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Optimal physical and chemical environment for vegetative gametophyte culture of *Saccharina latissima*

- with emphasis on nutrient composition and light quality

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Abstract

When cultivating *Saccharina latissima* the production of young sporophytes is a bottle-neck because of the seasonality of spore production. Establishing a vegetative gametophyte culture provides a constant supply of sporophytes, thereby enabling a year-round cultivation independent of the availability of natural spores. In the present study, it was desirable to establish optimal fysical and chemical environments for vegetative gametophyte cultures of *S. latissima* which can be used in large scale cultivation systems. This were examined by five different experiments evaluating the effect of nutrient composition and light quality on growth and development on *S. latissima* gametophytes. The two most commonly used media when cultivating laminaria; Provasoli's Enriched seawater (PES) and Guillard's f/2 medium (f/2), were examined. The nutrient compisiton were studied through an addition of nitrogen (N) and phosphorous (P) in different concentrations and ratios in steralized seawater (SSW). The growth hormones kinetin (KIN) and Indole-3-acetic acid (IAA) were added to PES in a concentration gradient and the seaweed extract AlgeaFert was added in different concentrations to half strength PES (PES/2) due to its assumed presence of several plant growth hormones. The growth of gametophytes under red LED lights preventing fertility and under white florescence lights was compared. White light contains blue wavelengths, thereby inducing fertility. Altering the nutrient ratio can be used to manipulate gametophyte cultures to grow vegetatively. The experiments conducted in white light therefore had a gradient of N:P ratios to evaluate the effect on fertility.

The experiments showed that the growth of *S. latissima* gametophytes were highly affected by the chemical composition of the medium it was grown in and it had a significantly higher growth in PES compared to f/2. An increased growth was strongly correlated by; the presence of chelating agents, an increased concentration of N and P and a low ratio between them, and the addition of a low concentration of seaweed extract. The present study demonstrated that an alternation of light quality from red LED light to warm-white fluorescence light gave a significantly higher growth of *S. latissima* gametophytes. Since none of the treatments in this study resulted in fertility it couldn't be concluded which treatments that inhibited fertilization.

Contents

1 INTRODUCTION

1.1 Norwegian and Global macroalgal aquaculture

Aquaculture is the fastest growing food production sector in the world (2012). In 2011 the wet weight of aquatic plants accounted for 25 % of the global aquaculture production and over 52 % of the marine production in aquaculture (FAO, 2011). The marine plant production is dominated by marine macroalgae, almost exclusively by *Saccharina japonica* (FAO, 2012). In the 2000s the aquatic algae aquaculture has had an annual increase of 7.4 % (FAO, 2012). Today the vast majority of seaweed are used for human consumption and for hydrocolloid production (Burton et al., 2009) and 99.5 % of the total aquaculture production of aquatic plants takes place in Asian countries (FAO, 2012).

Asian countries have a long tradition of seaweed utilization and the majority of their seaweed resources are cultivated (FAO, 2012). In contrast, the European macroalgae industry is based on the harvesting of natural stocks (Burton et al., 2009), with a decrease in production the last 10 years (Meland and Rebours, 2012). A further expansion of the seaweed industry in Europe are dependent on a stable access to farmed raw material and the development of more valuable products (Meland and Rebours, 2012). Cultivation of seaweeds enable improvement by genetic strain-selection and it provides a seaweed bed consisting of a single species in the same development stage, and thereby over-coming many of the disadvantages associated with the utilization of natural seaweed beds (Kain and Dawes, 1987). During the 1970s, many kelp farming systems were tested in the U.S. but failed because they were premature and faced to many challenges in the sea (Kraan, 2013). Modifications of long-line systems have been tested in Europe, showing that there is a potential for development of large-scale ocean cultivation of seaweed (Kraan, 2013).

In Norway seaweed has a long history used as fertilizer, feed and food and today the Alginate industry is by far the most important sector (Meland and Rebours, 2012). Seaweed aquaculture is under development, but still produces very small amounts of biomass (Meland and Rebours, 2012). The coastal water domain of Norway are large, uncontaminated and are relatively sheltered, which supplies very good areas for production

of food and cultivation of marine plant biomass (DKNVS-NTVA, 2012). Norway is today among the three largest fish and fishery product exporting nations in the world (FAO, 2004) and with 1 million tonnes fish produced in aquaculture annually, fish farming makes a big contribution to the national economy (FAO, 2004). However, during the last decade or so, there has been an increasing public recognition about the environmental impact of the aquaculture industry. This include a concern on coastal eutrophication caused by large fractions of organic and inorganic nutrients released from the farms into the surrounding waters (Troell et al., 1999). Seaweeds can act as a useful element in recycling of nitrates and phosphates (Kraan, 2013, John et al., 2011). Seaweeds can help to maintain a healthy coastal seawater environment if they are reared in a nearby locality to finfish production, as a component in so called Integrated Multi-trophic Aquaculture (IMTA) (Troell et al., 2009, Chen, 2004). To manage a successful seaweed cultivation, it is necessary with adequate amounts of nutrients (Handå et al., 2009). Therefore a combination with the already existing finfish-aquaculture in Norway can create a "win-win" situation where the seaweed utilizes the dissolved nutrients released from the farms (Handå et al., 2009, Troell et al., 2009).

1.2 Macroalgae biomass as a source to biofuel

Biomass is considered to be a suitable alternative source to fossil fuel (John et al., 2011, Nigam and Singh, 2011). Unlike ancient carbon unlocked by the burning of fossil fuels, the carbon in biofuels comes from the atmosphere, captured by plants during the growing season. This makes in theory the burning of biofuels carbon neutral. Currently the biomass comes from land-based crops in forms of sugar or starch crops (first generation biofuels) and lignocellulosic biomass (second generation biofuels) creating debates about "food versus fuel", fresh water and land use (Nigam and Singh, 2011). Marine biomass, such as macro algae, is an interesting source of biomass, serving as a third generation of biofuels (Nigam and Singh, 2011, John et al., 2011). Compared to terrestrial utilization, the oceans are barely applied and marine biomass can provide a high-yield source of biomass without compromising food supplies, forests, fresh water or cultivable land (Jensen, 1993, Lerat, 2011, DKNVS-NTVA, 2012).

Water, as compared to air and land-plants, enables nutrient absorption over the entire surface area of seaweeds and provides a supportive growth medium (Sze, 1993). Seaweeds require therefore less energy for production of supporting tissue, enhancing fast growth and high productivity, with a measured maximum productivity of 1.8 kg C m^{-2} yr^{-1}. This is comparable of a dense terrestrial forest and up to around five times higher than phytoplankton production (Chynoweth et al., 2001, Lüning and Pang, 2003, Adams et al., 2009). The scarcity of structural polymers in seaweeds, such as hemicellulose and lignin (found in higher plants), simplifies the process of ethanol production by eliminating the chemical and enzymatic pre-treatment steps to breakdown these biopolymers into fermentable sugars (John et al., 2011, Adams et al., 2009). Macroalgae are therefore capable of producing high yields of material when compared to the most productive land-based plants (John et al., 2011, Adams et al., 2009). Seaweed contains 80-85 % water, making it more convenient with microbial conversion (fermentation) than direct combustion or thermochemical conversion processes (Kraan, 2013). Normally 20-60 % of seaweed dry weight is carbohydrates which can be fermented to produce either biogas or alcohol-based fuels (Burton et al., 2009, Kraan, 2013, Handå et al., 2009). The most important carbohydrates present in seaweed are mannitol, laminaran and alginate (Handå et al., 2009, Adams et al., 2009, Jang et al., 2012). The composition of them varies throughout the year (Adams et al., 2009), with the maximum composition of the storage compounds, mannitol and laminaran, in late-summer and autumn and in the spring the reverse is true and there are a high content of alginate (Adams et al., 2009, Bartsch and al., 2008). The structural component, alginate, has a quite complex structure (Lobban and Harrison, 1994) that pose a challenge in fermentation (Handå et al., 2009, Tang et al., 2009). Laminarin and mannitol are more easily fermentable, therefore the seaweeds are harvested preferably during the autumn (Lobban and Harrison, 1994, Handå et al., 2009). It is of high importance to derive an efficient method to ferment alginate in order to get a year-round production (Handå et al., 2009). A year-round supply of biomass is of utmost importance to be able to meet the fuel demand (John et al., 2011, Forbord et al., 2012).

The production of seaweeds has to be made cost-efficient to compete with oil prices. Production of fuel from algal biomass can be made economically feasible if valuable co-products can be produced during the fuel generation process (Kraan, 2013, Reith et al.,

2005, John et al., 2011). This can be done by producing high-cost products; such as human foods, fertilizers, cosmetics, and the extraction of other industrial gums and chemicals and using the residues for energy production (Kraan, 2013).

1.3 Experimental organism: *Saccharina latissima*

In the northern hemisphere, the genus Laminaria is one of the most important macroalgae genera of the order Laminariales (kelp) found from the temperate to polar rocky coastal ecosystems (Bartsch and al., 2008). From winter to summer, they are growing fast, and from summer to winter, they are growing slow. This is due to accumulation of carbohydrates, available as energy when the light irradiance is low (Handå et al., 2009, Forbord et al., 2012). *Saccharina latissima* (former *Laminaria saccharina*) has a high content of carbohydrates and are one of the fastest growing species in European waters. The specie appears to be very well suited for large-scale cultivation (Handå et al., 2009, Kraan, 2013, John et al., 2011).

S. latissima (sugar kelp) is growing in protected areas, with a distribution from the sub littoral fringe to 30 meters depth (Handå et al., 2009). It has a wide circumpolar distribution in the northern hemisphere and in Europe it grows from Svalbard to Portugal at temperatures below 19° C with an average life span of 2-4 years (Forbord et al., 2012). A wide range of species inhabits the dense *S. latissima* forests and it therefore has an important ecological function (Bekkby and Moy, 2011). Since 2006 *S. latissima* has been red-listed by The Norwegian Biodiversity Information Centre, because of weakened stocks along the south- and west coast of Norway (Artsdatabanken, 2006, Handå et al., 2009).

S. latissima has a heteromorphic life-cycle (Figure 1), typical to the order Laminariales. It consists of a macroscopic, diploid, sporophyte phase and a microscopic haploid, gametophyte phase (Kain, 1979). Special cells in the adult sporophyte plant produce sporangias, zoospore producing cells. Dense areas of sporangia are called sori and are produced on plants in Norwegian waters usually from October to December (Forbord et al., 2012). In the late autumn and winter the seaweed disperse free-swimming zoospores produced by meiosis. After a period in the water column they settle on a suitable substrate were they germinate into a multicellular gametophyte stage. Three stages of gametophyte

development has been recognized; starting with the germination of the spore to form the gametophyte, thereafter vegetative growth of the gametophyte occurs to form either a male or female gametophyte. The last part is the reproductive phase where they develop either female reproductive structures (oogonia) or male reproductive structures (antheridia) (Kain, 1979). The reproductive phase is induced by the right biotic and abiotic conditions, if not, vegetative growth continues and form profusely-branched, filamentous plants (Cuijuan et al., 2005, Lüning, 1980). The formation of filamentous gametophytes might be expected at depths or under algal canopy that alters the composition of the light and nutrients. This strategy enables survival and growth under suboptimum conditions, awaiting more favorable conditions (Izquierdo et al., 2002). In nature fertility occurs during the spring months. Under optimal conditions female gametophytes of the northeast Atlantic Laminaria species become fertile in less than two weeks (Kain, 1979). When the fertilization has taken place a zygote is developed and this develops into a new sporophyte (Edwards and Watson, 2011, Handå et al., 2009).

Due to the small size of Laminarian gametophytes, most studies have been conducted in the laboratory (Bartsch and al., 2008, Kain, 1979). In the sea the dominant stage of the gametophytes seem to be a unicellular stage in female gametophytes and a few-celled stage in male gametophytes (Lüning and Neushul, 1978, Lüning, 1980). This is also the morphology expected under optimal conditions, and multiple-celled female gametophytes are believed to be an indication of non-optimal conditions (Izquierdo et al., 2002, Lüning and Neushul, 1978, Kain, 1979). Vegetative and reproductive development are antagonistic pathways, so when fertilization is induced in all cells, vegetative growth of the gametophyte stops (Lüning and Neushul, 1978). The optimal conditions for fertility, i.e. when the necessary substances are present and the inhibitory substances are absent, can be disturbed by the presence/absence of a single physical or nutritional factor (i.e. light temperature, trace metal etc.) (Kain, 1979, Cuijuan et al., 2005). Any cell in a gametophyte has the potential to develop reproductive structures and vegetative growth therefore increases the gametophytes fecundity, creating a sort of vegetative production (Kain, 1979). The sexual viability of vegetative gametophytes can be retained after a long time of cultivation in non-fertilization conditions, enabling seedlings to be cultured directly from expanded gametophytes (Druehl et al., 2005).

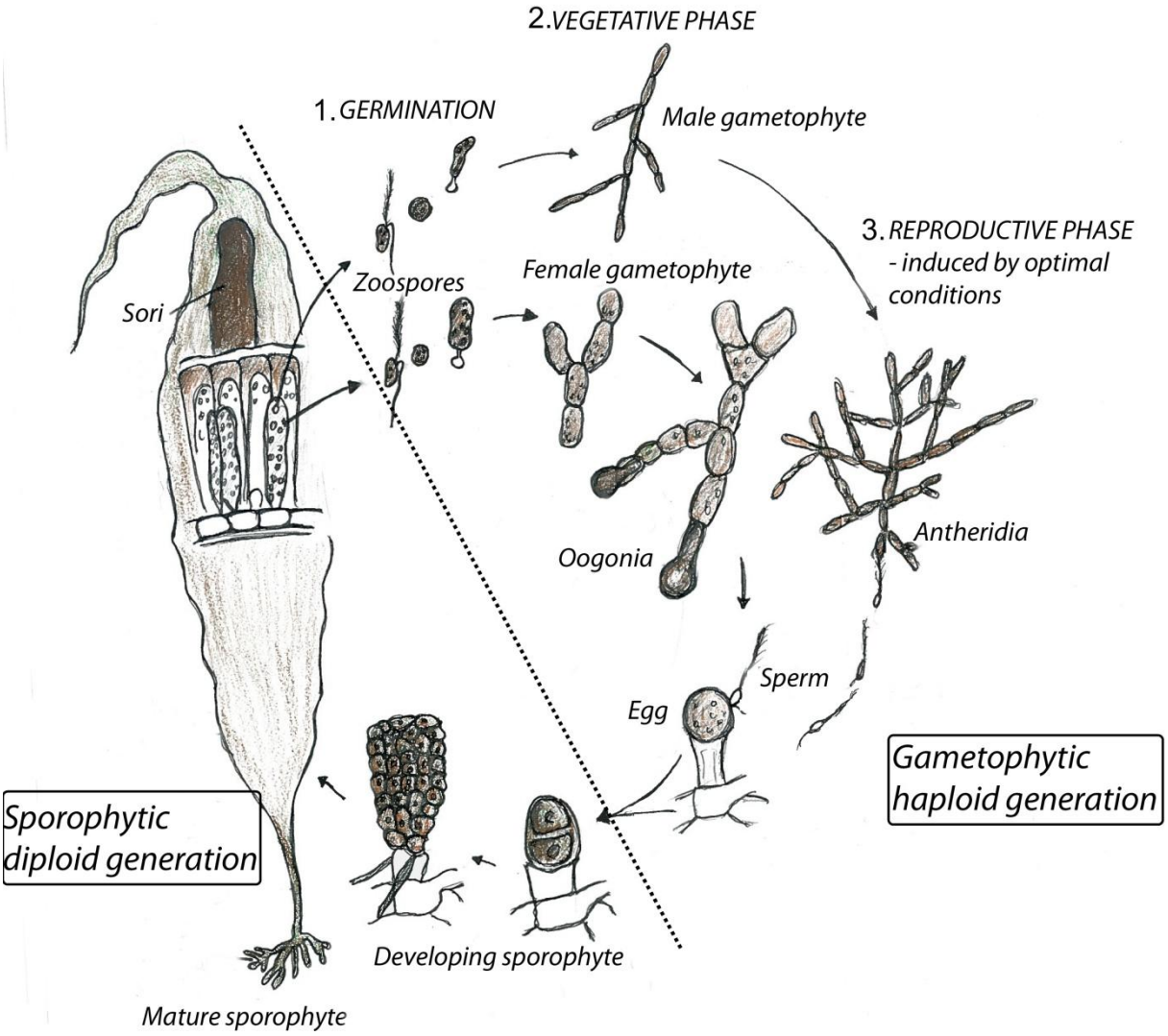

Figure 1 The heteromorphic life cycle of *Saccharina latissima* consisting of the macroscopic sporophyte phase and the three stages of microscopic gametophytes. Mature sporophytes develop sori with large numbers of haploid zoospores, which are released into the waters and settle on a substrate where they germinate into female and male gametophytes. The reproductive phase is initiated by optimal abiotic and biotic conditions and the gametophytes produce oogonia and antheridia that develop eggs and sperm. A fertilized zygote develops into a diploid sporophyte.

The cultivation of the genus Laminaria are usually a four step process; collection and settlement of zoospores onto seed strings (long-lines), production of seedlings, transplantations and outgrowing of seedlings and harvesting of grown out plants (Sahoo and Yarish, 2005). For a successful large-scale cultivation of seaweed, today's cultivation and

harvesting technologies need to be optimized to reduce cost and energy demands (Troell et al., 2009, John et al., 2011, Forbord et al., 2012). Controlling all stages in the life-cycle of *S. latissima* is essential, with a constant supply of healthy sporelings as a key to success for commercial cultivation (Wu et al., 2004, Xu et al., 2008, Kraan, 2013, Li et al., 1999). This can be done by a controlled production of zoospores; either aided by the induction of sporophytes or by the maintenance of a permanent filamentous gametophyte-stage kept in a culture (Handå et al., 2009, Forbord et al., 2012, Xu et al., 2008, Zhang et al., 2008). In Asian countries sporeling production has mostly depended upon naturally occurring sori and thereby been restricted by seasons. More recent developments have led to a focus towards establishment of vegetative gametophyte stocks (Xu et al., 2008, Zhang et al., 2008). A culture of gametophytes provides a major progress in the cultivation of seaweed, and it may reduce the sporeling raising times by up to 50 %, with an accompanied reduction in production costs (Sahoo and Yarish, 2005, Li et al., 1999). Gametophyte cultures can be established and maintained under unialgal conditions diminishing biotic contaminants (Westermeier et al., 2006). An onset of fertility will then provide kelp sporelings attached to culture ropes, which can be transferred from the land-based kelp hatcheries to the sea throughout the whole year independent of the availability of natural spores (Forbord et al., 2012, Westermeier et al., 2006).

1.4 Physical and nutritional experimental conditions

1.4.1 Light

Research has revealed an important role of blue light in the life cycle of laminariales and its presence is necessary in gametophyte reproduction (Lüning and Dring, 1972, Lüning and Dring, 1975, Cuijuan et al., 2005, Lüning, 1981). After culturing in the absence of blue light exposure of blue light for only a short time (6 hours or less) is necessary to induce fertility. It takes 5-6 days before any visible eggs can be seen, suggesting that the blue light is specifically affecting the reproductive development as a photomorphogenetic response, rather than acting through its effect on the photosynthetic system (Lüning and Dring, 1972, Lüning and Dring, 1975). The mechanism for blue-light induced fertilization remains unclear (Cuijuan et al., 2005)

Growth and development of the genus Laminaria are greatly influenced by the quality and intensity of light (Lüning and Dring, 1972, Hsiao and Druehl, 1971, Cuijuan et al., 2005, Lüning and Dring, 1975). Photosynthesis varies with the spectral composition of light, depending on the pigment composition of the plant. Pigments discriminate between different wavelengths and light with different spectral composition (quality) will therefore yield different growth responses (Sakshaug et al., 2009, MacIntyre and Cullen, 2005). Sublittoral marine algae like *S. latissima* are adopted to the greenish or bluish color of the underwater light due to their accessory pigments, like fucoxanthin, which absorb green and blue-green wavelengths more efficiently than the chlorophylls (Dring, 1981, Dring, 1986). Therefore it can be expected to show a better growth in light containing blue wavelength band (Wang et al., 2010). Research has shown that blue wavelengths stimulate photosynthesis in Laminaria and thereby giving a higher growth rate of the gametophytes (Wang et al., 2010, Lüning and Dring, 1972, Forster and Dring, 1992, Schmid and Dring, 1996, Dring et al., 1994, Cuijuan et al., 2005).

1.4.2 Growth medium

The increase in mass is a consequence of metabolic pathways that construct molecules from smaller units, so called anabolism. For anabolism to occur, a cell must be situated in an environment that supplies all resources needed. More than 50 known elements and a large number of organic compounds make up natural seawater (Harrison and Berges, 2005). When using seawater as a base for algal culturing, it often becomes necessary to enrich nutrients and trace metals to increase the yield of algae when grown for culture maintenance or laboratory experiments (Harrison and Berges, 2005). The enrichments made are usually essential macronutrient elements (carbon, nitrogen, phosphorous), major ions (Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, and SO_4^{2-}), micronutrient metals (iron, zinc, cobalt, and molybdenum), and vitamins (vitamin B_{12}, thiamine and biotin) (Harrison and Berges, 2005). The chemicals are added in different amounts and forms in the different media, but most recipes have basic similarities. Comparisons between them are complicated because of many factors (Harrison and Berges, 2005). Among the many media, there are two that dominate citations when working with seaweed; Provasoli's Enriched Seawater medium (PES), and Guillard's f/2 medium (f/2). The media have evolved over time and can also

change substantially between different publications. Because many media work well enough for growing algae, few have studied which media that are best suited for their specific undertaking (Harrison and Berges, 2005).

Chelates

Trace elements play a crucial role in many metabolic pathways, and without them the algae have problems to utilize light, nitrogen, phosphorous and CO_2 (Sunda et al., 2005), but at too high concentrations many essential trace metals are toxic (Rich and Morel, 1990). The metal ion concentrations in biological systems are controlled by the use of chelates. Chelating agents occur naturally, such as humic substances and amino acids, but can also be added to the solution through soil extracts, or as a synthetic chelating agent, such as ethylenediaminetetraacetate (EDTA). Extracted seaweeds are a source of organic matter, such as polyphenols, working as a chelating agent (Ragan et al., 1980). Metal ions and other essential micronutrients are bound by the chelating agent, thereby remaining in the solution, but with a reduced reactivity (Manahan, 2004). When the metals are in a metal chelating form they are not susceptible to formation of insoluble precipitates nor are they directly available for cellular uptake by phytoplankton. The non-chelated forms of the metal, including free ions and inorganic metal complexes, are available to phytoplankton. When these forms are removed due to algal uptake, they are replaced by dissociation of an equivalent concentration of the metal chelate. Through this mechanism the chelating complex is serving as a buffer system and therefore helps to regulate the availability of metal ions in a culture. The availability of chelating agents may be the factor determining algal growth (Sunda et al., 2005, Gerringa et al., 2000, Manahan, 2004). The growth of some algae may be limited by insufficiency or toxicity of certain trace metal ions at ion activities occurring naturally in sea water (Sunda et al., 2005), changes in the ion-chelator balance could be of immediate ecological significance.

Nutrients

The Redfield ratio is a concept referring to the relationship between an organism composition and water chemistry in form of Carbon : Nitrogen : Phosphorous (C:N:P) ratio (Geider and La Roche, 2002). Biologists use a molar C:N:P ratio of 106:16:1 as the average elemental composition of marine organisms, the same ratio that are usually found naturally in the deep seawater. The conventional interpretation of the Redfield stoichiometry is that the upper and lower limit for marine algae is tightly controlled by these values and it is used as a standard to differentiate between nitrogen (N)-limitation and phosphorous (P)-limitation in organisms (Geider and La Roche, 2002). In plants the N:P ratio of the plant tissue is strongly correlate with the N:P supply ratio, and the N and P content in plant tissue is mainly determined by the supply of N and P and plant physical properties (Koerselman and Meuleman, 1996). It is therefore the ratio between the nutrients supplied that decides which nutrient that become limiting, not the absolute content in plant tissue (Koerselman and Meuleman, 1996). Studies of the optimal C:N:P ratio for growth has shown that the biochemically fixed C:N:P ratio vary markedly from the defined Redfield ratio (Geider and La Roche, 2002), and that bethic macroalgae show a great range of C:N:P (Atkinson and Smith, 1983). However, if this is consistent also for gametophytes can be questioned, since little attention has been paid to the physiological differences between gametophytes and sporophytes (Wang et al., 2012, Forbord et al., 2012). It has been shown that gametophytes of *Saccharina japonica* have a significantly higher phosphorous uptake than sporophytes (Wang et al., 2012), and their optimum N:P ratio might therefore differ. Due to the small size of gametophytes they have a low area-to-volume ratio and might be similar to phytoplankton when it comes to N:P ratio and uptake rates. The average N:P found in phytoplankton biomass under nutrient replete conditions is found to be well below the Redfield ratio of 16N:1P (Geider and La Roche, 2002). In nature, a nutrient ratio of *S. latissima* of 6.8 has been documented (Komfeldt, 1982) and the optimum for absorbing nutrients from seawater by *Laminaria japonica* was found to be 7.4, when this was the lowest N:P examined (Xu et al., 2011).

As mentioned earlier, there is a great complexity of what factors trigger fertility of the gametophytes, and one factor is the medium used for cultivation, principally the nutrients (Kain, 1979). The development of gametophytes are affected by the interacting effect of

different amounts of nitrate and phosphate (Hoffmann et al., 1984, Cosson and Gayral, 1977). Altering the nutrient ratio can be used to manipulate gametophyte cultures to grow vegetatively and the technique is emerging in Asia (Xu et al., 2009b, Shao-Jun and Chao-Yuan, 1996). The method of changing the nitrogen supply are already widely used when culturing microalgae, where a reduction of available nitrogen promotes gametogenesis and an increase in nitrogen repress gametic activity (Andersen, 2005). The effect on the metabolic pathways is not understood (Andersen, 2005).

Research has been done on different species in the order laminariales, yielding contradictory results. Both Harries (1932) and Cosson and Gayral (1977) found that a decrease in nitrogen supply decreased the amount of reproductive organs in gametophyte cultures, whereas Hoffmann (Hoffmann and Santelices, 1982) and Hsiao (Hsiao and Druehl, 1973) found, quite opposite, that an increase of nitrogen decreased the amount of reproductive organs in the gametophyte cultures.

These findings are interesting to further investigate because of the presumed higher growth in white light compared to red light conditions. In addition cultivation in white light might reduce the costs for biomass production and also make the cultivation-process less complicated.

Hormones

In higher plants phytohormones (growth regulators) help to maintain or increase the growth of sensitive tissue (Evans and Trewavas, 1991). Essentially all known phytohormones of higher plants (abscisic acid, auxins, cytokinins etc.) are present in algae (Tarakhovskaya et al., 2007, Stirk et al., 2003, Kingman and Moore, 1982), suggesting that they play a physiological role in the regulation of growth and development in algae as they are known to do in higher plants. The knowledge of the algal hormone system is very limited and few studies have been done to investigate the physiological responses in algae of phytohormones, and most of what has been done has yielded inconclusive results (Stirk et al., 2003, Tarakhovskaya et al., 2007, Khan et al., 2009). Bradley showed that the addition of some exogenous plant hormones affected the growth and development in some seaweeds (Bradley, 1991). The addition of a single hormone might not give any enhanced growth or development of algae.

Several studies (Provasoli, 1958, De-lin et al., 1995, Bradley and Cheney, 1990, Khan et al., 2009) conducted with the use of more than one exogenous hormone showed an improved growth of algae, likely exhibiting a synergetic activity (Khan et al., 2009). Two important phytohormone classes are auxin and cytokinin. Indole-3-acetic acid (IAA) is a natural auxin. IAA is a plant signaling molecule involved in plant organogenesis and growth control. One of its primary effects in higher plants is the activation of the plasma membrane H+-ATPase, affecting the growth by elongation of the cells, due to an economic and rapid usage of basic resources as light energy and mineral nutrients (Tarakhovskaya et al., 2007). Cytokinins are associated with nutrient partitioning and nutrient mobilization in plants (Khan et al., 2009) and are known to activate cell-division and stimulates photosynthetic processes (Tarakhovskaya et al., 2007). Kinetin (6-furfurylaminopurine) is an adenine-type cytokinin phytohormone. The effect of exogenous kinetin on algae primarily seem to be the indirect stimulation of cell division (Burkiewicz, 1987, Wood and Braun, 1967).

The use of seaweed extract as a biostimulant in agriculture has a long history. The enhanced growth and development are most likely, but not fully exploited, due to presence of several plant growth substances, in particular the plant hormones and their presumed synergetic effect (Crouch and Van Staden, 1993, Khan et al., 2009). The effect is believed to come from the content of both cytokinins and auxins and some other PGRs such as Abscisic acid (ABA) (Khan et al., 2009). This might require revision due to modern technology with new evidence that larger molecules, such as oligomers and polysaccharide elicitors can be biologically potent (Craigie, 2010). Seaweed extract may change the hormonal balance in the plants, favoring cytokinins and auxin and thereby enabling continuation of antioxidant production when stress occurs (Wood and Braun, 1967), making the plants more tolerant to abiotic and biotic stress. Seaweed extract might show similar plant growth stimulating effects in seaweed production as it is in higher plants. This is not well-studied, but the few experiments done have shown that seaweed extracts also enhances growth rate and development in seaweed sporophytes (Hurtado et al., Robertson-Andersson et al., 2006). As far as I am aware no articles have been published on the effect of seaweed extracts on seaweed gametophytes.

1.4.3 Exchange rate of media

When growing algae continuously in a medium the constituents of the medium will change; major and trace nutrients are taken up by the algae, various compounds are released by the algae into the medium and some redox reactions are catalysed on the surface of the algae (Sunda et al., 2005). These changes may alter the availability of metal-ion concentrations, change the pH and alter the trace metal uptake. Thus, time play a major role on the composition of the media. These changes are dependent on the cell concentration in a culture. Thus in a culture with a higher cell-concentration the medium will be altered faster (Sunda et al., 2005). Gametophyte growth might therefore be highly affected by the exchange-rate of the medium, at the same time as a more frequent exchange rate increases the labour-intensity and thereby also the production costs.

1.5 Measuring productivity

The usual criteria for algal performance in a culture are growth and reproduction. Detection of reproduction is apparent, while measurements of growth may not be easy. Growth measurements are dependent on construction and mode of growth and the method of determining growth depends on the particular study (Chapman, 1973). Growth is the addition of organic material which is the outcome of all growth processes; anabolic and catabolic (Hansmann, 1973, Sorokin, 1973). Measurements of growth can be done qualitatively or quantitatively. A qualitative measurement says something about the growth rate of individual cells and measures the quality of a system. Quantitative measurements reflect the activity of a unit of cell mass (Sorokin, 1973).

Quantitative analyses using the whole or parts of the suspension can be done by measuring wet weight, sedimentation (packed cell volume), optical density (OD) or dry weight. Wet weight, sedimentation techniques or OD can be used during an experiment, keeping the cells alive (Guillard, 1973). Wet weight has been used to monitor growth of gametophytes when cultivating *Laminaria* (Zhang et al., 2008, Xu et al., 2009a), all using big volumes of gametophytes, thereby minimizing the impact of water. Sedimentation is time-consuming and both whole-sample techniques are quite messy, especially when dealing with many

replicates. For parts of the suspension; OD, packed cell volume (PVC) or dry weight are suitable measuring techniques. Dry weight has been used by Gao et al. (2005) to measure growth of *Laminaria* gametophytes. According to Sorokin (1973) OD/turbidity measurements are particularly suitable for determinations of growth rates. This because it is an effective method that has the possibility to take repeated readings of the same batch, without killing and with minimal disturbance of the cells. OD measures the amount of light absorbed by a culture (or other suspension) with the use of a spectrophotometer at a given wavelength. Pigments can be used for this and chlorophyll*a* is the most accepted which is a common measure to express growth rates of algal populations in term of the rate of photosynthesis and the chlorophyll*a* content (Hansmann, 1973). There are a correlation between chlorophyll*a* concentrations and cell numbers (Hansmann, 1973).

Filament length and counting cell number can be used to estimate the growth in cultures with filamentous types of algae, such as *S. latissima* gametophytes. However, with larger filaments with many cells, it is not possible to count the high cell numbers involved (Chapman, 1973). Growth of an algal culture does not necessarily imply cell division, but cell division usually accompanies it (Hansmann, 1973). Accumulation of nitrogen, carbon, protein or by measuring the products of cell metabolism, and plant pigments are other indices of growth that can be measured (Chapman, 1973).

1.6 Study aims and approach

The aim of this study was to establish an optimal cultivation method for vegetative growth of *S. latissima* gametophytes, from spores to large filamentous gametophytes. Growth and over-all fitness and fertility of *S. latissima* gametophytes were evaluated with the intention to present a culture method suitable for a large-scale production of *S. latissima*. An up-scaled supply with a high biomass of filamentous gametophytes will enable a year-round supply of seedlings that can be seeded in the sea at any time of the year.

Three sub-objectives were phrased:

- Evaluate the growth potential of *S. latissima* gametophytes in various conditions, primarily in regard to nutritional compositions of the growth medium and light quality, or more specifically; nutrient composition, chelating agents, growth hormones, seaweed extract, exchange-rate and light quality were examined.
- Evaluate the conditions giving vegetative (filamentous) growth as opposed to fertility of *S. latissima* gametophytes in relation to light quality and N:P ratios.
- Evaluate various growth-measuring methods to use as an estimate of productivity for *S. latissima* gametophytes in the various culture conditions.

Five different experiments were conducted to study the variance within these variables.

2 MATERIALS AND METHODS

2.1 Experiments conducted

Five experiments were conducted from February 2012 to March 2013 in order to characterize the effect of several variables on growth and fertility of *Saccharina latissima* gametophytes.

The aim of the first three experiments was to find an optimal medium composition for *S. latissima* gametophytes. This was examined by altering the medium composition and addition of hormones. They were carried out in red light conditions to prevent fertilization. The first three experiments were:

i. Comparing cultivation media; Provasoli's Enriched Seawater medium (PES) and Guillard's f/2 medium (f/2). The experiment was performed during a twelve week period to find out which of the two most commonly used media that gave the best growth of *S. latissima* gametophytes. These were compared to the growth in sterilized seawater (SSW).
ii. Evaluating the effect of growth hormones. The growth hormones kinetin (KIN) and Indole-3-acetic acid (IAA) were added in gradients to the most growth enhancing medium (from the above experiment); PES. The aim of the experiment was to see whether these growth hormones would further increase growth of *S. latissima* gametophytes compared to PES.
iii. Evaluating the effect of seaweed extracts. The seaweed extract AlgeaFert, more commonly used in agriculture, was added to half strength PES (PES/2) in different concentrations. This was done for the same reason as the hormone-experiment, but it gave a more multifaceted answer due to the more complex composition of hormones, nutrients and other constituents in AlgeaFert.

The aim of the last two experiments was to examine altered light quality and nutrient compositions in relation to growth and fertility. The reason for this was to try alternative cultivation methods; giving better growth and being more cost-efficient. They were carried out in red and white light conditions. The last two experiments were:

iv. Testing a gradient of nitrogen (N) and phosphorous (P) in the growth media (referred to as Experiment iv.). The nutrients N and P were added in different ratios to SSW to investigate growth and fertility. In addition PES with iodide added (PESI), and inorganic phosphorous were tested. The groups were compared to the growth in PES and SSW.

v. Evaluating the effect EDTA together with an N:P gradient (referred to as Experiment v.). This represented a variation of the fourth experiment, using Enriched Seawater medium (ESW) with nitrate, phosphate and EDTA added to SSW. ESW with double nitrogen concentration and ESW with double phosphorous concentration were used to examine growth and fertility of the gametophytes. The groups were compared to PES and SSW.

Figure 2 presents an overview of the five experiments conducted, with the roman numbers showing the correspondence to the sections above.

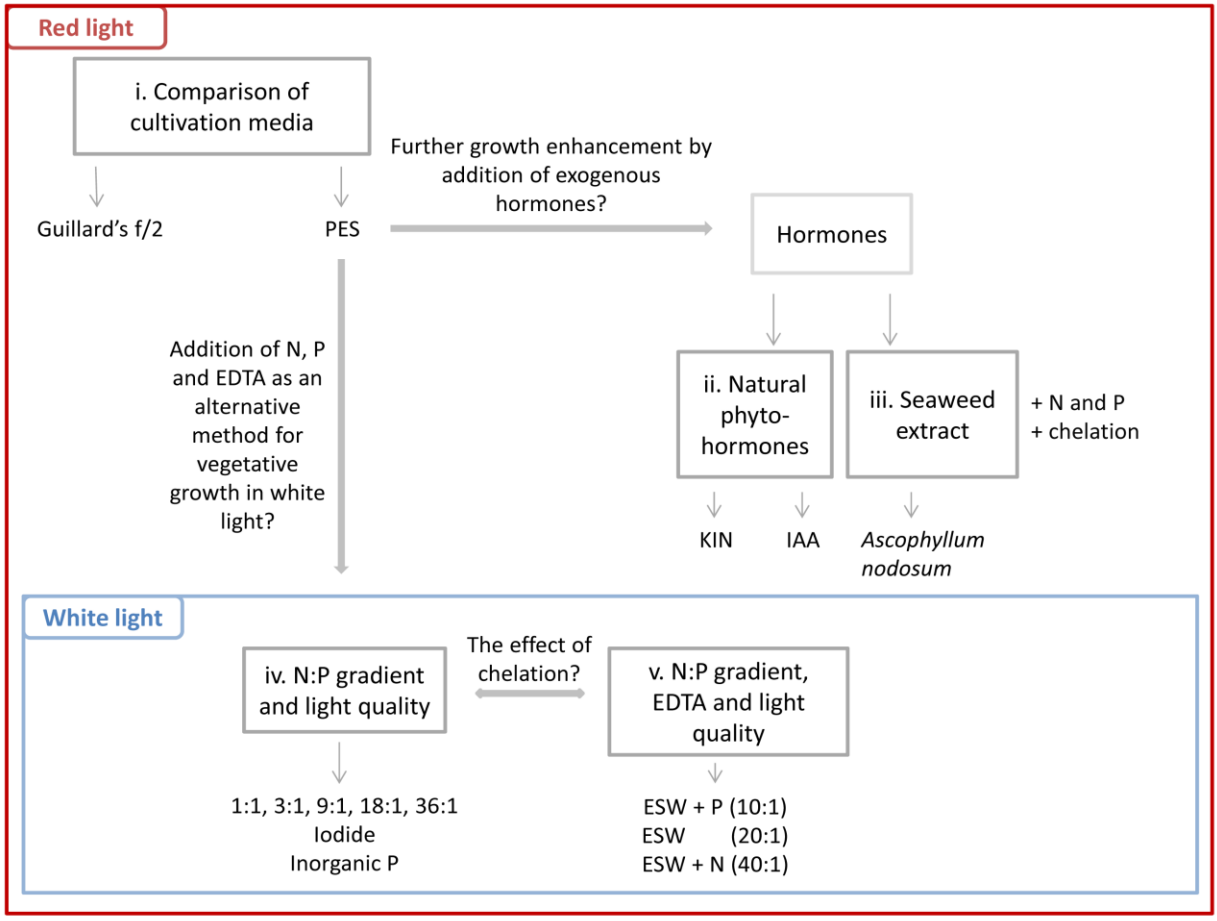

Figure 2 Five experiments were conducted, three (at the top) under red light and two (at the bottom) under red and white light conditions. The first experiment compared the two most commonly used media for growing algae. To see if the growth could be enhanced any further; hormones were added to PES in two experiments; natural phytohormones (KIN and IAA) and seaweed extract (*Ascophyllum nodosum*). Seaweed extract contains chelating agents and has a high composition of N and P in addition to many other constituents. An alternative cultivation method with less expensive chemicals and light source was tested comparing the growth in PES with a gradient of N and P in two experiments; without addition of a chelating agent (left) and with the addition of the chelating agent EDTA (right).

2.2 Methods for measuring growth

The qualitative growth of the algae was measured in terms of dry weight (DW), optical density (OD) or by cell counts. This gave an estimation of the population size and growth of the gametophytes, at the same time as the experiments were running.

Dry weights were used as a measure of growth when there was a sufficient amount of gametophytes in the culture. After mixing the gametophyte solution with a hand blender, a pipette was used to take a sample for dry-weight. GF/F glass microfiber filters (Whatman 47 mm Ø) were pre-dried in marked aluminum foil in 80 °C for 48 hours in a drying oven (Fermaks). They were cooled for 30 minutes and pre-weighted with an analytical balance (A6204 Delta range Mettler toledo) to three digits. A sample of the solution was taken with a pipette and the water was removed by a vacuum pump (230 V Neuberger). The filter papers were returned to the aluminum foil and dried for another 48 hours. It was cooled and weighted. The weight of the filter paper was subtracted from the final weight.

When cultured in multi-well plates with sufficient gametophyte density, quantitative measurements were done with a plate reader (BioTek Epoch Microplate Spectrophotometer) measuring the absorbance at two different wavelengths; 750 nm expressing the turbidity and 676 nm, expressing the chlorophyll*a* concentration.

When cultured in multi-well plates or petri dishes with low gametophyte density, quantitative measurements were done in an inverted microscope by counting number of cells per gametophyte and register sex.

The specific growth rates can be calculated:

$$\text{SGR}= \frac{ln\ W_t/W_0}{t_t - t_0},$$

where W_0 and W_t were the initial and final dry weights in grams and t_0 and t_t were the initial and final times in days (Evans, 1972). This can also be applied for cells per gametophytes.

Qualitative observations were made to observe the overall development of the cultures; including evaluations of contamination, fertility and pigmentation. Qualitative surveillance was carried out using an inverted microscope or an electron microscope.

2.3 General experimental conditions

2.3.1 Experimental organism: *S. latissima*

Sporophytes of *S. latissima* were collected in Vanvikan (63° 52' N, 9° 37' E) during the winter 2011/2012 (November, December and January) and at Storsteinan (63° 27' N, 10° 15' E) in 2013 (February) in the rocky sub littoral zone in the Trondheimsfjord. Sori production was induced by removal of the meristem and light manipulation (Forbord et al., 2012) or by natural induction. Sporophytes were kept in tanks (160L) at SINTEF Sealab holding sand filtered seawater from 90m depth at a constant temperature of 10 °C. A constant light regime of 100 µmol $m^{-2}s^{-1}$ was kept at the surface of the water in the tanks.

2.3.2 Method for sori disinfection, spore release and spore counting

Parts of the plants with mature sori were collected prior to the start of the experiments and spore release was induced by following procedure: The excess tissue was cut away around the brown sorus and the remaining sorus was mechanically cleaned to remove visible ongrowing organisms. The sorus was put in a sodium hypoclohride (NaHCl) (20mL NaHCl/3L water, 600ppm) bath for 5 minutes (Rød, 2012) to eliminate diatoms and other organisms. It was mechanically cleaned and put in a sterilized seawater (SSW) bath for 5 minutes, repeatedly three times. The sorus was put in a plastic bag and put in a 4 °C refrigerator overnight to stress spore release. On day two the sorus was put in a SSW bath for 30 minutes with constant stirring to enhance spore-release. The solution was filtered with a 200 µm mesh to remove unwanted particles. A part of the spore-solution was fixated with Lugol's iodine solution, and the spore density was determined by using a haemocytometer (Neubauer improved haemocytometer, 0.100 mm depth).

2.3.3 Water treatment

The seawater used in all experiments was taken from the Trondheimsfjord (63° 44' N, 10° 40' E) holding sand filtered seawater from 90m depth. It was filtered with a 230 V Neuberger vacuum pump and Whatman GF/F glass microfiber filters and thereafter autoclaved with a

high-pressure stream sterilizer (TOMY SX-700E) for 20 minutes at 120°C. The concentration of nutrients in the seawater was measured to be 15.5 µg PO_4^{2-} /L and 110 µg NO_3^-/L (N:P ratio of 15.7) made in December 2011 and 23.9 µg PO_4^{2-} /L and 140 µg NO_3^-/L (N:P of 12.9) made in April 2012 and four measurements throughout a whole year had an average N:P molar ratio of 14.6:1.

When working with impure cultures it is difficult to completely eliminate other algae (particularly diatoms), protozoa and bacteria. Germanium dioxide (GeO_2) was added to the treatments to prevent growth of diatoms (Harrison and Berges, 2005, Lewin, 1966), a method developed by Lewin (1966). A solution of GeO_2 was made with 1 g of GeO_2 dissolved into 1 L of distilled H_2O (Lewin, 1966) and 2.5 ml of the solution was added per liter of culture.

2.3.4 Abiotic conditions

Light sources used in the experiments were red light (630 nm LED) and white light (warm white fluorescent lamp, L35W-830) using the same intensity for both light qualities (20-60 µmol m^{-2} s^{-1}). The temperature conditions were kept at 10°C, as recommended in Aquaculture Explained No 26 (Edwards and Watson, 2011). All procedures used aseptic techniques.

2.4 Comparison of cultivation media; PES and Guillard's f/2

Two different media, Guillard's $f/_2$ medium (f/2) and Provasoli's Enriched Seawater medium (PES), were used in addition to sterilized seawater (SSW). The media were chosen because of their wide use when growing algae (Edwards and Watson, 2011). SSW was used as a comparison. Since the media differ between publications and have evolved over time, the media were made according to Aquaculture Explained no. 26 (Edwards and Watson, 2011) (see Appendix 1).

Experimental setup

After spore release the spores were transferred to eighteen pre-autoclaved 1L Erlenmeyer flasks and cultivated in semi-continuous batch cultures (Figure 3). The flasks were replaced with new ones each second week. The openings of the culture vessels were sealed with silicone tops with two drilled holes, one for air in, and one for air out. The cultures were bubbled with filter-sterilized air, pumped with a hiblow HP60, 51W and filtered with hydrophobic PTFE membrane filter (Acro 50 filter,Pall Gelman Acro® 50 Filter). The air-bubbles provided air with naturally occurring carbon dioxide and created a mixing of the gametophytes. In half of the replicates, the medium was exchanged every 7th day, in the other half the medium was exchanged every 14th day, giving three parallels for each treatment (see Table 1). Medium exchange was aided by a 25 µm mesh to avoid losses of gametophytes. Each 14th day the gametophyte-solution was mixed with a 120 V hand blender (Braun MR 300) for 1 minute to homogenize the cultures. pH was measured in the end of the experiment (pHenomenal VWR pH 1000 L).

Table 1 Comparison of cultivation conditions for Experiment i. "Comparison of cultivation media".

Exp. medium	**Dilution 7 days (no. replicates)**	**Dilution 14 days (no. replicates)**
SSW	3	3
PES	3	3
f/2	3	3
Experiment conditions:		
Spore density		$20 \cdot 10^6$ spores L^{-1}
Culture vessel		Erlendmeyer flasks (1 L)
Length of exp.		15 weeks
Light quality and intensity		Red light 60 µmol $m^{-2}s^{-1}$
Light regime		24 h
GeO_2		From week 5
Quantitative growth estimation		Dry weight, n=3, n= 9
Qualitative growth estimation		Pictures (electron microscope)

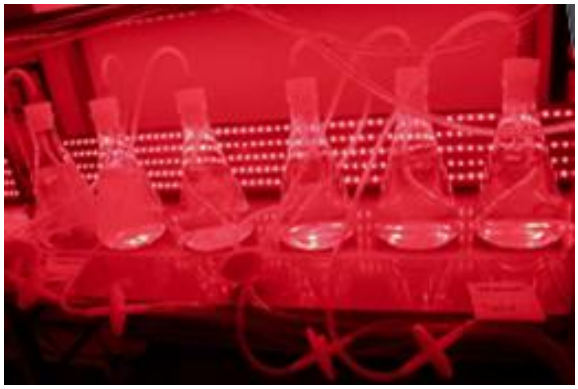

Figure 3 Experimental set-up of the experiment comparing cultivation media. 1 L Erlendmeyer flasks with rubber top and rubber tubes providing air bubbles through a filter to the treatments.

2.5 The use of hormones to enhance growth

Two different methods were used to study the effect of hormones on gametophyte growth; the addition of two natural phytohormones and the addition of extracted seaweed. The latter consist of many constituents, posing a possible synergetic effect between many growth hormones, but also from other molecules and nutrients.

2.5.1 Effect of growth hormones: Addition of natural phytohormones

Two different hormones, kinetin, 6-furfurylaminopurine, (KIN) and indolyl-3-acetic acid (IAA), were added in different concentrations to PES (see Table 2). The hormones were chosen because they are known to enhance the growth in plants, especially in higher plants.

Experimental setup

Spore release was conducted in PES solution the 30th of august and placed in 5 ml 12-well plates (Becton Dickinson MULTIWELL^{TM} 12 well). Hormones were added in concentration gradients, kintein (KIN) in concentrations in concentrations from 0-10 000 µg KIN/L solution (0µg KIN/L, 0.1µg KIN/L, 1µg KIN/L, 10µg KIN/L, 100µg KIN/L, 1 000µg KIN/L and 10 000µg KIN/L); and Indole-3-acetic acid (IAA) from 0-1 000 µg IAA/L solution (0µg IAA/L, 0.1µg IAA/L, 1µg IAA/L, 10µg IAA/L, 100µg IAA/L and 1 000µg IAA/L), all together twelve different

treatments. The hormones were prepared according to instructions at Sigma Aldrich homepage (see Appendix 2). Six replicates of each treatment added up to six 12 well plates.

Table 2 Comparison of cultivation conditions for Experiment ii." Effect of growth hormones".

Hormones	**Concentration gradient (µg L^{-1})**
KIN	0 – 0.1 – 1 – 10 – 100 – 1 000 – 10 000
IAA	0 – 0.1 – 1 – 10 – 100 – 1 000
Experiment conditions:	
Spore density	$135 \cdot 10^6$ spores L^{-1}
Culture vessel	12 well plates (5 ml)
Replicates	6
Length of exp.	5 weeks
Light quality and intensity	Red light 30 µmol $m^{-2}s^{-1}$
Light regime	24 h
Exchange rate	Each week
GeO_2	From week 2
Quantitative growth estimation	OD, n=6
Qualitative growth estimation	Pictures (inverted microscope)

2.5.2 Effect of seaweed extract

Extracted seaweed was added in different concentrations to half strength PES (PES/2). An extract of *Ascophyllum nodosum*, AlgeaFert, was used due to its assumed effect of plant growth promoting substances.

Experimental setup

Spore release was performed in SSW the 11th October 2012 and placed in 5 ml 12-well plates (Becton Dickinson MULTIWELL™ 12 well) with a concentration gradient; 0.250 g AlgeaFert/L, 0.125g AlgeaFert/L and 0.063 g AlgeaFert/L, in PES/2 medium (see Table 3). A solution of PES/2 was used as a control and a solution of PES media as a comparison. Six replicates of each treatment were used to test the impact of the addition of AlgeaFert-fertilizer on growth rate on *S. latissima* gametophytes. The plates were placed on an orbital shaker, stirring the solutions and mixing the nutrients.

Table 3 Comparison of cultivation conditions for Experiment iii. "Effect of seaweed extract".

Medium	**Description**
AlgeaFert (mg L^{-1})	0 – 6.3 – 125 – 250
PES	Comparison
Experiment conditions:	
Spore density	$126 \cdot 10^6$ spores L^{-1}
Culture vessel	12 well plates (5 ml)
Replicates	6
Length of exp.	9.5 weeks
Light quality and intensity	Red light, 30 µmol $m^{-2}s^{-1}$
Light regime	24 h
Exchange rate	Every week
GeO_2	From week 2
Quantitative growth estimation	Counting cells gametophyte^{-1}, n=120
Qualitative growth estimation	Pictures (inverted microscope)

2.6 Evaluation of fertility and light quality

Two experiments were conducted to evaluate any growth-enhancing effect of white light compared to red light and if an alternation of nitrogen (N) or phosphorous (P) ratio could prevent fertilization and affect growth. In the first experiment (Experiment iv.) the effect of iodide and inorganic phosphorous was additionally looked into. In the second experiment (Experiment v.) EDTA was added to the treatments to see if this would further enhance growth.

2.6.1 Experiment iv.; N:P gradient and light quality

A wide gradient of N:P ratios in the medium was tested to study effects on growth and fertility. Dissolved inorganic phosphorous (inorg P) was compared to dissolved organic phosphorous (P) to investigate a possible effect between f/2 and PES. Iodide is non-essential for growth, but was tested because small additions might increase the growth of gametophytes (Harries, 1932). Iodide is commonly used in studies to grow laminaria, and is used together with PES, called PESI (De-lin et al., 1995).

Experimental set-up

Spore release was performed in sterilized seawater on 11th October 2012 and mixed with the treatments in 5 ml 12-well plates (see Table 4). Six replicates of each treatment were treated with white light and six replicates were treated with red light. The first 9 weeks had a 24:0 light:dark regime and the last four weeks a 16:8h light:dark regime to see if this could induce fertility. NO_3^- and β-glycerophosphate were tested in different ratios; 18:1 (the same quantities as in PES), 36:1 (two times N), 9:1 (two times P), 3:1 (six times P), 1:1 (eighteen times P), 18:1 with inorganic phosphate (PO_4^{3-}) as used in f/2 medium and iodide (potassium iodide, KI) (100µg/L) was added to PES. PES was used as a control. The plates were placed on an orbital shaker, stirring the solutions and mixing the nutrients.

Table 4 Comparison of cultivation conditions for Experiment iv. evaluating the effect of an N:P gradient and light quality.

Medium	**Description**
SSW	Control
N:P ratio	1:1 – 1:3 – 1:9 – 1:18 – 1:36
PES	Comparison
PESI	Iodide + PES
N:Inorg. P	1:18
Experiment conditions:	
Spore density	$126 \cdot 10^6$ spores L^{-1}
Culture vessel	12 well plates (5 ml)
Replicates	6
Length of exp.	13 weeks
Light quality and intensity	Red and white light, 30 µmol $m^{-2}s^{-1}$
Light regime	Week 1-9; 24 h, week 10-13; 16:8h (light:dark cycle)
Exchange rate	Every week
GeO_2	From week 2 to week 9
Quantitative growth estimation	Counting cells gametophyte^{-1}, n=120
Qualitative growth estimation	Pictures (inverted microscope)

2.6.2 Experiment v.; Effect of EDTA, N:P gradient and light quality

The growth of some algae may be limited by insufficiency or toxicity of certain trace metal ions of ion activities occurring naturally in sea water (Sunda et al., 2005). An addition of a chelating agent could therefore alter the growth of *S. latissima* gametophytes. EDTA was added to SSW in addition to nitrate (NO_3^-) and phosphate (PO_4^{3-}) in different amounts (Appendix I) to evaluate the effect on growth and fertility.

Experimental set-up

Spore release was performed in sterilized seawater on the 7th February 2013 and placed in 25 ml petri dishes (see Table 5). Six replicates of each treatment were used to test the effect of nutrients and their ratio on growth rate and fertility on *S. latissima* gametophytes. Three of replicates were treated with white light (30 µmol m^{-2} s^{-1}, warm white fluorescent lamp, L35W-830) and the other three were treated with red light (30 µmol m^{-2} s^{-1}, 630 nm LED). A 16:8 light:dark regime was used. The nutrients were tested as in Hoffman and Santelices (1982) with Enriched Seawater (ESW), ESW with double concentration of phosphorous (ESW+P), ESW with double concentration of nitrogen (ESW+N). PES was used as a control and growth of gametophytes in SSW as a comparison. In the recipe used by Hoffmann and Santelices (1982) Tris was added to the medium, but it was omitted in this experiment because the necessity of Tris has been questioned by Andersen (2005).

Table 5 Comparison of cultivation conditions for Experiment v. Effect of EDTA, N:P gradient and light quality.

Medium	**Description**
SSW	Control
PES	Comparison
ESW - N:P ratio	1:10 – 1:20 – 1:40
Experiment v.	
Spore density	$25 \cdot 10^6$ spores L^{-1}
Culture vessel	Petri dish (25 ml)
Replicates	3
Length of exp.	5 weeks
Light quality and intensity	Red and white light, 30 µmol $m^{-2}s^{-1}$
Light regime	16:8h (light:dark cycle)
Exchange rate	Every 10th day
GeO_2	From week 2 to week 3
Quantitative growth estimation	Counting cells gametophyte^{-1}, n=60 n=120
Qualitative growth estimation	Pictures (inverted microscope)

2.7 Statistical Analysis of data

2.7.1 Parametric tests

Parametric tests were conducted on normal distributed data; in the experiments "Comparison of cultivation media" and "Effect of growth hormones". Both experiments used trimmed means by removing the lowest and the highest measurement in each treatment. Treatment means were compared using one-way "Analysis of Variance" (ANOVA). Prior to applying ANOVA, "Exploratory Data Analysis" (EDA) was done with Shapiro-Wilk's test, used to check for normality, and Levene's test, used to check for homogeneity of variance. The results are presented at a significant level of $p \leq .05$.

In the "Comparison of cultivation mediums" experiment, Welch's F was used because Levene's test showed the homogeneity of variance assumption was broken. Games-Howell post hoc test were performed due to unequal variances testing between-group means. Polynomial contrasts were performed for trend analyses, examining a linear trend or quadratic trend, connecting the ordered conditions with a line. If this line is straight (i.e. no curve) there are a linear trend, and if the line is curved (U-shaped) in one place there are a quadratic trend. Planned contrasts/comparison examines specific questions about the data that are based on theory. This enables comparisons between several groups against another group testing specific hypothesis. The theory that hormones have a growth enhancing effect was tested using planned contrasts, comparing all groups with addition of KIN and the groups with the addition of IAA towards the control group.

2.7.2 Non-parametric tests

In the last three experiments; "Effect of seaweed extract", "Experiment iv." and "Experiment v.", normality and transformed (log-, square root-) normality was rejected by a Shapiro-Wilk's test at a significant level of $p \leq .05$. Hence a Kruskal-Wallis test, a non-parametric substitute for the parametric one-way ANOVA, was used to compare treatment means. A significance level of $p \leq .05$ was used. Kruskal-Wallis tests were followed up by visual inspection of graphs and Mann-Whitney test. A Bonferroni correction was applied by

dividing the α-level by the amount of tests conducted to ensure that the cumulative Type 1 error is below .05. This because multiple significance tests were conducted (Field, 2009).

Effect sizes (ω) are presented when necessary and are an objective and standardized measure of the magnitude of an observed effect (Field, 2009). ω≤.01 small effect, ω≤.06 medium effect ω≤.14 large effect size.

Statistical analyses were carried out and figures created by the use of IBM SPSS 19.0.

3 RESULTS

3.1 Comparison of cultivation media; PES and Guillard's f/2

Saccharina latissima gametophytes were grown in two different media; Provasoli's Enriched Seawater medium (PES) and Guillard's f/2 medium (f/2). Growth in sterilized seawater (SSW) was used for control. Figure 4 show pictures taken in the end of the experiment after 15 weeks of growth. A clear difference can be seen between the media used, showing the best biomass in PES and more contamination of cyanobacteria in f/2. The cultures in PES and SSW consisted of purer *S. latissima* gametophytes as confirmed by images taken in the microscope. An increase in biomass due to exchange rate can also be seen in PES, with a higher density of gametophytes when exchanging the medium every 7th day (top picture) compared to every 14th day (bottom picture).

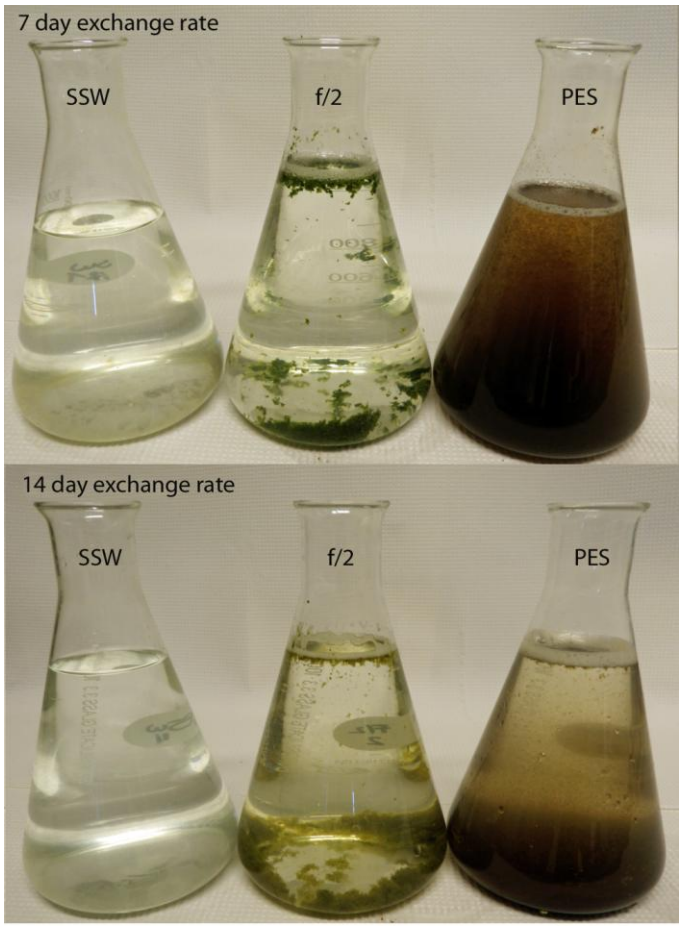

Figure 4 Pictures taken in the end of the experiment at week 15 of gametophytes treated in SSW (left), f/2 (middle) and PES (right) with the two different exchange intervals; 7 day media exchange rate (top picture) and 14 day media exchange rate (bottom picture).

Table 6 shows the specific growth rates, μ, for the treatments in "comparison of cultivation media" Experiment. They were calculated in SigmaPlot 10.0. SSW with a 7-day exchange interval had the lowest μ of 0.0008 d^{-1} and PES with a 7-day exchange interval had the highest μ of 0.062 d^{-1}.

Table 6 The specific growth rates (μ) from week 6 (n=3) to week 15 (n=9) ± standard error are shown for the different treatments for the "Comparison of cultivation media" experiment.

Treatment	**μ (d^{-1}) ± SE**
PES, 7-day exchange interval	0.062 ± 0.0089
PES, 14-day exchange interval	0.039 ± 0.0041
f/2, 7-day exchange interval	0.012 ± 0.00030
f/2, 14-day exchange interval	0.012 ± 0.00090
SSW, 7-day exchange interval	0.00080 ± 0.00037
SSW, 14-day exchange interval	0.0028 ± 0.0013

Figure 5 show grams dry weight per liter during the fifteen-week treatment time. The growth rates showed a similar trend throughout the whole experiment, with a clear difference between the three various media at all measurements; PES yielding the highest biomass throughout the growth period and SSW the lowest. The differences between the dilution rates were most prominent in PES, were an increase in exchange rate showed a positive effect on gametophyte biomass. The opposite seemed to be true for SSW, where an increase in exchange rate showed a non-significant ($p>.05$) decrease in gametophyte biomass. The very small biomasses of the SSW treatments appeared to be negatively affected by a more frequent exchange rate.

Visual inspection of the data showed an exponential growth right from the start, but due to a too small density no measurements are done during the first 5 weeks. Therefore it is hard to say anything about the initial development. The growth rate seems to be decreasing after week 6, and is followed by a constant growth to the end of the experiment. More measure points might have revealed stagnation in growth prior/after week 15.

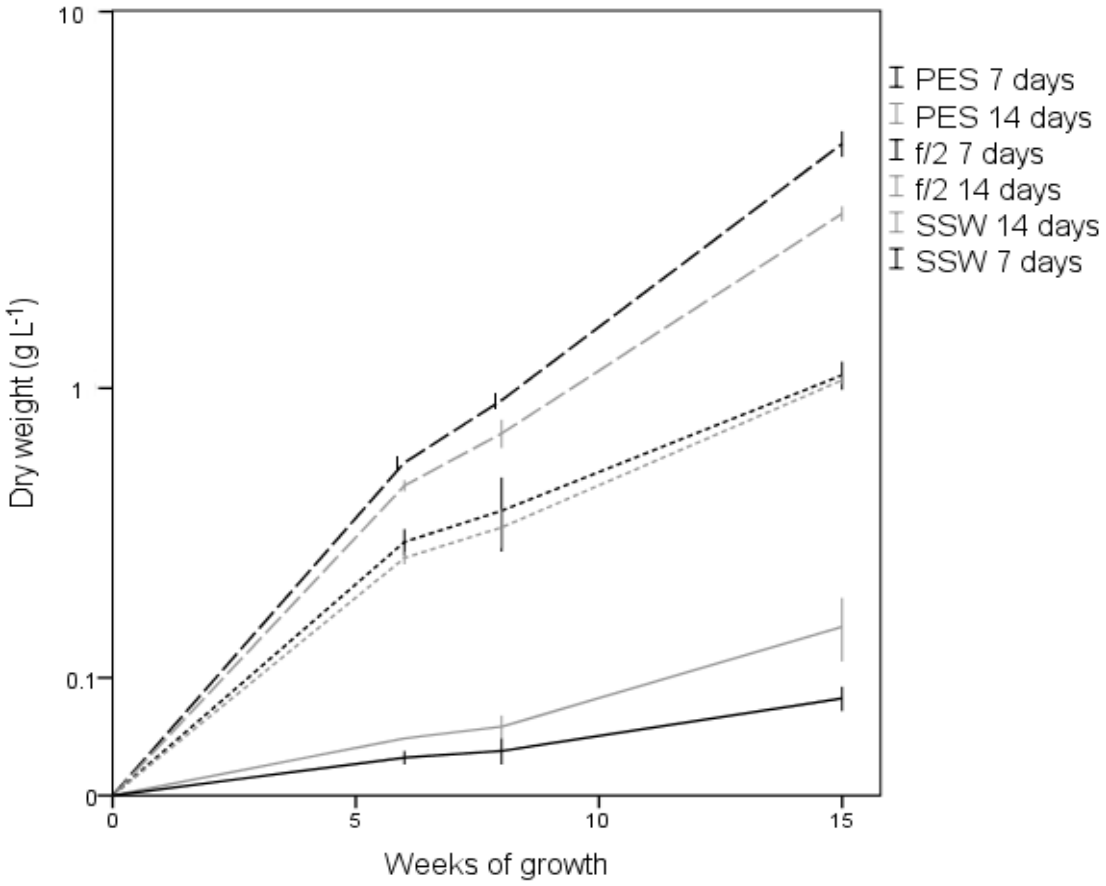

Figure 5 Dry weight in grams per liter of *S. latissima* over 15 weeks of growth. PES is represented by large stippled lines, f/2 with small-stippled lines and SSW by continuous lines. 7 days exchange rates are represented by black and 14 days exchange rate by grey. The values are mean (n=3 in week 6 and 8, n=9 in week 15) dry weight in grams per liter. Error bars indicate ± 1SE.

Figure 6 shows the resulting mean dry weight, as grams per liter for the treatments in the end of week fifteen on the left y-axis (bars) and the pH of the medium in the end of the treatment time on the right y-axis (line). From Fig. 6 it can be seen that the growth of gametophytes were markedly affected by the growth medium. Moreover the exchange rate has a significant impact for the cultures grown in PES, with PES "7-day exchange interval" showing the highest biomass (M=4.52, SE=1.01) and SSW "7 day exchange interval" showing the lowest biomass (M=0.08, SE=0.01). A one-way ANOVA confirmed significant differences, $F_W(5, 19)=154.54$, $p \leq .001$. The treatments had a very strong effect on the biomass of the gametophytes, this is supported by the large effect size of $\omega = .95$ (>>.14, which shows a large effect size). A higher biomass was followed by an increase in pH. A Games-Howell test proved significant differences in biomass between the treatments as shown by letters in Fig.

6. Different letters indicate significant differences between treatments, whereas equal letters indicate that no significant difference could be revealed.

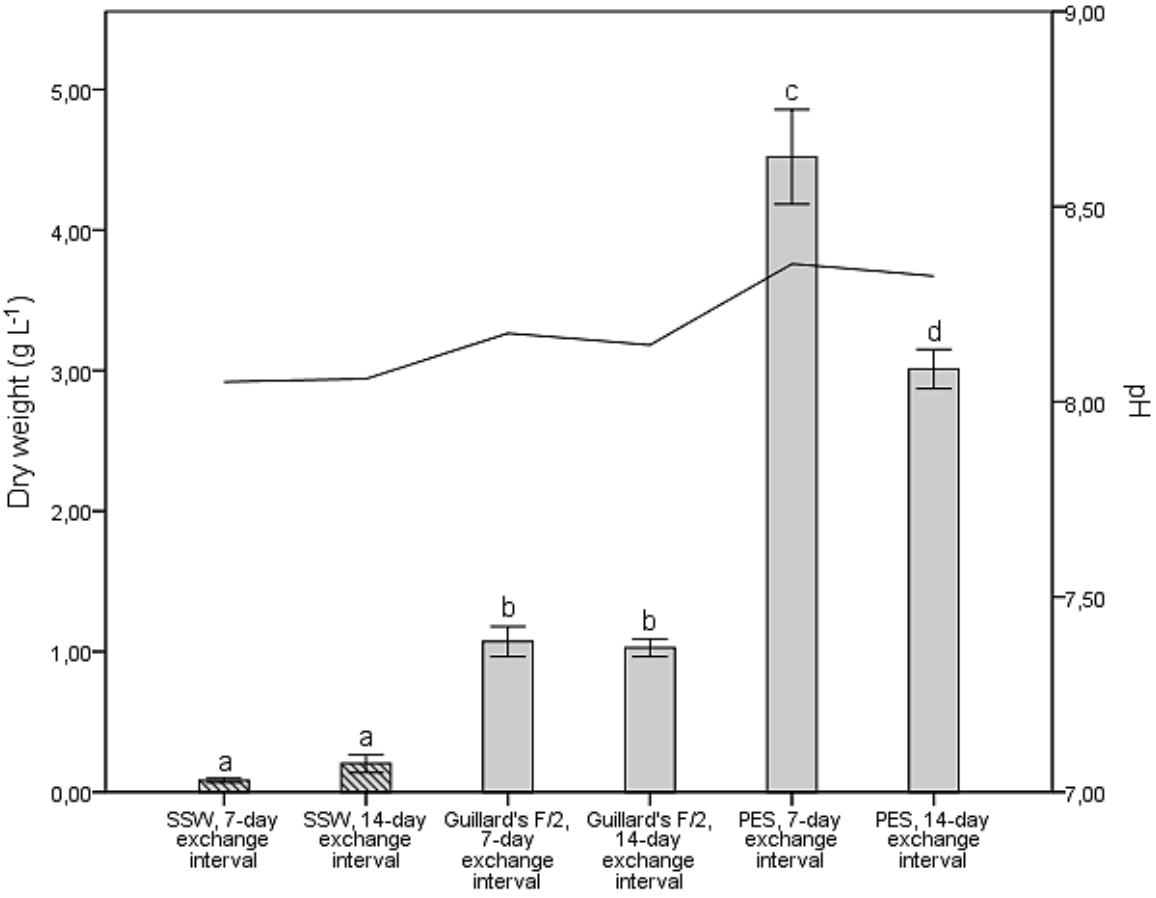

Figure 6 Dry weight for *S. latissima* gametophytes grown in the two medium; Guillard's f/2 and PES, and the control treatment, SSW, after fifteen weeks of growth. Values are mean of three measurements from each of the three replicates (n=9) ± 1SE. Letters indicate significant difference between treatment, all p≤.001 except between c and d, p≤.05. Treatments with similar letters are not significantly different. The mean pH (right y-axis) in the end of the treatment-period is shown as a line.

3.2 The use of hormones to enhance growth

3.2.1 The effect of growth hormones: Addition natural phytohormones

S. latissima gametophytes were treated with the hormones IAA and KIN in different concentrations added to PES, with 0 µg hormone L^{-1} as a control. Figure 7 shows growth curves of the gametophytes treated with IAA (left) and KIN (right). Gametophyte growth was

measured with a spectrophotometer (OD, 676nm) during a five-week treatment time. Visual inspection of the OD data of both IAA and KIN shows an exponential growth right from the start, but due to a too small density no measurements were done during the first 2 weeks. Therefore it is hard to say anything about the initial development. The growth rate seemed to decrease after week 4. The growth probably stagnated earlier in this experiment compared to the "Comparison of cultivation media" experiment due to a higher density of gametophytes in this experiment which probably inhibited further development. The gametophytes in this experiment did not get damaged by mixing with a Waring blender, as in the "Comparison of cultivation media" experiment. Since the gametophytes in this experiment could develop undisturbed another possibility might be that they reached the stagnation phase more rapidly due to a more rapid growth in the initial phase.

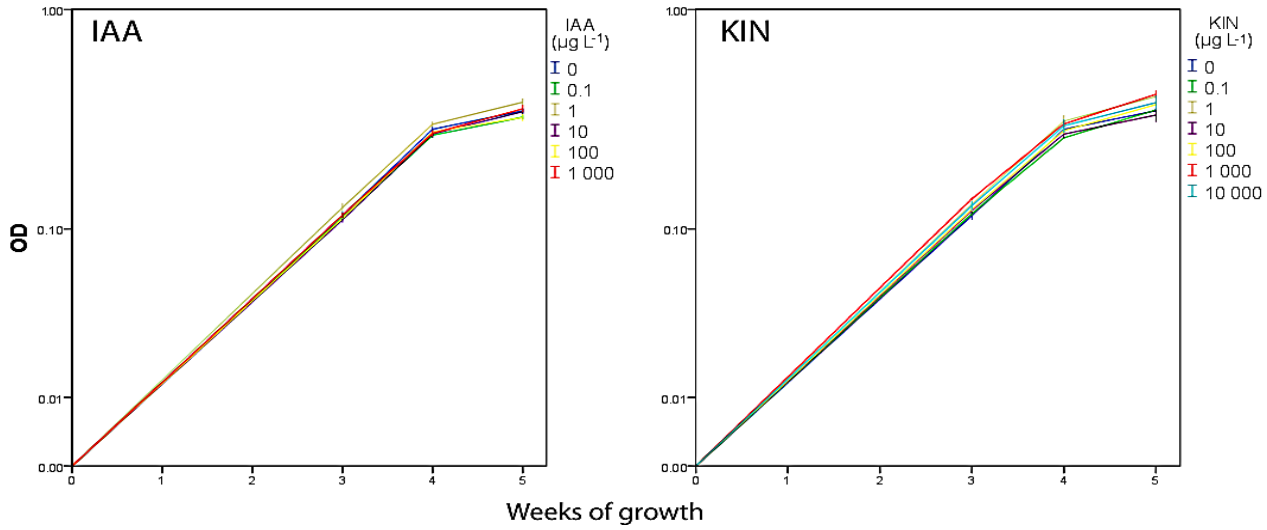

Figure 7 Optical density (OD, 676nm) of gametophytes treated with different concentrations of IAA (left) and KIN (right) over five weeks of growth (mean values ± 1SE, n=6). OD measurements were done at week three, four and five.

The OD was measured at two different wave-lengths; 676nm and 750nm, indicating chlorophyll*a* activity and turbidity respectively. Those were used as a measure of growth of the gametophytes treated with hormones. Figure 8 shows the OD of IAA (top) and KIN (bottom). The line at the left y-axis shows the mean absorbance at 676nm (n=6) and the line at the right y-axis gives the mean absorbance at 750nm (n=6) at week five of growth. The different treatments are given on the x-axis.

The addition of 1 µg IAA L^{-1} showed the highest absorption at 676 nm (M=0.38, SE=0.02), but an independent t-test did not reveal significant difference from the control group (M=0.35, SE=0.01), t(6)=-1.24, p(one-tailed)=0.17. One-way ANOVA for 676nm showed that there were no significant differences of any of the treatments, F(5,18)=2.32, p=.09, neither was any linear, F(1,18)=.015, p=.90, or quadratic trend found in a trend analysis, F(1, 18)=.004, p=.95. Planned contrasts enabling comparisons between several groups against another group tested the hypothesis of increased growth due to the addition of hormones, did not reveal an increased turbidity of the gametophytes when IAA was added to the medium compared to the control group, t(18)=-.014, P=.84.

However, one-way ANOVA for 750nm showed that there were significant differences between the treatments, F(5,18)=3.58, p<.05. A Post-hoc test revealed the difference to be between 0.1µg IAA L^{-1} and 100 µg IAA L^{-1} to 1 µg IAA L^{-1}. No linear trend, F(1,18)=.013, p=.91, or quadratic trend were found in a trend analysis, F(1, 18)=.11, p=.75. Planned contrasts did not reveal an increased growth of the gametophytes when IAA was added to the medium compared to the control group, t(18)=.001, P=.97.

The results of OD measurements in week 5 for the hormone KIN treatments are shown in Figure 8 (bottom). At 676 nm absorbance the addition of 1000 µg KIN L^{-1} to the medium showed the highest growth (M=0.41, SE=0.01), but an independent t-test did not reveal significant difference from the control group (M=0.35, SE=0.01), t(6)=-3.71, p(one-tailed)=0.33. One-way ANOVA for 676nm showed that there were significant differences between the treatments, F(6, 21)=2.65, p<.05, but no linear trend, F(1,21)=3.75, p=.06, or quadratic trend were found in a trend analysis, F(1, 21)=2.42, p=.07. Planned contrasts did not reveal an increased growth of the gametophytes when KIN was added to the medium compared to the control group, t(21)=.16, p=.19.

One-way ANOVA for 750nm showed that there were significant differences between the treatments, F(6, 21)=2.71, p<.05. A linear trend, F(1, 21)=7.68, p<.05 was found in a trend analysis. Looking at the graph (Fig. 8 bottom) it can be seen that the trend is not very consistent. No quadratic trend was found, F(1, 21)=.002, p=.96. Planned contrasts did not reveal an increased growth of the gametophytes when KIN was added to the medium

compared to the control group, t(21)=.07, P=.32. A Post-hoc test revealed the difference to be between 0.1 µg KIN L^{-1} to 1 000 µg KIN L^{-1}.

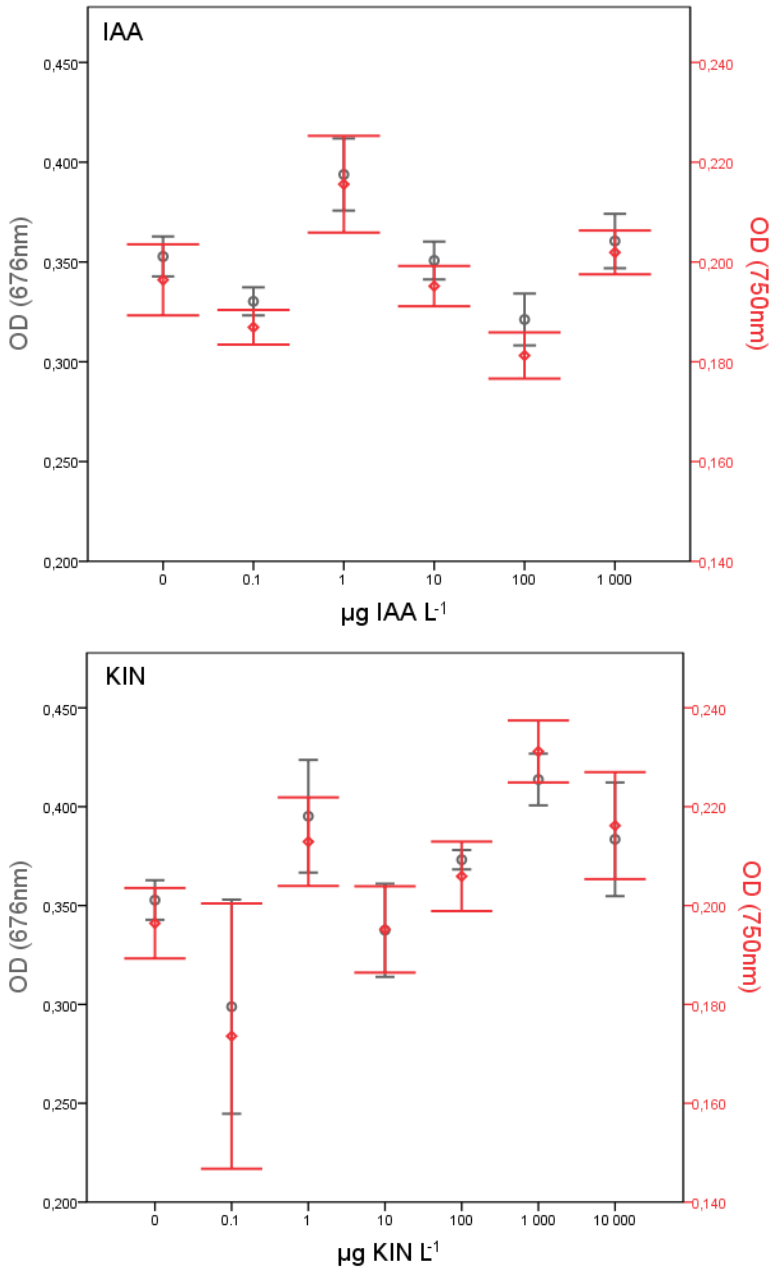

Figure 8 Black circles represent absorbance (OD) of gametophytes at 676nm (left y-axes) and red diamonds represent OD at 750nm (right y-axes) after five weeks of growth (mean values ± 1SE, n=6) in IAA (top) and KIN (bottom). No trends are apparent.

Figure 9 shows representative pictures of the treatment yielding the highest growth in IAA (1 µg IAA L^{-1}) and KIN (1 000 µg KIN L^{-1}) compared to the control treatment in week 5 of the

experiment. No specific differences are apparent. Fig. 9 show that the cultures were relative contamination-free, and therefore the increased turbidity can be expected to reveal a higher growth of gametophytes treated with KIN.

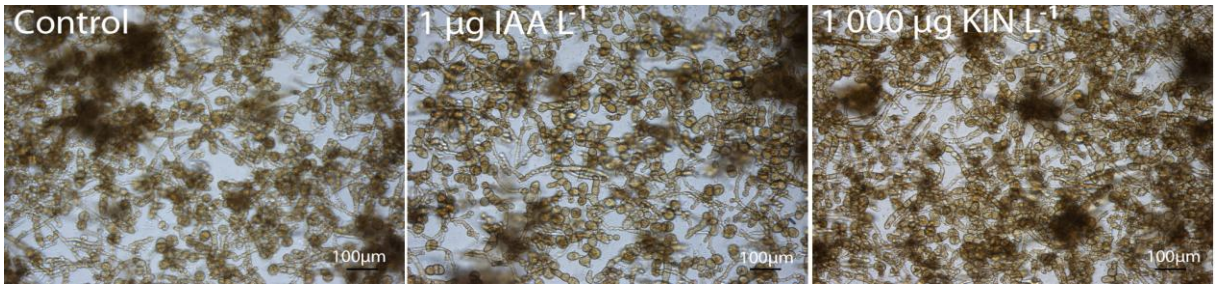

Figure 9 Gametophytes of *S. latissima* after five weeks of growth with addition hormones to PES medium. A: 0 µg L^{-1}, B: 1 µg IAA L^{-1}, C: 1 000 µg KIN L^{-1}. The bar represents 100µm (magnification 20x).

3.2.2 The effect of seaweed extract

S. latissima gametophytes were treated with the seaweed extract AlgeaFert in a low, medium and high concentration added to half strength PES medium (PES/2). PES/2 was used as control treatment. In addition the gametophyte growth treated with PES was investigated as comparison. Figure 10 shows mean number of cells per gametophyte as a function of treatment (n=120) in the end of the 67-day treatment time. As the gender has a clear relation to cell number, male and females are shown separately. A clear trend can be seen, with the addition of low AlgeaFert concentration giving the highest number of cells per gametophyte males and females together (M=18.83, SE=0.90), significantly higher than in the control group treated with PES/2 (M=5.71, SE=0.34).

A Kruskal-Wallis test revealed significant differences between the treatments, $H(4) = 244.44$, $p \leq .001$. Mann-Whitney tests revealed significant differences between all treatments except PES and a medium concentration of AlgeaFert. Significant differences are shown as letters in Figure 10, showing that there were large effects of all treatments, when compared with the control treatment.

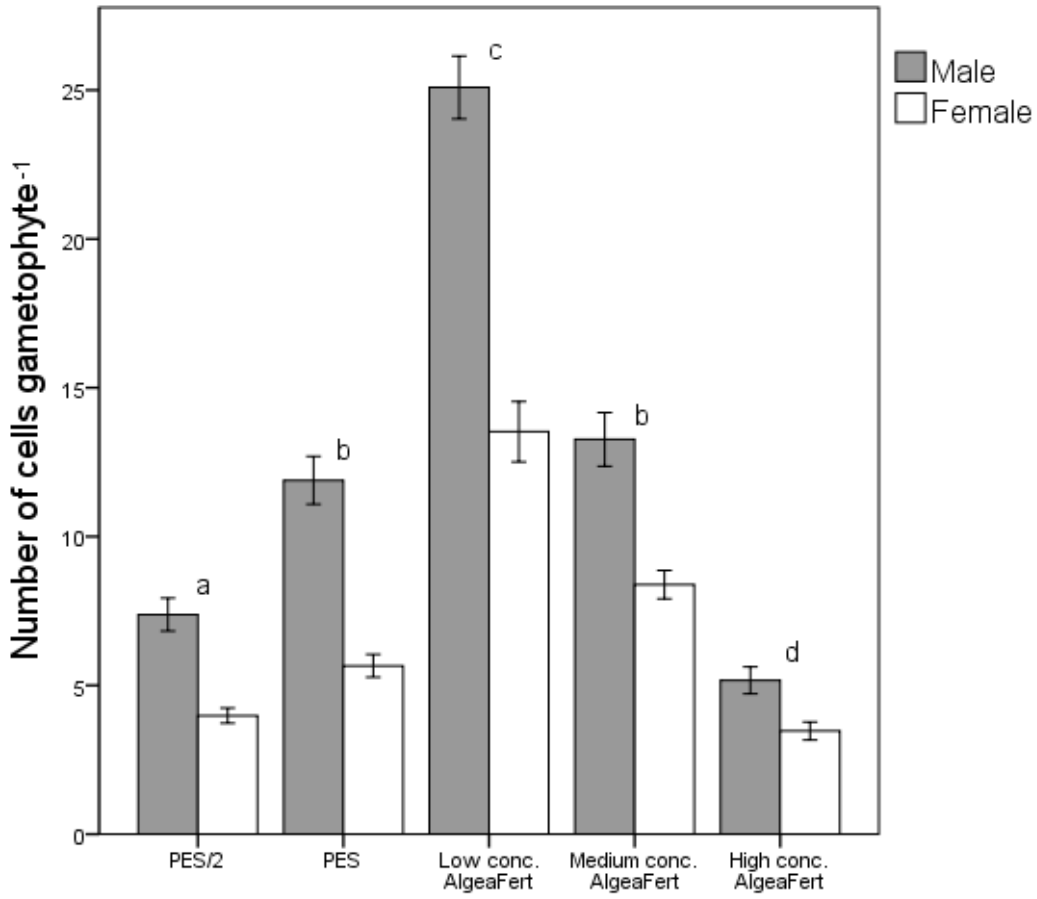

Figure 10 Number of cells per gametophyte of gametophytes treated with different concentrations of AlgeaFert and PES medium over 67-days of growth. PES/2 was used as a control. The values are mean values ± 1SE, n=120. Letters indicate significant difference between treatments, female and males together, at a p≤.05 level of significance. Treatments with equal letters are not significantly different.

Figure 11 shows representative pictures taken at day 67, showing the development of the gametophytes in the five treatments. Color, thickness of the cells and branching was varying with treatment and were most developed in a low addition of AlgeaFert. An increase in AlgeFert concentration caused an increase in precipitation and gave a higher coloration of the gametophytes.

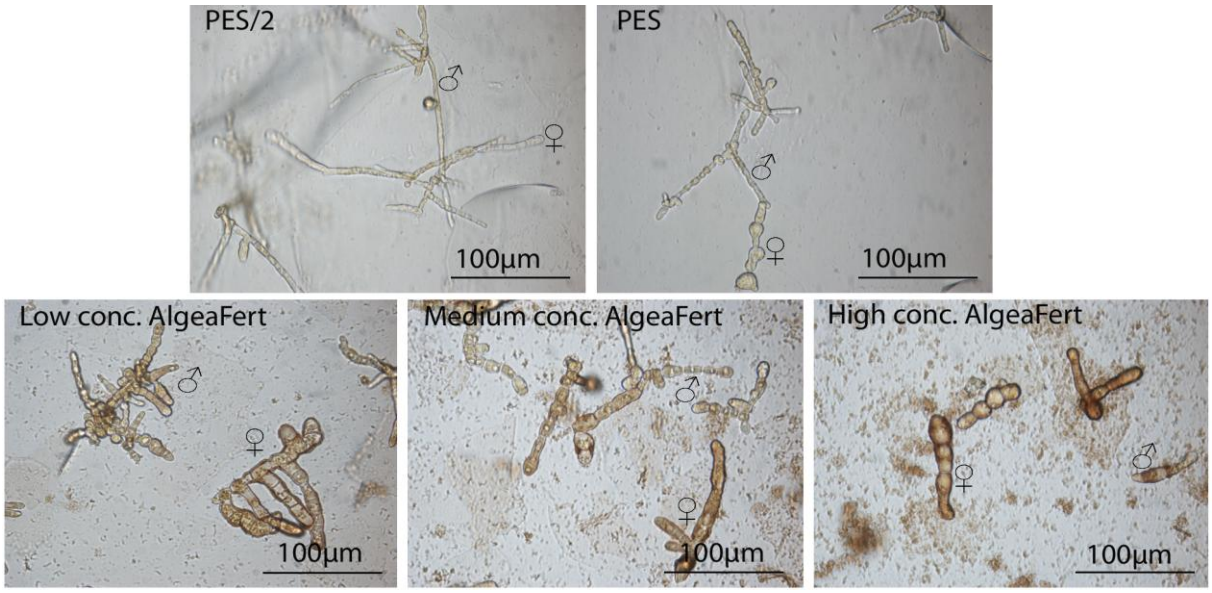

Figure 11 Gametophytes of *S. latissima* after 67 days of growth under red light. Showing from top left; PES/2, PES, and from bottom left; low conc. of AlgeaFert, medium conc. of AlgeaFert, high conc. of AlgeaFert. ♂=male gametophyte. ♀= female gametophyte. The bar represents 100µm (magnification 40x).

3.3 The effect of light quality and nutrient composition on growth and fertility

Two experiments (Experiment iv. and Experiment v.) were conducted to examine differences in growth and fertility due to the alternation of light quality and N:P ratio. In Experiment iv., a gradient of N:P was added without the addition of EDTA. In addition the effect of iodide + PES (PESI) and inorganic P (inorg P) were examined. The growth of *S. latissima* gametophytes in PES and SSW was studied as comparison.

3.3.1 Experiment iv.; N:P gradient and light quality

Figure 12 shows the mean cell number per gametophyte in the end of week thirteen of growth, where an over-all higher cell number of gametophytes grown in white light compared to red light could be seen, and a higher cell number of gametophytes grown in PES and PESI compared to the other treatments. A Kruskal-Wallis test showed that there were significant differences between the groups grown in white light, $H(8)=575.48$, $p \leq .001$. A Mann-Whitney test revealed significant difference between the treatments under white light

and are shown as capital letters in Figure 12. The significant differences were between PES and PESI with all other treatments, in addition N:P ratios of 1:1, 9:1, 18:1 and 36:1, and inorg P showed significant differences from sterilized seawater (SSW). Inorg P was significantly different from all treatments but 9:1. A Kruskal-Wallis test showed that there were significant differences between the groups in red light, $H(8)=339.60$, $p\leq.001$. A Mann-Whitney test showed significant difference between the treatments under red light and are shown as small letters in Figure 12. The significant differences were between PES and PESI with all other treatments, in addition 1:1 and 3:1 showed significant differences from SSW. All effects are reported at a $p\leq.05$ level of significance. No significant differences could be revealed with a Mann-Whitney test between PES medium and PES with 100 µg Iodide L^{-1}, $U_{Red\ light}=7162$, $p=.47$, white light $U_{White\ light}=7150$, $p=.93$. No significant differences could be revealed with a Mann-Whitney test between the addition of organic phosphorous (18:1) and inorganic phosphorous in red light conditions (Inorg. P), $U=7009$, $p=.36$. However a significant difference was revealed in white light conditions, $U=4982$, $p<.01$.

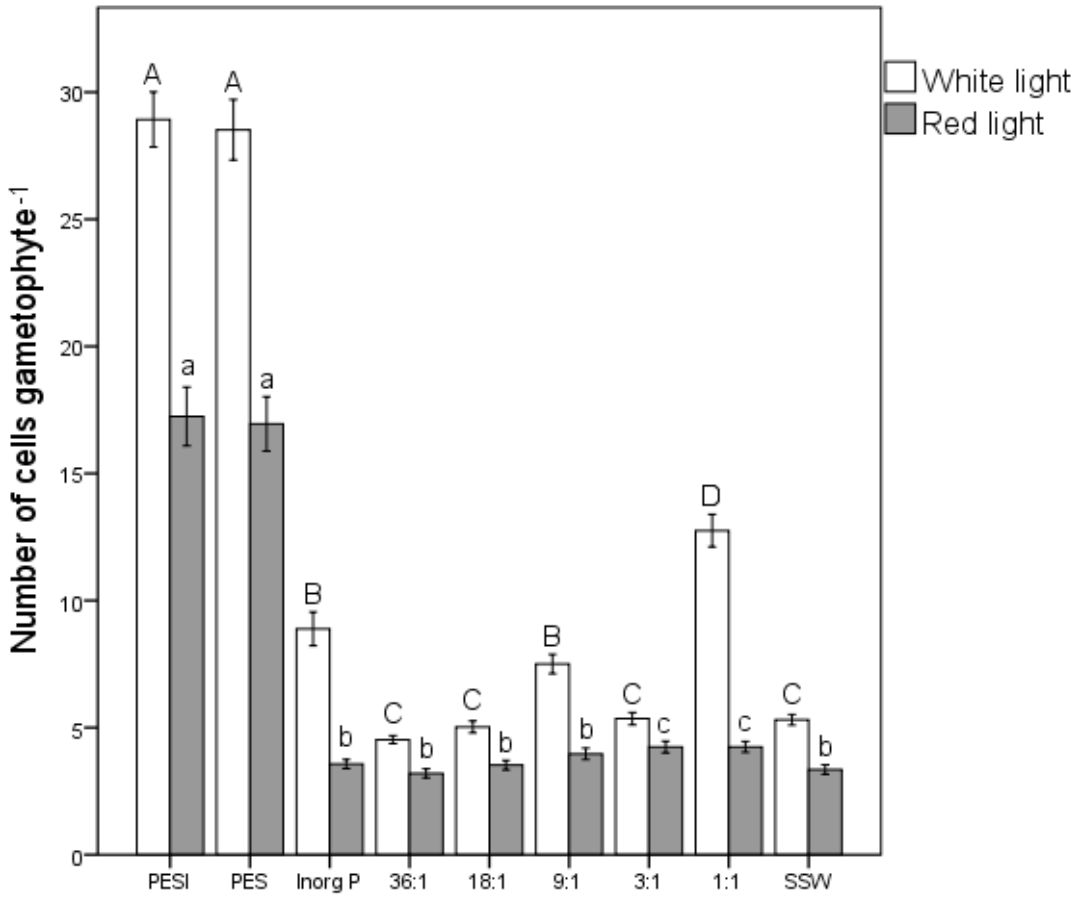

Figure 12 Number of cells per gametophyte after thirteen weeks of growth in treatments; PES + iodide (PESI), PES, 18N: 1inorg.P, N:P of; 36:1, 18:1, 9:1, 3:1, and 1:1. The values express mean values for n=120, ± 1SE. Capital letters show significant differences in white light conditions and small letters show significant differences in red light. Treatments with similar letters are not significantly different, p≤.05.

Figure 13 and Figure 14 show representative pictures of *S. latissima* gametophytes taken with an inverted microscope after thirteen weeks of growth in red and white light conditions, respectively. An increase in cell numbers in white light conditions can be seen comparing Figure 13 to Figure 14. A higher pigmentation can be seen in the gametophytes grown in red light conditions. No signs of antherida or oogonia can be seen in either of the light qualities.

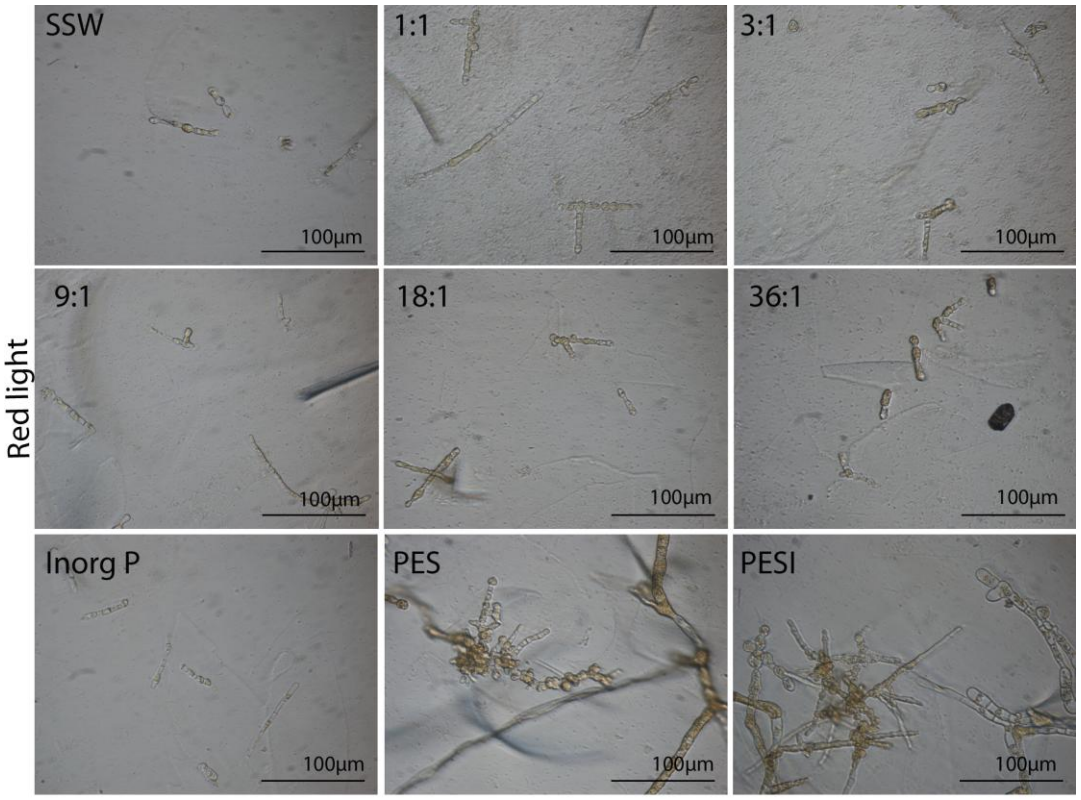

Figure 13 Gametophytes of *S. latissima* after thirteen weeks of growth under red light in the different treatments as specified in the pictures. The bar represents 100µm (magnification 40x).

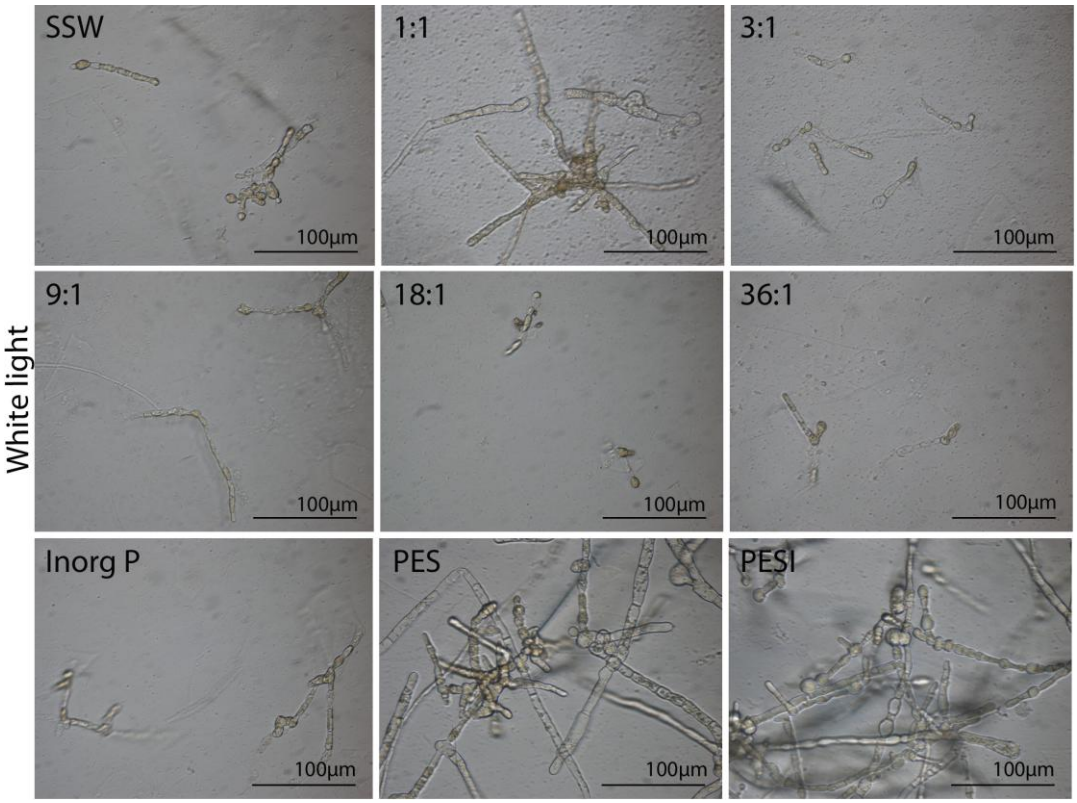

Figure 14 Gametophytes of *S. latissima* after thirteen weeks of growth under white light in the different treatments as specified in the pictures. The bar represents 100µm (magnification 40x).

3.3.2 Experiment v.; N:P gradient, EDTA and light quality

S. latissima gametophytes were treated with enriched seawater medium (ESW), ESW with double concentration of nitrogen (ESW + N) and ESW with double concentration of phosphorous (ESW +P). Figure 15 shows the number of cells per gametophyte during a five week treatment time under white light (Fig. 15 left) and red light (Fig. 15 right). Fig. 15 shows higher numbers of cells per gametophyte grown in white light compared to gametophytes grown in red light. ESW+P showed the highest number of cells per gametophyte in white light conditions (Fig. 15 left), whereas in red light conditions (Fig.15 right) all treatments except SSW seemed to stagnate towards the end if the experiment. SSW showed the slowest growth in both light conditions. The growth of gametophytes in the PES control treatment had lower growth than all ESW treatments in both light qualities. The growth of gametophytes in SSW, which was used as a reference control, had a lower growth than all other treatments.

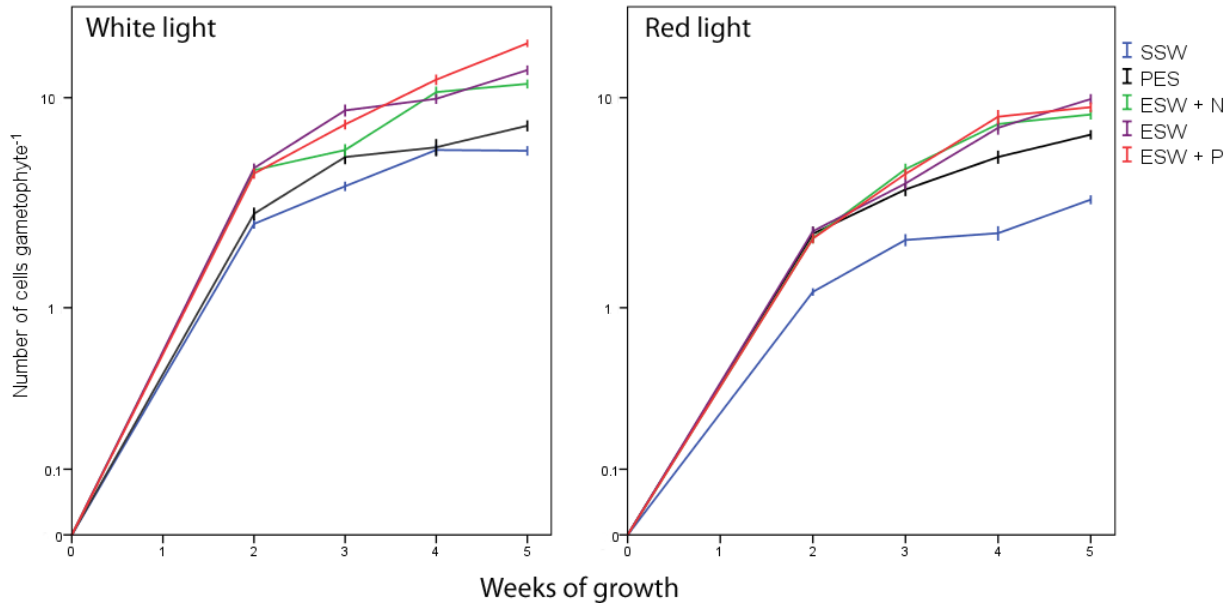

Figure 15 Number of cells per gametophyte in different treatments during a five week treatment-time in white light (left) and red light (right). The values are mean values ± 1SE, n=60 (week 2-4) and n=120 (week 5).

Figure 16 shows the mean number of cells per gametophyte as a function of the different treatments in the end of week five. Treatments under white light are represented by white bars and red-light treatments by grey bars. A Kruskal-Wallis test showed that growth in cell numbers was significantly affected by which treatment they received, $H(4) = 571.78$, $p<.001$ for white light and $H(4)161.60$, $p<.001$ for red light. Mann-Whitney tests revealed significant differences between all treatments in white light except between ESW and ESW+N. Significant differences are represented by capital letters in white light and small letters in red light in Fig. 16. Under red light, SSW showed a significantly lower growth than all other treatments and PES showed a significantly lower growth than ESW and ESW+P. Under white light ESW + P had significantly higher growth than all other treatments, and ESW and ESW + N showed significantly higher growth than PES. All effects are reported at a level of $p\leq.05$.

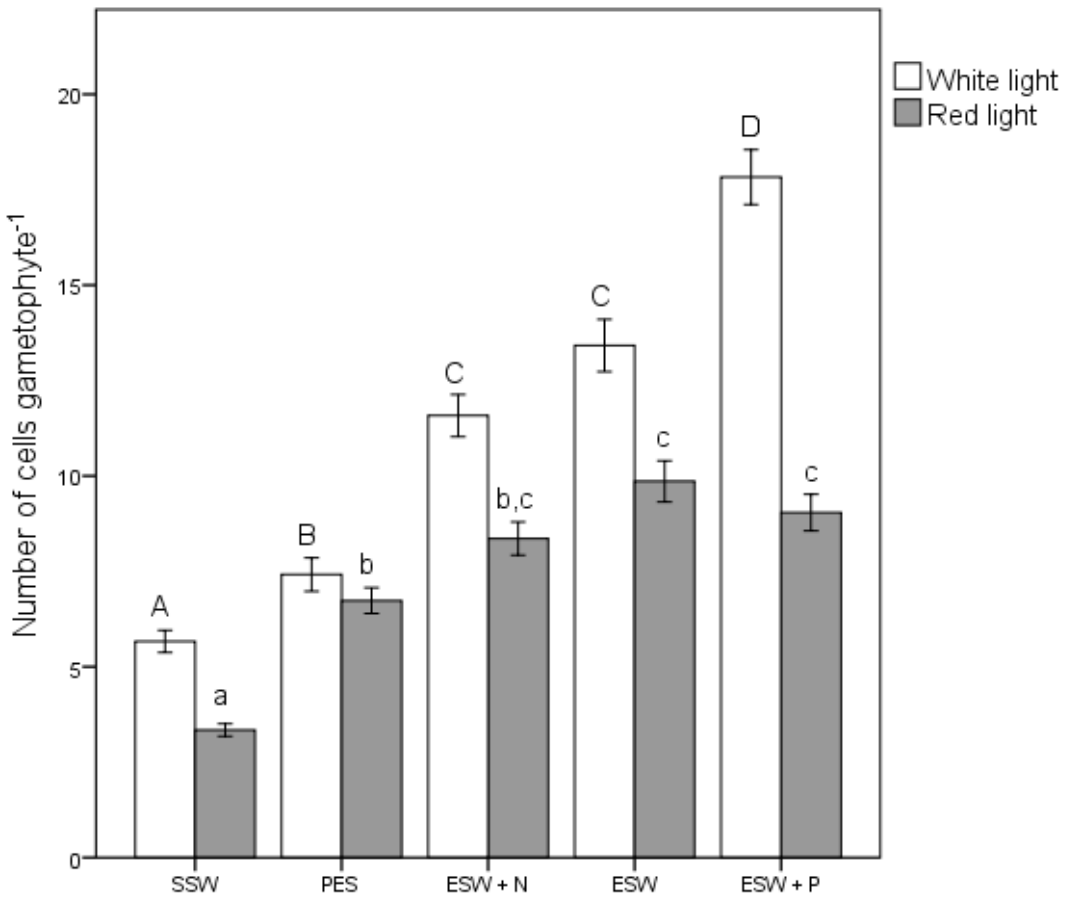

Figure 16 Number of cells per gametophyte in different treatments in the end of week 5. Capital letters represent significant differences between treatments receiving white light, and small letters represent significant differences between treatments receiving red light at $p<.05$. Bars that have the same letters are not significantly different. The values express mean values ± 1SE, n=120.

Table 7 shows the specific growth rates, μ, for the treatments in Experiment v., calculated in SigmaPlot 10.0. SSW has the lowest μ in both light treatments with 0.102 d^{-1} in red light and 0.148 d^{-1} in white light. ESW had the highest μ with 0.357 d^{-1} in red light and ESW + P had the highest μ, with 0.638 d^{-1} in white light. μ for gametophytes grown under white light is 36 % higher than μ for the gametophytes grown under red light.

Table 7 The specific growth rates, μ, ± standard error are shown for the different treatments from week 2 (n=60) to week 5 (n=120) under red and white light conditions in Experiment v.

Treatment	**Red light** $\mu \pm SE$ **(d^{-1})**	**White light** $\mu \pm SE$ **(d^{-1})**
SSW	0.102 ± 0.011	0.148 ± 0.020
PES	0.211 ± 0.024	0.217 ± 0.030
ESW + N	0.293 ± 0.029	0.333 ± 0.038
ESW	0.357 ± 0.037	0.415 ± 0.047
ESW + P	0.327 ± 0.033	0.638 ± 0.049
Light quality	0.258 ± 0.014	0.350 ± 0.021

Effect of light quality on gametophyte growth

The effect of light quality on gametophyte growth from the two expermints examining light quality; Experiment iv. and Experiment v. are presented together. Figure 17 shows a comparison of the mean number of cells of all treatments in the two different light regimes (white light and red light) in Experiment v. The numbers of cells in white light are between 1-1.5 weeks ahead of the red light, but appears to level off beyond 4 weeks of growth.

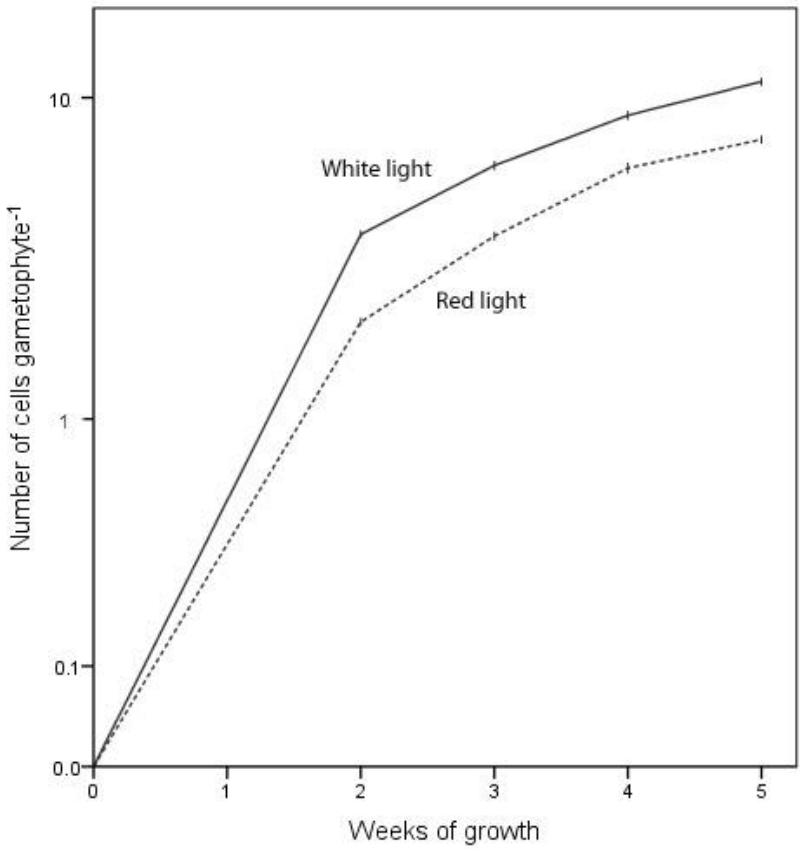

Figure 17 Number of cells per gametophyte of all treatments during a five week treatment-time under white light (solid line) and red light (stippled line). The values are mean values ± 1SE, n=60 (week 2-4) and n=120 (week 5).

Figure 18 show a serie of pictures taken in the end of Experiment v. There is a decrease in the N:P ratio with ESW + N, ESW and ESW + P and an increase in cellnumber was found with the decreasing N:P ratio (from left to right). The top row of pictures shows treatments in red light and the bottom row shows treatments in white light. A higher pigmentation of the gametophytes grown in red light is apparent. No signs of antheridia or oogonia can be seen.

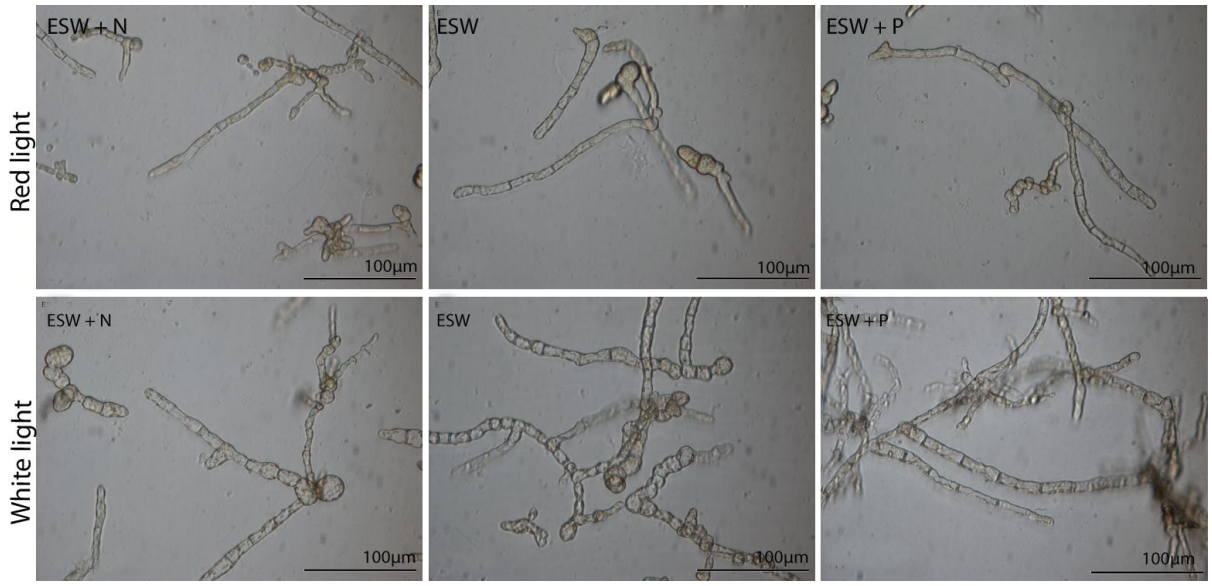

Figure 18 Gametophytes of *S. latissima* after five weeks of growth in red (top) and white (bottom) light conditions. They were grown from left to right in: ESW + N, ESW and ESW + P (magnification 40x). No signs of antheridia or oogonia can be seen in any of the treatments.

The effect of light quality on the numbers of cells per gametophyte from Experiment iv. are shown in Figure 19A and from Experiment v. in Figure 19B. Male and female cell numbers are separated. The mean number of cells per gametophyte was significantly higher under white light conditions compared to red light conditions. A Kruskal-Wallis test showed a significant effect of white light on of the cell number of the gametophytes, $H(1) = 266.55$, $p<.001$. A Mann-Whitney test revealed a significant difference of number of cells per gametophyte between the two light qualities, $U=347624$, $r=-.35$, $p<.001$.

At the end of week thirteen of Experiment iv., the mean male gametophyte length was 8.96 ±0.42 cells per gametophyte in red light, and 13.7 ±0.53 cells per gametophyte in white light, corresponding to an increase of 53 % in white light. The mean female gametophyte length was 4.41 ±0.23 cells per gametophyte in red light and 9.74 ±0.45 cells per gametophyte in white light, corresponding to an increase of 121 % of the mean cell number of the gametophytes grown under white light. At the end of week five in Experiment v., the mean cell number of male gametophytes were 11.0 ±0.33 cells per gametophyte in red light, and 14.0 ±0.50 cells per gametophyte in white light, giving an increase of 27 % in white light. The mean female gametophyte length was 4.63 ±0.13 cells per gametophyte in red light and 9.00 ±0.34 cells per gametophyte in white light, giving an increase of 94 % of the mean cell number in white light.

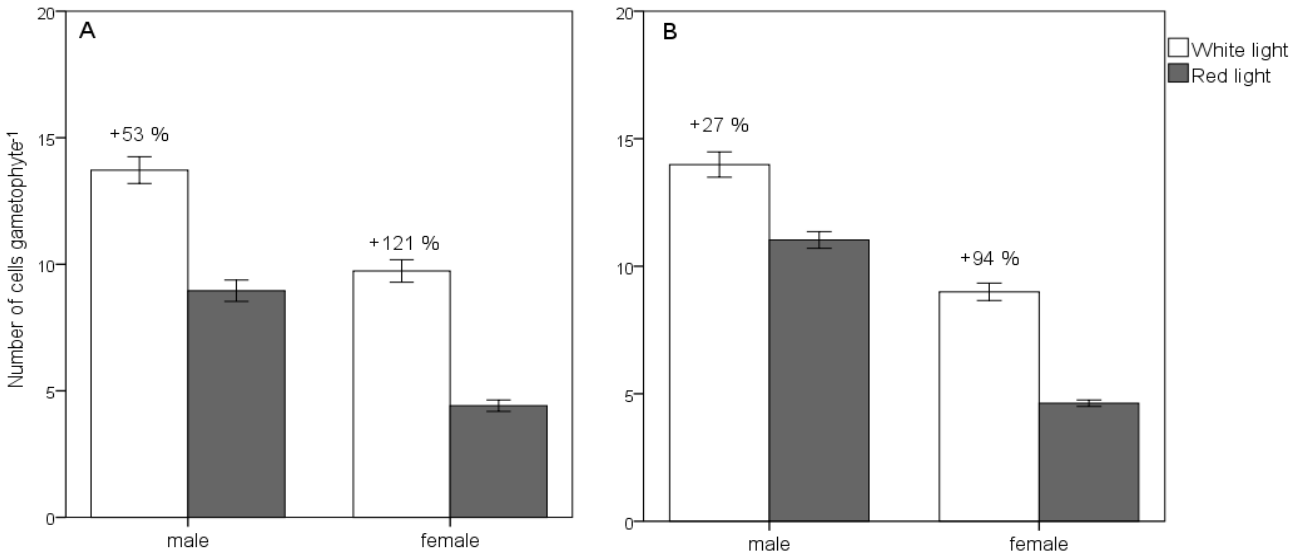

Figure 19 Numbers of cells per male and female gametophyte in red and white light regimes in the end of treatment times. (A) The effect of light quality on amount of cells per gametophyte in Experiment iv., and (B) the effect in the Experiment v. The increased numbers of cells for gametophytes treated under white light compared to gametophytes treated under red light are shown as percentage on top of the bars. The values are mean values of the number of cells per gametophyte, with error bars showing ±1SE.

Effect of EDTA and N:P ratio on gametophyte growth

Two experiments (Experiment iv. and Experiment v.) were conducted to study the effect of N:P on fertility and growth. The result of the nutrient ratio on gametophyte growth obtained in the two experiments are presented together in Figure 20A and Figure 20B. The results are presented without any of the other treatments (PES, SSW etc.) that were studied in these two experiments. Figure 20 shows the growth in cells per gametophyte of *S. latissima* as a function of N:P in; A, Experiment iv. and B, Experiment v. Circles represent number of cells per gametophyte and the trend seen as a result of the nutrient ratio and light quality is represented by regression lines. Gametophytes treated under white light is represented by grey circles and regression lines, and gametophytes treated under red light by red circles and regression lines. Presenting number of cells per gametophyte will always show a wide range of cell numbers because some gametophytes stop developing after only a few cells. This can therefore give an impression of a large variation, as seen in Fig. 20. Many

gametophytes with the same amount of cells are represented by thicker circles which might be hard to distinguish in the figure.

As the nutrient ratio decreases an increased growth in cell number per gametophyte can be seen in both Fig. 20A and Fig. 20B, treated under white light. In Experiment iv. (Fig. 20 A) there was a significant relationship between the N:P ratio of the medium and the number of cells per gametophyte in white light, r_s=-.442, p≤.01 (Spearman's rho). R^2=.201, indicating a 20 % effect of nutrient ratio on number of cells per gametophyte. In Experiment v. (Fig. 20B) there was a significant relationship between the N:P ratio of the medium and number of cells per gametophyte in white light, r_s=-.334, p≤.001 (Spearman's rho). R^2=.112, indicating an 11 % effect of N:P on number of cells per gametophyte. The trend was not clear under red light treatment; Experiment iv. r_s=-.181, p≤.01 (Spearman's rho). R^2=.033 indicating a 3 % effect of nutrient ratio on number of cells per gametophyte. No significant relationship in Experiment v. under red light was seen, r_s=-.049, p=.117 (Spearman's rho). R^2=.003. A higher overall biomass can be seen in Fig. 20B compared to Fig. 20A even though these gametophytes only had been growing for five week compared to 13 weeks in Fig. 20A. This is probably due to the chelating capacity of EDTA.

These results show that the light quality alters the effect that the nutrient ratio has on gametophyte growth, and that red light might inhibit growth and development of the gametophytes.

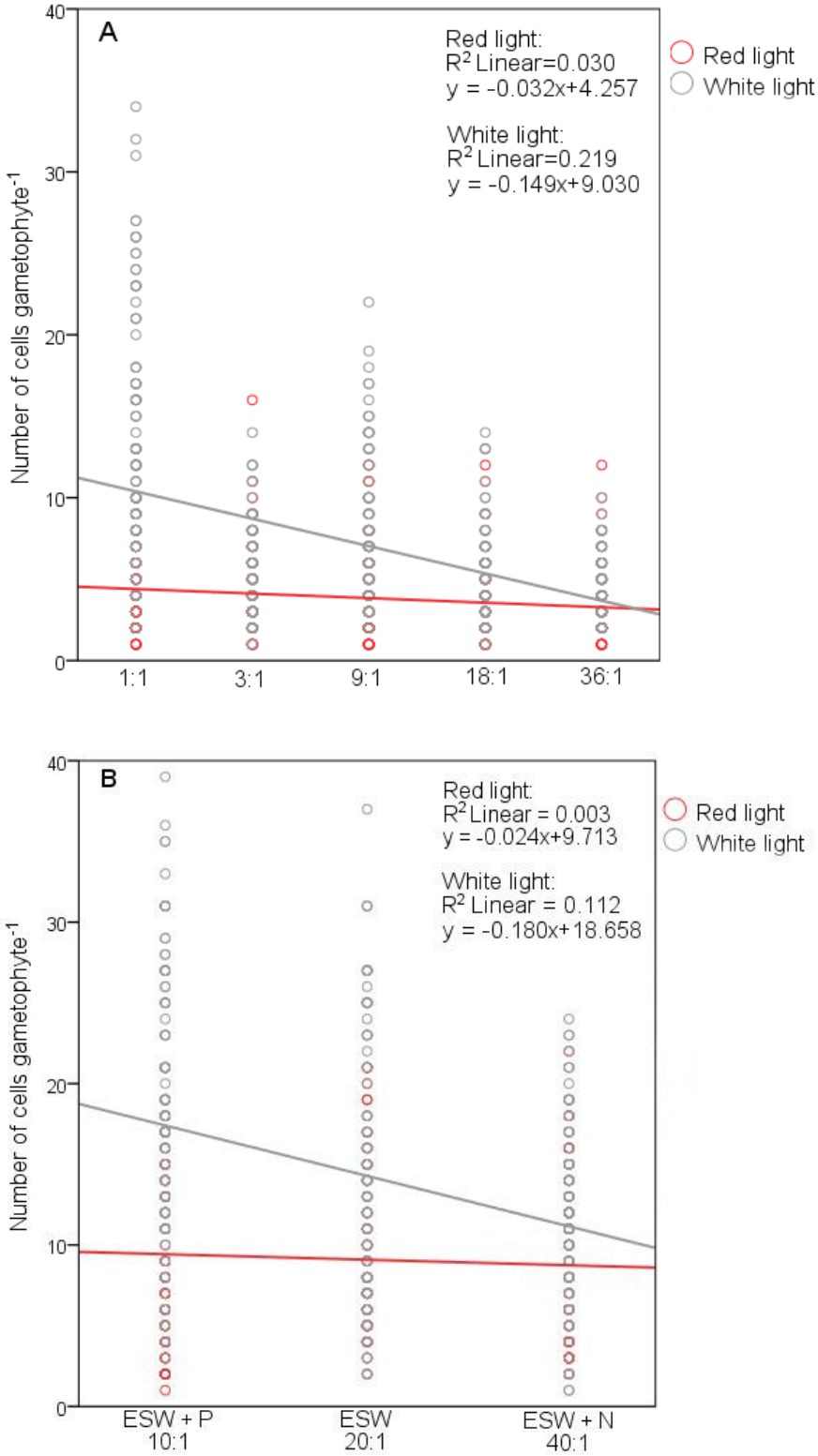

Figure 20 The effect of N:P ratio on number of cells per gametophyte in red and white light conditions, in (A) the results from Experiment iv. and (B) the results from Experiment v. The circles represent number of cells per gametophyte, red circles representing gametophytes grown in red light, and grey circles representing gametophytes grown in white light. Gametophytes with the same cell number are shown by thicker circles. A linear regression line is added to each of the light treatments, a red line showing the trend for cell numbers of gametophytes grown in red light, and a grey line showing the trend in white light.

The effect of N concentrations on the growth of gametophytes under white light is shown in Figure 21 and Figure 22. In Experiment iv. (Fig. 21) a regression line showed no significant ($p=.203$) linearity. In experiment v. (Fig. 22) a regression line showed significant ($p\leq.001$) linearity, where an increase in N concentration was followed by an increase in number of cells per gametophyte. R^2 showed a 15.8 % relationship between N concentration and gametophyte growth in Experiment v.

Figure 23 and Figure 24 shows the effect of P concentration on gametophyte growth under white light. A significant increase in growth of the gametophytes can be seen as a consequence of the increasing P concentrations in both graphs ($p\leq.001$). A regression line showed a relationship between the P concentrations and the gametophyte cell number in Experiment iv. of 20.6 % (Fig. 23), and in Experiment v. a relationship of 30.5 % (Fig. 24). The effect of P concentrations on gametophyte growth showed a stronger effect than the N concentrations.

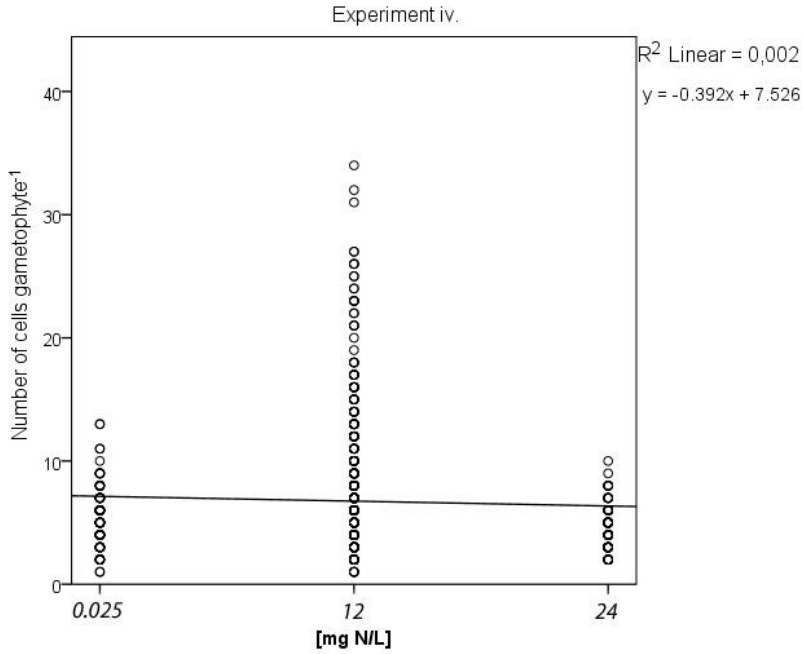

Figure 21 The effect of N concentration in mg N per liter on the number of cells per gametophyte (n=120) in white light conditions from Experiment iv. The circles represent number of cells per gametophyte. Gametophytes with the same cell number are shown by thicker circles. A linear regression line is added showing the trend for cell numbers of gametophytes grown in white light.

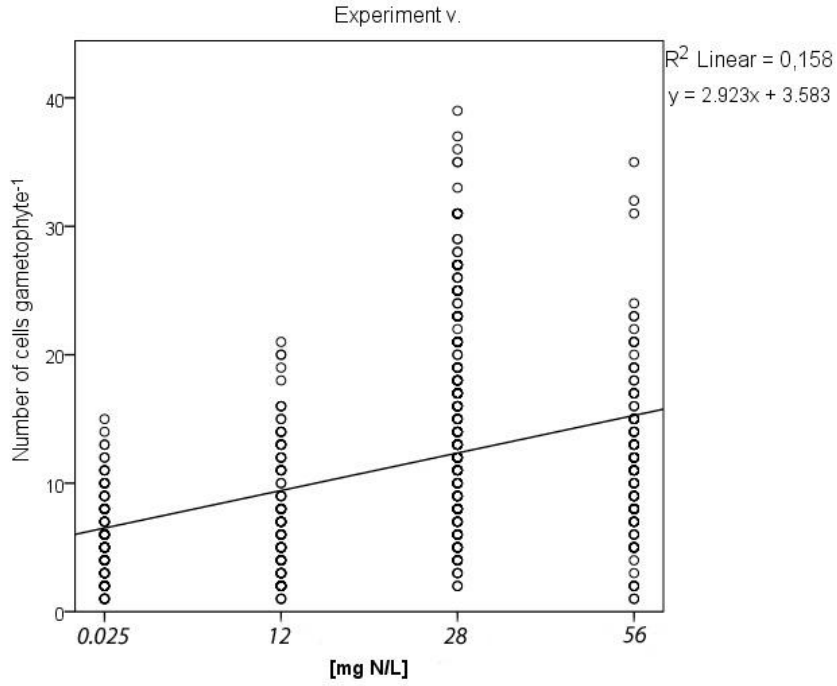

Figure 22 The effect of N concentration in mg N per liter on the number of cells per gametophyte (n=120) in white light conditions from Experiment v. The circles represent number of cells per gametophyte. Gametophytes with the same cell number are shown by thicker circles. A linear regression line is added showing the trend for cell numbers of gametophytes grown in white light.

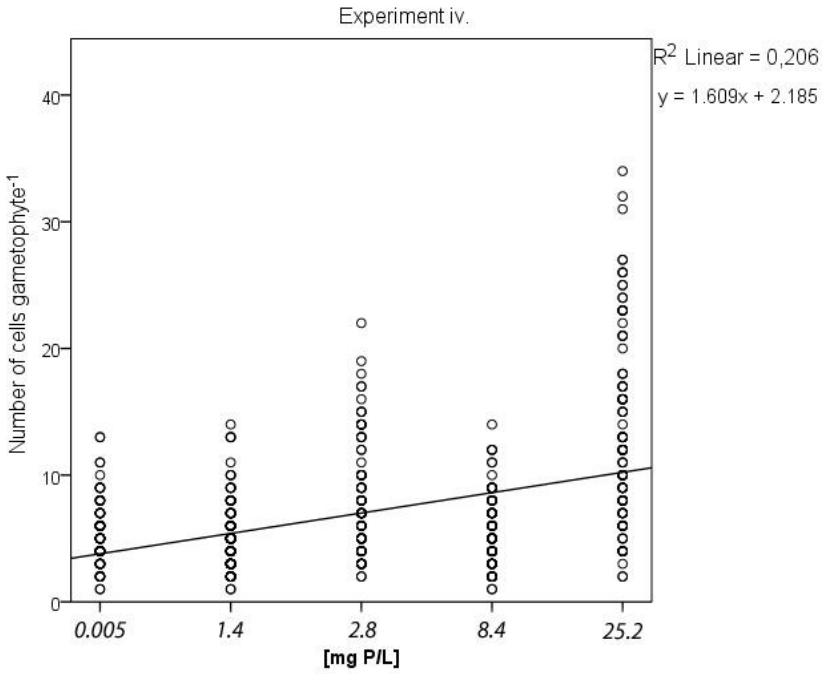

Figure 23 The effect of P concentration in mg P per liter on the number of cells per gametophyte (n=120) in white light conditions from Experiment iv. The circles represent number of cells per gametophyte. Gametophytes with the same cell number are shown by thicker circles. A linear regression line is added showing the trend for cell numbers of gametophytes grown in white light.

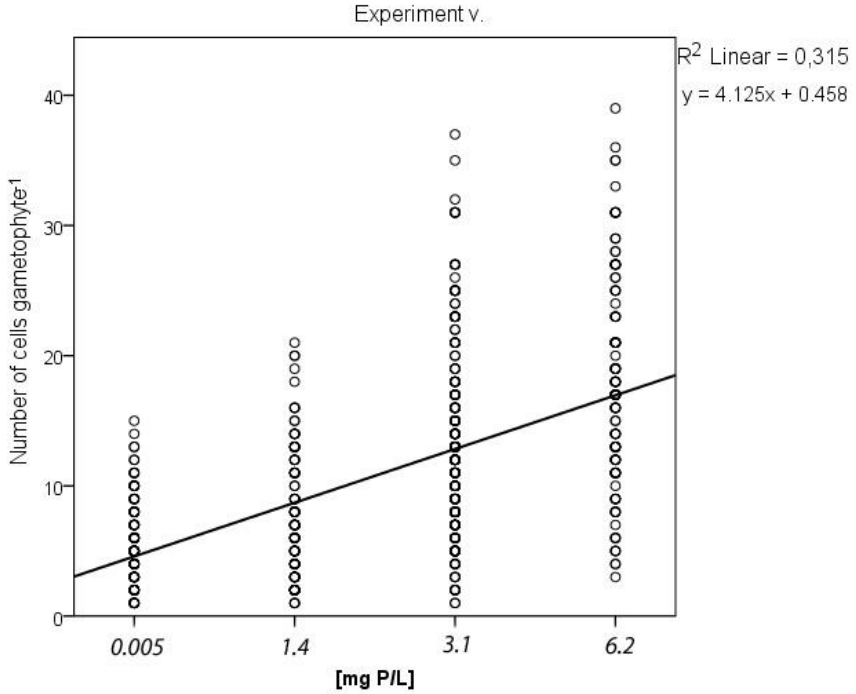

Figure 24 The effect of N concentration in mg N per liter on the number of cells per gametophyte (n=120) in white light conditions from Experiment v. The circles represent number of cells per gametophyte. Gametophytes with the same cell number are shown by thicker circles. A linear regression line is added showing the trend for cell numbers of gametophytes grown in white light.

4 DISCUSSION

4.1 Evaluation of growth estimation methods

To estimate the productivity in the different culture conditions in this study an evaluation of various growth-measuring methods was necessary. The most difficult situation when measuring growth are when the algae grow largely attached to the culture vessel walls and the cells aggregate into lumps (Guillard, 1973). This is the situation for *Saccharina latissima* gametophytes. Finding a method suitable for growth measurements of gametophytes were difficult and different approaches based on the culturing technique had to be used, including cell counts, dry weight and optical density (OD).

Quantitative measurements can be undertaken by counting the increase in cell numbers, as cell number per gametophyte or a specific area. This is an approach that can be used where the cultures have a low density, the cells are clearly visible and the density are too low for using techniques such as measurements of dry weight or OD as a measure of growth. Counting the number of cells per area as compared to cells per gametophyte, would have given a better estimation of the concentration of cells in the medium. Since *S. latissima* gametophytes were prone to cluster and settled unevenly on the substrate, this would have given highly variable results. The estimation of cells per gametophyte overlooked the increase in biomass caused by growth of individual cells of the gametophytes; cells increasing in mass, length and diameter without dividing. However, when culturing *S. latissima* gametophytes the number of sporophytes produced per unit of biomass are of interest because all individual cells can develop sexual organs (Kain, 1979). Counting the number of cells per gametophyte appeared therefore to be a suitable method for measurement of growth of *S. latissima* gametophytes. Hsiao and Druehl (1973) and Hoffman (1984) used this method to estimate growth in laminarian gametophytes.

In dense *S. latissima* gametophyte-cultures, individual cells were hard to distinguish even after homogenization and it were difficult to count individual cells of the gametophytes. Therefore the use of dry weight as a measurement method for growth of bigger cultures was therefore preferred. There were many variables that had to be taken into careful consideration when using dry weight as a measure of growth, and a standard procedure

were developed. For example, the process of taking a representative sample was one of the crucial steps for a reliable estimation of dry weight of cells (Chapman, 1973) when homogenization and fast pipetting to prevent cell settling in the process of sampling were needed. Moreover the sizes of the samples were altered depending on the density to get reliable results. For cultures cultivated in wells with high density, measurements of OD with a plate-reader were a convenient method for biomass estimation. This method enabled measurements without disturbing the cultures. Comparing the three quantitative measuring techniques used in this study would have been interesting. This would have made it possible to compare the results from the various experiments, but was difficult by many means and comparing low density methods with high density methods with aggregated cells made it impossible to compare the techniques. The growth measuring techniques used in this study showed to be appropriate for the specific experiments conducted.

4.2 Light quality

The two experiments (Experiment iv. and Experiment v.) undertaken with *S. latissima* gametophytes showed an increased growth of the gametophytes under white light conditions compared to red light (Figs. 17, 19, 20). The effect was most prominent in the female gametophytes, with a 121 % increase in Experiment iv. and 47 % in Experiment v. When cultivating gametophytes the males produce sperm and only a small number of cells are necessary for reproduction, whereas an increase in female cell number has a higher impact on the sporophyte formation. Since any cell of the gametophytes had the potential to develop reproductive structures (Kain, 1979) these experiments show that white light increased the gametophytes fecundity.

Figure 25 show an absorbance spectrum of *S. latissima* (green line) together with the available wavelengths of the two light qualities used in the experiments (Experiment iv. and Experiment v.). The red LED light (red line) had a peak at 630 nm, and contained no blue wavelengths. The white light (grey line) used in these experiments were so called “warm-white” with two peaks in green and red, but also two small peaks in the blue wave-length band. Any photons with wavelengths in the visible spectrum (400 to 700 nm) are in principle

available for photosynthesis. However, the probability of a given photon of being captured by the photosynthetic apparatus will vary with the wavelengths of the photons and the pigments present in the algae (Kirk, 1994). An absorbance spectrum shows how efficient photons of different wavelengths are absorbed by the pigments in a plant and thus can be used in photosynthesis. The fraction of light energy converted into chemical energy through photosynthesis is thereby determined by the absorption capabilities of the pigments within the cells in the algae. Chromophytes (including the class Phaeophyceae) has the chlorophyll *a*/*c*-fucoxanthin protein complex as the major light harvesting complex, with the main absorption peak in blue light (Wang et al., 2010, MacIntyre and Cullen, 2005). *S. latissima* therefore showed a high photosynthetic efficiency and absorption in blue light. The white light used in this study included more of photons that were absorbed by the pigments in *S. latissima*, compared to the red LED light (Fig. 25). The higher gametophyte growth in white light in this study was therefore expected.

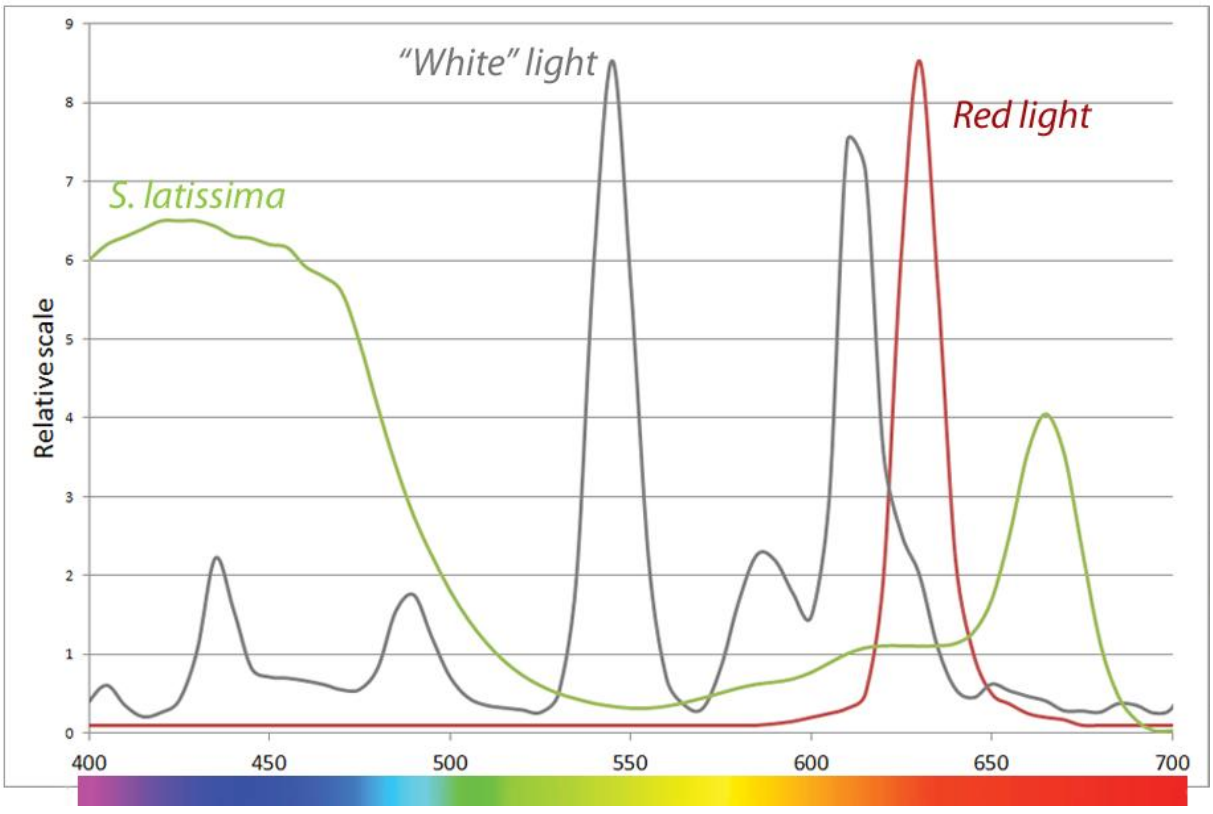

Figure 25 The green line represents the absorbance spectrum of *S. latissima* sporophytes from Fuglefjellet, Svalbard, in 2010. Measurements were made using a unicam Spectrofotometer (data made available by Inga Arnesen Aamot). The grey line represents the irradiance curve from the white light used in the experiment (data made available by Zsolt Volent). The red line represent the irradiance curve in red LED light as seen in literature with a peak in 630 nm.

Earlier research undertaken on growth of Laminariales in white/blue and red light qualities has given different results. Lüning and Dring (1975) have reported that *S. latissima* gametophytes showed almost identical relative growth rates in blue, white and red light qualities. Contrary to these results, Cuijuan et al. (2005) found that the growth of *Saccharina japonica* gametophytes was significantly higher under blue light than under red light. Lüning and Dring (1975) used glass filters and interference filters to filter away unwanted wavelengths, whereas Cuijuan et al. (2005) used LED lights. Glass filters might have a tendency of letting through unwanted wavelengths giving an increase in high-energy photons affecting growth (pers. communication with Zsolt Volent).

The gametophytes grown in red light showed higher cell pigmentation (Fig. 13) compared to the gametophytes grown in white light (Fig. 14). The reason for the increased pigmentation in red light is probably due to the lower amount of photons available for photosynthesis (Fig. 25). The gametophytes grown under red light therefore experienced lower light condition than the gametophytes grown in the same light intensity under white light. The rate of light absorption co-varies with the chlorophyll content of the cells (MacIntyre et al., 2000). Gametophytes are small photosynthetic structures with a low potential to alter their pigment composition in accordance to the fractional absorption of PAR, the so called package effect (Raven and Hurd, 2012). Gametophytes therefore have to synthesize new pigments in order to alter their absorbance of photons. The gametophytes grown under red light might have had a higher energy investment in synthesizing new pigments in comparison to the gametophytes grown under white light where more of the energy could be used for growth (Raven and Hurd, 2012, Neori et al., 1984).

The effect of blue light on the photosynthetic capacity appears to involve an increase in the rate of carbon dioxide assimilation to the plant (Forster and Dring, 1992, Wang et al., 2010). The photosynthetic carbon metabolism is initiated by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) and the maximum rate of carbon dioxide fixation co-varies with the concentration of Rubisco (MacIntyre et al., 2000). Both photosynthesis and the activity of Rubisco vary with light intensity by changing between active and inactive forms of the enzyme. A higher number of absorbed photons increases the amount of active forms of

Rubisco (Zhang et al., 2002). Hence blue wavelengths gives an increased inorganic carbon uptake (Wang et al., 2010), and the gametophytes grown under red light probably experienced a lower relative CO_2 assimilation rate than the gametophytes grown under white light and the growth might have been carbon-limited (Brown et al., 1995, Forster and Dring, 1992). Forster and Dring (1992) found that the maximum photosynthetic rates in brown alga were carbon-limited in red light.

The results in this thesis suggest that the use of a light quality containing more photons in the blue-wavelength band obtained a higher growth of *S.latissima* gametophytes. The reason for this seems to be due to higher energy investment into growth compared to synthesizing pigments and a higher CO_2 uptake rate. It is of high importance for optimal growth conditions to relate the quality of light source and the pigment composition in gametophytes.

4.3 Medium

The growth of *S. latissima* gametophytes were highly affected by the chemical composition of the medium it was grown in. The five experiments conducted in this study showed that the growth was strongly affected by the addition of chelating agents (Figs. 20, 16, 6), the concentration of N and P and the ratio between them (Figs. 20, 22, 23, 24), and the addition of seaweed extracts (Fig. 10). The effect was less protruding by the addition of a single phytohormone (Fig. 8). The two most commonly used media; Provasoli's Enriched seawater medium (PES) and Guillard's f/2 medium (f/2) also gave a significant difference in growth response (Fig. 6).

4.3.1 Chelating agents

The presence of chelating agents emerged as a crucial factor for the growth of *S. latissima* gametophytes (Figs. 16, 20). Chelating agents control the metal ion concentrations in biological systems, serving as a buffer system, regulating the availability of metal ions in a culture. Therefore the availability of chelating agents may be the factor which affects algal

growth (Sunda et al., 2005, Gerringa et al., 2000, Manahan, 2004). There were similar growths of gametophytes grown in sterilized seawater (SSW) and gametophytes grown in SSW enriched with N and P, without any chelating agent added to the medium in Experiment iv. (Fig 12). Seawater enriched with only nitrates and phosphates is not an adequate medium for the majority of algal species (Provasoli et al., 1957). Experiment v. showed that the addition of EDTA significantly increased the growth of the gametophytes compared to the growth in SSW (Fig. 16) and surprisingly also in PES ($p \leq .05$).

Other studies have shown that an addition of EDTA to seawater is as effective as an addition of an EDTA-chelated trace metals mixture for phytoplankton cultures (Johnston, 1964, Johnston, 1962). This is probably due to the chelation of labile forms of Cu^{2+} present in the seawater, limiting growth and/or the presence of adequate trace-metals in the natural seawater (Johnston, 1964, Johnston, 1962). Because most trace-metals in seawater are in a particulate or unionized form, unavailable to the plants, it need to be made available to the gametophytes as a chelating agent like EDTA (Jackson and Morgan, 1978). The results from this study suggest that there might have been enough trace-metals in the natural seawater and/or that there were metals present in the water that were toxic to the gametophytes when they were in an un-chelated form.

The chemical composition of PES and f/2 are relatively similar (Appendix I). One essential difference between the two media is the ratio between the chelating agent (EDTA) and the trace-metals added to the solution. A chelating agent should in theory combine with a metal in a 1:1 molar ratio, but in practice it is necessary to add an excess of chelate to ensure adequate chelation. A chelate-metal ratio of 1.5-3:1 is commonly used to obtain efficient chelation (McLachlan, 1973). Sunda et al. (2005) state that most of the iron added to the medium results as a precipitate of hydrous ferric oxide that adsorb other trace metals and further reduces their availability if the chelate-metal ratio is too low. According to Sunda et al (2005), this is probably what happened in Guillard's f/2 medium because of the low ratio (<1) of added EDTA to added trace metals. The same is unlikely to have happened in the Enriched Seawater medium (ESW) and PES due to a higher ratio (>2) of EDTA to trace metals. The high amount of EDTA in PES and ESW may also have reduced the bioavailability of toxic metals that might otherwise been too high and might have inhibited the development of the gametophytes (pers. communication with prof. F.M.M. Morel).

There was an increased growth of *S. latissima* gametophytes in medium containing low concentration of seaweed extracts (Fig. 10). The main reason for this is believed to be due to the hormones present in the seaweed extract (Crouch and Van Staden, 1993, Khan et al., 2009). Other constituents of the extract might also give growth enhancing effects and studies have shown that high amount of organic matter in the extracts have chelating effects (Johnston, 1964, Wang et al., 2009). Johnston (1964) found that the addition of plankton extracts gave a higher growth than an EDTA-trace metal solution. The high binding capacities of algal components, such as polysaccharides and phenolic compounds, to heavy metals are well documented (Wang et al., 2008, Wang et al., 2009). The stimulatory effects of low concentrations of the brown algal polyphenols are directly related to their ability to detoxify heavy metal ion (Ragan et al., 1980). The chelating ability of the seaweed extract used in this study *Ascophyllum nodosum* has been documented as very high, but still much lower than for EDTA (Wang et al., 2009, Saiga et al., 2003, Andjelković et al., 2006). Therefore the increased growth with AlgeaFert added is assumed mostly to be an effect of other substances present, such as growth hormones, or a combination of several factors, including chelation. EDTA has a low rate of biodegradation and it therefore remains for long time in natural waters. The presence of EDTA increases the leakage of heavy metals and therefore have detrimental effect in the natural waters (Manahan, 2004). Extracted seaweed may be a possible organic alternative to EDTA due to its proved chelating abilities.

From the present study it seem like there were enough trace-metals present in the seawater and that an addition of chelating agents together with the nutrients N and P supports high growth rates. When trace metals are added to the solution it is of great importance to provide a high enough amount of chelating agent to provide sufficient trace-metals to the gametophytes and/or to avoid toxicity of too high concentrations.

4.3.2 Nutrients

Increased phosphorous (P) concentrations in the growth media resulted in significantly ($p \leq .001$) higher gametophyte growths (Figs. 23, 24). An increase in the supply of nitrogen (N) in the growth media increased the number of cells in the gametophytes in Experiment v. (Fig. 22), but not in Experiment iv. (Fig. 21). An addition of N and P are almost always

necessary in order to prevent nutrient limitation of plant growth in cultures maintained in natural seawater (Berges et al., 2001, Harries, 1932).

The gametophytes grown in the ESW had a higher growth compared to the gametophytes grown in PES (Fig. 16). This was especially apparent in the treatments where extra P was supplied (ESW + P). PES is a more complex medium containing trace-metals, vitamins and nutrients, and a higher growth of the gametophytes would have been expected in this medium. ESW has a higher concentration of N and P (times 2.4 N and times 2.2 P) compared to PES. Therefore this may suggest that the growth of the gametophytes were to some extend limited by both N and P in PES or by the fact that ESW contains inorganic P instead of organic P as in PES. It is known that a deficiency of phosphorous in cultures generally may prohibit cell division (Thomas and Dodson, 1968).

The N and P contents of the gametophytes and in the media were regrettably not measured in the end of the experiment. This would have allowed an evaluation of the nutritional state of the plants and their optimal limiting factor for growth. There have been very little work on the nutritional requirements of brown algae (Chapman et al., 1978) and the literature on nutrients has mainly been focusing on nitrogen since this is the nutrient known to limit growth of macroalgae in natural waters (Lobban and Harrison, 1994). There are even less information available on the N and P contents of gametophytes and using data for sporophytes might be inappropriate because gametophytes may differ in many aspects from sporophytes. Studies show that the nutrient uptake rates depend on the size of the organism due to the differences in area-to-volume ratio, altering the area where the nutrients are taken up into the algae and made available for metabolism (Hein et al., 1995). The uptake kinetics in small algae have been shown to be significantly higher than for large algae (Hein et al., 1995). The mean uptake rate of N of *S. latissima* sporophytes have been measured to be 6.5 µmol g·dry weight $^{-1}$ h^{-1} over 9 days of growth (Subandar et al., 1993). Applying these data to the gametophytes in this study would give an uptake of 4.94 mmol $week^{-1}$ L^{-1} of N in the densest cultures (4.52 g·dry weight L^{-1}). This can be compared to the weekly additions in PES of 0.82 mmol N L^{-1}, in f/2 of 0.88 mmol N L^{-1} and in ESW of 2.0 mmol N L^{-1}, all being lower than the hypothetic weekly uptake. Hsiao and Druehl (1973) concluded that high concentrations of N and P produced *S. latissima* gametophytes with a higher cell number and were more profusely branched. Contrary the results in this study Hsiao and Druehl

(1973) found that the optimal concentrations of N and P in the media for gametophyte growth were 0.59 mmol N L^{-1} and 15 µmol P L^{-1}, which also is a lower addition than the nutrient levels in PES. It can therefore not be concluded that the levels of N and P in PES are too low for optimal growth of *S. latissima* gametophytes. The higher growth in ESW compared to PES may be because the nutrient uptake was limited by a water film surrounding the algal cells in culture thereby requiring a higher level of nutrients than if the cultures would have been mixed.

The N:P ratio had a significant ($p \leq .01$) relationship with the growth of *S. latissima* gametophytes under white light conditions (Fig. 20), but this effect was small or absent ($p=117$) under red light. A lower N:P ratio resulted in an increased number of cells per gametophyte, this was apparent in both of the two experiments conducted (Experiment iv. and Experiment v.). The N:P ratio will determine which of these nutrients that will become limiting for growth and drive the changes in chemical composition and physiological state of the algae (Berges et al., 2001). According to Berges et al (2001) experimentalists usually pay little attention to the N:P ratios in the medium they are using. Few data are available concerning the proportion in which nitrogen and phosphorous are utilized by the marine macroalgae (Kain, 1979). Three studies on N:P ratio and gametophytes show different results. Harries (1932) found a relationship between a decreasing N:P ratio in the medium (by increasing the phosphorous concentration in equal quantities of nitrogen) and a corresponding increase in the length of *S. latissima* gametophytes. Hoffmann et al. (1984) came to a similar conclusion where an N:P ratio of 10:1 gave comparably larger *Lessonia nigrescens* gametophytes than a higher N:P ratio of 40:1. These two studies agree with the results obtained in this thesis. Quite opposite, Hsiao and Druehl (1973) found an optimal N:P ratio of 40:1 for the number of cells per gametophyte. Guillard's f/2 medium has a ratio of 24N:1P and the gametophytes may have been phosphorous limited while N were still in excess (Harrison and Berges, 2005). The increased growth in media with lower N:P ratios found in this study (Fig. 20) may suggest that the optimal N:P of *S. latissima* gametophytes were lower than those in the f/2 and PES media. Another hypothesis from the results in this study is that an increased concentration of N and P to the media might be followed by an increased growth of *S. latissima* gametophytes.

4.3.3 Most commonly used media

The results from this study showed that there were significant differences ($p \leq .001$) in growth between the two most commonly used media; PES and Guillard's f/2 (Fig. 6). This stresses the importance of comparative studies and challenges the wide-spread assumption that many of the most common media work equally well when growing algae and *S. latissima* gametophytes (Harrison and Berges 2005, and pers. comm. Dr. Delin Duan). There was a higher degree of contamination of other algae and cyanobacteria in f/2 compared to both SSW and PES (Fig. 4). The cultures in PES and SSW consisted of purer *S. latissima* gametophytes. The slower growth of *S. latissima* gametophytes in f/2 (Fig. 5) might have allowed other algae to compete better for the available resources. The basic chemicals are relatively similar between the two media (see Appendix 1). However, three differences are apparent; the species of phosphorous, the ratio between N and P and the amount of added chelating agent (EDTA) (see Appendix 1). In PES organic phosphorous (Na_2 β-glycerophosphate) is used as a P, whereas f/2 contains an inorganic P (NaH_2PO_4). The higher growth in PES is probably not due to of the source of P because organic P did not give any increase growth (Fig. 12), but rather the contrary. PES has an 18:1 molar ratio of N and P and f/2 has a molar ratio of 24:1. The difference in N:P ratio between the two media had most likely a small influence on growth, but the ratio between chelating agent and trace metals in PES and f/2 probably have a higher effect on the gametophyte growth. The slower growth in f/2 compared to PES were probably due to the better nutrient composition and/or chelating abilities in PES (McLachlan, 1973, Sunda et al., 2005).

There was a significant difference in gametophyte biomass ($p \leq .05$) between a 7 day exchange interval and a 14 day exchange interval was seen in the cultures with high densities (Fig. 6), but this trend was absent in cultures with lower densities. An inversed trend could in fact be seen at very low densities. Therefore it seems like a frequent supply of new growth medium is necessary when the cultures are dense, because of the more rapid alternation and consumption of the nutrients of the medium (Sunda et al., 2005). A more frequent exchange rate might be needed in a production with increased volumes.

4.3.4 Hormones

Phytohormones such as IAA and KIN help to maintain or increase the growth of sensitive tissue in higher plants (Evans and Trewavas, 1991). A similar effect was expected in gametophytes. A low addition of seaweed extract gave an increased growth of *S.latissima* gametophytes (Fig. 10). The effect of addition of the phytohormone IAA to the media showed no effect. There was a relationship between the additions of KIN to the media and an increased growth of the gametophytes, but the trend was not very consistent (Fig. 8). An extended treatment time might have given stronger results, but this is doubtful since reports on similar studies have gotten results within the same time-range (Provasoli, 1958, Bradley and Cheney, 1990). Hormones are only necessary in small amounts and literature show that the amount of IAA (De-lin et al., 1995, Provasoli, 1958) and KIN (Provasoli, 1958, De-lin et al., 1995, Burkiewicz, 1987, Wood and Braun, 1967) used in this study should be enough to detect any effect. The most probable reason for the absence of any effect of phytohormones in the media was due to the non-sterile culture conditions. The phytohormones might have been broken down by the microorganisms in the seawater. The microbial community in the seawater might also have produced a variety of hormones in sufficient quantity to saturate growth of the gametophytes (prof. Matthew Dring, personal communication). This is supported by Bradley (1991) who states that an investigation of plant hormone effects should use axenic plant material in artificial seawater because marine bacteria are known to release plant growth promoters and seawater may contain active compounds that could interfere with the results.

The addition of a low concentration of extract of *Ascophyllum nodosum* gave a significantly higher growth rate compared to that in PES (Fig. 10). Plant growth regulators (PGRs) usually work in combination giving an enhanced effect in land plants. The same seem to be true for algae (Bradley and Cheney, 1990, De-lin et al., 1995) and a combination of PGRs (e.g. auxins and cytokinins) might be necessary to enhance growth and cell-division of cultured seaweed cells. The higher growth in AlgaeFert might therefore be due to the synergetic effect generated by the growth hormones present in seaweed extract (Crouch and Van Staden, 1993, Khan et al., 2009), and/or by larger molecules, such as oligomers and polysaccharide elicitors (Craigie, 2010). *Ascophyllum nodosum* are known to contain IAA, and concentrations as high as 50 mg IAA per gram of dry weight have been measured (Khan et al., 2009),

yielding a concentration of 315 µg IAA/L in the lowest addition of AlgeaFert. This would correspond to the intermediate concentrations added in the first hormone experiment and the effect of seaweed extract is therefore probably not only due to the presence of IAA. Seaweed extracts contains minerals and nutrients, but are unable to provide all the nutrients needed in higher plants (Schmidt et al., 2003). This might be true for macroalgae as well and additions of nutrients are most likely necessary.

Extracted seaweed is known to enhance the chlorophyll content of plants due to betaine present in the extract (Blunden et al., 1996). A higher concentration of seaweed extract gave an increased coloration of the gametophytes (Fig. 11). The higher growth of the gametophytes grown in low additions of AlgeaFert suggests that parts of PES can be exchanged with seaweed extracts as biostimulant giving an increased growth of the gametophytes and possibly other beneficial effects such as stress tolerance.

4.4 Fertility

Two experiments (Experiment iv. and Experiment v.) were conducted to evaluate the conditions preventing fertility. Unfortunately, there were no fertility in any of the treatments, even in the treatments were fertility was expected. Fertilization is the process requiring the most precise conditions (Hoffmann et al., 1984) and the production of vegetative plants, especially multiple-celled female gametophytes, is an effect of non-optimal conditions (Hsiao and Druehl, 1971, Izquierdo et al., 2002, Lüning and Neushul, 1978, Kain, 1979). Since all treatments in Experiment iv. (Fig. 14) and Experiment v. (Fig. 18) consisted mostly of unfertile multiple-celled female and male gametophytes, some variable seem to inhibit fertility. No signs of fertility were found after thirteen weeks of growth under conditions assumed to be optimal for fertility (Fig. 14). After week 9 the light regime was changed to 16:8h (light:dark cycle) and the weekly addition of GeO_2 were terminated, without any effect on the fertility. In other studies, *S. latissima* gametophytes grown in PES medium, has been shown to become fertile within a short period of time (Brinkhuis et al., 1984). Therefore, for the gametophytes grown in PES under white light at 30 µmol $m^{-2}s^{-1}$ at a constant temperature of 10 °C fertilization within a couple of weeks was expected (Edwards

and Watson, 2011). Cutting the gametophytes into smaller pieces are sometimes used to induce gametophyte fertility, this because the shorter fragments with a bigger surface area are believed to receive more light, carbon and/or nutrients (Zhang et al., 2008). The small size of the culture vessels used in this experiment might have created a micro-environment with limited supplies of light, carbon and/or nutrients might explain why no reproductive organs were produced. In this experiment the cultures were agitated to avoid establishment of any micro-environments, but apparently without any success in inducing fertility. There were no signs of fertility in the gametophytes in Experiment v. and after five weeks of growth no signs of sex organs were observed (Fig. 18). After the experiment was ended, the light intensity was increased to 60 µmol $m^{-2}s^{-1}$ and the gametophytes were transferred to 100 ml petri dishes, to see if this would induce fertility. This had no influence on the fertility of the gametophytes after 4 additional weeks of treatment. Cuijuan et al. (2005) found that fertility due to different light qualities in *Saccharina japonica* gametophytes differed significantly depending on what time of the year the zoospores where collected. Some gametophytes only grew vegetatively and never formed oogonia or antheridia, independent of the light quality and intensity. Cuijan et al. (2005) argued that the reason for this may be seasonal differences in the patterns of the gene expressions, resulting in different reactions to blue light. If this is true, this might also explain the absence of sexual organs in this study.

Since none of the treatments in this study resulted in fertility it cannot be concluded which treatments that inhibit fertilization and promote vegetative growth. The process of reproduction is a delicate balance of many factors that has to be further studied.

4.5 Future research

The genetic improvement of *Saccharina japonica* has contributed to the fast development of cultivation in China (Wang et al., 2012) and today almost all of the harvests comes from genetically modified strains (Li et al., 1999). Usually this is done through successive inbreeding and selection using meiosis as the basic process of spore formation. This is a time-consuming method. An alternative is to use gametophyte clones, which can be propagated vegetatively under controlled conditions, saving time, money and avoid

degeneration of selected strains (Westermeier et al., 2006, Li et al., 2007). The culture method described in this study, with vegetative growth due to mitosis, provide a good base to initiate systematic breeding programs. This can be used to establish a genetically defined and stable gametophyte culture with selection to yield a higher growth rate, reduced tissue abrasion, increased stress tolerance, increased disease resistance and tolerance of temperature and irradiance etc. (Westermeier et al., 2006). This can also be used as a base for hybridization between species for a combined agronomic effect, which is widely used in China (Li et al., 2007, Druehl et al., 2005).

From the results presented above, it can be seen that the quality of light affected the growth of *S. latissima* gametophytes. A higher growth might be possible if the light composition match the absorption spectrum of *S. latissima* better, with a higher proportion of wavelengths in the blue wavelength band than the white light used in this study. Finding a more suitable light source would also further rationalize the energy consumption in the on-land cultivation step in a large scale production of seaweed, this because less energy have to be used to produce photons that are not used for photosynthesis. Using light containing blue wavelengths has to be accompanied by a method to prevent fertilization alternatively to red light. This study aimed to find a method for this, but without any positive results. More knowledge about the fertility process is of great importance to avoid a sudden outburst of fertile gametophytes at the wrong time. This is also important when it comes to knowledge about how to induce fertility in the gametophytes to produce sporelings.

The two N:P ratio experiments showed that a lower N:P ratio gave a higher growth rate. This should be further studied in order to find the optimal concentration of nitrogen and phosphorous the ratio between them for vegetative growth.

5 CONCLUSION

The present study demonstrated that an alternation of light quality from red LED light to warm-white fluorescence light gave a significantly higher growth of *Saccharina latissima* gametophytes. From these observations white light appeared to have a spectral composition more corresponding with the pigments in *S. latissima* gametophytes, compared to the red light. The potential of higher growth of the gametophytes grown in a light source containing blue wavelength seem to be due to higher energy investment into growth compared to synthesizing pigments and a higher CO_2 uptake rate.

The five experiments conducted in this study showed that the growth of *S. latissima* gametophytes were largely affected by the chemical composition of the medium it was grown in. The growth were affected by an addition of chelating agents, the concentration of nitrogen (N) and phosphorous (P) and the ratio between them, and the addition of seaweed extracts. The effect was less protruding by the addition of a single phytohormone. The two most commonly used media; Provasoli's Enriched seawater medium (PES) and Guillard's f/2 medium (f/2) also gave a significant difference in growth response.

In this study the presence of chelating agents emerged as a crucial factor for the growth of *S. latissima* gametophytes. Comparison between experiments showed that a sufficient chelation is necessary for a satisfactory supply of trace metals and to reduce the bioavailability of toxic metals. This is probably the reason why PES was a more suitable medium than f/2. There was a significant increase in growth of the gametophytes when the chelating agent EDTA was added to the media together with the nutrients N and P compared to sterilized seawater (SSW). It seems like enough chelation and an addition of N and P, are the only additions necessary for a high growth of *S. latissima* gametophytes, and chemically complex and expensive mediums, such as PES, may be exchanged by a simpler medium such as Enriched Seawater medium (ESW).

Extracted seaweed increased the growth significantly compared to PES, probably due to a desirable constitution of hormones, nutrients and chelating properties. The effect was small or absent in the experiment evaluating the addition of the natural phytohormones; IAA and KIN.

The nutrient ratio showed a significant relationship with gametophyte growth where a decrease in N:P ratio was followed by an increased growth. The optimal N:P ratio for *S. latissima* gametophytes may therefore differ from the N:P found in conventional mediums based on the Redfield ratio, but this should be examined more thoroughly.

Since none of the treatments in this study resulted in fertility it cannot be concluded which treatments that inhibit fertilization and promote vegetative growth. The process of reproduction is a delicate balance of many factors that has to be further studied.

Due to the complicated growth patterns of *S. latissima* gametophytes various methods had to be used when estimating growth depending on culture conditions. The growth measuring techniques used in this study showed to be appropriate for the specific experiments conducted.

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Appendix I

Provasoli Enriched Seawater Medium (PES)

– Version as in Harrison and Berges. In Aquaculture explained No. 26 Cultivating Laminaria digitata (Edwards and Watson, 2011)

Iron-EDTA solution was prepared by taking 900 ml distilled water and dissolve the components shown (in the same order as shown) in the table below. Distilled water was added to bring the final volume to 1 liter. The solution was pasteurized in a 90 °C water bath for 4 hours. The solution was stored in a sterile dark glass bottle and kept refrigerated.

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

The Trace Metals Solution was prepared by adding 900 ml distilled water to a volumetric flask. The EDTA was dissolved first and thereafter the rest of the ingredients in the same order as shown below. The final volume was brought to 1 liter by distilled water. It was thereafter pasteurized and refrigerated.

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

The Enrichment Stock Solution was prepared in a volumetric flask. 500 ml distilled water was used and the components in the list below were added. 0.5 g of Na_2 β-glycerophosphate · H_2O was exchanged with 0,654 g of Na_2 β-glycerophosphate · $5H_2O$. The vitamins were added last after all of the other ingredients have been added. The contents were stirred with

a magnetic stirrer and a little heat was added. Distilled water was added to bring the final volume to 1 liter. The solution was pasteurized in a 90 °C water bath for 4 hours. The solution was stored in a sterile dark glass bottle and kept refrigerated.

Component Stock Solution	**[g L^{-1} dH_2O]**	**Quantity Used**	**Concentration in Final Medium**
Tris Base	-	5.0 g	$8.26 \cdot 10^{-4}$ M
$NaNO_3$		3.5 g	$8.24 \cdot 10^{-4}$ M
Na_2 β-glycerophosphate $\cdot 5H_2O$	-	0.654 g	$4.63 \cdot 10^{-5}$ M
Iron-EDTA solution (see following recipe)		250 ml	-
Trace Metals solution (see following recipe)		25 ml	-
Thiamine · HCl (vitamin B1)	-	0.500 mg	$2.96 \cdot 10^{-8}$ M
Biotin (vitamin H)	0.005	1 ml	$4.09 \cdot 10^{-10}$ M
Cyanocobalamin (vitamin B12)	0.010	1 ml	$1.48 \cdot 10^{-10}$ M

When used: The enriched seawater medium was prepared by adding 20 ml enrichment stock solution to 980 ml autoclaved and filtered seawater.

Guillard's f/2 medium

-Version as in Aquaculture explained No. 26 Cultivating Laminaria digitata (Edwards and Watson, 2011).

f/2 Trace Metals Solution was prepared by dissolving EDTA and the other components from the list below in 950 ml distilled water and thereafter brought to 1L.

Component 1° Stock Solution	**[g L^{-1} dH_2O]**	**Quantity Used**	**Concentration in Final Medium**
$FeCl_3 \cdot 6H_2O$	-	3.15 g	$1.17 \cdot 10^{-5}$ M
$NaEDTA \cdot 2H_2O$	-	4.36 g	$1.17 \cdot 10^{-5}$ M
$MnCl_2 \cdot 4H_2O$	180	1 ml	$9.10 \cdot 10^{-7}$ M
$ZnSO_4 \cdot 7H_2O$	22	1 ml	$7.65 \cdot 10^{-8}$ M
$CoCl_2 \cdot 6H_2O$	10	1 ml	$4.20 \cdot 10^{-8}$ M
$Na_2MoO_4 \cdot 2H_2O$	6.3	1 ml	$2.60 \cdot 10^{-8}$ M

The f/2 Vitamin solution was prepared by adding the vitamins below in 950 ml distilled water, and thereafter bringing the volume to 1 liter. The solution was filter-sterilized and frozen.

Component Stock Solution	**[g L^{-1} dH_2O]**	**Quantity Used**	**Concentration in Final Medium**
Thiamine · HCl (vitamin B_1)	200 mg	-	$2.96 \cdot 10^{-7}$ M
Biotin (vitamin H)	1.0	1 ml	$2.05 \cdot 10^{-9}$ M
Cyanocobalamin (vitamin B_{12})	1.0	1 ml	$3.69 \cdot 10^{-10}$ M

When used: 1 ml of each of the first three components from the list below was added to 1L of filtered seawater. The solution was autoclaved and 0.1 ml of the vitamins solution was added per liter of solution.

Component 1° Stock Solution	**[g L^{-1} dH_2O]**	**Quantity Used**	**Concentration in Final Medium**
$NaNO_3$	75 g	1 ml	$8.82 \cdot 10^{-4}$ M
NaH_2PO_4	5 g	1 ml	$3.62 \cdot 10^{-5}$ M
Trace Metals Solution (See recipe above)		1 ml	-
Vitamins solution (See recipe above)		0.1 ml	-

Enriched Seawater (ESW)

As used by Hoffmann and Santelices (Hoffmann and Santelices, 1982) (minus Tris).

Component 1° Stock Solution	**[g L^{-1} dH_2O]**	**Concentration in Final Medium**
$NaNO_3$	8.5 g	$2.0 \cdot 10^{-3}$ M
$NaH_2PO_4 \cdot H_2O$	0.69 g	$0.1 \cdot 10^{-3}$ M
$Na_2EDTA \cdot 2H_2O$	3.7×10^{-3} g	$2.0 \cdot 10^{-6}$ M

To ESW+P it was added double amount of phosphate. To ESW+N it was added double amount of nitrate. The chemicals were added to 900 ml distilled water. Distilled water was added to bring the final volume to 1 liter and the solutions were then autoclaved.

When used: The ESW was prepared by adding 20 ml enrichment stock solution to 980 ml autoclaved and filtered seawater.

Appendix II

Hormone stock solution

1 mg/ml stock solution was prepared by adding 100 mg of plant growth regulator (IAA, KIN) to a 100 ml volumetric flask. 5 ml of solvent (1 N NaOH) was added to dissolve the powder. Once it was completely dissolved the volume was (during stirring) brought to 100 ml with distilled water. 1.0 ml of the stock solution was added to 1L medium giving a final concentration of 1.0 mg hormone/L solution. It was thereafter sterilized with 0.20 µm filter (Millipore membrane) into sterile containers and frozen for preservation (sigma Aldrich)