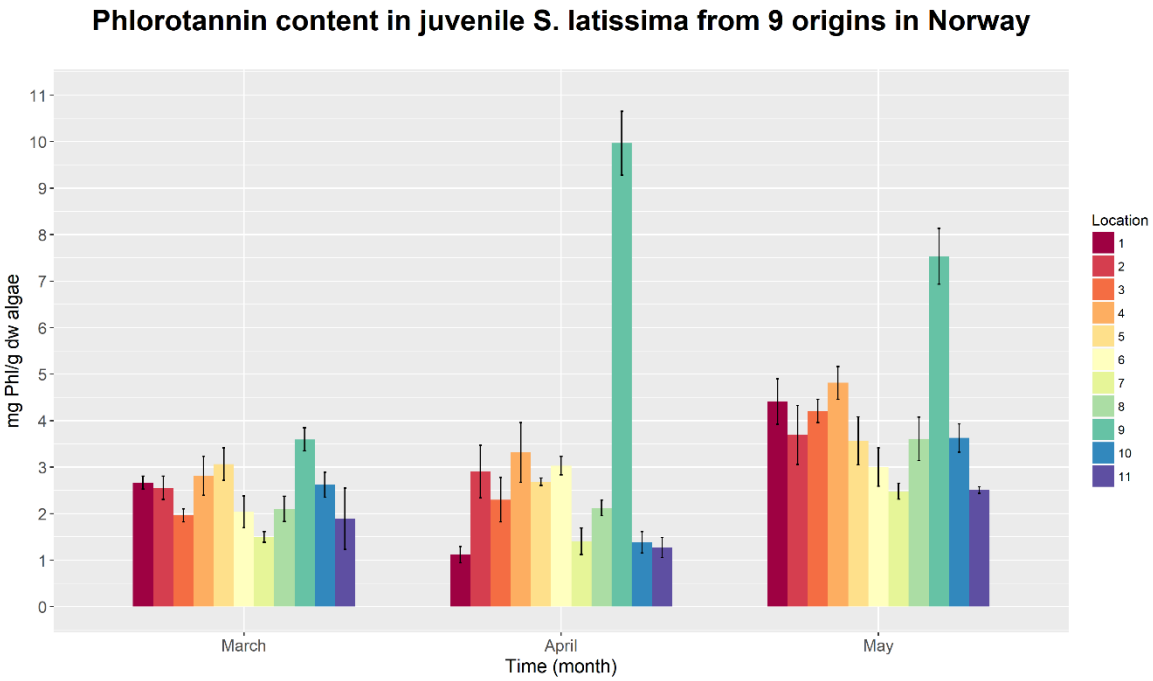

Figure 25. Phlorotannins content in juvenile S. latissima from 9 origins in Norway. Values are means ± SD (n=3).

The lipid removal was mandatory before the phlorotannin analysis. As a complement, the lipid phase was analyzed and a number of lipid candidates were identified (Appendix IV: Supplemental table 4).

The qualification of the phlorotannins was performed to identify different phlorotannins present on the samples of *A. esculenta* and *S. latissima*. The mass spectrometry data were collected in a non-targeted approach, which the full spectrum data were acquired in negative ion mode from m/z 50 to 1200. The data were then analyzed by searching for the theoretical masses corresponding to all possible phlorotannins in the recorded mass spectra (Table 4). The search was performed either by 1) comparing phlorotannin masses described in the literature and tentatively assigning peaks in the mass spectra to these when matched, or 2) by selecting the most dominant masses with highest intensities present on samples, followed by the identification of these compounds based on the literature and other database platforms (Metlin, KEGG, Pubchem and other). The later was not accomplished successfully.

Table 4. List of phlorotannins referred in the literature found in the samples. (s) sporophyte, (v.g.) vegetative gametophyte and (f.g.) fertile gametophyte

Phlorotannin compounds		Experiments				
		E3 (s)	E4 (v.g.)	E4 (f.g)	E4 (s)	WP2 (s)
Phloroglucinol mmz=126.0317	Average Intensity	1205,61	4507,56	3151,94	0	33787,72
Dihydrophloroglucinol mmz=128.0473		166421,90	55344,79	11775,35	44298,01	1865292,37

Two forms of the phloroglucinol were identified: phloroglucinol (PG), and dihydrophloroglucinol (DHPG). The building structure of phlorotannins was identified both in *Alaria* and Saccharina experiments, excepting in E4. The DHPG was the phlorotannin compound found with the highest intensity in both species. Sporophytes from E3 had the highest intensity within *Alaria* experiments. Both PG and DHPG had the highest intensity in *S. latissima* comparing with *A. esculenta.* The figure 26 shows the peaks of PG and DHPG of the mass spectrum of the different seaweed species.

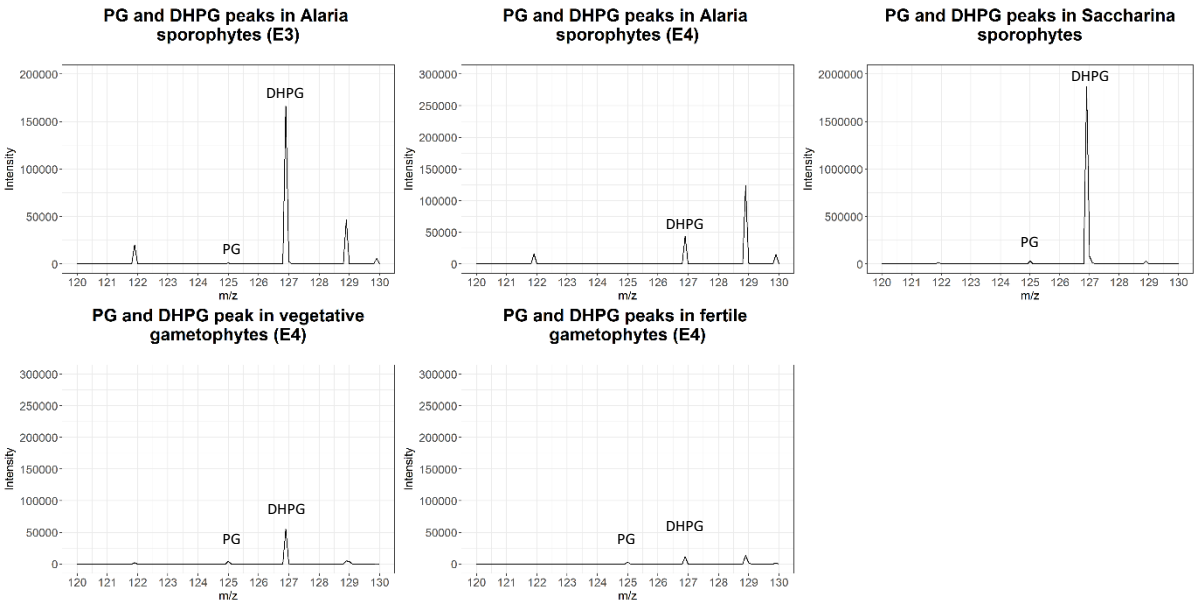

Figure 26. PG and DHPG peaks of the mass spectrum of the extracts of *A. esculenta* and *S. latissima.*

A correlation was made between phlorotannins content from the Folin-ciocalteu method and the intensity of PG and DHPG from mass spectrometry for *A. esculenta* (Fig. 27) and for *S. latissima* experiments (Fig. 28). The data on the individual phloroglucinols are semi quantitative.

The PG and DHPG correlation showed two distinct clusters matching with the experiments group. The gametophytes from E4 had a negative correlation between phlorotannin content and PG intensity, where the increase of content results in the decrease of PG ($y = -942{,}19x + 5070{,}2$; $R^2 = 0{,}538$). The PG decrease seems to be related to the synergy of the fertilization induction and the addiction of the growth enhancer, where the latter seems to reduce PG intensity. The sporophytes from E3 (highest point excluded) and E4 had no correlation between phlorotannin content and PG intensity ($y = 0$; $R^2 = \#N/A$). The PG intensity was zero, meaning other type of phlorotannins was quantified by the FC method. No relationship was found between treatments.

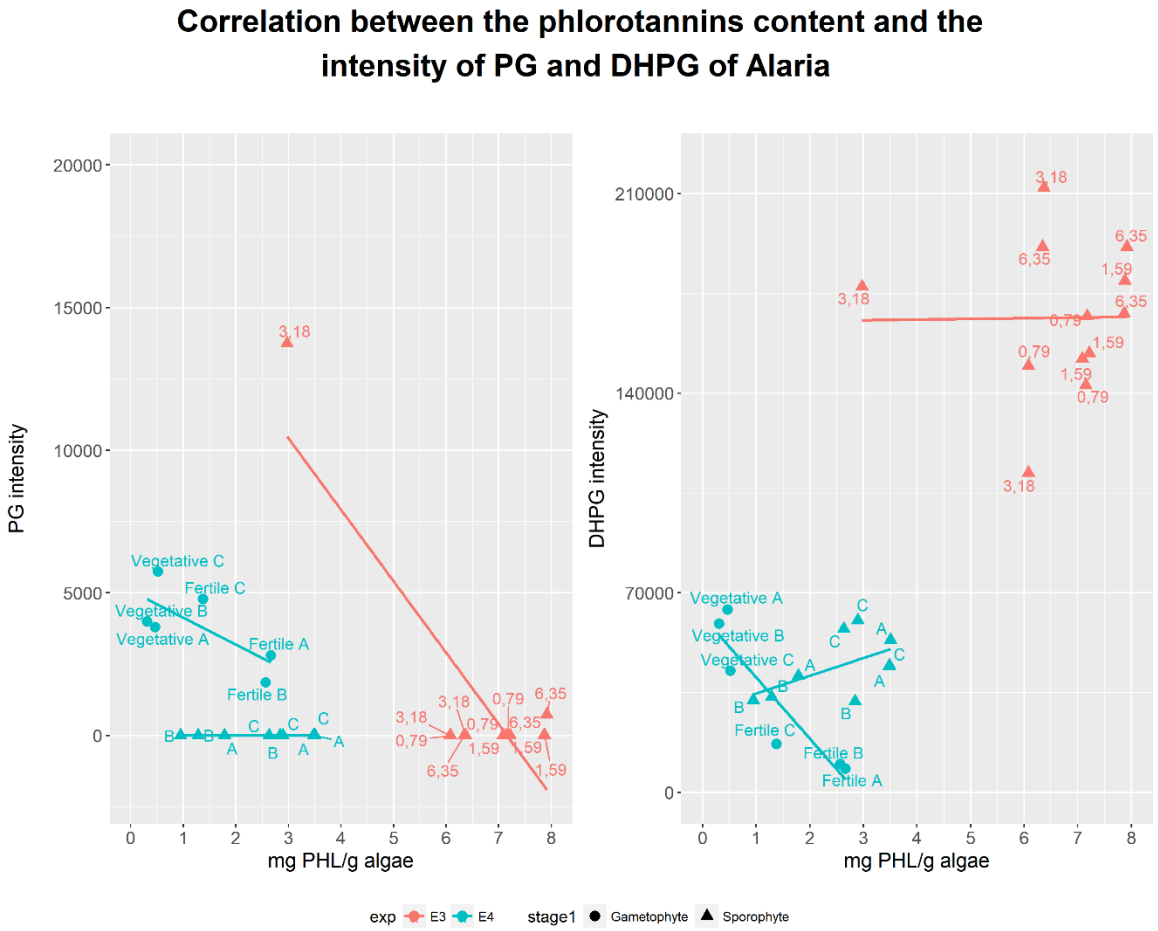

Figure 27. Correlation between the content of phlorotannins obtained by the FC method and the intensity of PG and DHPG from mass spectrometry for *A. esculenta*

The DHPG correlation on the gametophytes from E4 also had a negative correlation, which DHPG intensity decreased with the increase of phlorotannins content (y= -21456x+61808; R^2=0,8443). The fertilization induction seems to decrease DHPG intensity and the addiction of the growth enhancer seems to have no influence. The DHPG correlation on the sporophytes from E3 had no correlation between phlorotannin content and intensity (y=9817,9x+96579; R^2=0,063). The different densities seem to have no effect DHPG content. Yet, the sporophytes from E4 had a positive correlation between phlorotannins content and DHPG intensity (y=6206,2x+28536; R^2=0,3135) with no apparently effect caused by the putative gametophyte qualities that started the seedlings.

The Saccharina sporophytes (Fig. 28) showed no correlation, with a high degree of dispersion, between the phlorotannins content and intensity of PG and DHPG. Sporophytes from location 9 had a decrease in PG intensity and an increase in DHPG with the phlorotannin content. Besides that, no evident effect by the time and the location was noticed.

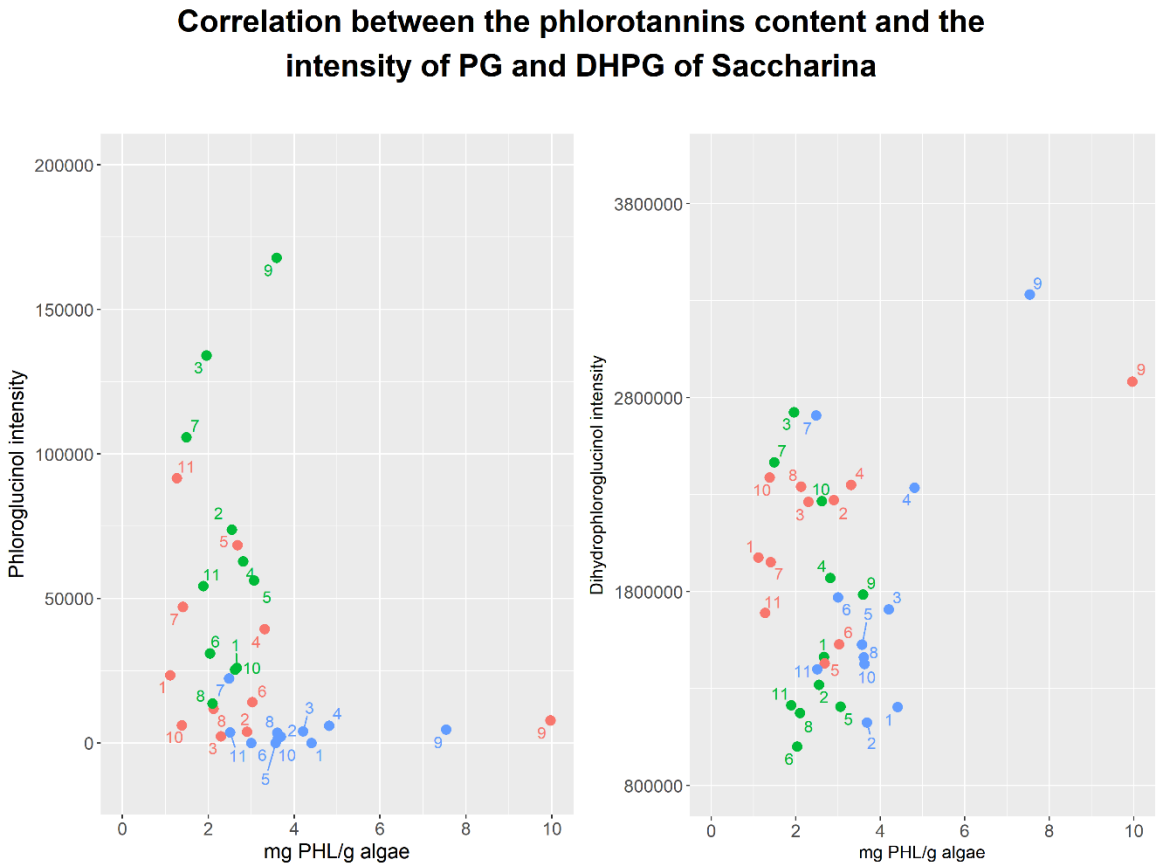

Figure 28. Correlation between the content of phlorotannins obtained by the FC method and the intensity of PG and DHPG from mass spectrometry for *S. latissima*

The sum of the phloroglucinol and dihydrophloroglucinol intensity was made to correlate the phlorotannin content with the phlorotannins intensity identified in each kelp specie (Fig. 29). Two clusters were observed belonging to the two species. The correlation of phlorotannins content and intensity of *A. esculenta* was positive, where the intensity of the identified phlorotannins increased with the content (y=20999x+11925; R^2 = 0,6686). However, the intensity of these compounds was very low compared with *S. latissima*, suggesting that the PG and DHPG are not the only phlorotannins present in *Alaria* samples. The *S. latissima* correlation showed a weak relationship between phlorotannins content and intensity (y=107501x + 2000000; R^2=0,1013). As already mentioned, *S. latissima* had higher intensities than *A. esculenta*, even with similar concentrations.

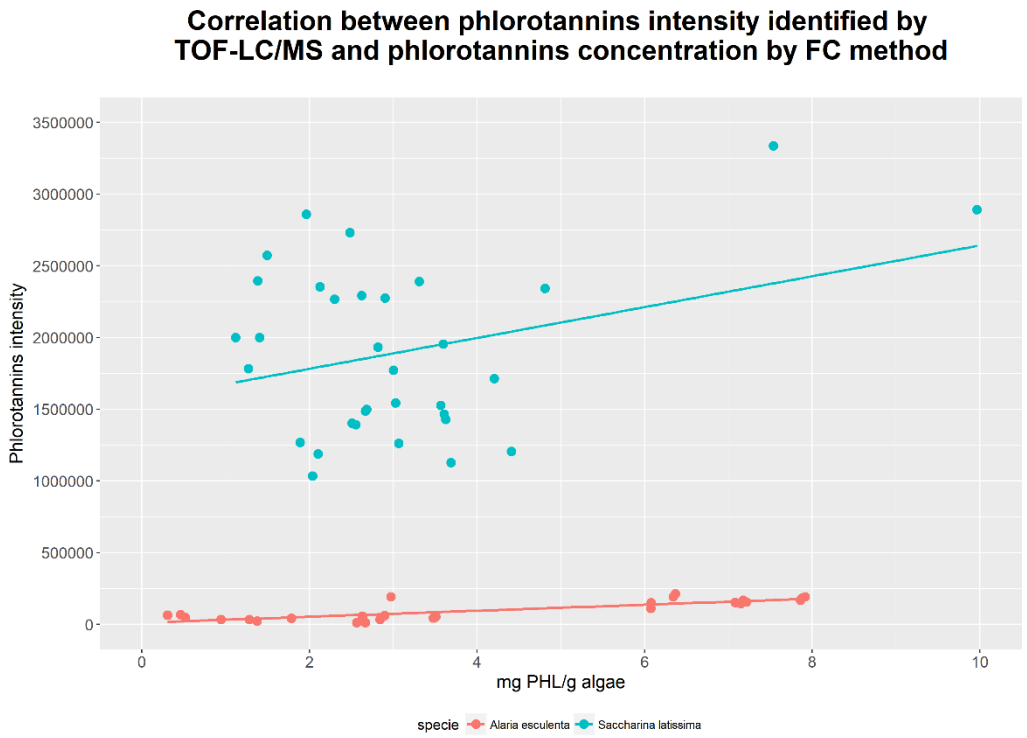

Figure 29. Correlation between the content and the intensity of phlorotannins (PG and DHPG) identified for *A. esculenta* and *S. latissima*

7.4. Discussion

Phlorotannins are specific to brown macroalgae. Most of the studies involving their characterization have been done in seaweeds from the Fucales order due to the high levels of phlorotannins found on these seaweeds (Isaza Martínez and Torres Castañeda, 2013). The phlorotannins are present in *Alaria esculenta* and *Saccharina latissima* (Zhang et al., 2006; Nwosu et al., 2011; Lopes et al., 2012; Kim et al., 2013) however no characterization has been done on these species. The present study performed a qualification and quantification of phlorotannins present in juvenile stages of those kelp species using FC method and TOF-MS, respectively.

The content of phlorotannins present in the sporophytes that were cultivated at from different seedings densities (E3) was apparently not affected by the density, as the lower and the highest density had the same phlorotannin content. A crowded growth brings limitation to the cultures in the form of competition for light and nutrients, and may cause an abiotic stress. The phlorotannin production in macroalgae is often related to stressful environments (Dethier et al., 2005; Akula and Ravishankar, 2011), and similar phlorotannin content at all tested seeding densities may thus suggest that all seedlings either were equally stressed or that the environmental conditions experienced by the sporophytes in the different tanks not varied enough to induce differences in the phlorotannin synthesis.

The 4 weeks old sporophytes produced from gametophyte cultures treated with growth enhancers (E4) had lower phlorotannin content than the nine weeks old sporophytes from the seeding density experiment (E3). This finding illustrates that the total content of phlorotannin increases with the age of the sporophytes. The seedling development period is recommended for 4 weeks. After nine weeks of growth the sporophytes were probably highly limited in light and possibly nutrients by the volume of the tanks used in this experiment and this possible stressful environment could also have affected the increased phlorotannin content.

The sporophytes (E4) obtained from the gametophyte cultures B showed lower phlorotannin content than the sporophytes from the gametophyte culture C and A (Fig. 24). Since the light intensity and temperature were constant within the replicates, no evident explanation is found on this observation.

The phlorotannin content present in vegetative, non-fertile gametophytes was lower than in the fertile gametophytes, indicating that the changing of the quality and intensity of light influences this content (Fig. 23). The phlorotannin content of the non-fertile gametophytes was independent of the growth enhancer. Contrarily, the fertile gametophytes showed an increase of the phlorotannin content in the cultures supplemented with growth enhancer. As no differences were found between cultures treated with 25 or 50 mg/L of growth enhancer it is expected that the possible phlorotannin in the growth enhancer, an extract from *Ascophyllum nodosum,* not influenced significantly on the concentration in the growth medium. Potin and Leblanc (2006) reviewed the phenolic-based adhesives in brown algae, where there is a correlation between the phenolic polymers secreted and the attachment process of the zygotes in Fucus spp. Thus, the gametophyte medium could also have been analyzed to evaluate the presence of possible phlorotannin excreted by the *Alaria* gametophytes and how the cultivation conditions possibly affect this excretion (Steinhoff et al., 2011).

The species from the Laminariales order with more in-depth phlorotannin characterization studies are *Laminaria ochroleuca* (Glombitza et al., 1976; Koch et al., 1980), *Pleurophycus gardneri* (Glombitza and Kno, 1992), Eisenia spp. (Okada et al., 2004; Kim et al., 2013) and Ecklonia spp. (Kim et al., 2009). In the present study just two forms of phloroglucinol were found: Phloroglucinol (PG) and dihydrophloroglucinol (DHPG), where DHPG was the compound with the highest intensity in both *A. esculenta* and *S. latissima*.

The phlorotannins content of the sporophytes from E3 and E4 showed no correlation in any of the compounds, excepting the DHPG of the sporophytes in E4 with a weak correlation. The treatments with growth enhancer had apparently no effect on the synthesis of either of the three compounds.

In the gametophytes that were used to study effects of the growth enhancer (E4), both PG and DHPG had negative correlations with the total phlorotannin content. The induction of fertility of the gametophytes seems to provoke a decrease of PG and DHPG. The growth enhancer did not influence on the content of DHPG, but PG seems to be reduced by the growth enhancer concentration. The decrease of PG and DHPG suggests a polymerization of these compounds into other phlorotannin compounds but due to the lack of studies related with phlorotannin characterization in *A. esculenta* it is difficult to identify other compounds in the samples.

The PG and DHPG compounds had no correlation with the phlorotannin content in *S. latissima*. As in the experiments with *Alaria* DHPG was the dominant compound also in Saccharina (Supplement figure 4). The sporophytes from location 9 showed a particular behavior compared to the other locations. The PG intensity decreased and the DHPG increased with the phlorotannin concentration, which suggests that the PG was transformed into DHPG by the addition of two hydrogens. Besides this, no evident effect was noticed by the time of the year and the location.

The sum of the two phloroglucinol forms showed two clear cluster when the two species were compared. *A. esculenta* had a positive correlation between the total phlorotannin content and the content of identified phloroglucinol or phloroglucinol derivates, with median relationship (R^2=0.67) and *S. latissima* a weak relationship (R^2=0.1). In *S. latissima* the PG seems to be converted into DHPG increasing the total phlorotannin content. For *A. esculenta* both PG and DHPG seemed to be transformed into other phlorotannin compounds, that not were identified. The experiments also demonstrated lower intensity in all phloroglucinol forms in *A. esculenta* compared to S. latissima, even with a similar phlorotannin content.

Other phlorotannins definitely seemed to be present in these data, however, due to the lack of studies of these species it was impossible to make identification. It is also important to remember that the mass spectrometry data when collected as fingerprints are semi quantitative and if there had been access to more reference standards, positive (as opposed to tentative) assignments could have been made also using chromatographic separation of the phloroglucinols. This should be pursued in further studies.

This study represents a first description of the polyphenols in juvenile stages of *A. esculenta* and S. latissima. The results suggest that more studies are needed for a more complete explanation of the dynamics of these compounds during the development from gametophytes to sporophytes and as sporophytes grow, and as a response to different environmental conditions during the early life stages.

8. Conclusions and future perspectives

In this study it was demonstrated that it is possible to measure the biomass of *Alaria* gametophyte cultures either by optical density or *in vivo* fluorescence and convert to dry weight. However, there will always be an error associated to these measurements and for that reason the choice between the three methods will depend on time limitations and investment of the laboratory. The addition of a putative growth enhancer did not affect the growth of gametophyte cultures, where the PES medium is enough for a maximum growth performance.

The study related with gametogenesis showed that not only the fertility was induced in almost all gametophytes but also that it was possible to reduce the induction time by applying a photoperiod regime with 23 hours light + 1 hour darkness. The growth enhancer had no influence on the fertility of the gametophytes. However, the volume and the surface area demonstrated to have a high influence over the fertility induction, being directly related to the amount of light received by the gametophytes. The fertility development of gametophytes also seemed to be influenced by the addition of GeO_2, where a faster development was obtained by the addition of this compound.

The study of seeding densities showed an optimal number of sporophytes using the lower density tested, however the number of seaweeds per twine was probably still higher than the needed. The deployment of the sporophytes at the sea could be important to evaluate the survival rate of sporophytes produced with high and low densities. Nevertheless, these experiments were a starting point to find the ideal seeding density for *Alaria* cultivation.

The number of sporophytes on twine were influenced by the treatment with growth enhancer used in the gametophyte cultures but not the length. The seedlings made from treated gametophytes had lower sporophyte density and this is probably the explanation of a higher number of seedlings with a length over 5 mm. This result was probably not directly related to the growth enhancer application but because of the lower density of the seedling.

The manual measurements of sporophytes on the twine is an accurate method but extremely time-consuming as the counting and length measurement needed to monitor the growth can take hours. However, the estimation of the seedlings growth was possible using image analysis. This method could be interesting to the seaweed industry because by these digital images the growth can be estimated within a few minutes, giving a green-light to deploy the seedlings on the sea.

A. esculenta and *S. latissima* showed similar quantities of phlorotannins. From the qualitative analysis only two forms of phloroglucinol (PG and DHPG) were found in the species. The correlation between these two analysis showed a clear cluster by species, which means the

phlorotannins composition were different between them. The most dominant phlorotannin in *S. latissima* was DHPG, whereas for *A. esculenta* a most dominant phlorotannin was not identified.

The content and composition of phlorotannins of *A. esculenta* sporophytes were independent from the seeding density and the growth enhancer addition, except for seedling B where no apparent conclusion could be drawn. The content of phlorotannins and the identified compounds were also dependent on the incubation period, increasing with the time. The phlorotannins content of *S. latissima* sporophytes showed high variance within locations and between different ages. This was most pronounced for one location. The increase of phlorotannins content was accompanied with the increase of DHPG and the decrease of PG, appearing that the PG was being converted into DHPG.

The phlorotannins content on the gametophyte cultures increased during fertility induction, especially in the cultures with addition of growth enhancer. The production of phlorotannin thus showed to be dependent of the white light exposure and to be stimulated by growth enhancer addition. The qualitative analysis shows that the PG and DHPG decreased with this induction. The PG and DHPG seemed to be polymerized in other phlorotannins, that not were identified in this study.

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Appendix I

Provasoli Enriched Seawater Medium (PES)

Version in Harrison and Berges (Harrison and Berges, 2005)

The Enrichment Stock Solution was prepared in a volumetric flask with 500 mL of distilled water. The remaining ingredients were added in the order shown (Supplemental table 1). All components were dissolved with a magnetic stirrer and with a little heat. Distilled water was added to bring the final volume to 1 liter. The solution was pasteurized, stored in a sterile glass bottle and kept refrigerated.

Supplemental table 1. Components of the Enrichment Stock Solution

Component	Stock solution [g L^{-1} dH_2O]	Quantity Used	Concentration in Final Medium (M)
Tris Base	-	5 g	8.26×10^{-4}
$NaNO_3$	-	3.5 g	8.24×10^{-4}
Na_2 β-glycerophosphate $\cdot 5H_2O$	-	0.7 g	4.63×10^{-5}
Iron-EDTA solution	See following recipe	250 mL	-
Trace Metals solution	See following recipe	25 mL	-
Thiamine · HCl (vitamin B1)	-	0.5 mg	2.96×10^{-8}
Biotin (vitamin H)	0.005	1 mL	4.09×10^{-10}
Cyanocobalamin (vitamin B12)	0.010	1 mL	1.48×10^{-10}

Iron-EDTA solution was prepared in 900 mL of distilled water and the components were dissolved in the same order as shown (Supplemental table 2). Distilled water was added to bring the final volume to 1 liter. The solution was pasteurized, stored in a sterile glass bottle and kept refrigerated.

Supplemental table 2. Components of the Iron-EDTA solution

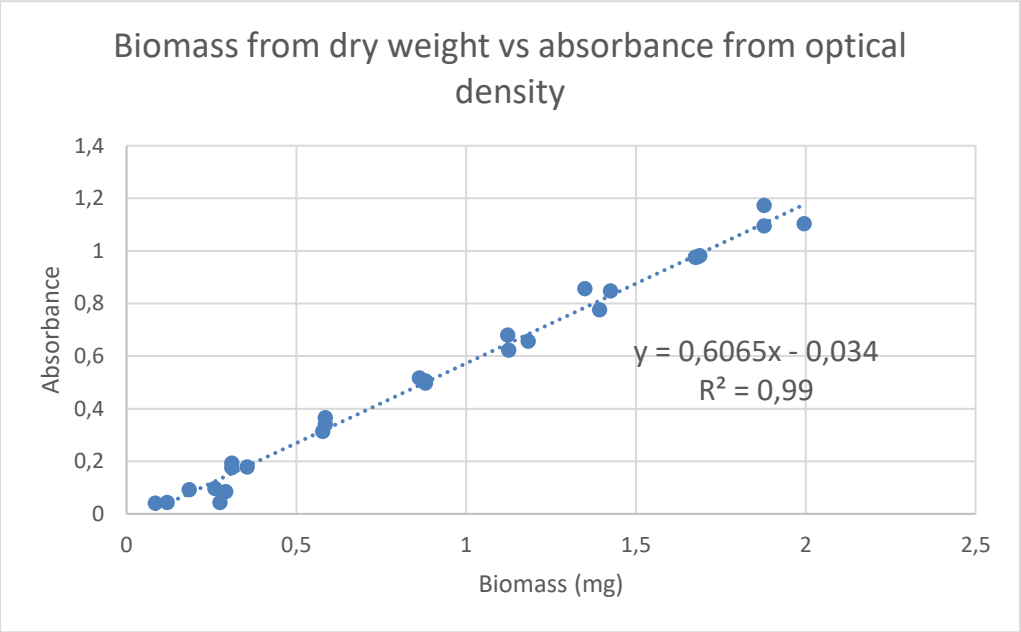
Component	Stock solution [g L^{-1} dH_2O]	Quantity Used (g)	Concentration in Final Medium (M)
$Na_2EDTA \cdot 2H_2O$	-	0.841	1.13×10^{-5}
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	-	0.702	1.13×10^{-5}

The Trace Metals Solution was prepared by adding 900 ml distilled water to a volumetric flask. The EDTA was dissolved and then the remaining ingredients were added in the order shown (Supplemental table 3). The final volume was brought to 1L by distilled water. The solution was pasteurized, stored in a sterile glass bottle and kept refrigerated.

Supplemental table 3. Components of the trace metals solutions

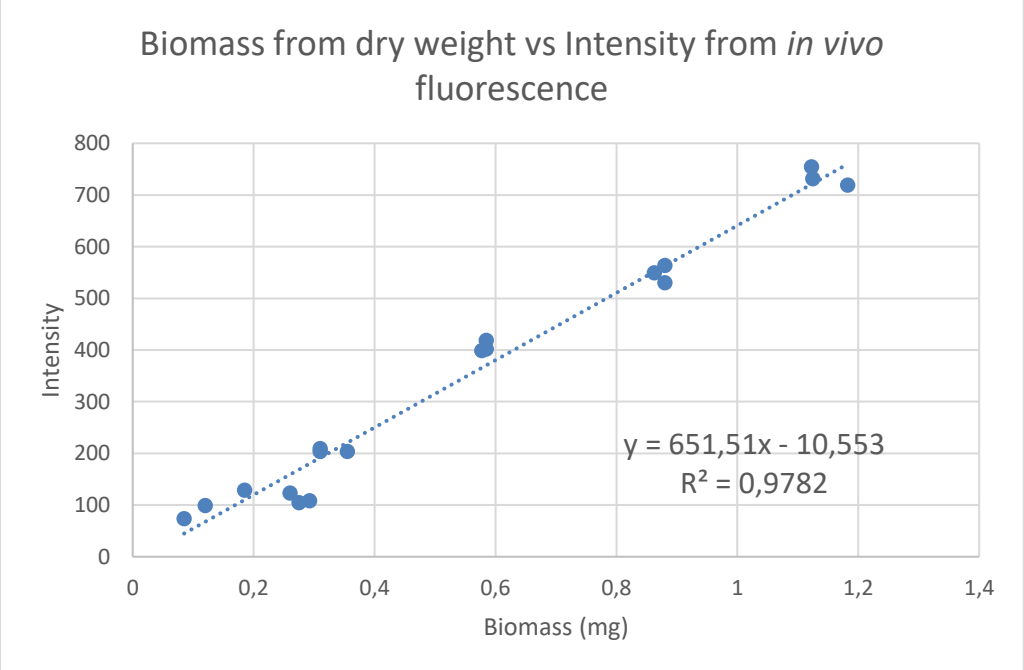
Component	Stock solution [g L^{-1} dH_2O]	Quantity Used (g)	Concentration in Final Medium (M)
$Na_2EDTA \cdot 2H_2O$	-	12.74	1.71×10^{-4}
$FeCl_3 \cdot 6H_2O$	-	0.484	8.95×10^{-6}
$MnSO_4 \cdot 4H_2O$	-	1.624	3.64×10^{-5}
$ZnSO_4 \cdot 7H_2O$	-	0.220	3.82×10^{-6}
$CoSO_4 \cdot 7H_2O$	-	0.048	8.48×10^{-7}

Appendix II



Supplemental figure 1. Standard curve for optical density

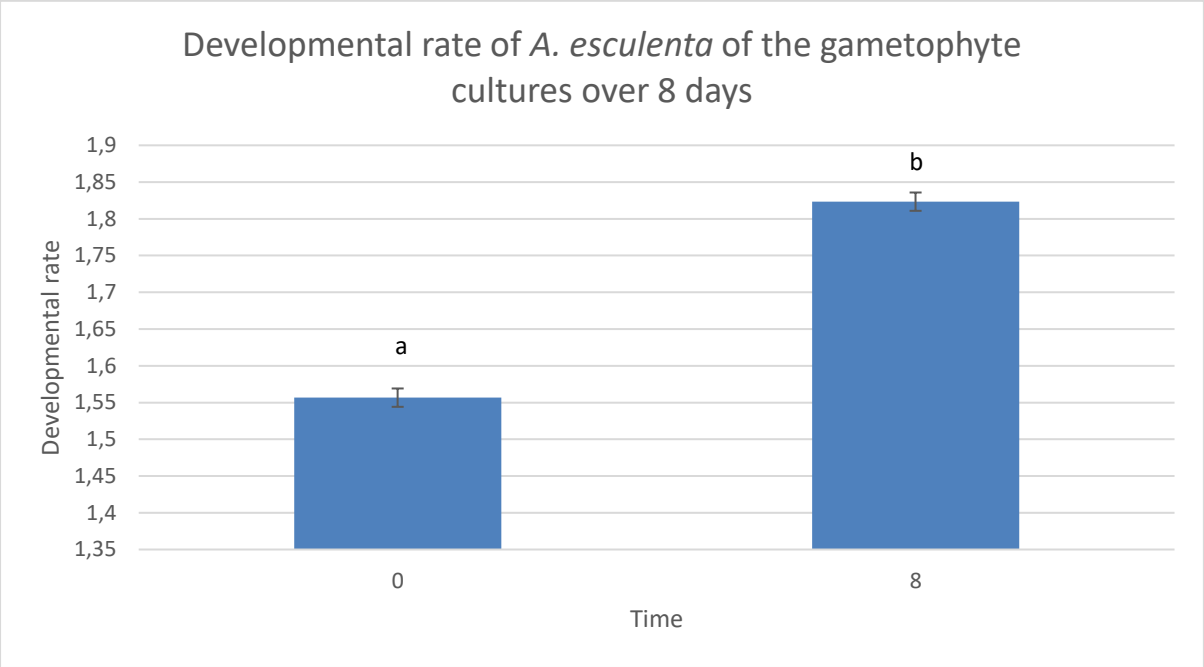
Supplemental figure 2. Standard curve for in vivo fluorescenceSupplemental figure 3. Standard curve for optical density



Supplemental figure 4. Standard curve for in vivo fluorescence

Supplemental figure 5. Standard curve for in vivo fluorescence

Appendix III



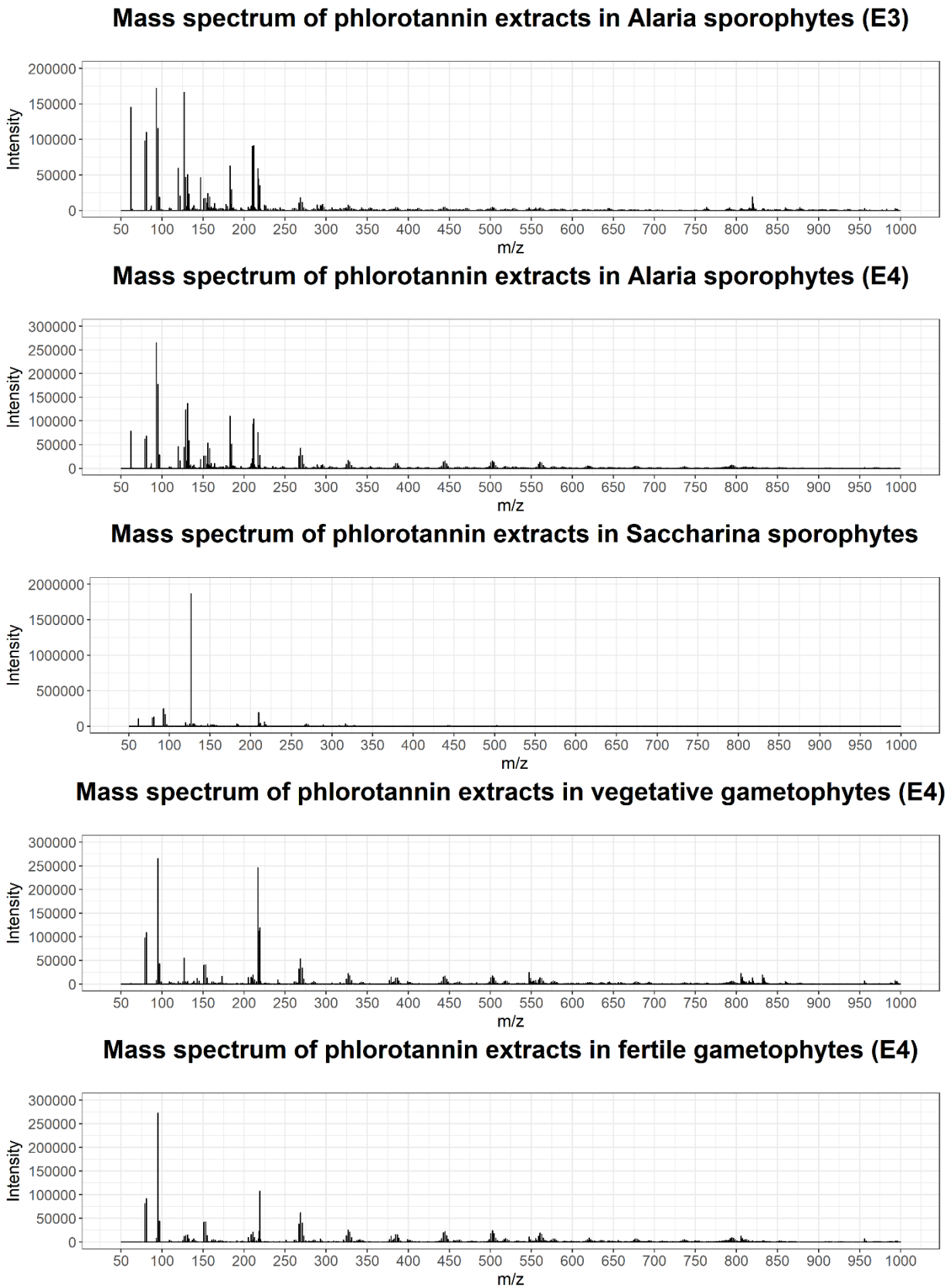
Supplemental figure 7. Developmental rate of *A. esculenta* of the gametophyte cultures C:0mg/L under the 23:1 photoperiod regime over 8 days. Values are means ± SD (n=3). Similar letters denote no statistical significant differences between treatments and days.

Supplemental figure 8. Mass spectra of putative phlorotannins in *A. esculenta* and *S. latissima*Supplemental figure 9. Developmental rate of *A. esculenta* of the gametophyte cultures C:0mg/L under the 23:1 photoperiod regime over 8 days. Values are means ± SD (n=3). Similar letters denote no statistical significant differences between treatments and days.

Appendix IV

Supplemental table 4. Possible lipids identified in the lipid fraction

Mz-H	Lipid Name	Formula	Summary Name
227,2	Myristic acid	C14H28O2	Myristic acid
255,2	Octadecatetraenoic acid	C18H28O2	Octadecatetraenoic acid
548,3	PS(20:2(11Z,14Z)/0:0)	C26H48NO9P	PS(20:2)
671,4	PA(22:0/12:0)	C37H73O8P	PA(34:0)
766,5	PS(O-16:0/20:5(5Z,8Z,11Z,14Z,17Z))	C42H74NO9P	PS(O-36:5)
805,5	PG(17:1(9Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	C45H75O10P	PG(39:7)
806,5	PS(16:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	C44H74NO10P	PS(36:6)
807,5	PI(16:0/16:1(9Z))	C41H77O13P	PI(32:1)
817,5	PG(18:2(9Z,12Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	C46H75O10P	PG(40:8)
818,5	PS(17:1(9Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	C45H74NO10P	PS(39:7)
819,5	PI(13:0/20:2(11Z,14Z))	C42H77O13P	PI(33:2)
830,5	PS(18:4(6Z,9Z,12Z,15Z)/22:4(7Z,10Z,13Z,16Z))	C46H74NO10P	PS(40:8)
831,5	PI(14:0/20:3(8Z,11Z,14Z))	C43H77O13P	PI(34:3)
841,5	PG(20:4(5Z,8Z,11Z,14Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	C48H75O10P	PG(42:10)
843,5	PI(13:0/22:4(7Z,10Z,13Z,16Z))	C44H77O13P	PI(35:4)
855,5	PI(14:1(9Z)/22:4(7Z,10Z,13Z,16Z))	C45H77O13P	PI(36:5)
865,5	PG(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	C50H75O10P	PG(44:12)



Supplemental figure 10. Mass spectra of putative phlorotannins in *A. esculenta* and *S. latissima*

Supplemental figure 11. Mass spectra of putative phlorotannins in *A. esculenta* and *S. latissima*