(A)biotic lifecycle controls of multiannual delayed gametophytes of *Laminariales* (brown seaweeds)

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Alla luce del nostro futuro, un po' di saggezza del passato.

"Il piu grande pericolo per noi non é che miriamo troppo in alto e non riusciamo a raggiungere il nostro obiettivo, ma che miriamo troppo in basso e lo raggiungiamo." *Michelangelo Buonarroti*

* In light of our future, some wisdom from the past

"The greatest danger for us is not that our aim is too high and we miss it, but that it is too low and we reach it."

Summary

The lifecycle of the brown seaweed species *Saccharina latissima* and *Alaria esculenta* (*Laminariales*) have been studied for decades. Most studies have been done on the macroscopic diploid sporophyte part while gametophytes, which are the haploid microscopic part of the lifecycle, are relatively understudied. The main reason why gametophytes are understudied is because it takes a lot of effort to reliable study gametophyte cultures under in-vitro conditions, while in-vivo observations of gametophytes are practically impossible to do. The vast majority of gametophyte studies use "newly-formed" gametophytes to conduct their studies. These newly-formed gametophytes are gametophytes that recently formed from zoospores. A second characteristic of these newly-formed gametophytes is that they did not have a significant period of vegetative growth before sexual reproduction took place. Here we will look more closely to those gametophytes that did actively delay their sexual reproduction, by growing vegetatively instead.

Understanding how and when "delayed" gametophytes initiate sexual reproduction or stay vegetative is gaining interest in recent years. The main reason for this uptick in interest is the way delayed gametophytes can be used as "seed-stock" for an innovative cultivation method that is meant to serve an ever expanding seaweed industry. There are two main cultivation methods that lie at the heart of kelp cultivation. The traditional "uncontrolled reproductive method" primarily uses zoospores to seed, while the more novel "controlled reproductive method" primarily uses delayed gametophytes and the subsequent young sporophytes to seed. Multiannual delayed (MAD) gametophytes are especially off interest within the controlled reproductive method, since these gametophytes can grow vegetative for years, are easy to clone, and ideal to use for breeding purposes. However, how and when MAD gametophytes initiate sexual reproduction is an very understudied subject and not well understood. There are still a lot of unknowns that govern the sexual reproduction of MAD gametophytes and thus hamper the reliability in which viable sporophytes can be produced. In order to reliable produce sporophytes, using MAD gametophytes, we need to better understand how to control the sexual reproduction of these delayed gametophytes using different (a)biotic factors, which in this case will be called (a)biotic lifecycle

In order to reliable produce sporophytes, using MAD gametophytes, we need to better understand how to control the sexual reproduction of these delayed gametophytes using different (a)biotic factors, which in this case will be called (a)biotic lifecycle controls.

This thesis delves deeper into how (a)biotic lifecycle controls govern delayed gametophytes, for either the initiation of sexual reproduction or vegetative growth. Focus is placed on the interactive effects of several major (a) biotic lifecycle controls that influence the state of a gametophyte culture at any given moment (i.e. light, temperature, culture density, sex-ratio, and nutrients). In order to quantitively compare the reproductive success between experiments and species we quantified gametophyte biomass in all our experiments. Understanding how much gametophyte biomass is used for sexual reproduction allowed us not only to quantify the reproductive success (sporophytes · mL^{-1}), but also the relative reproductive success (sporophytes · mg^{-1} gametophyte DW) in all our cultures. The quantification ultimately allowed us to observe the effects that culture density has on the sexual reproduction of delayed gametophytes.

Culture density influenced the reproductive success of delayed gametophytes the most predictable of all (a)biotic lifecycle controls we tested. An inverse correlation between reproductive success and culture density was observed in all experiments, regardless of other (a)biotic factors, after an initial reproductive optimum was reached. At what gametophyte density the reproductive optimum specifically was reached depended on the species, location, and sex-ratio, but the trend was always there and observable. Especially the quantity of female gametophyte biomass appeared to be a strong determinant of reproductive success, with optimal reproductive success in *Saccharina latissima* (0.013 mg · mL^{-1} DW) and *Alaria esculenta* (0.025 mg · mL^{-1} DW) being reached at similar female gametophyte biomass densities.

Light, as abiotic lifecycle control, can be subdivided into light quantity (light intensity) and light quality (light colour). Here we show that light quality only influenced the reproductive success in limited ways, while light intensity strongly influenced the reproductive success. The function of light quality

while light intensity strongly influenced the reproductive success. The function of light quality becomes especially apparent when light intensity and light quality are combined, into the overarching photosynthetically usable radiation. Our results suggest that light quality might not be used as a qualitative signal for the initiation of sexual reproduction by delayed gametophytes, but that the amount of photons that are absorbed by the gametophyte are key to determine whether sexual reproduction is initiated or not.

Temperature was also a strong determinant for the reproductive success, with clear reproductive optima (10.4 – 12.6°C) observable. Clear reproductive minima were also observed, especially at higher temperatures (>14.0°C) where sexual reproduction was practically being halted altogether. What was especially intriguing was how temperature interacted with light intensity. We observed that sexual reproduction increased at lower temperatures when light intensity was high, while sexual reproduction increased at higher temperatures when light intensity was low. This initiated a search for an hypothesis that might explain these results and the subsequent birth of the seasonal-lag hypothesis. The seasonal-lag hypothesis revolves around the yearly cycle of daily radiation and sea surface temperatures, resulting in increased discrepancies between the two abiotic factors during autumn and spring conditions. The seasonal-lag hypothesis suggests that the increased discrepancy between light and temperature is used by MAD gametophytes to precisely time and align their sexual reproduction with each other, and maybe even the surrounding adult sporophytes.

Understanding these (a)biotic lifecycle controls that govern the sexual reproduction of MAD gametophytes ultimately helps us towards our goal to further domesticate kelp as a crop. However, crop domestication is not only achieved through theory, it also needs an applied component that translates theory into a tangible, operational task that can be successfully executed by a farmer. Here is where the SeaCoRe system comes into play, with a sequential set of bioreactors that are compatible with each other, plug-and-play, and all have their own specific function in the overarching process of producing enough sporophytes for a seaweed farm. Here we describe the blue prints and validation of the system, and subsequently do the calculations needed

sporophytes for a seaweed farm. Here we describe the blue prints and validation of the system, and subsequently do the calculations needed to assess how much gametophyte biomass is needed for large scale kelp farming, using the controlled reproductive method.

Content

Underwater view of a seaweedfarm with direct seeded *Alaria Esculenta* and *Saccharina latissima* kelp.

We are all screwed
So don't tell us that
We can imagine a healthy planet
Because at the end of the day
It's too late to fix the climate crisis
And we refuse to believe
We need to demand a liveable future
Because we don't have a choice

Now read this bottom up

Chapter 1

General Introduction

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CHAPTER 1

INTRODUCTION

This thesis aims to better understand the lifecycle of the kelp (*Laminariales*), and in particular the lifecycle of the species *Saccharina latissima* (Fig. 1-1) and *Alaria esculenta*. Especially the life cycle transition where kelp gametophytes initiate gametogenesis, and through sexual reproduction form sporophytes is of interest (Fig. 1-2). We use multi annual delayed (MAD) gametophytes to study this, since these understudied gametophytes have many intrinsic characteristics that make them ideal as seed stock for seaweed aquaculture. These specific gametophytes delayed their sexual reproduction by growing vegetative for prolonged periods of time (Pang et al., 1996), often in artificial environments, changing the way they react to the lifecycle controls that govern lifecycle transitions (Carney and Edwards 2011). The better our fundamental understanding becomes on this part of the lifecycle of the *Laminariales*, the better we are able to control MAD gametophytes. The better we are able to control MAD gametophytes, the better we can use them in our domestication of the *Laminariales*, as a crop. The better we domesticate *Laminariales*, the better we can cultivate them, closing the gap towards a collectively aspired reality of large scale seaweed aquaculture.

The lifecycle of the *Laminariales*

Species of the order *Laminariales* (Phaeophyceae, brown seaweeds), have a heteromorphic lifecycle that alternates between haploid gametophytes and diploid sporophytes (Fig. 1-1). The Gametophytes grow vegetatively through fracturing (Destombe and Oppliger, 2011), can persist for prolonged periods of time (Carney, 2011), even up to years (Zhao et al. 2016), and remain highly sensitive to changes in environmental quality (Edwards, 2000; Carney and Edwards, 2006). The asexual reproduction, growth and increase of gametophyte biomass is regarded to be the adaptive form for stressful environments (Dieck 1993). To date the (a)biotic factors, or lifecycle controls, that determine whether delayed gametophytes persist in their asexual vegetative forms or begin gametogenesis remains open for exploration. A better understanding of the lifecycle controls of this microscopic part of the

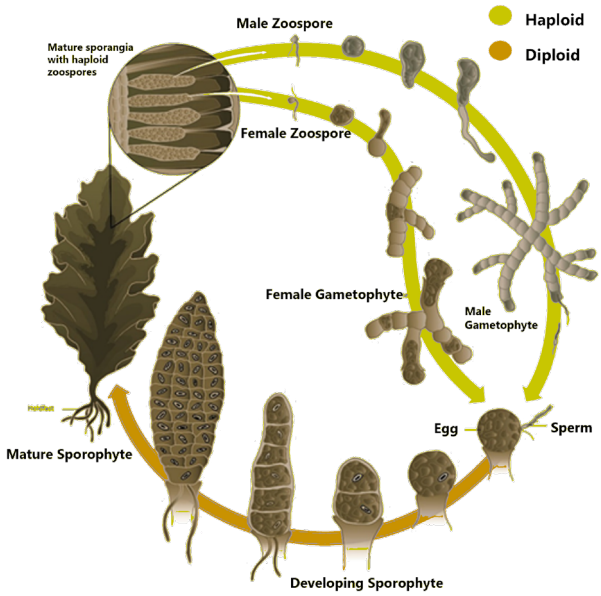

Figure 1-1. The lifecycle of *Saccharina latissima* (*Laminariales*)

lifecycle of the *Laminariales* is highly needed, as this phase largely determines their recruitment success (Wiencke et al. 2006; Fredersdorf et al. 2009). The transition to the generative phase is furthermore thought to be highly susceptible to environmental perturbations, and hence a critical process in determining the distributional limits of the species (Destombe and Oppliger 2011). Whether gametophytes initiate gametogenesis may be influenced by a range of abiotic lifecycle controls such as temperature (Morita et al. 2003), light intensity (Bolton and Levitt 1985), photoperiod (Choi et al. 2005), and nutrient availability (Martins *et al.* 2017).

Especially the spectral composition of light has often been described as a major influencer of gametogenesis, with blue light acting as a major inducer of gametogenesis (Lüning and Dring 1972; Lüning and Dring 1975; Ratcliff et al. 2017). Biotic factors have also been identified as potential lifecycle control mechanisms for gametogenesis (Pohnert et al. 2002; Frenkel et al. 2014). Most studies on the *Laminariales* have focused on sexual pheromones like “ectocarpene”, “fucoserratene” or “lamoxirene”. Ectocarpene, is the first brown algal pheromone that was identified and functions as a male attracting substance (Müller 1971), while lamoxirene

and fucoserratene are excreted by the eggs (oogonia) and are involved in the fertilization with sperm (Müller and Jaenicke 1973; Marner et al. 1984), with lamoxirene inducing the active release of sperm from the antheridia of male gametophytes (Kinoshita, Nagasato and Motomura 2017). In the end, it is the interaction between these (a)biotic factors, as lifecycle controls, that result in either the initiation of gametogenesis, or the delaying of it by prolonging their vegetative growth.

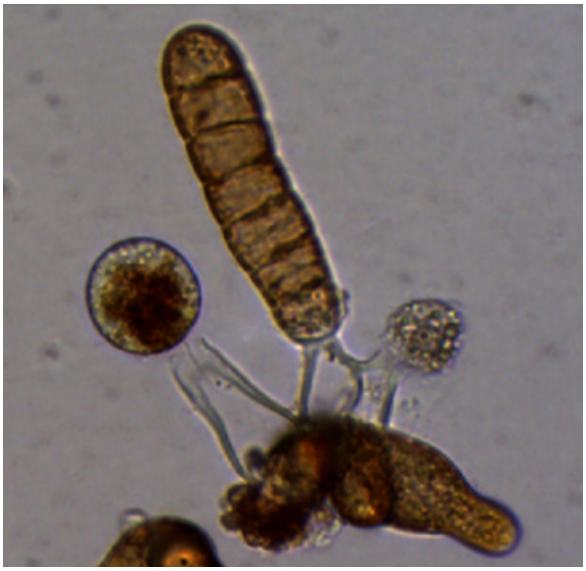

Figure 1-2. juvenile *S. latissima* sporophyte (middle), a fertilized oogonium (left), and a dying oogonium (right), developing from the same female gametophyte

Delayed gametophytes are gametophytes that actively delayed sexual reproduction by growing vegetatively. These delayed gametophytes are relatively understudied compared to gametophytes that were newly formed from zoospores (Bartsch 2008; Carney 2011). The need to better understand delayed gametophytes is apparent from a scientific point of view, since delayed gametophytes may be an integral component of the lifecycle of both annual (Lüning 1980, Klinger 1984, Blanchette 1996, Edwards 2000, McConnico and Foster 2005), and perennial species of brown seaweeds (Ladah et al. 1999, Hernandez-Carmona et al. 2001). MAD gametophytes, which are gametophytes that transgressed any seasonally induced reproduction by

remaining vegetative for more than a year, are of particular interest in our research into the (a)biotic lifecycle controls. From a research perspective this is mainly interesting because of the possibility of the *Laminariales* to possess an endogenous annual clock (Lüning and Diecks 1989), and by remaining vegetative for more than a year it transgresses any circannual rhythms that could influence/hamper gametophyte reproduction, and thus interfere with our research into the (a)biotic lifecycle controls of delayed gametophytes. MAD gametophytes are also interesting from a practical point of view, since they can be stored relatively easily, can be reused in experiments, and the fact that they grow vegetatively through fracturing (Destombe and Oppliger 2011) creates the possibility to do repeating studies on the reproduction of gametophytes with genetically identical gametophyte cultures.

Understanding the lifecycle controls of delayed gametophytes on a fundamental level will give us a better understanding of their role in their natural environment. It may answer why these gametophytes evolved to delay their reproduction in the first place, but also how the delay of reproduction translates to the seasonality that is observed in sporophytes. The same lifecycle controls also function as tools that can be used in the domestication of the *Laminariales*, as a crop, and can therefore be utilized by seaweed farmers for the cultivation of seaweeds using delayed gametophyte cultures as seed stock.

The controlled reproductive method as fundamental basis for the domestication of kelp

Crop domestication, commonly defined as the anthropogenic separation of the domesticate from free-living populations, requires humans to be master over all aspects of its lifecycle (Zeder 2015). Crop domestication is also an integral part when cultivating *Laminariales*, which after this will be described as kelp aquaculture. This mastery allows the kelp farmer to cultivate kelp the way they want, on farms they designed, on locations of their own choosing (Broch et al. 2019). In the case of kelp aquaculture it is still difficult to pinpoint how far we progressed on the domestication ladder. Vodouhè and Dansi (2012) describe 7 cultivation phases in the domestication process of crops. The progression starts at phase 1, with plants that are still wild and only collected when needed. The last phase is phase 7, where income

generation of the farmer is more clearly taken care of using species varieties that meet specific consumer preferences (Table. S3-2). Kelp aquaculture hovers between the 4th and 6th phase.

> *Phase 4* is the phase in which the reproductive biology of the species is known but mass production still needs to be mastered.
>
> *Phase 5* is when the crop is cultivated and harvested using traditional practices.
>
> *Phase 6* is the phase where farmers are able to adopt specific criteria to select plants that better satisfy the needs of consumers.

Even though some kelp farmers are already selecting plants that are able to better satisfy the needs of consumers (Zhang et al. 2017; Valero et al. 2017), many phase 4 aspects of mass production are still researched today. Examples of phase 4 research are the identification of the date of planting (Peteiro and Freire 2009), substrate optimization (Kerrison et al. 2018), identifying optimal farm locations (Matsson et al. 2019), and development of disease management (Peng and Li 2013). It might seem weird that the cultivation of a crop can hoover between different domestication phases, but the reality is that as of today there is still no consensus of what the best cultivation method for large scale kelp farming is (Goecke 2020). On the surface this might seem a more applied question, but in order for us to scale the domestication ladder of kelp, we need to further our fundamental understanding in the lifecycle of kelp (Fig. 1-1) and the corresponding lifecycle controls (i.e. (a)biotic factors) that are used by kelp to transition between life stages.

To date, two different cultivation methods are being practised in kelp aquaculture (Kim et al. 2017): the uncontrolled reproductive method versus the controlled reproductive method (Chapter 3).The uncontrolled reproductive method originates from Japan Fig. 1-3, and is considered to be the traditional method (Shan, Pang and Gao 2013) and is done by releasing substantial amounts of zoospores in large tanks. These zoospores first develop into gametophytes, after which the newly-formed gametophytes sexually reproduce and form young sporophytes on twine strings (Su et al. 2017), all happening without the direct interference of the farmer.

Figure 1-3. Visiting a seaweed farm that uses the traditional method of cultivation (Sendai region, Japan).

In contrast, in the controlled reproductive method gametophyte reproduction is directly controlled by the farmer, by inducing gametophyte reproduction under laboratory conditions (Ratcliff et al. 2017), preferably using delayed gametophytes. The induced gametophytes, often already showing juvenile sporophytes (Fig. 1-2) are then attached to seed strings using a method called paintbrush-seeding (Redmond et al. 2014a). In fact, these two methods symbolise the fundamentally different ways of how kelp is getting domesticated, i.e., whether gametophyte reproduction is directly controlled by humans (the controlled reproductive method) or whether reproduction happens without our direct interference (the uncontrolled reproductive method). Although the differences at first glance might seem trivial, using the controlled reproductive method can change how kelp is farmed on a fundamental level, like the possibility to direct seed your sporophytes on ropes and nets (Fig. 1-4), without the need for a mandatory hatchery phase in between (Kerrison et al., 2018). However, there are a couple immediate hurdles to the adoption of the controlled reproductive method : (1) the method is more labour intensive then the uncontrolled reproductive method, and (2) shows a higher detachment from the seed

strings when applied (Xu et al. 2009). This probably explains for a large part why using the uncontrolled reproductive method is still the common practice in many parts of the world (Goecke et al. 2020).

Towards the future however, using the controlled reproductive method might be the more promising method because it provides the basis for a novel way of kelp cultivation and breeding. A method that revolves around using delayed gametophytes as seed stock. Delayed gametophyte cultures are promising as a seed stock since *i)* these gametophytes can be kept in cultures for prolonged periods of time (Barrento et al. 2016; Carney 2011; Wade et al. 2020), *ii)* can successfully be cryopreserved for long term storage (Visch et al. 2019, *iii)* gametophyte reproduction can successfully address the reproductive yields needed for large scale kelp aquaculture (Ratcliff et al. 2017), and *iv)* the crossing of distantly related unialgal male and female gametophyte clone cultures may evoke intraspecific hybrid vigour for future F1 hybrid cultivars (Shan et al. 2016) and (Zhao et al. 2016). However, delayed gametophytes and their role in kelp aquaculture is still a largely understudied subject and using these cultures is only achievable by fully understanding the lifecycle and lifecycle controls of these larger volumes of delayed gametophytes (Zhang et al. 2008).

The controlled reproductive method can only function optimally in combination with delayed gametophytes as its seed stock. This is why we need more studies that focus on delayed gametophytes since, to this day, the large majority of gametophyte studies have been focussing on newly formed gametophytes (Bartsch et al. 2008; Carney et al., 2011). The idea that the controlled reproductive method can be based on research conducted with newly formed gametophytes contains certain misassumptions. First, the misassumption that newly formed gametophytes react the same way to (a) biotic factors as delayed gametophytes. Second, the misassumption that the artificial environments where delayed gametophytes reside do not influence the behaviour of these delayed gametophytes in the long run. A lot of progress in the field of delayed gametophyte research is still needed, especially since it is not only research into delayed gametophyte reproduction that is necessary, but also research into the cultivation tools that allow for the utilization of these delayed gametophytes.

Not only a fundamental understanding of kelp is necessary in the

quest for domestication, but also the applicability of the tools that allow for cultivation. Any successful domestication effort is the result of both the theoretical understanding of the lifecycle of the crop and the applicability of the cultivation method that is conducted by the farmer. It must allow farmers, of all walks of life, to control and utilize the lifecycle of kelp as easy and optimally as possible. Here is where novel kelp propagation systems come into play, and in particular systems that govern the vegetative growth and reproduction of delayed gametophytes. Its only when fundamental science and applied science merge that progress becomes tangible and large scale kelp cultivation can become a reality, allowing for its potential to come into fruition.

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Figure 1-4. Direct seeded net structure with *S. latissima* sporophytes. Several pollock fish are visible, taking refuge in the young artificial kelp forest.

The potential role of kelp aquaculture in the years to come

Interest in seaweed aquaculture is rising around the world for both food and non-food applications (Holdt and Kraan, 2011), and the cultivation of

seaweed is growing rapidly, resulting in more than a doubling in aquaculture production since 2000, with no indications of slowing down (FAO 2015). The *Phaeophyceae* (kelp) belong to the most cultivated seaweed species and in 2018 cultivated kelp species accounted for approx. 15 million tonnes wet weight (ww), corresponding to 45%, of global seaweed production (FAO 2020). The main driver for the increased interest in seaweed aquaculture and in particular kelp aquaculture is the search for novel renewable sources of food (van der Burg, 2021), feed (Carrier et al. 2017), bioplastics (Lim et al., 2021), fertilizer (Gutierrez et al,. 2006), or as a scalable carbon sink for the mitigation of climate change (Chung et al. 2013). Although this form of oceanic farming is relatively new, it is recently gaining traction due to its perceived potential as one of the low-impact crops of the 21st century.

The need for low-impact crops coms from the increasing pressures on the earths ecosystem, by a growing human population, who simultaneously also increases its environmental footprint. All this in combination with a looming climate crisis, asks us to look for alternative low-impact crops that can be utilized. Shortages in fresh water and food, in combination with a changing climate, irreversible tipping points, and ecological decimation culminates into one of the grand challenges of the 21st century: The search for a cultivatable crop that;

- uses minimal amounts of fresh water
- does not compete with conventional agriculture
- can grow fast
- can be grown all year around
- does not need damaging fertilizers
- can ultimately can be grown in all corners of the planet.

This might sound like a daunting search, and it is, but this thesis delves deeper into a cultivatable crop that ticks all the boxes. Kelp, that as a crop does not need fresh water or fertilizers, does not compete with conventional agriculture, can grow incredibly quickly and all year around (Bak et al. 2020), and is found in all corners of the earth (Jayathilake 2020).

Grown on large enough scales it has the added potential of deacidifying the ocean (Hirsch et al., 2019), function as a sanctuary for oceanic wildlife (Theuerkauf et al. 2021), thereby restoring and rewilding the ocean. It can especially become renewable when placed in combination with other forms of aquaculture, called integrated multi-trophic aquacultures (Chopin et al. 2001), where different types of farms can function synergistically (Fig. 1-5). The most promising and yet daunting potential of kelp is that some suggest that it might be the only scalable solution we have at our disposal to combat and reverse human induced climate change (Flannery 2017).

Kelp will have a prominent place in the role-out of oceanic farming, with large scale endeavours already on their way as we speak (Cascadia Seaweed, Ocean harvest, Ocean Rain Forest, Kelp blue, Greenwave). But first, in order to make sure that kelp really fulfils its promise as the crop of the future, we need to progress our understanding of them, by researching their lifecycle and the multiannual delayed gametophytes that govern it, on a fundamental level.

Synopsis of this thesis

This thesis contains 4 published research (Chapters 2 to 5), following the steps to better understand MAD *S. latissima and A. esculenta* gametophytes and their application in large scale seaweed aquaculture. The experiments were set out to answer the following fundamental questions that lie at the heart of our understanding of MAD gametophyte reproduction and the domestication of kelp (*Laminariales*).

- LIGHT - Does light, as a lifecycle control, primarily function as a qualitative signal or as a quantitative signal ? – *Chapter 2 (S. latissima)*

- CULTURE DENSITY - How does culture density influence the reproduction of delayed gametophyte cultures that have grown vegetatively for prolonged periods of time? – *Chapter 2 & 3 (S. latissima)*

- TEMPERATURE - Are MAD gametophytes sensitive to temperature, and how does temperature interact with light intensity? – *Chapter 3* (*S. latissima*)

- AGE - Can MAD gametophytes reliably sexually reproduce after more than a year of vegetative growth? – *Chapter 3 & 4* (*S. latissima & A. esculenta*)

- SEX RATIO - What is the influence of changing sex ratios on MAD gametophyte reproduction and what consequences does this have, over time, on aging MAD gametophyte cultures? – *Chapter 4* (*S. latissima & A. esculenta*)

- APPLICATION - Can we build a system that facilitates the controlled reproductive method by allowing a starting farmer, without fundamental knowledge or laboratory facilities, to utilize clean MAD gametophyte cultures for their farm? – *Chapter 5* (*S. latissima & A. esculenta*)

Chapter 2 (DOI: 10.1111/jpy.12976) starts with an analysis on the interactive effects between some of the more influential lifecycle controls influencing the sexual reproduction of multi annual delayed gametophytes of *S. latissima*. There are still unclarities on how light and culture density influence the sexual reproduction of gametophytes. Light, as an abiotic lifecycle control, can be subdivided into how light intensity and light quality, both influencing reproduction, while culture density (biotic factor) can halt reproduction altogether when it becomes too high. Here we performed a full factorial experiment on the (interactive) influences of light intensity, light quality, and the Initial Gametophyte Density (IGD) on delayed *S. latissima* reproduction.

Chapter 3 (DOI: 10.1111/jpy.13191) expands the research into major lifecycle controls governing the reproduction of multi annual delayed *S. latissima* gametophytes, by adding temperature to the mix. A full factorial experiment was done, to assess the interactive influences of light intensity, temperature, and culture density on the reproduction of multi annual delayed gametophytes of *S. latissima*. This chapter subsequently translates the results to seasonal patterns, in order to assess whether the observed reproductive data can be linked to natural seasonal cycles.

Chapter 4 (DOI: 10.3390/jmse9111250) focusses on the element of vegetative growth and how this can change gametophyte cultures of *S. latissima* and A. esculenta over time. The different growth rates between male and female gametophytes were of interest, since this could result in

skewed male:female ratios in time. Whether the changing male:female ratio of older cultures can change the reproductive yields of aging cultures was of special interest. Using both *A. esculenta* as well as *S. latissima* we looked at whether the observed reproductive patterns were observable in both species within the order of *Laminariales*. We furthermore propose solutions to the observed inevitable decline in fertility of aging gametophyte cultures, creating a link to the 5th chapter.

Chapter 5 (DOI: 10.1007/s10811-021-02638-2) introduces the SeaCoRe system that is specifically tailored for seaweed farmers that want utilize the controlled reproductive method at their farm. The blueprints and work protocols of the system are all included. The main goals of this system include *i)* the maintenance of clean gametophyte clonal cultures in non-sterile environments over prolonged periods of time, *ii)* the production of large numbers of juvenile sporophytes, and *iii)* effective transportation of gametophyte and sporophytes.

The synthesis of the thesis can be found in *Chapter 6*, distilling the general take home messages subdivided into the following four subchapters:

- The (a)biotic lifecycle controls of multi annual delayed gametophytes of *S. latissima* and *A. esculenta (Laminariales).*
- The role of delayed gametophytes in their natural environment.
- The facilitation of the seaweed farmer using the controlled reproductive method
- The broader implications of large scale seaweed aquaculture

Petra Steenhoek in search of ripe kelp blades for Hortimare

This chapter is in memory of Jacco Kromkamp, without whom we would never have solved a 50 year old kelp mystery.

Chapter 2

HOW LIGHT AND BIOMASS DENSITY INFLUENCE THE REPRODUCTION OF DELAYED SACCHARINA LATISSIMA GAMETOPHYTES (Phaeophyceae)

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Abstract

The lifecycle transitions of kelp's are complex and susceptible to various (a)biotic controls. Understanding the microscopic part of the kelp's lifecycle is of key importance, as gametophytes form a critical phase influencing, amongst others, the distributional limits of the species. Many environmental controls have been identified that affect kelp gametogenesis, whose interactive effects can be subtle and counterintuitive. Here we performed a full factorial experiment on the (interactive) influences of light intensity, light quality, and the Initial Gametophyte Density (IGD) on *S. latissima* reproduction and vegetative growth of delayed gametophytes. A total of 144 cultures were followed over a period of 21 days. The IGD was a key determinant for reproductive success, with increased IGDs (≥0.04 mg DW · mL^{-1}) practically halting reproduction. Interestingly, the effects of IGDs were not affected by nutrient availability, suggesting a resource-independent effect of density on reproduction. The Photosynthetically Usable Radiation (PUR), overarching the quantitative contribution of both light intensity and light quality, correlated with both reproduction and vegetative growth. The PUR furthermore specifies that the contribution of light quality, as a lifecycle control, is a matter of absorbed photon flux instead of colour signaling. We hypothesize that i) the number of photons absorbed, independent of their specific wavelength, and ii) IGD interactions, independent of nutrient availability, are major determinants of reproduction in *S. latissima* gametophytes. These insights help understand kelp gametophyte development and dispersal under natural conditions, while also aiding the control of *in vitro* gametophyte cultures.

Introduction

Kelp species of the family *Laminariaceae* have a heteromorphic lifecycle that alternates between haploid gametophytes and diploid sporophytes. In contrast to the macroscopic sporophytes, the haploid gametophytes are of a microscopic nature and especially delayed gametophytes are relatively understudied (Bartsch et al. 2008). Delayed gametophytes remain vegetative under limiting conditions (Kinlan et al. 2003), disperse through fracturing (Destombe and Oppliger 2011), can persist for prolonged periods of time (Carney 2011), even up to years (Zhao et al. 2016), and remain highly sensitive to changes in environmental quality (Edwards 2000; Carney and Edwards 2006). The asexual reproduction, growth and increase of gametophyte biomass is regarded to be the adaptive form for stressful environments (Dieck 1993). To date, the lifecycle controls that determine whether delayed gametophytes persist their asexual vegetative growth or rather start gametogenesis (i.e. sexual reproduction to form sporophytes) remains open for exploration. A better understanding on this microscopic part of the kelp's lifecycle is highly needed, as this phase largely determines their recruitment success (Wiencke et al. 2006; Fredersdorf et al. 2009). The transition to the generative phase is furthermore thought to be highly susceptible to environmental perturbations, and hence a critical process in determining the distributional limits of the species (Destombe and Oppliger 2011).

Whether kelp gametophytes initiate gametogenesis may be influenced by a range of abiotic factors such as temperature (Lüning and Neushul 1978; Morita, Kurashima and Maegawa 2003), light intensity (Hsiao and Druehl 1971; Bolton and Levitt 1985), photoperiod (Hsiao and Druehl 1971; Choi et al. 2005), and nutrient availability (Harries 1932; Martins et al. 2017). Light intensity has been described as a generic abiotic factor controlling gametogenesis, with broad light intensity gradients in which gametogenesis was successfully induced (Lüning 1980; Lee and Brinkhuis 1988). Especially the spectral composition of light is considered a major influencer of gametogenesis, with blue light acting as a major inducer of gametogenesis (Lüning and Dring 1972; Lüning and Dring 1975; Ratcliff et al. 2017). The combination of light intensity and light quality can be functionally integrated as the Photosynthetically Usable Radiation (PUR; Fig. 2-1). PUR as

an abiotic lifecycle control has never been assessed in kelp gametophytes. Integrating light intensity and light quality into PUR, as a single variable, further elaborates how light quality functions as a gametogenesis inducer, as PUR consists out of the light quality dependent photon flux of absorbed photons by an organism (Orefice et al. 2016).

Biotic factors have also been identified as potential lifecycle control mechanisms for gametogenesis, especially within the *Phaeophyceae* (Pohnert and Boland 2002; Frenkel et al. 2014). Most studies on the *Phaeophyceae* have focused on sexual pheromones like ectocarpene (Müller 1971), fucoserratene (Müller and Jaenicke 1973) or lamoxirene (Marner et al. 1984). Culture density has been shown to influence reproduction, with higher densities resulting in lower reproductive success (Reed 1990; Reed et al. 1991; Choi et al. 2005; Carney et al. 2010). Culture density was hereby always described as an indirect biotic factor, with population size also affecting other primary abiotic factors like nutrient availability or light intensity. No studies have looked at gametophyte population density as a direct biotic factor regulating reproduction, independent of nutrient availability or light intensity. Since density-dependent behavioral mechanisms (*e.g.* quorum sensing) are found widespread within the eukaryotic kingdom (Amin et al. 2012), including the sporophytes of the *Phaeophyceae* (Dayton, Currie and Gerrodette 1984), such density-dependent mechanisms might also affect gametophytes. In the case of gametophytes, population density (mg DW · mL^{-1}) might be at the heart of whether gametophytes initiate gametogenesis or keep growing vegetatively.

Since the gametophyte life phase is considered to be the adaptive form for stressful environments, gametophyte vegetative growth may be expected to be promoted under sub-optimal conditions (Lüning 1980). The Initial Gametophyte Density (IGD) may therefore have a substantial influence on whether a single gametophyte perceives its environment optimal or as sub-optimal. If a higher IGD simulates suboptimal conditions it would especially influence the reproduction of delayed gametophytes, since prolonged periods of vegetative growth prior to gametogenesis automatically results in higher IGDs, therefore lowering reproductive success. Understanding the

direct influence of IGD on delayed gametophytes is especially important for the seaweed industry, where genetic strain development is still considered a major challenge (Kim et al. 2017). Strain development in kelp is established using gametophyte clone cultures that have grown vegetatively for prolonged periods of time, hence resulting in artificially increased IGDs to levels that might be considered sub-optimal for reproduction.

Light intensity, light quality, and their overarching abiotic factor (PUR), combined with the IGD as direct biotic lifecycle control, have to our knowledge never been investigated in a full factorial design for delayed gametophytes. Here we address the question on how the interaction of such environmental factors influences reproduction and the vegetative growth of delayed kelp gametophytes, using the economically important North Atlantic species *Saccharina latissima.* We hypothesize that lifecycle control drivers include (i) IGD as a direct biotic control, with higher gametophyte densities inhibiting reproduction, thus promoting vegetative growth; and (ii) PUR as an abiotic lifecycle control that functionally integrates the influence of both light quality and light intensity.

Methods

Saccharina latissima sporophyte collection. Ripe *S. latissima* sori were collected along the coast of Flekkefjord Norway (58.2983751, 6.1107353 °E) on 1 December 2016. Ten parental individuals were pooled, where the ripe sori were cut out of the blade and cleaned thoroughly using absorbent paper. The sori were submerged in hypochlorite 0.15% (ClO^-) and subsequently washed in pasteurized seawater (80˚C for 5 hours in three cycles). The cleaned sori were then placed in an incubator (12˚C) overnight in order to dry. The next day the sori were placed in flasks (400mL) filled with pasteurized seawater for zoospores to be released, after which the zoospores developed into gametophytes through time. The gametophyte stock cultures were hereafter incubated at (12˚C) under red light (30 µmol photons $\cdot$ $m^{-2} \cdot s^{-1}$; 12:12 hours), using f/2 medium (Guillard and Ryther 1962). These cultures were incubated for 343 days prior to the start of the experiment in high density cultures (> 0.08 mg DW $\cdot$ mL^{-1}). During this period the cultures grew vegetatively and were monitored and refreshed on a monthly basis.

Light conditions. Randomly filled 24-well plates (n=36) with a volume of 3mL were placed under 5 different light intensities (5, 10, 30, 60, and 80 µmol photons · m^{-2} · s^{-1}) and 4 different light qualities (White-, Blue-, Red-, Yellow light, Fig. 2-1). The light qualities in this experiment were provided through either fluorescent tube lights (warm white) or LEDs. Tube lights were used for the colours white, red, and yellow. The colors red and yellow were achieved using specially designed color sleeves (Eurolite). It was impossible to achieve high irradiances of blue light using tube light sleeves, therefore we had to use blue LEDs in this experiment. We choose to use a different light intensity gradient for red light because we had no material at our disposal to increase the light intensity above 60 µmol photons · m^{-2} · s^{-1}. Spectral distributions were measured using a modular multispectral radiometer (TriOs Ramses ARC, Germany; Heuermann et al. 1999; Fig. 2-1). Variations in light intensity were achieved through specific placements of the cultures in respect to the light sources.

Gametophyte culture measurements. Part of the stock gametophyte culture was diluted at the start of the experiment, to four Initial Gametophyte Densities (0.01, 0.02, 0.04, and 0.08 mg DW · mL^{-1}). Fluorometry was used to estimate the biomass for IGD as well as further measurements through time, using the chlorophyll-a concentration [Chl a] as a proxy for phytoplankton biomass (Huot et al. 2007). This was done by extrapolating measurements from a Chl-a calibration line (Fig. S2-1), to the fluorometry measurements (Fast Ocean/Act2 FRRF, Chelsea Technologies Group Ltd), relating this to freeze-dried gametophyte dry weight (DW) measurements using 21 gametophyte cultures (60mL). This extrapolation was necessary because of the very low quantities of gametophyte biomass in the 3 mL wells. The maximum PSII photosynthetic efficiency (F_v/F_m, Suggett et al. 2009), a proxy of cell viability, was furthermore measured using the FRRF and was followed during the experiment. The samples were dark-adapted overnight before these measurements were taken (Fig. S2-3).

Reproductive success. Reproductive success, i.e. number of successfully formed young sporophytes (≥25 µm length) per mL, was determined on day 21 (*cf* Choi et al. 2005; Martins et al. 2017). Microscopic observations showed that the young sporophytes only developed on the bottom of the well-plates, and all were counted per triplet of the experimental conditions.

After 21 days, all fertilized oogonia had developed into small sporophytes and the sizes of the sporophytes were still small enough for accurate counting of the single individuals.

Photosynthetically Usable Radiation. A Spectrophotometer (Agilent Cary 100 UV-VIS fitted with a Labsphere DRA –CA-3300 integrating sphere) was used to measure the absorbance spectrum of the gametophytes (Fig. 2-1). The absolute absorbed light per specific wavelength was then used for the calculation of PUR under the Photosynthetic Active Radiation spectrum (400nm – 700nm), using the following equation:

$$PAR(\lambda)a(\lambda)d\lambda$$

where a(λ) is described as the probability that a photon of a given wavelength will be absorbed by the cells, which is derived from the absorption spectrum of gametophytes at the given wavelength (λ) and cell size (d) (Orefice et al. 2016).

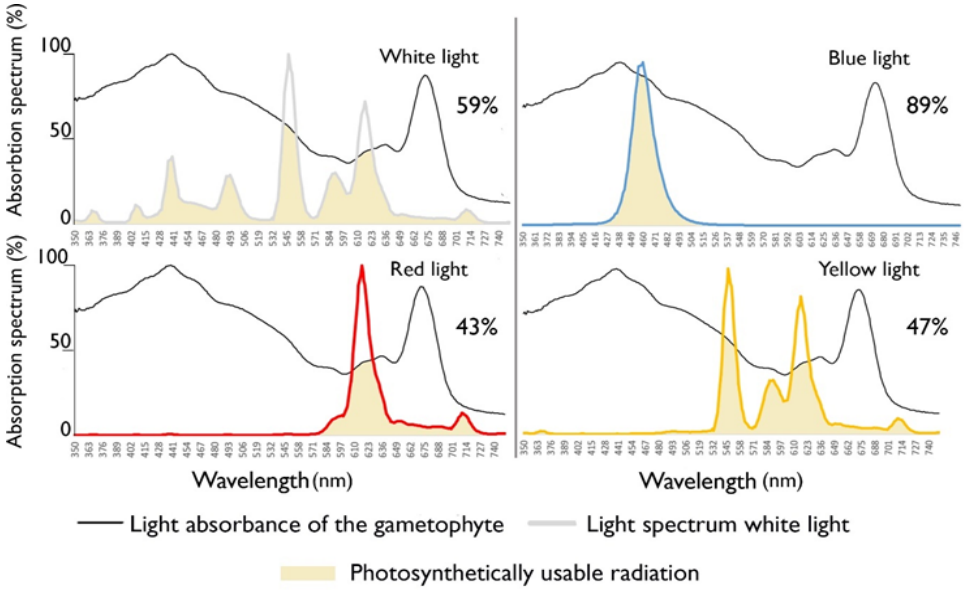

Figure 2-1. The light absorbance spectrum of *S. latissima* gametophytes (black line) projected over the spectral distribution of four light qualities (White, Yellow, Red and Blue), produced by different experimental sources. Light was measured at different wavelengths from 400nm until 700nm, and peak emission strength was normalized to 1 and plotted against the absorbance of the culture (%).

Nutrient experiment. A nutrient experiment was conducted to investigate the effects of nutrient availability on reproduction, using identical experimental protocols as the full factorial experiment described above (12˚C; 30 µmol photons · m^{-2} · s^{-1} white light). Cultures in this experiment were either placed in pasteurized seawater (nutrient poor) or in seawater enriched with f/2 medium (Guillard and Ryther 1962). The experiment was done using a dilution gradient of six IGDs (0.007, 0.012, 0.22, 0.038, 0.07 and 0.12 mg DW · mL^{-1}). We plotted the relative reproductive success (sporophytes · mg^{-1}) on the y-axis instead of the reproductive success (sporophytes · mL^{-1}), by calculating the amount of sporophytes that were produced per mg dry weight IGD instead of mL culture.

Statistical analysis. All statistical analysis was done using SPSS 20.0.0 statistical package (SPSS Inc. Chicago, USA) and Sigmaplot 13.0 (Systat software inc, London, UK). A linear regression using a second-order polynomial (parabola) was fit over both the effects of IGD and PUR on the reproductive success in R (Team R Core 2018) after log transformation of reproduction, IGD, and PUR values. This function was chosen through Akaike information criterion (AIC) model comparison of linear, parabolic, and log-log parabolic functions (Akaike 1969). Predictors for the vegetative growth were evaluated using a stepwise linear regression (fixed factors: light intensity, light quality, and IGD). All data was normally distributed and analyzed for homogeneity using the Levene's test of variance. In case of unequal variances, a robust test of equality of means for unequal variances was applied (Welch t-test). A Games-Howell Nonparametric Post-Hoc comparison was subsequently applied to test for significant differences between the subgroups (light qualities, light intensities, Nutrients and IGDs). If the data was found to be homogeneous a one-way ANOVA was applied followed by the conservative Scheffe post-hoc test to determine which factor level was responsible for the specific treatment differences. All tests were run with a significance level of 0.05%. Data of the reproductive success of the gametophytes (n=102) and their vegetative growth (n=144) are presented as mean ± SD. A Contour plot is also added on the bottom of the 3-d scatterplot in order to increase the clarity of the data. These contour plots consist out of smoothed averages of the displayed z-axis of the scatterplots (Loess smoother, sampling portion = 0.8, interval = 6).

RESULTS

S. latissima reproductive success. Reproduction was induced under different Initial Gametophyte Densities (IGD), light intensities, and light qualities (Fig. 2-2 and 2-3) and quantified as the number of sporophytes formed. Reproductive success (sporophytes · mL^{-1}) became visible after 14 days, and was significantly influenced by all three environmental factors (Table. S2-3, S2-4, & S2-5), ranging from 336 sporophytes (white light; 5 µmol photons · m^{-2} · s^{-1}; 0.02 mg DW · mL^{-1}) to 1 sporophyte (red light; 5 µmol photons · m^{-2} · s^{-1}; 0.093 mg DW · mL^{-1}; Fig. 2-3). White light led to the highest reproductive success of all light qualities tested under optimal IGD conditions (0.01 mg · mL^{-1}), whereas cultures in blue light had the lowest reproductive success, especially at higher light intensities (Fig. 2-2A). Cultures placed under yellow and red light gave, apart from the clear absence of reproduction under low red light conditions (5 µmol photons · m^{-2} · s^{-1}), average results in terms of reproduction (Fig. 2-2A). High light intensities (≥ 80 µmol photons · m^{-2} · s^{-1}) resulted in significantly lower reproduction under all light qualities (Table. S2-5). The inhibitory effect of high light intensities on reproduction became more pronounced when plotting reproductive success against PUR. This analysis reveals systematically lower reproduction at a calculated PUR exceeding 26.8 µmol photons · m^{-2} · s^{-1}, independent of light quality (Fig. 2-2B). Importantly, the PUR range is built up from a variety of light intensities and light qualities, accurately predicting reproduction irrespective of how specific PUR values were composed (regression in Fig. 2-2B; Table. S2-2).

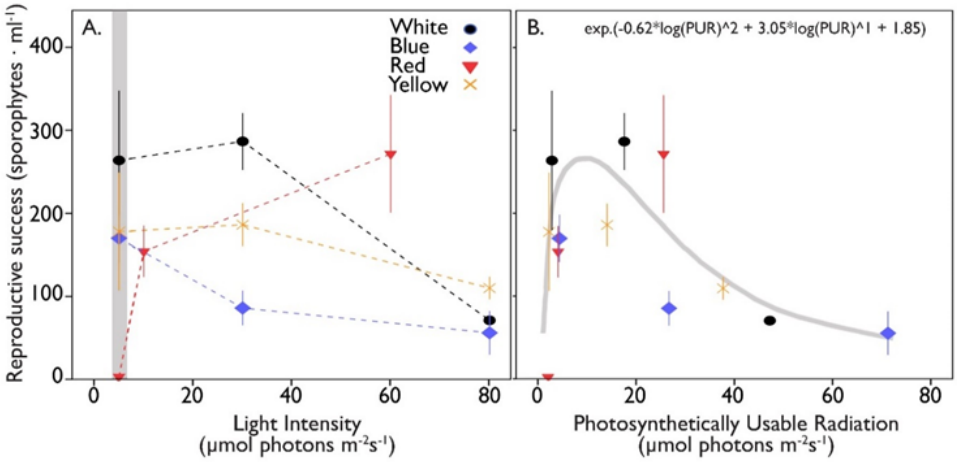

Figure 2-2. The influence of light intensity (µmol photons · m^{-2} · s^{-1}) and PUR (µmol photons · m^{-2} · s^{-1}) on the reproductive success of *S. latissima* gametophytes using an IGD of 0.01 mg · mL^{-1}. The influence of light intensity (x-axis, µmol photons · m^{-2} · s^{-1}) and light quality (Legend) on reproductive success (sporophytes · mL^{-1}) is depicted on side A, with the dotted lines representing the linear interpolation between the different data points. A grey bar is depicted on the left side in order to highlight the low light intensity environments described in the discussion. The influence of PUR (x-axis, µmol photons · m^{-2} · s^{-1}) on reproduction (sporophytes · mL^{-1}) is depicted on side B, with the colour of the data points corresponding to the light qualities (legend). A regression (grey line) is fitted through these data points and the equation describing the regression is written in the upper right corner. Values are expressed as mean ± SD, n = 3

Reproduction is influenced positively as well as negatively by the combination of IGD and PUR, resulting in an interaction of these two factors determining an IGD optimum between 0.02 and 0.01 mg DW · mL^{-1} and a PUR optimum between 14.2 µmol and 25.7 µmol photons · m^{-2} · s^{-1} (i.e., see 2d scatterplots A & B of Fig. 2-3). There was furthermore a pronounced decrease in reproductive success when PUR went above 26.8 µmol photons · m^{-2} · s^{-1}, regardless of IGD. The regression describing the influence of IGD and PUR on the reproductive success was fitted (Table. S2-1; Linear regression: $F_{4,97}$ = 40.88, R^2 = 0.628, n = 102, $p < 0.001$). The representation of the interaction between IGD and PUR on the reproduction of *S. latissima* is shown as a contour plot on the bottom of Fig. 2-3. Note that the interactive effects of both the IGD and PUR (contour plot) resulted in higher average reproductive

optimums than represented by the regressions on the sides. At (*) for example, at an IGD of 0.01 mg DW · mL^{-1} interacting with a PUR of 26 µmol photons · m^{-2} · s^{-1} red light a reproductive success of 190 sporophytes · mL^{-1} was observed, which is higher than what is calculated in both regressions.

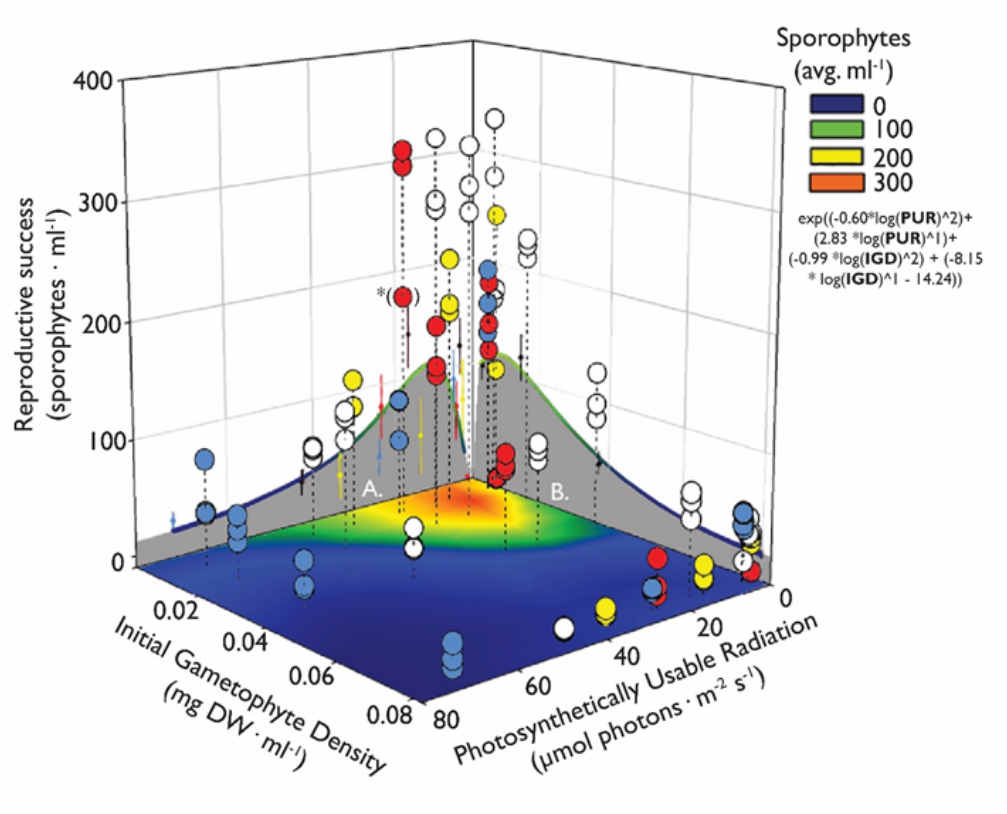

Figure 2-3. The interaction between IGD (x-axis; mg DW · mL^{-1}) and PUR (y-axis; µmol photons · m^{-2} · s^{-1}) on the reproductive success of *S. latissima* (z-axis; sporophytes · mL^{-1}), with the colours of the dots representing the used light qualities. A regression is fitted over the 3-d scatterplot and the corresponding equation can be found under the legend. The effects of the two separate lifecycle controls are depicted on the sides in the form of regressions, with PUR (grey surface, side A) or the IGD (grey surface, side B) as single variables. The interaction between PUR and the IGD as lifecycle controls is further clarified in the form of a smoothed contour plot. Colours match the colours in the legend. Error bars on the sides = ± SE, n = 102

Reproduction was also followed to investigate the role of nutrients in interaction with the IGD as a direct influence on reproduction. Both pasteurized seawater (no added nutrients) as well as the f/2 medium (added nutrients) showed similar rates of reproduction (Fig. 2-4, Table. S2-6; ANOVA: $F_{1,35} = 0.047$, $p \geq 0.05$), with decreasing IGDs resulting in increased levels of reproduction, independent of nutrient availability. Only at the lowest IGD (0.007 mg · mL^{-1}) did the cultures without added nutrients show a decrease in relative sporophyte density. Although the observed reproduction was very similar between the treatments, the sizes of the individual sporophytes differed visually, with the treatments with added nutrients containing larger sporophytes. This last observation is purely anecdotal, since we did not quantitatively measure sporophyte size during this experiment.

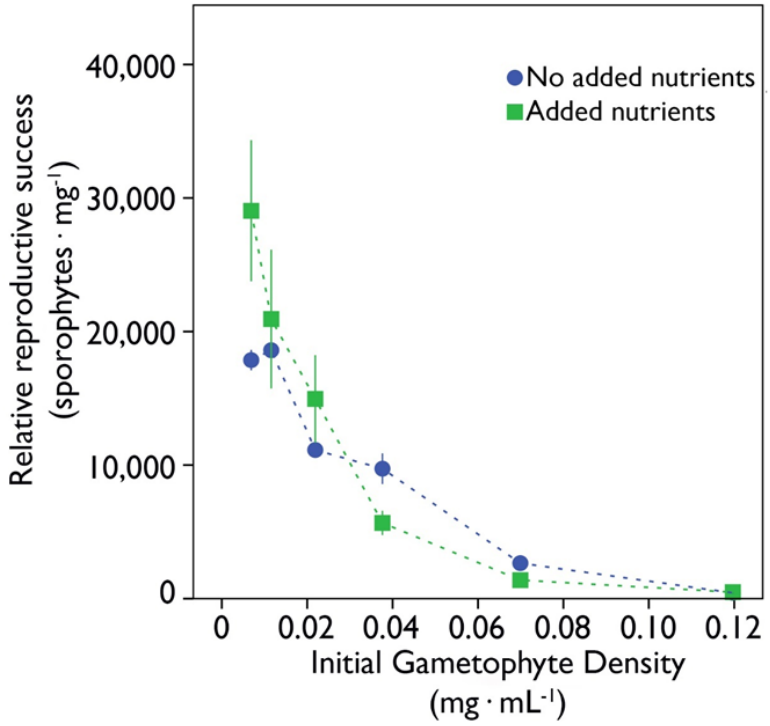

Figure 2-4. The influence of IGD (mg DW · mL^{-1}) on the relative reproductive success (sporophytes · mg^{-1}) of *S. latissima* gametophytes. The relative reproductive success of cultures with f/2 medium (added nutrients) and cultures in regular seawater (no added nutrients) are depicted as mean ± SD; n = 3

Vegetative growth. Gametophytes grew vegetatively in all cultures under all experimental conditions (Fig. 2-5). Primary predictor for the vegetative biomass accumulation in Fig. 2-5A was light intensity ($R^2 = 0.477$), followed by IGD ($R^2 = 0.235$) and subsequently light quality ($R^2 = 0.054$; Table. S2-7 & S2-8). Low light intensities (<30 µmol photons $\cdot m^{-2} \cdot s^{-1}$) reduced the vegetative growth the most (Table. S2-9; Welch ANOVA: $F_{4,41} = 50.37$, $p < 0.05$), with significantly lower biomass found when grown at 10 and 5 µmol photons $\cdot m^{-2} \cdot s^{-1}$ (Games-Howell, $p < 0.05$). Gametophytes grew significantly more at 30 µmol photons $\cdot m^{-2} \cdot s^{-1}$, after which biomass accumulation of the gametophytes levelled off with only slight further increases in biomass at 80 µmol photons $\cdot m^{-2} \cdot s^{-1}$. While light quality under comparable light intensities had limited influence on the vegetative growth of *S. latissima* gametophytes (Fig. 2-5; Table. S2-11; ANOVA: $F_{2,105} = 2.970$, $p \geq 0.05$) some distinctions can be made. The highest growth was achieved under white light 80 µmol photons $\cdot m^{-2} \cdot s^{-1}$, whereas growth under blue light already started to plateau at 30 µmol photons $\cdot m^{-2} \cdot s^{-1}$. PUR as abiotic factor (Fig. 2-5B) was also plotted against the observed vegetative growth, with a resulting correlation of $R^2 = 0.53$, irrespective of the light quality used. The large spread of the data points in the scatterplot is, among other things, a result of grouping the different IGDs. Plotting the IGDs separately resulted in higher correlations between PUR and vegetative growth for all light qualities, apart from cultures places under blue light (Fig. S2-4).

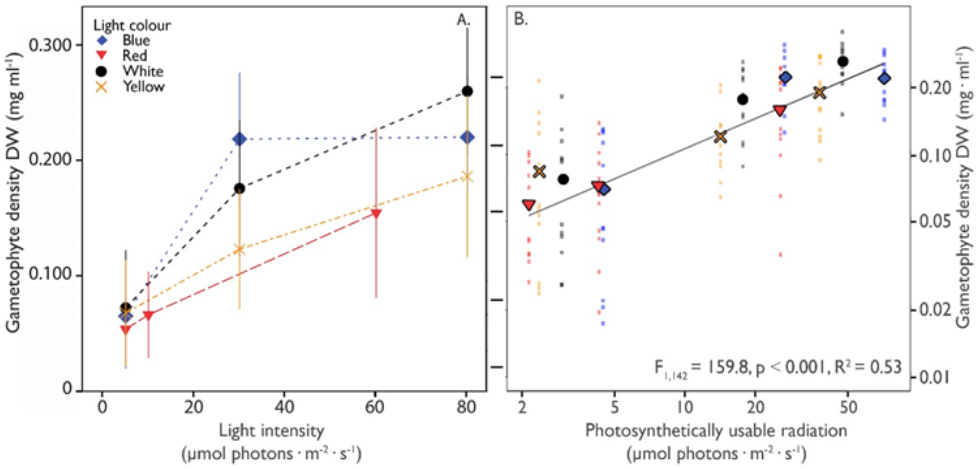

Figure 2-5. The influence of light intensity and the PUR on the vegetative growth of *S. latissima* gametophytes. Gametophyte biomass (mg DW · mL^{-1}) on day 21 under different light intensities (µmol photons · m^{-2} · s^{-1}) following different light qualities (legend) is depicted in A, with the dotted line representing the linear interpolation between the different data points. Side B depicts the gametophyte biomass (mg DW · mL^{-1}) on day 21 under different PURs (µmol photons · m^{-2} · s^{-1}) following different light qualities (legend), with the line representing the correlation between the different data points (R^2 is depicted in the lower right side). Note that both axis of B. are on a log scale. Values in A. are expressed as mean ± SD, while values in B. are expressed as a scatterplot with the mean (symbols), n = 12.

DISCUSSION

Initial Gametophyte Density (IGD) as a direct biotic lifecycle control. This study presents the results for the effects of the (a)biotic factors *i)* IGD, *ii)* light intensity, *iii)* light quality, and the overarching *iv)* PUR on reproduction and the vegetative growth of delayed *S. latissima* gametophytes during a 21 days experimental period. Reproduction became visible after 14 days in treatment, coinciding with periods found in other studies with Laminariaceae (Morelissen et al. 2013; Ratcliff et al. 2017). Reproduction decreased with increasing IGDs, under all light intensities and light qualities . These results are in agreement with data obtained by Choi et al. (2005) and Reed et al. (1990; 1991), where increasing spore densities of *Undaria pinnatifida* and *Macrocystis pyrifera* resulted in lower sporophyte counts. Carney and Edwards (2010) found similar negative correlations between reproduction

and culture density of non-delayed *Macrocystis pyrifera* gametophytes. Interestingly, these authors also studied delayed gametophytes (88 days), and found no significant difference in reproduction in three of their four starting zoospore densities. Although their study reported gametophyte density as the number of gametophytes per area, rather than gametophyte biomass per volume as used here, similar trends could be observed between our highest starting densities. Indeed, their highest density treatment of 212 gametophytes · mm^2 (± 15.3) showed a significant decrease in reproduction, comparable to what we observed in our higher IGD samples (≥ 0.04 mg · mL^{-1}) of *S. latissima* gametophytes.

The experimental data supports our hypothesis that density has a direct influence on *S. latissima* reproduction, with high IGDs (> 0.04 mg · mL^{-1}) practically halting reproduction. Nutrient addition had no significant influence on reproduction and the reproductive success did not follow the observed differences in F_v/F_m ratio's, a proxy for cell viability (Suggett et al. 2009; Fig. S2-3). These data demonstrate that the negative effects of gametophyte density on reproduction is not likely occurring via putative density-associated nutrient deficiency. Self-shading (i.e. light dependent effects) can also be ruled out because of the low culture densities, top-down light placement of the light source and homogeneity of the cultures. Our results showed furthermore that light limitation did not negatively influence reproduction, apart from cultures placed under 5 µmol photons · m^{-2} · s^{-1} red light. This is in agreement with results by Lee and Brinkhuis (1988), who found no decrease in the reproductive success of female gametophytes under low light conditions (6 µmol photons · m^{-2} · s^{-1} white light). The exact mode of action of IGD as a direct biotic factor remains to be investigated. Whether the observed density dependent behavior is controlled pheromonally or is more similar to the autoinducers found in quorum sensing bacterial communities, is not yet known. It might even be possible that density dependent reproduction is the result of interkingdom signaling between gametophytes and bacteria, a phenomenon already studied within diatom communities (Amin et al. 2012).

Multiple hormones related to reproduction (i.e. ectocarpene, lamoxirene, and fucoserratene) have already been described for kelp gametophytes (Müller 1971; Marner et al. 1984; Müller and Jaenicke

1973), making it feasible that one of these previously mentioned or novel compounds secreted by the gametophytes can accumulate in high density cultures, thereby suppressing reproduction. Suppressing reproduction under higher gametophyte densities could have benefits for their offspring, as the inverse correlation between IGD and reproductive success would prevent any future competition between sporophytes living in high-density populations due to their competition for space (Dayton et al. 1984). The vegetative growth and the subsequent fragmentation of gametophyte branches therefore becomes the alternative option for dispersal (Destombe and Oppliger 2011). Moreover, in vitro work looking into reproduction, or gametogenesis in general, should take into account the IGD as a relevant biotic lifecycle control, especially regarding delayed gametophytes. The older a delayed gametophyte is, the more time it had to grow vegetatively, and the longer their vegetative growth period was, the higher the IGD automatically becomes, suppressing reproduction.

Photosynthetically Usable Radiation as abiotic lifecycle control. There were interactions between light intensity and quality in determining reproduction, calling for a proxy that integrates both: PUR. Indeed, PUR seems to regulate reproduction in our delayed gametophyte cultures very tightly. Previous studies on light quality as a lifecycle control did not incorporate PUR (Lüning and Dring 1972; Lüning and Dring 1975; Ratcliff et al. 2017), so a comparison is complicated, especially because gametophyte densities were not quantified in the same way as done here. It is likely that gametophyte densities in the previously mentioned studies were in the lower range of the ones used here, as either the gametophytes were countable (Lüning and Dring 1972; Lüning and Dring 1975), or the cultures were diluted substantially into larger volumes of seawater (Ratcliff et al. 2017). Moreover, the light intensities reported were in the lower range of what we used here (6-15 umol photons m^{-2} s^{-1}). Interestingly, zooming into the low light intensities, low IGD region in Fig. 2-2A (grey bar) reveals that a low intensity of red light resulted in very poor reproduction, whereas a similarly low intensity of blue light gave clear reproduction. This is entirely consistent with literature findings, such as by Lüning and Dring (1972; 1975). However, these conclusions shift when higher light intensities were used.

Using higher light intensities of red light resulted in higher reproductive success and suggests that not so much light quality but the absorbed photon flux (PUR), irrespective of their wavelength, appears to be the important determinant regulator of reproduction. Importantly, when gametophyte densities become very high, the density effects overrule the effects of PUR and suppresses reproduction altogether.

Light intensity by itself was a strong predictor for the vegetative growth of gametophytes, with optima at 80 µmol photons $\cdot m^{-2} \cdot s^{-1}$, under all light qualities and IGDs . Interestingly, biomass growth started to level off between 30 µmol photons m^{-2} s^{-1} and 80 µmol photons $\cdot m^{-2} \cdot s^{-1}$. This corroborates with results of other studies, finding no effects on growth in gametophytes at irradiances of 30 µmol photons $\cdot m^{-2} \cdot s^{-1}$ or higher (Lüning and Neushul 1978; Izquierdo et al. 2002; Choi et al. 2005). The influence of light quality was more limited , where it's role on the vegetative growth is better explained through the usage of PUR as a parameter . Average gametophyte density (DW $\cdot mL^{-1}$) on day 21 correlated well with PUR (R^2= 0.53), especially considering the interactive effects that were still present due to the different IGDs used. The correlation between vegetative growth and PUR, independent of light quality, becomes especially apparent when the interactive effects of IGD are taken out of the equation (Fig S4). In this case, overall higher correlations were found under all light qualities except for cultures incubated under blue light, showing consistently lower correlations. The lower correlation under blue light is likely due to the plateauing biomass growth of cultures grown at a PUR of 71.4 µmol photons $\cdot m^{-2} s^{-1}$, irrespective of IGD. These high light intensities of blue light subsequently lowered the maximum quantum yield of the PSII substantially (Fig. S2-3), suggesting that photo inhibition was taking place (Gevaert et al. 2002).

To our knowledge, the gametophyte dry weight (mg $\cdot mL^{-1}$) of these small cultures (3mL), have never been followed through time before. Using these small cultures was necessary for the feasibility of this full factorial experiment of such a large sample size. This makes it difficult, if not impossible, to compare our vegetative growth rates with cultures grown in similar condition. Furthermore, most research into the vegetative growth of gametophytes followed the surface area, the number of cells, or the length of gametophytes (Bolton and Levitt 1985; Carney and Edwards 2010;

Morelissen et al. 2013; Martins et al. 2017). Ratcliff et al. (2017) used similar parameters to ours, looking at much larger volumes of gametophyte biomass dry weight ($g \cdot L^{-1}$), and found similar growth rates under comparable light conditions, also using f/2 medium. The difficulty of quantitatively comparing our results to other data is showing the need for concise and comparable methods of following gametophyte biomass in future studies.

Future work on the lifecycle controls in kelp will benefit from the inclusion of IGD and PUR in interaction with other lifecycle controls (*e.g.* temperature, day length, or other (a)biotic factors). The interaction between these lifecycle controls are also interesting from a more applied perspective, where finding the reproductive optimum can result in better production cost estimates and lower production costs. Advancements that are crucial in order to make large-scale seaweed aquaculture economically feasible (van den Burg et al. 2016).

CONCLUSIONS

Although there are clear interactive effects, two individual factors were identified as the most important determinants of reproduction and vegetative growth. The Initial Gametophyte Density was shown to be a dominant biotic factor influencing reproduction, outweighing light intensity or light quality. The Photosynthetically Usable Radiation, indicating the absorbed photon flux through the integration of both light intensity and light quality, is a second dominant (abiotic) determinant explaining the results on reproduction and the vegetative growth of kelp gametophytes. Light quality appears to act primarily through the efficiency in photon absorbance, as calculated through PUR. Light quality has hereby shown to be an abiotic factor that should be interpreted quantitatively instead of qualitatively as a colour signal.

ACKNOWLEDGEMENTS

The authors want to thank Hortimare BV and their staff for the usage of their *S. latissima* gametophyte cultures. We furthermore want to thank Greg Fivash for his help and patients in the challenging journey of full factorial data analysis. Lastly, we want to thank the reviewers and editor for their constructive criticism, increasing the quality of the manuscript

Supplementary data

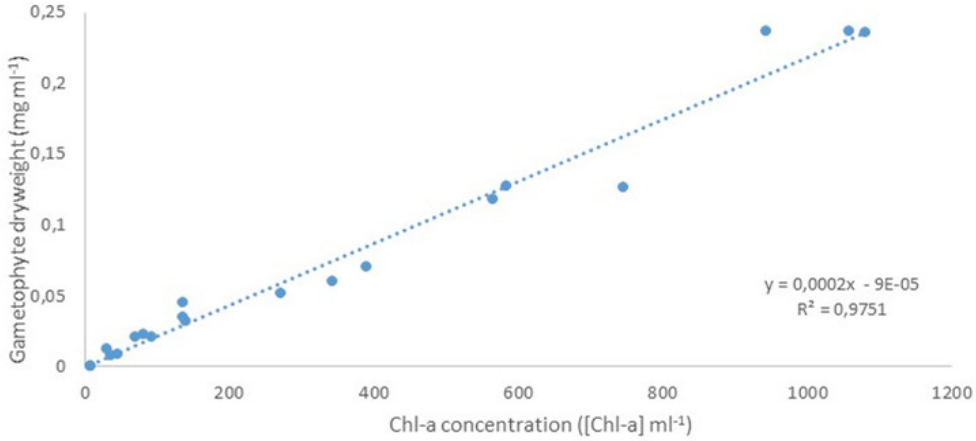

Figure S2-1. Calibration curve between the chlorophyll a concentration (mg Chl · m^{-3}), and *S.latissima* gametophyte dry weight per ml (mg DW · ml^{-1}). Gametophyte dry weights are extrapolations from 60ml cultures, whose [Chl] concentration were measured using a FRRF fluorometer. The linear regression and correlation coefficient were y=o,ooo2x – 9e-05 and 0,975 respectively.

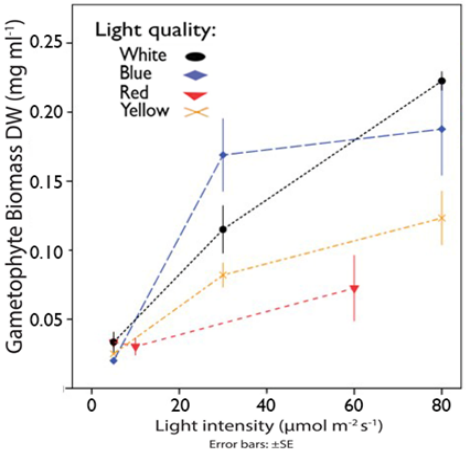

Figure S2-2. The interaction between light intensity (µmol photons · m^{-2} · s^{-1}) and the light quality (white, blue, red and yellow) on *S.latissima* gametophyte biomass (mg DW · ml^{-1}) of cultures starting with the Initial Gametophyte Density of 0.01 mg DW · ml^{-1}. Biomass was measured on day 21 and the error bars are ± SE, n = 36

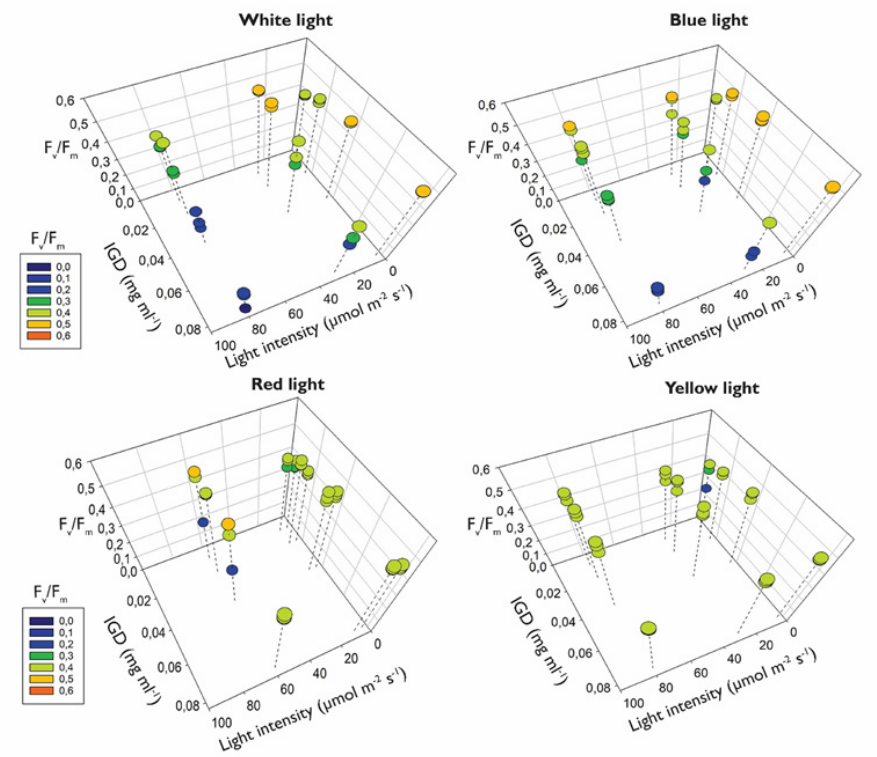

Figure S2-3. The 3d-scatter plot showing the interaction between the Fv/Fm, the IGD (mg · ml^{-1}), and light intensity (µmol photons · m^{-2} · s^{-1}) of *S. latissima* gametophyte cultures grown under four different light qualities. The color of the dots correspond with the legend (white, blue, red and yellow), thus corresponding with the Fv/Fm value of the sample n = 144

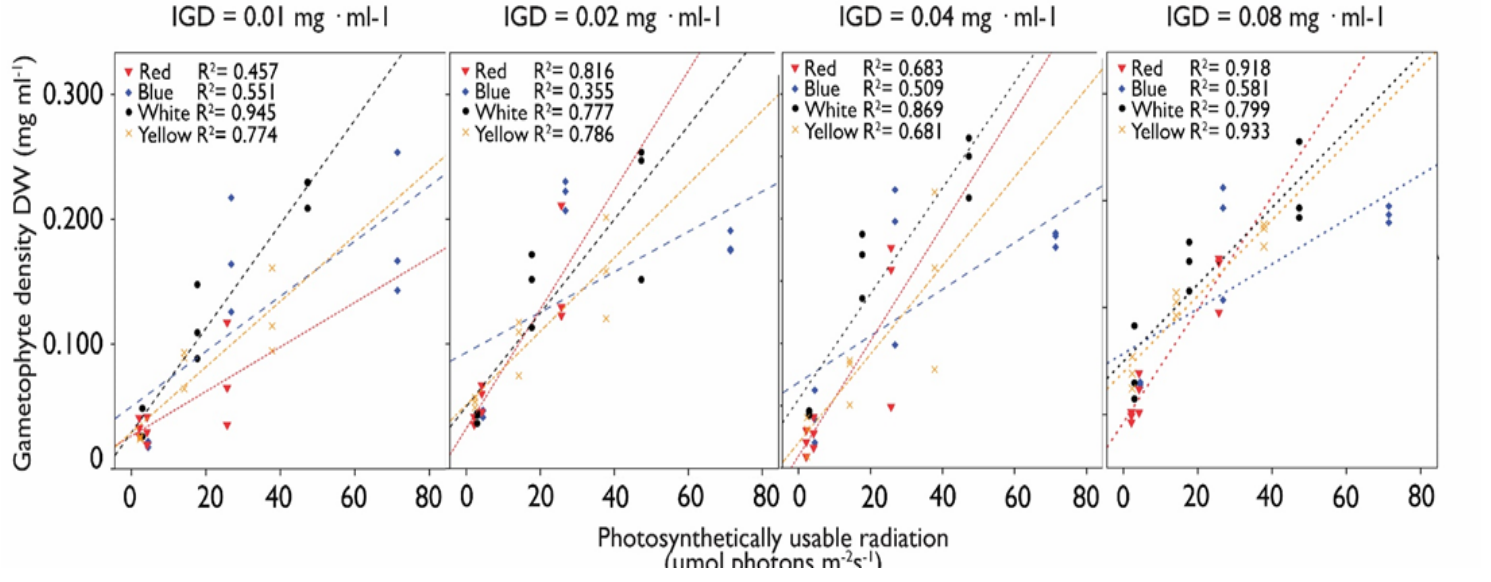

Figure S2-4. Scatterplots depicting the *S. latissima* gametophyte biomass measured on day 21 (y-axis) under different levels of Photosynthetically Usable Radiation (µmol photons · m^{-2} s^{-1}). Four different light qualities (white, blue, red and yellow) were used to grow out gametophyte cultures starting with four different Initial Gametophyte Densities (0.01, 0.02, 0.04 and 0.08 mg DW . ml-1). Values are "as is" , n = 36

Reproduction

Table S2-1. Predictors for the regression describing the correlation of the IGD and PUR on the reproduction of *S.latissma* gametophytes in Fig.3 (n=102). Included is the R^2 of the primary (PUR) and secondary (IGD) predictor combined.

	Dependent variable		
Predictors	***Estimates***	***CI***	***p***
(Intercept)	-14.24	-19.64 – -8.84	<0.001
poly(log(d$PUR), 2, raw = T)1	2.83	1.92 – 3.74	<0.001
poly(log(d$PUR), 2, raw = T)2	-0.60	-0.79 – -0.42	<0.001
poly(log(d$IGD), 2, raw = T)1	-8.15	-11.34 – -4.96	<0.001
poly(log(d$IGD), 2, raw = T)2	-0.99	-1.44 – -0.54	<0.001
Observations			102
R^2 / adjusted R^2	0.628 / 0.612		

Table S2-2. Predictors for the regression describing the correlation of PUR and the reproduction of *S.latissima* gametophytes in Fig.2, using an IGD of 0.01 mg · ml^{-1}.

	Dependent variable		
Predictors	***Estimates***	***CI***	***p***
(Intercept)	1.85	0.24 – 3.46	0.031
poly(log(d$PUR[filter]), 2, raw = T)1	3.05	1.48 – 4.62	0.001
poly(log(d$PUR[filter]), 2, raw = T)2	-0.62	-0.94 – -0.30	0.001
Observations			35
R^2 / adjusted R^2	0.313 / 0.270		

Table S2-3. Games-Howell post hoc analysis for the influence of light quality on gametogenesis after we found significant differences using the robust test of variance. The mean difference is significant at $p < 0.05$.

Dependent Variable: Sporophyte density (No. ml-1)

						95% Confidence Interval	
(I) colour			**Mean Difference (I-J)**	**Std. Error**	**Sig.**	**Lower Bound**	**Upper Bound**
Games-Howell	white	blue	75,54630*	20,66946	**,003**	20,7590	130,3336
		red	43,75463	26,27572	**,352**	-25,9382	113,4475
		yellow	44,20370	26,21918	**,343**	-25,8759	114,2833
	blue	white	-	-	**-**	-	-
		red	-31,79167	22,14952	**,486**	-91,4677	27,8843
		yellow	-31,34259	22,08243	**,499**	-91,7638	29,0786
	red	white	-	-	**-**	-	-
		blue	-	-	**-**	-	-
		yellow	,44907	27,40110	**1,000**	-73,0661	73,9642
	yellow	white	-	-	**-**	-	-
		blue	-	-	**-**	-	-
		red	-	-	**-**	-	-

*. The mean difference is significant at the 0.05 level.

Table S2-4. Games-Howell post hoc analysis for the influence of the IGD on gametogenesis after we found significant differences using the robust test of variance. The mean difference is significant at $p < 0.05$.

Dependent Variable: Sporophyte density (No. ml-1)

(I) IGD (mg ml-1)		(J) IGD (mg ml-1)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Games-Howell	0.01	0.02	-28.444	87.312	**.988**	-267.62	210.73
		0.04	276.756*	55.139	**.000**	130.08	423.43
		0.08	386.556*	49.071	**.000**	254.85	518.26
	0.02	0.01	-	-	**-**	-	-
		0.04	305.200*	78.088	**.005**	84.37	526.03
		0.08	415.000*	73.928	**.000**	201.34	628.66
	0.04	0.01		-	**-**	-	-
		0.02	-	-	**-**	-	-
		0.08	109.800*	29.703	**.008**	26.20	193.40
	0.08	0.01	-	-	**-**	-	-
		0.02	-	-	**-**	-	-
		0.04	-	-	**-**	-	-

*. The mean difference is significant at the 0.05 level.

Table S2-5. Games-Howell post hoc analysis for the influence of light intensity on gametogenesis after we found significant differences using the robust test of variance. The mean difference is significant at $p < 0.05$.

Dependent Variable: Sporophyte density (No. ml-1)

(I) Light intensity (umol m-2 s-1)		(J) Light intensity (umol m-2 s-1)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Games-Howell	5	10.00	13.60000	33.93687	**.994**	-99.4917	126.6917
		30	-21.59444	28.75857	**.943**	-103.1918	60.0029
		60	-20.70556	35.03790	**.975**	-125.9136	84.5024
		80	61.77778*	19.34050	**.022**	6.4124	117.1431
	10	5	-	-	**-**	-	-
		30	-35.19444	36.53035	**.866**	-151.6722	81.2833
		60	-34.30556	41.65411	**.919**	-163.8637	95.2525
		80	48.17778	29.68949	**.537**	-65.5780	161.9335
	30	5	-	-	**-**	-	-
		10	-	-	**-**	-	-
		60	.88889	37.55542	**1.000**	-110.0355	111.8133
		80	83.37222*	23.59682	**.012**	14.5801	152.1644
	60	5	-	-	**-**	-	-
		10	-	-	**-**	-	-
		30	-	-	**-**	-	-
		80	82.48333	30.94202	**.118**	-15.7825	180.7492
	80	5	-	-	**-**	-	-
		10	-	-	**-**	-	-
		30	-	-	**-**	-	-
		60	-	-	**-**	-	-

*. The mean difference is significant at the 0.05 level.

Table S2-6. Robust test of variance for the effects of nutrients on the gametogenesis of *S.latissima* gametophytes (Fig. 4; Welch and Brown-Forsythe), after not passing the test of homogeneity of variances.

Robust Tests of Equality of Means

	Statistic[a]	df1	df2	Sig.
Welch	,376	1	28,165	,545

a. Asymptotically F distributed.

Vegetative growth

Table S2-7. Stepwise linear regression for the correlation between the gametophyte biomass on day 21 (mg DW· ml^{-1}), the IGD, light intensity, and light quality (n=144)

Correlations

		[Chl]	Light intensity (µmol m-2 s-1)	IGD (m g m l-1)	Colour
Pearson Correlation	[Chl]		-,426	-,348	-,052
	Light intensity (µmol m-2 s-1)			0,000	,047
	IGD (mg ml-1)				0,000
	Colour				
Sig. (1-tailed)	[Chl]		,000	,000	,065
	Light intensity (µmol m-2 s-1)			,500	,082
	IGD (mg ml-1)				,500
	Colour				

*. The mean difference is significant at the 0.05 level.

Table S2-8. Predictors that significantly influence gametophyte growth. Included is the R^2 of the primary (IGD) and secondary predictor (light intensity) combined.

					Change Statistics				
Model	R	R Square	Adjusted R Square	Std. Error of the Estimate	R Square Change	F Change	df1	df2	Sig. F Change	Durbin-Watson
1	,691[a]	,477	,474	884,29732	,477	129,628	1	142	,000	
2	,844[b]	,712	,708	659,05674	,234	114,646	1	141	,000	
3	,875[c]	,766	,761	596,14326	,054	32,331	1	140	,000	1,185

a. Predictors: (Constant), Intensity
b. Predictors: (Constant), Intensity, Biomass
c. Predictors: (Constant), Intensity, Biomass, Colour
d. Dependent Variable: Chl

Table S2-9. Games-Howell post hoc analysis for the influence of light intensity on the growth of gametophyte biomass (chlorophyll-a concentration) on day 21 after we found significant differences using the robust test of variance. The mean difference is significant at $p < 0.05$.

Dependent Variable: [Chl]

	intensity (µmol photons m-2 s-1)		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Games-Howell	5	10	-,001467	,012159	**1,000**	-,03809	,03515
		30	-,105217*	,012524	**,000**	-,14053	-,06990
		60	-,087818*	,021659	**,010**	-,15611	-,01952
		80	-,153758*	,012240	**,000**	-,18825	-,11926
	10	5	-	-	**-**	-	-
		30	-,103750*	,015267	**,000**	-,14765	-,05984
		60	-,086351*	,023352	**,014**	-,15772	-,01498
		80	-,152291*	,015035	**,000**	-,19560	-,10898
	30	5	-	-	**-**	-	-
		10	-	-	**-**	-	-
		60	,017399	,023544	**,944**	-,05398	,08877
		80	-,048541*	,015332	**,019**	-,09147	-,00561
	60	5	-	-	**-**	-	-
		10	-	-	**-**	-	-
		30	-	-	**-**	-	-
		80	-,065940	,023395	**,076**	-,13704	,00516
	80	5	-	-	**-**	-	-
		10	-	-	**-**	-	-
		30	-	-	**-**	-	-
		60	-	-	**-**	-	-

*. The mean difference is significant at the 0.05 level.

Table S2-10. Scheffe post hoc analysis for the influence of the different IGDs on the growth of gametophyte biomass (chlorophyll-a concentration) on day 21 after we found significant differences using a one-way ANOVA. The mean difference is significant at $p < 0.05$.

Dependent Variable: [Chl]

	(I) biomass		Mean Difference (I-J)	Std. Error	Sig.	Interval Lower Bound	Interval Upper Bound
Scheffe	.010	.020	-,024768	,017706	**,583**	-,07487	,02534
		.040	-,063898*	,017706	**,006**	-,11400	-,01379
		.079	-,110307*	,017706	**,000**	-,16041	-,06020
	.020	.010	-	-	**-**	-	-
		.040	-,039130	,017706	**,186**	-,08923	,01097
		.079	-,085540*	,017706	**,000**	-,13564	-,03544
	.040	.010	-	-	**-**	-	-
		.020	-	-	**-**	-	-
		.079	-,046410	,017706	**,081**	-,09651	,00369
	.079	.010	-	-	**-**	-	-
		.020	-	-	**-**	-	-
		.040	-	-	**-**	-	-

*. The mean difference is significant at the 0.05 level.

Table S2-11. Games-Howell post hoc analysis for the influence of light quality on the vegetative growth of gametophyte biomass (chlorophyll-a concentration) on day 21 after we found significant differences using the robust test of variance. The mean difference is significant at $p < 0.05$.

Dependent Variable: [Chl]

(I) colour			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Games-Howell	White light	Blue light	,001359	,021165	**1,000**	-,05435	,05707
		Red light	,075981*	,018835	**,001**	,02628	,12568
		Yellow light	,042628	,019516	**,138**	-,00881	,09406
	Blue light	White light	-	-	-	-	-
		Red light	,074622*	,018195	**,001**	,02664	,12260
		Yellow light	,041269	,018899	**,138**	-,00851	,09105
	Red light	White light	-	-	-	-	-
		Blue light	-	-	-	-	-
		Yellow light	-,033353	,016247	**,179**	-,07612	,00942
	Yellow light	White light	-	-	-	-	-
		Blue light	-	-	-	-	-
		Red light	-	-	-	-	-

*. The mean difference is significant at the 0.05 level.

Figure S2-5; A photo of the starting culture in a well plate (IGD = 0.01mg DW · ml^{-1}).

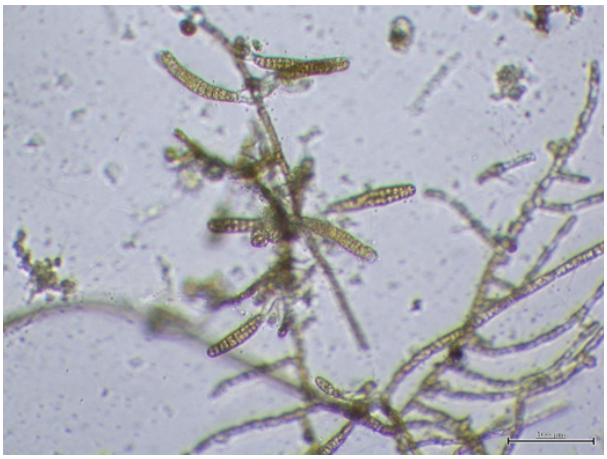

Figure S2-6. A photo of a culture on day 21 (IGD=0.01 mg DW · ml $^{-1}$, 30 µmol · m^{-2} · s^{-1}, white light). Sporophytes only formed on the bottom with gametophyte biomass being a bit blurry since it grew upward towards the light, out of focus.

View of the “laboratory” where most gametophyte cultures were created that have been used in the experiments described in this thesis. Look carefully, it’s the small red wooden shed with the two boats docked in front of the door.

Chapter 3

THE ROLE OF SEASONALITY IN REPRODUCTION OF MULTIANNUAL DELAYED GAMETOPHYTES OF *SACCHARINA LATISSIMA*

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Abstract

Delayed gametophytes are able to grow vegetatively for prolonged periods of time. As such, they are potentially very valuable for kelp aquaculture given their great promise in opening up novel opportunities for kelp breeding and farming. However, large-scale application would require more in-depth understanding of how to control reproduction in delayed gametophytes. For newly formed gametophytes, many environmental factors for reproduction have been identified, with key drivers being light intensity, temperature, and the initial gametophyte density. However, the question of whether delayed gametophytes react similarly to these life cycle controls remains open for exploration. In this study, we performed a full factorial experiment on the influences of light intensity, temperature, and density on the reproduction of multiannual delayed gametophytes of *Saccharina latissima*, during which the number of sporophytes formed were counted. We demonstrate that delayed gametophytes of *S. latissima* can reliably reproduce sexually after more than a year of vegetative growth, depending on the effects between light intensity and temperature. Under higher light intensities (≥29 µmol photons m^{-2} s^{-1}), optimal reproduction was observed at lower temperatures (10.2°C), while at lower light intensities (≤15 µmol photons m^{-2} s^{-1}), optimal reproduction was observed at higher temperatures (≥12.6 °C). Given the seasonal lag between solar radiation and sea surface temperature in natural systems, these conditions resemble those found during spring (i.e. increasing light intensity with low temperatures) and autumn (i.e. decreasing light intensity with higher temperatures). Seasonality, that can be used as an aquaculture tool to better control the reproduction of delayed gametophytes.

Introduction

Crop domestication requires humans to understand and exert control over the life cycles of target organisms (Zeder 2015), and involves building multi-generational relationships (Valero et al. 2017). Vodouhè and Dansi (2012) describe seven cultivation phases in the domestication process of crops (Table. S3-1). Kelp aquaculture currently hovers somewhere between phase 4 and phase 6 of these cultivation phases. Phase 4 is the phase in which the reproductive biology of the species is known but mass production still needs to be mastered. Phase 5 is when the crop is cultivated and harvested using traditional practices. Phase 6 is the phase where farmers are able to adopt specific criteria to select plants that better satisfy the needs of consumers, and even though some kelp farmers are already selecting plants that are able to better satisfy the needs of consumers (Zhang et al. 2017; Valero et al. 2017), many phase 4 aspects of mass production are still researched today. These include identifying the most suitable date of planting (Peteiro and Freire 2009), substrate optimization (Kerrison et al. 2018), year-round zoospore production (Forbord et al. 2012), identifying optimal farming locations (Matsson et al. 2019), and development of disease management (Peng and Li 2013). For further progress in the domestication process of kelp, we need to increase our fundamental understanding of the life cycles of kelp, and apply this knowledge in improving the cultivation methods.

To date, two different cultivation methods are practised in kelp aquaculture (Valero et al. 2017; Forbord et al. 2018; Goecke et al. 2020): the uncontrolled reproductive method (URM) and the controlled reproductive method (CRM). In traditional URM (Shan et al. 2013), substantial numbers of zoospores are released in large tanks. These zoospores first attach on strings and develop into gametophytes, after which the newly formed gametophytes form gametes. The female gametophytes become fertilized, subsequently forming juvenile sporophytes, which then also attach to these strings (Su et al. 2017), all without direct intervention of farmers. In CRM, gametophyte reproduction is directly controlled under *in vitro* laboratory conditions (Ratcliff et al. 2017; Forbord et al. 2020). The induced gametophyte cultures, often already containing juvenile sporophytes, are then attached to seed strings using a method called paintbrush seeding (Redmond et al. 2014) or deployed immediately through direct seeding (Kerrison et al. 2018; Forbord

et al. 2020). CRM is more labour-intensive than URM, and results in lower attachment to seed strings (Xu et al. 2009). This probably explains why the URM is still commonly practiced in many parts of the world (Goecke et al. 2020).

In the future, using CRM might be more promising because *(i)* gametophytes can be kept in cultures for prolonged periods of time (Barrento et al. 2016; Carney 2011; Wade et al. 2020) and successfully cryopreserved (Visch et al. 2019, *(ii)* gametophyte reproduction still has room to be further optimized (Ratcliff et al. 2017), and *(iii)* this opens up new possibilities for breeding, by allowing for the crossing of distantly related unialgal male and female gametophyte clone cultures to increase intraspecific hybrid vigour in future F1 hybrid cultivars through heterosis (Shan et al. 2016; Zhao et al. 2016). It should be noted that the reliable use of gametophyte clones for large-scale aquaculture or breeding purposes can only be achieved by greater understanding of reproduction in larger volumes of delayed gametophyte cultures (Zhang et al. 2008). Presently, the great majority of gametophyte studies have been carried out using newly formed gametophytes (Bartsch et al. 2008). Large-scale application of CRM, necessitates working with gametophytes that have delayed their sexual reproduction by growing vegetatively through mitosis as undifferentiated filamentous cells for prolonged periods of time (Pang et al. 1996; Carney 2011; Zhang et al. 2019). Gametophytes can even delay reproduction for multiple years (Barrento et al. 2016), transgressing seasonally induced reproduction; these are hereafter referred to as multiannual delayed gametophytes. Hence, there is a need for a more in-depth understanding of how abiotic and biotic factors can trigger life cycle transitions and reproductive success in delayed gametophytes (Edwards 2000), particularly in multiannual delayed gametophytes, which is the focus of this study.

Members of the kelp order Laminariales have heteromorphic life cycles that transition between microscopic, haploid gametophytes and macroscopic, diploid sporophytes. This results in the spatiotemporal separation of meiosis and fertilization, with somatic development occurring at both the haploid and diploid stages (Bell et al. 1997). The transitions between these life phases is controlled by abiotic and biotic environmental factors (Lüning and Dieck 1989), hereafter described as life cycle controls

(Ebbing et al. 2020). Extensive studies on life cycle controls for newly formed *S. latissima* gametophytes have identified the following as the main drivers: light intensity (Hsiao and Druehl 1971; Bolton and Levitt 1985), temperature (Lüning and Neushul 1978; Morita et al. 2003), nutrients (Harries 1932; Martins et al. 2017), daylength (Choi et al. 2005; Martins et al. 2017), and initial gametophyte density (Choi et al. 2005; Carney and Edwards 2010; Ebbing et al. 2020).

Given the importance of light and temperature in reproductive timing and success, growth and reproduction in most Laminariaceae are strongly seasonal, since they are primarily found in subtidal areas at higher latitudes, where light and temperature vary strongly over the year (Krumhansl et al. 2016). Gametophyte reproduction in natural populations has been observed throughout the year, making it likely that there are multiple times during the year when conditions are suitable for gametophyte reproduction (Parke 1948; Druehl 1965; Tatsumi 2018). The influence of seasonality in the reproduction of newly formed gametophytes has been thoroughly studied, and indicates that interactions between light and temperature greatly influences the growth and reproduction of newly formed *S. latissima* gametophytes (Lüning 1980; Lee and Brinkhuis 1986; 1988; Park et al. 2017). This dynamic between light and temperature can be seen all around the world, with the yearly cycle of sea surface temperature always lagging behind the yearly cycle of daily radiation in the northern hemisphere resulting in increasing light intensities with low sea surface temperatures during spring and decreasing light intensities with high sea surface temperatures during autumn (Donohoe 2020). This consistent lag between these two abiotic factors might potentially function together as an important life cycle control mechanism within the life cycles of kelp. We hypothesize that multiannual delayed *S. latissima* gametophytes use this lag between the seasonal cycles of solar irradiation and sea surface temperature as a strong life cycle control in order to align gametogenesis with sorus formation, thus aligning gametogenesis to the dispersal of large quantities of zoospores. In addition, initial gametophyte density is included in this experiment, as it has been shown to be a strong biotic life cycle control mechanism in higher density gametophyte cultures (Reed 1990; Choi et al. 2005; Carney and Edwards 2010; Ebbing et al. 2020).

Methods

Culture collection. Ripe *Saccharina latissima* individuals were collected along the coast of Flekkefjord, Norway, (58.294270°N, 6.656618°E) in December 2016. The ripe sori of ten pooled individuals were excised from the blades and biofouling was thoroughly removed using absorbent paper. The collected sori were further cleaned by shortly (~1 minute) submerging them in hypochlorite 0.15% (ClO^-), followed by rinsing three times with pasteurized seawater. The cleaned sori were then dried using absorbent paper, followed by overnight drying in an incubator (12°C). Zoospores were released the next day in Erlenmeyer flasks, by submerging the sori in seawater, and these cultures were hereafter incubated at 12°C under red light (30 µmol photons $m^{-2} \cdot s^{-1}$, 12:12 hour light to dark cycle) in f/2 medium (Guillard 1962). These cultures were then incubated for 1.5 years prior to the start of the experiment. During this period, the medium was refreshed on a monthly basis and cultures were monitored for general health, infections, preliminary reproduction, and growth.

Gametophyte culture measurements. During the experiment, we used a dilution gradient of a stock solution to reach specific gametophyte biomass (0.01, 0.02, 0.04, 0.08 mg · mL^{-1}). In order to estimate initial gametophyte densities, we measured chlorophyll-a concentrations (Chl-a) with fluorometry (FastOCEAN/Act2 FRRF, Chelsea Technologies Group Ltd), and calibrated these against gametophyte biomass (Fig. S3-1; R^2= 0.975), similar to what has previously been done for phytoplankton biomass (Huot et al. 2007). The small volumes of the wells used in this experiment made it necessary to use biomass extrapolations of larger volumes of stock gametophyte culture (60 mL) to calculate relationships between fluorometry measurements and freeze-dried gametophyte dry weights (DW) that were weighted using a precision scale.

Modelled daily radiation and sea surface temperature. The seasonal cycles of daily radiation and sea surface temperature were collected close to the original environment of the parental sporophytes from publicly available datasets. These measurements were used to demonstrate the seasonal lag between light intensity and sea surface temperature, which characterizes the seasonal shift between spring and autumn. Modelled solar radiation derived

from a climatology model (ERA INTERIM) and represent 20-year averages (1993–2013) of a grid cell close to the origin of the parental sporophytes (57°N, 5.25°E). Note that estimates of mean surface solar radiation ($kJ \cdot m^{-2} \cdot hr^{-1}$) were not modelled using daily radiation but the 20-year average of peak solar radiation between 10:00–14:00 so that they could be directly translated into *in vitro* light intensities instead of acting as a proxy for day length. The sea surface temperature data consists of five-year averages of the North Sea and come from the weather station located at the Ecofisk oil field (56.5434°N, 3.2244°E).

Light and temperature conditions. The variations in light intensity were achieved through specific placement of the cultures with respect to light sources. The manual placement of the well plates resulted in slightly different light intensities between these incubators, which was taken into account in the analyses afterwards. Light intensities were measured at three points in the middle of the plate, after which an average light intensity was calculated per plate. The spectral distribution of white light was measured using a modular multispectral radiometer (TriOS Ramses ARC, Germany; Heuermann, Reuter and Willkomm 1999, Fig. S3-2). The temperature range in this experiment was achieved using 6 small-scale incubators (Polar CE202, Fig. S3-3). All incubators were adjusted for a specific temperature, with 10.4 °C being the lowest reliable temperature that could be achieved using this setup without strong temperature gradients forming inside the incubators. LED panels (LS LED, RGB + CCT 30 · 30cm 18W) were placed in front of the glass door at an angle of approximately 45°. Randomly filled well plates (n=24) were placed in each incubator along a light intensity gradient (10, 30, 60, and 80 µmol photons $\cdot m^{-2} \cdot s^{-1}$).

Gametophyte reproduction. Successful reproduction was determined on day 28, and quantified using a proxy for gametogenesis, namely the number of sporophytes (≥25 µm) per mL. To elaborate further, our focus is the gametophytic part of kelp reproduction, where kelp reproduction as a whole consists of two spatiotemporally separated reproductive events (sporophytic meiosis and gametophytic fertilization). The sporophytes developed on the bottom of the well-plates, and were counted in triplicate for each unique treatment (n = 96). After 28 days, all fertilized oogonia had developed into small sporophytes, which were still small enough to accurately distinguish

for counting single individuals.

Statistical analysis. All statistical analysis was done using the SPSS 20.0.0 statistical package (SPSS Inc. Chicago, USA), R version 3.6.0 (Team R Core 2018), and Sigmaplot 13.0 (Systat software Inc., London, UK). A linear regression using a 2^{nd} order polynomial (parabola) was fitted to quantify the effects of temperature on reproductive success under two contrasting light regimes using R version 3.6.0. The reproductive success was log-transformed before fitting these regressions so as to avoid passing through 0, as predictions of negative reproduction are meaningless. Reproductive success was log-transformed to achieve normal distributions and analyzed for homogeneity of variance using Levene's test. In case of unequal variances, a robust test of equality of means for unequal variances was applied (Welch t-test). Games-Howell nonparametric post-hoc comparisons were subsequently applied to test for significant differences between the subgroups (light intensities, temperature, and initial gametophyte densities). All tests were run with a significance level of 0.05. Data on the reproductive success of gametophytes (n = 288) are presented as means ± SD. Contour plots were also used to visualize the interactive influence of light intensity and temperature on the reproduction of multiannual delayed gametophytes at an initial gametophyte density of 0.01 mg · mL^{-1} (Loess smoother, sampling portion = 0.8, interval = 6).

Results

The interactive effect of light intensity, temperature, and initial gametophyte density on gametophyte reproduction. Reproduction of multiannual delayed *S. latissima* gametophytes was successfully induced under different light intensities (µmol photons · m^{-2} · s^{-1}), temperatures (°C), and initial gametophyte densities (mg · mL^{-1}). In Fig. 3-1A, we plotted the optima of the reproductive success (sporophytes · mL^{-1}) found at varying temperatures, along with the corresponding initial gametophyte densities. The highest reproduction success observed in this experiment was 469 sporophytes · mL^{-1}, which was achieved at 8.5 µmol photons · m^{-2} · s^{-1}, 12.6°C, and at an initial gametophyte density of 0.04 mg · mL^{-1}. The effect of light intensity on reproduction is shown in Fig. 3-1B for an initial gametophyte density of 0.01 mg · mL^{-1}. We only visualized results for the lowest initial gametophyte density (0.01 mg · mL^{-1}), as the interactive effects of light and temperature on gametophyte reproduction followed similar trends for all initial gametophyte densities, but were difficult to visualize on the same scale. This was mainly because higher initial gametophyte densities had strong negative interactive effects on gametophyte reproduction, thereby visually masking the relative influence of light and temperature on gametophyte reproduction (Fig. 3-2). The contour plots in Fig. 3-1B show that the gradient of gametophyte reproduction success varied with light intensity, depending on the temperature that was used. At 10.4°C the reproductive optimum was found at 60 µmol photons · m^{-2} · s^{-1}, while at ≥ 12.6°C the reproductive optima were found at lower light intensities (9 - 15 µmol photons · m^{-2} · s^{-1}). At a temperature of 11.2°C, an intermediate reproductive gradient was observed. Although light intensity did generally influence gametophyte reproduction (Welch ANOVA, $F_{3,4.07} = 7.483$, $p < 0.05$), no significant differences in reproductive success were found between light intensities (12.5-, 29.0-, and 58.0 µmol photons · m^{-2} · s^{-1}; Games - Howell, $p > 0.05$; Table. S3-2). Cultures with no gametophyte reproduction were also observed in this experiment (dark-blue color) and usually occurred at temperatures ≥ 14.0°C or at initial gametophyte densities > 0.04 mg · mL^{-1} (Fig. 3-2).

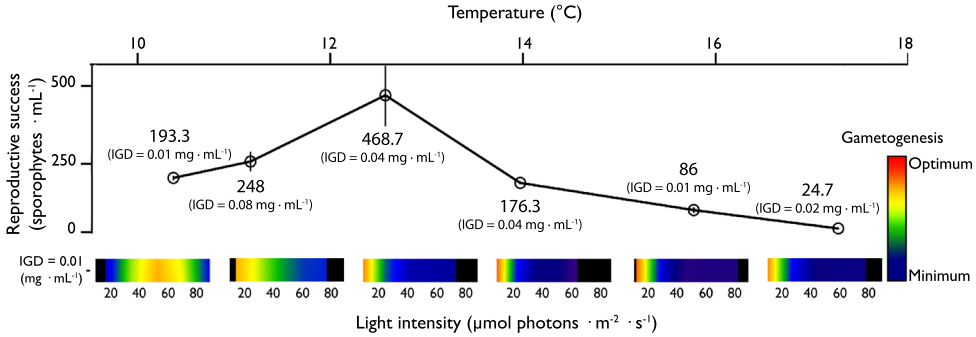

Figure 3-1. The interactive influences of light intensity (µmol photons · m^{-2}· s^{-1}) and temperature (˚C) on the reproductive success (sporophytes · mL^{-1}) of multiannual delayed gametophytes of *Saccharina latissima*. Fig. 3-1A shows the optimal reproductive success of *S. latissima* gametophytes at the different temperatures (top x-axis). The corresponding initial gametophyte density values were added next to the points, since optimal reproduction was found at different initial gametophyte densities. Fig. 3-1B contains 6 smoothed contour plots that visualize how light intensity influences the reproductive success in cultures placed at an initial gametophyte density of 0.01mg · mL^{-1} at the same six temperatures shown in Fig 3-1A. Error bars represent ±SD, n = 3.

The effect of initial gametophyte density *on gametophyte reproduction.* The relative reproductive success (sporophytes · mg^{-1}) was calculated by dividing the sporophytes that had formed (sporophytes · mL^{-1}) with the initial gametophyte density that had been used (mg · mL^{-1}). Increasing initial gametophyte density resulted in low relative reproduction success (Fig. 3-2). Optimal relative reproductive success was found at the lowest initial gametophyte density used in this experiment of 0.01mg · mL^{-1}, with three optima of similar reproductive success (18.247 sporophytes · mg^{-1} ± 604) at three different temperatures (10.4-, 11.2-, and 12.6 ˚C), depending on the light intensity used. This inverse correlation between initial gametophyte density and relative reproductive success was especially pronounced at higher light intensities (≥29 µmol photons · m^{-2} · s^{-1}), with relative sporophyte production at an initial gametophyte density of 0.08 mg · mL^{-1} mostly present at low light intensities (≤15 µmol photons · m^{-2} · s^{-1}).

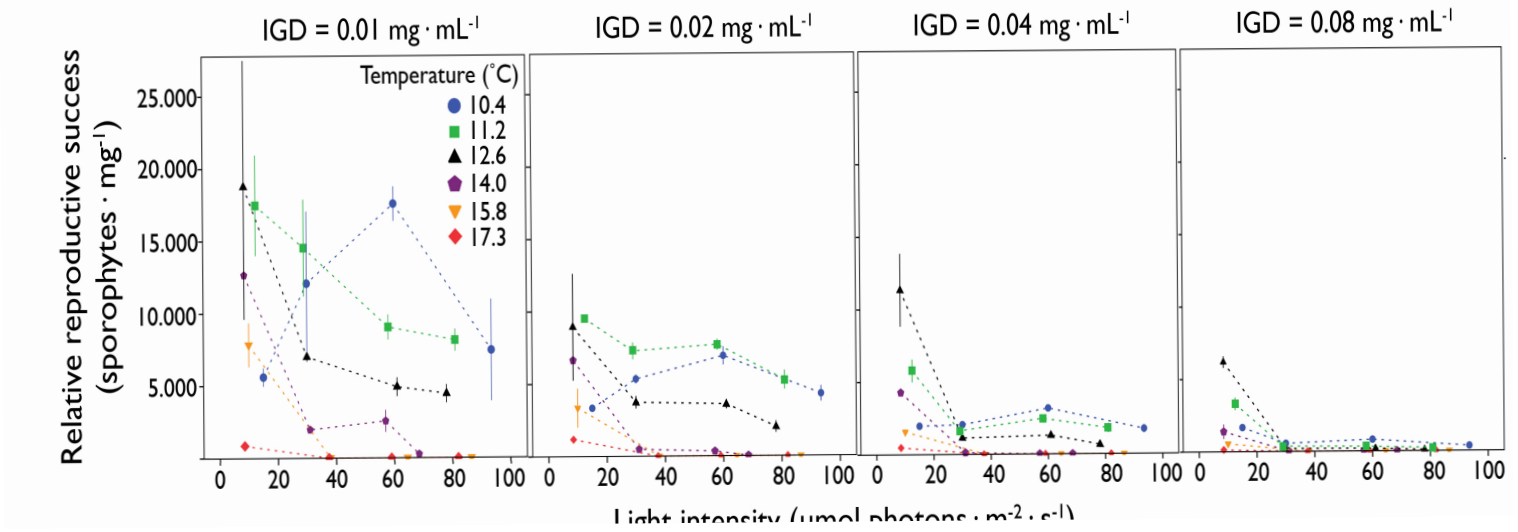

Figure 3-2. The influence of light intensity and temperature on the relative reproductive success of multiannual delayed *S. latissima* gametophytes. The four graphs represent the four initial gametophyte densities used in the experiment, with the relative reproductive success depicted as mean ± SD, n = 3.

Seasonality in multiannual delayed *gametophyte reproduction.* The relation between reproductive success and temperature was fitted with a polynomial linear regression for low (≤15 µmol photons · m^{-2} · s^{-1}) and high (≥29 µmol photons · m^{-2} · s^{-1}) light intensities (Fig. 3-3). Distinct optimal temperatures were found for reproduction under high and low light intensity regimes (ANCOVA, $F_{5,49}$ = 49, R^2 = 0.82, P ≤ 0.001; Table. S3-3). A similar disparity between light and temperature was also measured in a natural system, reflecting the seasonal lag between daily radiance and sea surface temperatures (Fig. 3-4). This seasonal lag between the two abiotic factors either result in *i)* lower temperatures in combination with higher light intensities during spring (○) or *ii)* higher temperatures in combination with lower light intensities during autumn (□). These two scenarios roughly resemble the two separate temperature optima found for the reproductive success of multiannual delayed *S. latissima* gametophytes as identified under high and low light intensities, respectively (Fig. 3-3).

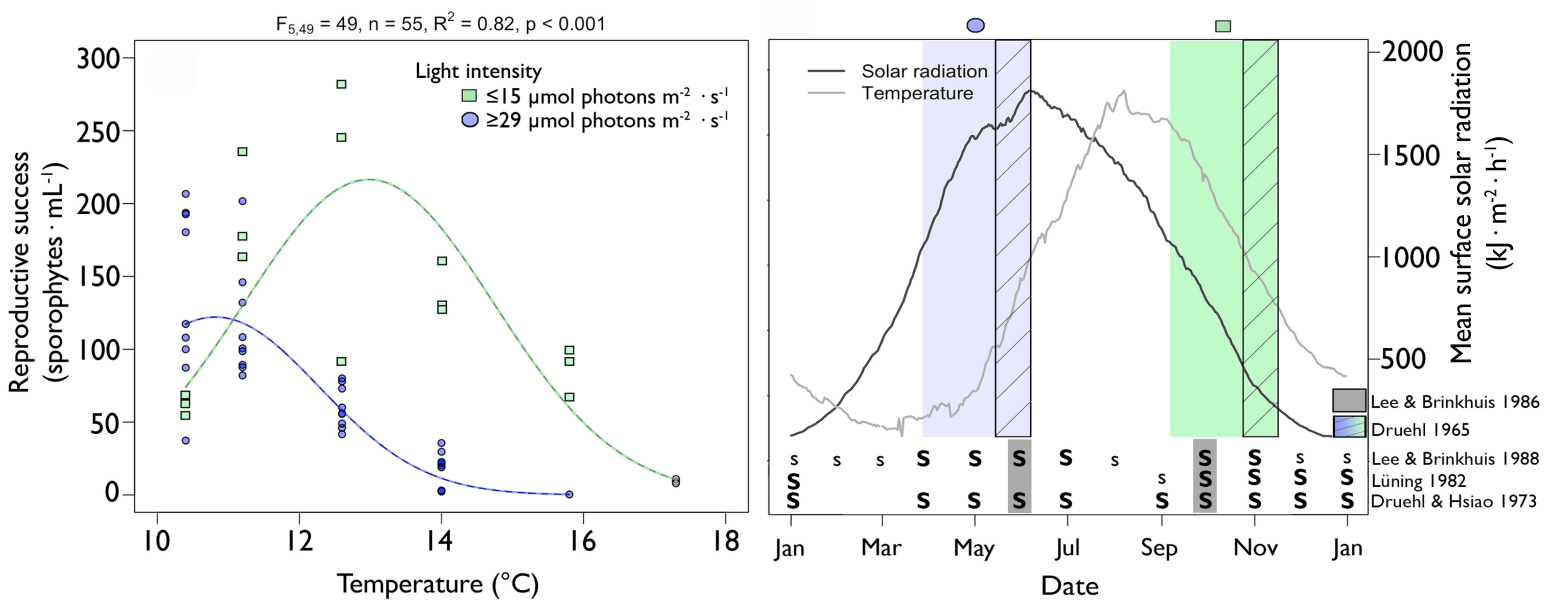

Figure 3-3. The *in vitro* interactive effect between light intensity and temperature on multiannual delayed *S. latissima* gametophyte reproduction. Values are expressed in a scatterplot, and two regressions were fitted through the data points of both low (≤15 µmol photons · m^{-2} · s^{-1}) and high (≥29 µmol photons · m^{-2} · s^{-1}) light intensities using ANCOVA. Both regressions (○ & □) are colour- and symbol-coded to match to the periods in the year with the highest disparity between mean solar radiation and sea surface temperatures, to show alignment with the seasonal lag hypothesis (Figure 3-4).

Figure 3-4. Representation of the seasonal lag between mean surface solar radiation and sea surface temperatures around Flekkefjord, Norway. *In vivo* proxies for higher reproduction of *S. latissima* gametophytes are also shown. Proxies include presumed peak zoospore releases (dashed bars), and observed peak sorus abundances (described below the graph). The abundance of sori is displayed visually with (**S**) representing ripe or abundant sorus material, (s) representing scarce sorus material, and blank spaces representing the absence of sorus material. Peak percentages of sporogenous *S. latissima* plants, observed by Lee & Brinkhuis 1986, are highlighted in grey.

Vegetative growth. Multiannual delayed gametophytes increased vegetatively in all cultures under all experimental conditions, including at the initial gametophyte density of 0.01 mg · mL^{-1} depicted here (Fig. 3-5). We only show results for the lowest initial gametophyte density here, since higher initial gametophyte densities had a strong negative interactive effect on reproduction, thereby masking the relative influence of light and temperature on reproduction. Gametophyte density at this initial gametophyte density increased with increasing light intensity at all temperatures. The increase in gametophyte density started to plateau at ≥38 µmol photons · m^{-2} · s^{-1}, and the slowest overall growth was observed in cultures placed at a temperature of 10.4°C and at 15 µmol photons · m^{-2} · s^{-1}. Note that at 10.4°C, gametophyte density was still positively correlating with light intensity.

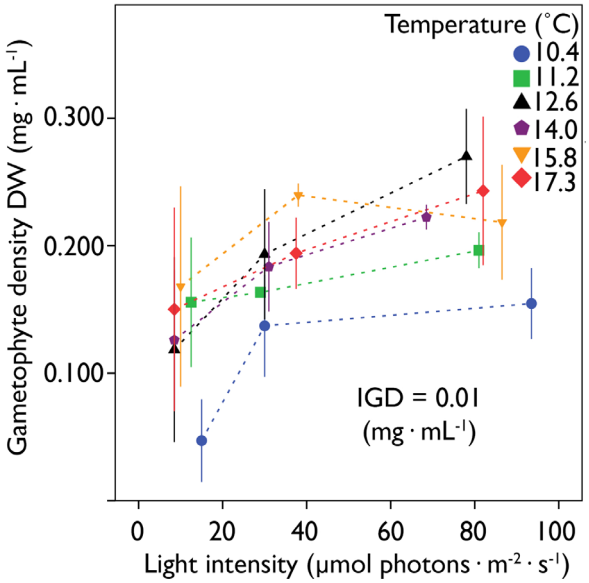

Figure 3-5. Multiannual delayed *S. latissima* gametophyte density (mg DW · mL^{-1}) on day 28 under different light intensities at different temperatures with the dotted lines representing linear interpolations between the different data points. Cultures started with an initial gametophyte density of 0.01 (mg DW · mL^{-1}). Values are expressed as mean ± SD, n = 3.

Discussion

In nature, kelp gametophytes can remain vegetative for years (Edwards 2000; Barrento et al. 2016), thereby passing multiple reproductive seasons (Druehl and Hsiao 1973) before initiating gametogenesis. In this study, we demonstrate that multiannual delayed *S. latissima* gametophytes can successfully become fertilized after more than a year of vegetative growth. We furthermore show that light intensity and temperature interact to control reproductive behaviour in multiannual delayed *S. latissima* gametophytes, which we suggest is most likely related to the lag between seasonal light and temperature cycles. As a result, multiannual delayed gametophytes reach optimal reproduction when the disparity between light intensity and temperature is high, such as found in spring and autumn.

The seasonal lag hypothesis. The influence of light and temperature as seasonal cues has already been well established for many autotrophs (Andrés and Coupland 2012; Singh and Singh 2015). The role of the seasonal lag between light and temperature as a life cycle control, however, has been understudied for most autotrophic organisms, including terrestrial plants (Kudoh 2019). Our experimental results on the combined effects of light, temperature and initial gametophyte density on reproduction of multiannual delayed *S. latissima* gametophytes may well be explained by the seasonal lag between daily radiation and sea surface temperatures observed around the world (Donohoe 2020). The reproductive success of multiannual delayed *S. latissima* gametophytes in our experiment increased when the disparity between temperature and light intensity increased. Multiannual delayed gametophytes appear to use the increasing disparity between solar radiation and sea surface temperature to reliably assess when spring and autumn starts, aligning their reproductive cycles with surrounding adult kelp that also might use this seasonal lag to initiate sporogenesis. If this complex response is there, it most likely uses an endogenous annual clock or circannual oscillator to synchronize with the seasons (Lüning and Dieck 1989). In evolutionary terms, the presence of an endogenous annual clock might be of fundamental importance, especially for a species that has spatiotemporally separate reproductive phases in their life cycle (Krueger-Hadfield 2020).

Alternatively, our experimental findings could be explained by the

phenomenon of vegetative growth of gametophytes increasing under higher light intensities (≥29 µmol photons · m^{-2} · s^{-1}) and temperatures (≥12.6°C), with this increase in vegetative growth translating into reduced reproductive potential. However, this does not explain all the results: for at 10.4°C the vegetative growth still correlates positively with increasing light intensities, while the reproductive success also correlates positively with increasing light intensities (Fig. 3-5). This indicates that vegetative growth and reproduction are not absolute antagonists, asking for a different hypothesis to complement or even override this explanation. Hence, we considered this alternative explanation less appropriate and henceforth continue to delve deeper into the seasonal-lag hypothesis using multiannual delayed gametophytes.

Multiannual delayed gametophytes have been observed *in vivo* in similar brown seaweeds with a kelp like life history (*Desmarestia ligulata*, Edwards 2000) and *M. pyrifera* forests have been found to have mixed-parent origins, suggesting periods of longer periods of reproductive dormancy (Carney et al. 2013). However, quantifying *in vivo* gametophyte reproduction remains notoriously difficult (Carney and Edwards 2006), making the use of proxies for gametogenesis necessary for further validation of the seasonal lag hypothesis in multiannual delayed gametophytes. *In vivo* juvenile sporophyte observations, indicating recent synchronized gametogenesis events, suggest that there are multiple reproductive peaks between autumn and spring for *S. latissima,* with a decrease in reproduction observed in midwinter (Parke 1948; Druehl 1965; Hsiao et al. 1973). Druehl and Hsiao (1973) even described seasonal events that are consistent with our described seasonal lag hypothesis, with two peaks in new macroscopic sporophyte production closely matching the periods in which increased gametophyte reproduction is predicted by our hypothesis. Furthermore, Nagasato et al. (2019) successfully quantified *in vivo S. japonica* zoospore releases for the first time, and found that over a period of two years, clear peaks occurred in October/ November with undulations and slight increases above baseline of zoospore concentrations during spring in both years, supporting the seasonal lag hypothesis. The possibility of biannual fertilization events is also supported by the fact that spores and the microscopic forms of many macroalgal species have strong tolerances to long periods of darkness (Santelices et al. 2002), suitable to survive winter conditions. We hypothesize that the increasing

light conditions, accompanied with low water temperatures during spring, help delayed *S. latissima* gametophytes to synchronize gametogenesis with surrounding zoospore releases.

Another proxy for gametogenesis is the presence of sorus patches as an indicator of sporogenesis (Alsuwaiyan et al. 2019) and thus, also for recently sporulated and newly formed gametophytes. Multiannual delayed gametophytes should in this case, hypothetically, synchronize their reproduction with peak occurrences of viable newly formed gametophytes. Thanks to thorough research by Bartsch et al. (2008), three temporal scenarios can be distinguished when analysing the observed occurrence of *in vivo S. latissima* sorus patches. In the first scenario, *S. latissima* sorus patches were observable throughout the year (Parke 1948), with peak percentages of sporogenous plants observed in October and June (Lee and Brinkhuis 1986). In the second scenario, sporophytes started to develop sorus patches in autumn, which gradually disappeared starting in spring (Harries 1932; Sears and Wilce 1975; Lüning et al. 1982). Last, sorus bands were observed during two distinct periods, divided by a mid-summer and late winter hiatus when sorus patches were practically absent (Druehl and Hsiao 1977; Lee and Brinkhuis 1988). Multiannual delayed gametophytes might successfully synchronize their reproduction using the seasonal lag between solar radiation and sea surface temperature in all the above scenarios. The last scenario most closely matches our seasonal lag hypothesis with peaks of sorus patches occurring when the disparity between light intensity and temperature is greatest. We interpret this as supporting our seasonal lag hypothesis, but both *in vitro* and *in vivo* information is needed on the influence of daylength and relative temperature changes on multiannual delayed gametophyte reproduction for further validation. Moreover, the need to quantify the microscopic components of the kelp life cycles in their natural environment is apparent since the artificial environment of multiannual delayed gametophytes in laboratory studies can influence their behaviour in ways that are not yet understood. This is why it is important that novel and improved molecular tools become available in the future, enabling us to further characterize these hidden components of *in vivo* algal communities (Hoffmann and Santelices 1991; Edwards 2000; Schoenrock et

al. 2020).

How light and temperature influence multiannual delayed gametophyte reproduction. Gametophyte reproduction was observable for the entire spectrum of light intensity and temperature, in accordance with the broad spectrums observed in previous studies using newly formed gametophytes. Successful reproduction was achieved using broad light intensity spectrums of white light in Lüning et al. (1980, 5 – 40 µmol photons m^{-2} s^{-1}) and Lee and Brinkhuis (1988, 6–120 µmol photons m^{-2} s^{-1}). Similarly broad temperature ranges that resulted in reproduction were also observed the same studies: Lüning et al. (1980, 5 - 15°C, Helgoland) and Lee and Brinkhuis. (1988, 7 - 14°C, Long island). Such results have not only been observed in *S. latissima* but also in other Laminariaceae, including *Laminaria ochroleuca* (12–18°C, Izquierdo, Pérez-Ruzafa and Gallardo 2002), *Laminaria digitata* (5–15°C, Martins et al. 2017), and *Undaria pinnatifida* (10–15°C, Morita et al. 2003). This indicates that reproduction in the Laminariaceae is possible during multiple times of the year, as previously observed (Parke 1946; Hsiao and Dreuhl 1973).

Despite the similarities in the broadness of effective spectrums of light intensity and temperature on gametophyte reproduction, large discrepancies have been observed on the interactive effects of light intensity and temperature in these same studies. Lüning et al. (1980) described a necessary increase in photon flux density in order to induce gametophyte reproduction at high temperatures (18°C), while Lee and Brinkhuis (1988) found no reproductive success at all using temperatures higher than 14°C, regardless of the applied light intensity. When addressing other members of the Laminariaceae, Izquierdo et al. (2002) found no reproductive success in *Laminaria ochroleuca* at 10°C, regardless of light intensity, while Deysher and Dean (1986) hardly observed any gametophyte reproduction in *Macrocystis pyrifera* under low light conditions (≥ 6.7 µmol photons $m^{-2} \cdot s^{-1}$), regardless of temperature. The novel insights described here add on the one hand to the already large discrepancies observed in literature described above. On the other hand, they fit perfectly well in our seasonal lag hypothesis, ultimately highlighting the need to delve deeper in lesser understood (a)biotic life cycle controls governing delayed gametophyte reproduction.

Biomass density (Ebbing et al. 2020) or in this study culture age, could

influence the reproduction of multiannual delayed gametophyte cultures in more profound ways than it would influence the reproduction of newly formed gametophyte cultures. This is because the prolonged periods of vegetative growth not only increases the age and biomass density of multiannual delayed gametophyte cultures, but it may also change genetic diversity and sex ratio of the cultures if genotypes and sexes have different vegetative grow rates (e.g., see Destombe and Oppliger 2011). This type of selection towards individuals with higher growth rates is well documented for microalgae cultures, causing changes in the frequencies of genotypes and thus the evolution of a culture (Lakeman et al. 2009). Such lesser understood factors might ultimately influence multiannual delayed gametophyte reproduction in profound ways, thereby becoming important life cycle controls that need to be understood to enable mass cultivation of kelp using the controlled reproductive method (CRM). Understanding these lifecycle controls involving CRM is important for kelp domestication as it opens up fundamentally new and different ways of kelp-farming and kelp-breeding.

ACKNOWLEDGEMENTS

The authors want to thank Hortimare BV and their staff for allowing use of their *S. latissima* gametophyte cultures. We furthermore want to thank Tim Hermans for his help in providing us with the modelled daily radiation around Flekkefjord, Norway, used in this study to support the seasonal lag hypothesis.

Supplementary data

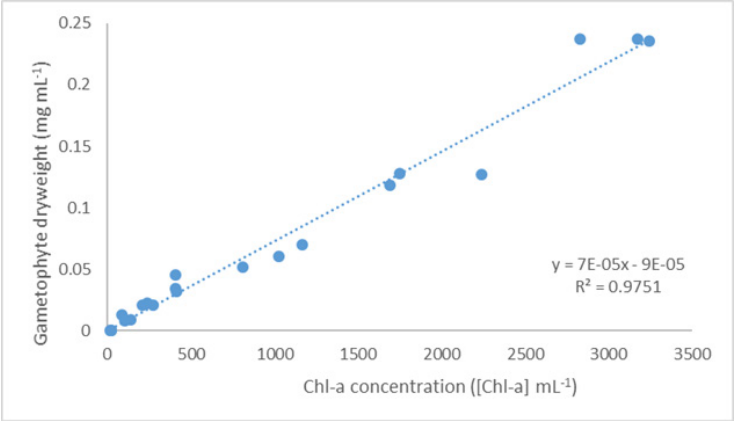

Figure S3-1. Calibration curve between the chlorophyll a concentration (mg Chl · m^{-3}), and *S.latissima* gametophyte dry weight per mL (mg DW · mL^{-1}). Gametophyte dry weights are extrapolations from 60mL cultures, whose [Chl] concentration were measured using a FRRF fluorometer. The linear regression and correlation coefficient were y = 7E-05x - 9E-05 and 0,975 respectively.

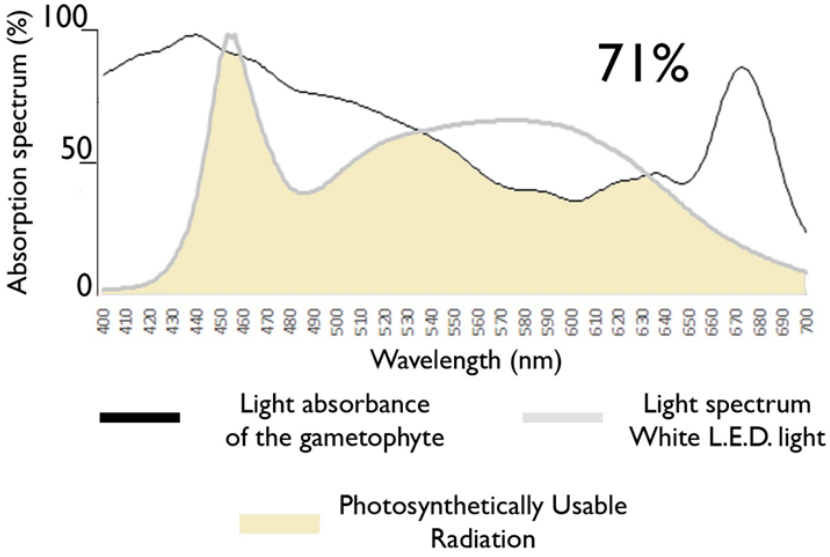

Fig. S3-2. The light absorbance spectrum of *S.latissima* gametophytes (black line) projected over the spectral distribution of White L.E.D. light. Light was measured at different wavelengths from 400nm until 700nm, and peak emission strength was normalized to 1 and plotted against the absorbance of the culture (%).

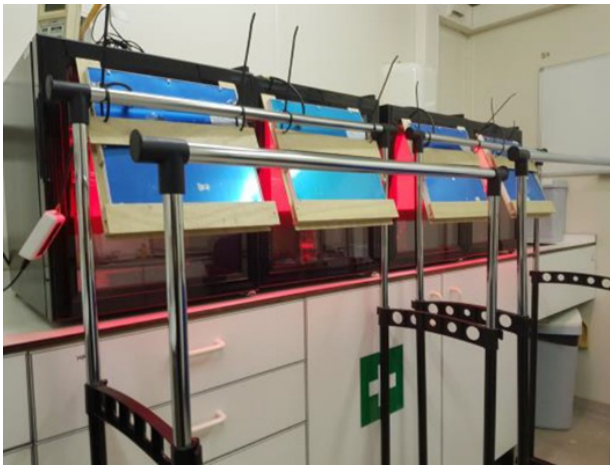

Fig. S3-3. Photo of the experimental setup with some of the incubators used. The L.E.D. panels were hanged in front of the see-through doors of the incubators, enlightening the gametophyte cultures with white light.

Table S3-1. The 7 cultivation phases described by Vodouhe et al., 2012 are used to see how far we have come in the domestication process of kelp as a aquatic crop. Based on a literature study we assessed that kelp aquaculture is hovering between the 4th and 6th cultivation phase, depending on the cultivation method used.

Cultivation phase	**Description**
Phase 1.	Species entirely wild and collected only when needed.
Phase 2.	Wild species maintained in the fields when found during land preparation (clearance, burning, and weeding) due to its proved utility and regular need, its scarcity around habitations, and the difficulties for getting it on time, in quality and in quantity. These preserved plants are subject to regular observations for the understanding oftheir reproductive biology.
Phase 3.	Farmers start paying more attention to the preserved plants (weeding, protection against herbivorous) for their survival and their normal growth. A sort of ownership on the plants start.
Phase 4.	The reproductive biology of the species is known, and multiplication and cultivation of the species in the home gardens or in selected parts of cultivated fields are undertaken by farmers or healers. At this stage, farmers tend to conduct diverse experiments (date of planting, sowing or planting density, pest and diseases management, etc.) in order to master mass production of the species in the future. The ownership on the plant is more rigorous.
Phase 5.	The species is cultivated and harvested using traditional practices.
Phase 6.	To improve the quality of the product, farmers adopt specific criteria to select plants that better satisfied people needs. The best cultivars/plants (good grain/fruit quality, resistant/tolerance to diseases and pests) are known, and technical package is adopted for their development and multiplication. At this stage, access to market is considered and some species benefit from traditional postharvest tech- nologies (method for processing, cooking or conservation, etc.) to meet consumers' needs.
Phase 7.	Selection initiatives continue with cooking qualities, protection against pests, and diseases in cultivation and storage. Income generation is more clearly taken care of: market demands (quantity and quality) are also taken into account, and species varieties that meet consumers' preferences are selected and produced.

Table S3-2. Games-Howell post hoc analysis for the influence of light intensity on gametophyte reproduction at an IGD of 0.01 mg · ml^{-1}, after we found significant differences using the robust test of variance. The mean difference is significant at $p < 0.05$.

	(I) Light intensity (µmol photons · m-2 · s-1)	(J)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Games-Howell	13	29	32.333	30.661	.731	-92.57	157.24
		58	92.778	22.757	.115	-46.63	232.19
		81	102.778	22.613	.096	-39.07	244.62
	29	13					
		58	60.444	21.980	.237	-73.12	194.00
		81	70.444	21.832	.182	-65.57	206.46
	58	13					
		29					
		81	10.000	7.375	.581	-20.24	40.24
	81	13					
		29					
		58					

*. The mean difference is significant at the 0.05 level.

Table S3-3. The ANCOVA analysis of the influence of light intensity (µmol photons · m^{-2} · s^{-1}) and temperature (°C) on the reproductive success of MAD gametophytes

lm(formula = log Hatchling density ~ poly(Temperature, 2) * Light intensity - Light intensity)

Residuals:

Min	1Q	Median	3Q	Max
-158.941	-0.2517	-0.02991	0.37515	1.13751

Coefficients:

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	-22.087557	4.147004	-5.326	2.39e-06 ***
10.4°C : ≥29 µmol photons · m-2 · s-1	4.982.671	0.674296	7.389	1.48e-09 ***
12.6°C: ≥29 µmol photons · m-2 · s-1	-0.230781	0.027224	-8.477	3.07e-11 ***
10.4°C : ≤15 µmol photons · m-2 · s-1	-0.752306	0.103034	-7.302	2.03e-09 ***
12.6°C : ≤15 µmol photons · m-2 · s-1	0.067931	0.007942	8.553	2.35e-11 ***

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

View over the Oosterschelde and the tidal tanks from the point of view of the seaweed centre at the NIOZ in Yerseke.

Chapter 4

IN-CULTURE SELECTION AND THE POTENTIAL EFFECTS OF CHANGING SEX RATIOS ON THE REPRODUCTIVE SUCCESS OF MULTIANNUAL DELAYED GAMETOPHYTES OF *SACCHARINA LATISSIMA* AND *ALARIA ESCULENTA*

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Abstract

Multiannual delayed gametophyte cultures can stay vegetative for years, while also having the ability to grow. This study aims to investigate if male and female multiannual delayed gametophyte strains of the species *Saccharina latissima* and *Alaria esculenta* grow at different rates in culture. We furthermore assessed how changing sex ratios can affect the reproductive yields of these cultures. The results indicate that the reproductive yield of cultures declined with decreasing male:female ratios. A correlation that becomes especially apparent at higher culture densities for both species. Female gametophyte densities in particular affected the observed reproductive yield of the cultures, with *S. latissima* cultures showing a clear reproductive optimum (sporophytes · mL^{-1}) at 0.013 mg · mL^{-1} DW female gametophyte biomass, while the reproductive success of *A. esculenta* peaked at a density of 0.025 mg · mL^{-1} DW of female gametophyte biomass, after which the reproductive yield started to decline in both species. The results show that the sex ratio of a gametophyte culture is an important biotic life cycle control, with higher amounts of female gametophyte biomass halting gametophyte reproduction. Understanding how these changing sex ratios in gametophyte cultures affect reproduction is especially important in aquaculture of kelp, where reliable preforming cultures are key to long-term success.

Introduction

Interest in using delayed gametophytes as seed stock for large scale aquaculture of brown seaweeds is growing around the world. The advantages of using delayed cultures are well understood Goecke et al. (2020): i) they can be kept healthy for prolonged periods of time (Barrento et al. 2016; Carney 2011; Wade et al. 2020) , *ii)* they can be successfully cryopreserved (Visch et al. 2019) and *iii)* gametophyte cultures can easily be cloned (Reddy et al. 2008). These characteristics allow for the crossing of distantly related unialgal male and female gametophyte clone cultures evoking hybrid vigour for future F1 hybrid cultivars (Shan et al. 2016; Zhao et al. 2016; Martins et al. 2019). However, in order to use these delayed gametophytes for large scale aquaculture, we first need a detailed understanding of the controlled growth and reproduction of larger volumes of these delayed gametophyte cultures (Zhang et al. 2008). This is especially important for multiannual delayed (MAD) gametophytes, which are gametophytes that delayed their sexual reproduction by more than a year, thereby transgressing seasonally induced reproduction (Ebbing et al. 2021).

MAD gametophyte cultures can be subdivided into two distinctly different types of cultures. The first culture type is created from large amounts of extracted zoospores (Nahlah et al 2019), contains both sexes (Choi et al. 2005), and is described in this study as “wildtype” cultures. Unialgal clonal cultures are the second culture type and start out from small isolated gametophyte individuals that are bulked up through fragmentation into larger cultures (Wu et al. 2004), creating genetically identical clonal cultures (Zhao et al. 2016). The advantages of working with MAD clonal gametophytes instead of regular MAD wildtype cultures are both practical and scientific in nature. In practice, clonal cultures are less prone to biofouling and culture infections compared to wildtype cultures, making them more reliable to use on the long term. The reason why clonal cultures are less prone to fouling is because they start from individually isolated gametophytes (Li et al. 2004). In contrast, wild type cultures start from zoospore extractions that inevitably come in contact with contaminated sorus material (Nahlah et al. 2019), increasing the chances for contamination on the long run. On a scientific level the advantages of individually growing out clonal gametophytes allows us

to optimize the vegetative growth of the gametophytes (Zhang et al. 2019). Using clonal cultures also allows us to manually change the genetic make-up of the gametophytes in the cultures, through clonal inclusion or exclusion for breeding purposes (Martins et al. 2019), or change the sex ratio (Zhang et al. 2008). Lastly, using unialgal clonal gametophyte cultures prevents in-culture selection to take place, a process that occurs when conditions favour the growth of one genotype over another in genetically diverse cultures (Lakeman et al. 2009), like wildtype gametophyte cultures.

Wildtype gametophyte cultures contain both male and female gametophytes, and when the sexes grow vegetatively at different rates, sexual in-culture selection can take place. For example, Destombe and Oppliger (2011) showed that *Laminaria digitata* female gametophytes grow at a different rate than their male gametophyte counterparts, suggesting that over time the sex ratio of *L. digitata* wildtype cultures will change. Not just growth rates, but also (a)biotic factors can change the sex ratio of a culture (Oppliger et al. 2012; Murua et al. 2021), with the changing sex ratios inevitable altering the reproductive yield of a culture, since the contribution of the limiting sex to the production of offspring will diminish over time. To illustrate, Zhang et al. (2008) showed that the sex ratio in *Saccharina japonica* cultures affect the reproductive yield of a culture, with decreasing male:female ratios corresponding to decreasing sporophytes being formed.

This study aims to investigate if male and female multiannual delayed gametophyte strains grow at different rates in culture. We furthermore separately look into how changing sex ratios affect the reproductive success rates (sporophytes · mL^{-1}) of MAD *S. latissima* and *A. esculenta* gametophyte cultures over time. We hypothesize that the sex ratio of the cultures can have a strong influence on the reproductive success rates of MAD gametophytes, with overarching similarities between *S. latissima* and *A. esculenta*. Sex specific clonal gametophyte are calibrated against Chlorophyll-a in order to quantitatively to allow the comparison for interspecific reproductive yields along gradients of sex specific gametophyte biomass. This ultimately allowed us to group the results of the two species together to assess whether the influence of changing sex ratios on the reproductive yield functions similarly in both species, proposing a common trait within the *Laminariaceae*.

Material and Methods

Cell culture and maintenance. Ripe *Saccharina latissima* sorus of 6 individuals from Leknesund, Norway (61.212994, 4.896644°E) and *Alaria esculenta* sorus of 8 individuals from Fuhreholmen, Norway (61.043292, 4.868790°E) were collected and pooled in 2016. The release of zoospores was done in the Netherlands, using a standard extraction protocol (protocol of Ebbing et al., 2020, similar to earlier methods described by Redmond et al. (2014) and Bartsch et al. (2018). Zoospores were isolated from the culture to set up clean clonal cultures, separating the sexes. The medium of the multiannual delayed (MAD) clonal cultures used in this experiment was refreshed once every other month with f/2 medium (Guillard and Ryther 1962) and the cultures grew vegetatively for approx. 2.5 years prior to the start of this experiment (11°C; 5 µmol · m^{-2} · s^{-1} photons of red light; 12:12 light:dark cycle). Isolated clonal gametophytes initially started out small in Petri dishes, eventually placing the clonal cultures in 2 Litre plastic barrels with filtered airlift systems when the gametophyte biomass became more voluminous. The sex-specific clonal cultures used here consisted out six individual clonal lines of the same sex that were grouped together. All clonal gametophytes were initially isolated from the same wildtype cultures of 6 (*S. latissima*) to 8 (*A. esculenta*) grouped individual motherplants. We grouped multiple clonal cultures together to increase the genetic diversity of the cultures, emulating the genetic diversity of wildtype cultures, by relying less on gametophytes on the individual level.

Gametophyte biomass calibration. We used fluorometry measurements (Fast Ocean/Act2 FRRF, Chelsea Technologies Group Ltd) to assess the Chl-a concentration [Chl-a] of the cultures. Assessing gametophyte biomass is done in a similar way as with phytoplankton, where Chl-a is used as a proxy for phytoplankton biomass (Huot et al. 2007). These Chl-a concentrations were calibrated against the dry weight (DW) of freeze-dried gametophyte biomass of both *S. latissima* and *A. esculenta* clonal cultures. The gametophyte biomass needed to accurately calculate calibration lines was much higher (Fig. 4-1), than the biomass needed for the assessment of the reproductive success rates, making it necessary for us to extrapolate the biomasses used in the well plates from the calibration lines. Caution is still

needed with interpreting Chl-a as a proxy for biomass, since there could still be unknown interspecific differences, and chlorophyll content might possible differ between individual gametophytes in ways we do not yet understand.

The vegetative growth of clonal gametophyte biomass. The biomass ($mg \cdot mL^{-1}$) of sex-specific clonal gametophyte cultures, consisting out of the six pooled clonal gametophytes of *S. latissima* and *A. esculenta*, grown in 2-litre aerated bottles, was measured over a time period of 21 days, in triplicate, under stock conditions (8 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photons of red light; 11°C; 12:12 light:day cycle) using f/2 medium [24]. The initial culture density was relatively low (~0.2 $mg \cdot mL^{-1}$ DW) and culture medium was refreshed daily, thereby limiting negative effects of space, light, and nutrient limitation.

Gametophyte reproduction. The sexual reproduction of the pooled clonal gametophytes cultures were induced after homogenization, using 30 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photons of white light (11°C; 12:12 light:day cycle), with f/2 medium (Guillard and Ryther 1962), using a gradient of gametophyte biomass density (0.004 – 0.55 $mg \cdot mL^{-1}$ DW). Successful reproduction, i.e. number of regular looking young sporophytes (≥25 µm) per mL was determined using an inverted microscope (40X magnification), 28 days after starting the induction for sexual reproduction. Each treatment (N = 30) was done in triplicate, placed in a 96 well plate (0.3 mL), culture medium was not refreshed, and all the young sporophytes were counted in each well. After 28 days, all fertilized eggs developed into small sporophytes that are reliable countable, with unfertilized eggs remaining an egg. Cultures with different sex ratios were manually created to assess the influence of sex ratio on the reproductive yield of a culture (Table. 4-1). The five ratios were specifically chosen to cover the entire male:female ratio spectrum using approximately equal steps.

Statistical analysis. All statistical analysis was done using SPSS 20.0.0 statistical package (SPSS Inc. Chicago, USA). All data was normally distributed and analyzed for homogeneity using the Levene's test of variance. In case of unequal variances, a robust test of equality of means for unequal variances was applied (Welch t-test). If the data was found to be homogeneous a one-way ANOVA was applied. Independent sample t-tests were used to assess

Table 4-1; The ratios of male:female biomass used in the experiments that are further described in Fig. 4-3 & 4-4.

Male gametophyte biomass (%)	Female gametophyte biomass (%)
5	95
25	75
50	50
75	25
95	5

whether biomass accumulation was significant or not (Fig. 4-2). The Levene's test for equal variances was also applied here in every analysis, and the statistical results are interpreted accordingly (Table. S4-1). The regressions that are described in Fig. 4-1 and Fig. 4-5 are calculated using R version 3.6.0 (Team R Core 2018). Data of the reproductive success of the gametophytes (n = 3) are presented as mean ± SD.

Results

Gametophyte biomass calibrations. The dry weight of gametophyte biomass (mg · mL^{-1}) was successfully calibrated against the measured chlorophyll-a content of the gametophytes for both *S. latissima* and *A. esculenta* (Fig. 4-1). Combining male and female biomass within the two species resulted in a calibration line for *S. latissima* ($F_{1,39}$=523, R^2= 0.929, $p < 0.0001$) and one for *A. esculenta* ($F_{1,35}$=646 R^2 = 0.947, $p < 0.0001$). Note that the calibration line of *S. latissima* culture was steeper than that of *A. esculenta*, indicating that per mg biomass there is less chlorophyll-a present in the gametophyte.

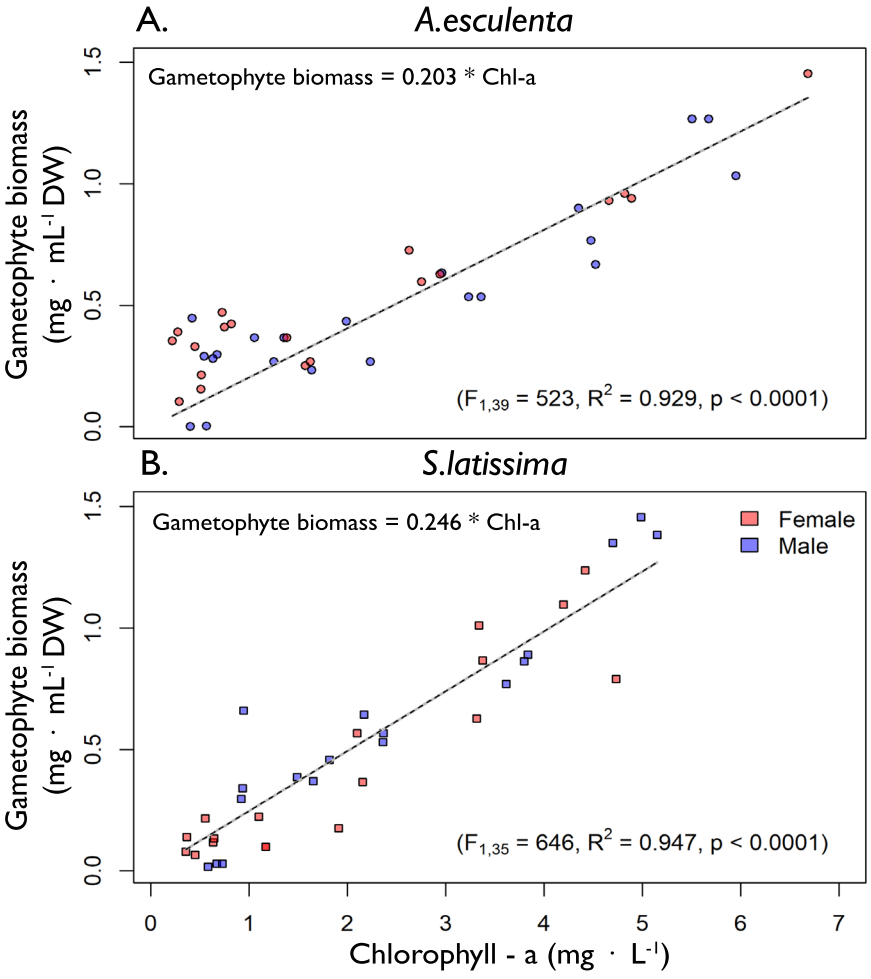


Figure 4-1 A and B. Chlorophyll a concentration (mg Chl · L^{-1}; x-axis) versus Initial Gametophyte Density (mg DW · mL^{-1}; y-axis) of *A. esculenta* (A) and *S. latissima (B)* and the corresponding calibration lines.

Gametophyte growth over time. The *A. esculenta* female culture (Independent samples T-test, t (4) = -16.7, p < 0.05), *A. esculenta* male cultures (t (4) = 6.5, p < 0.05), and female *S. latissima* cultures (t(4) = -3.5 , p < 0.05) all grew significantly in biomass (Table. S4-1). The male *S. latissima* cultures did not show significant increases in biomass (t(4) = -1.5 , p > 0.05). Mean male *A. esculenta* biomass increased by 322% and mean female biomass with 533% above the initial biomass, over a period of 21 days. The biomass increase of female *S. latissima* cultures was 165% above the initial biomass, while the male *S. latissima* cultures grew the least of all cultures, with an average biomass increase of 68% above the initial biomass.

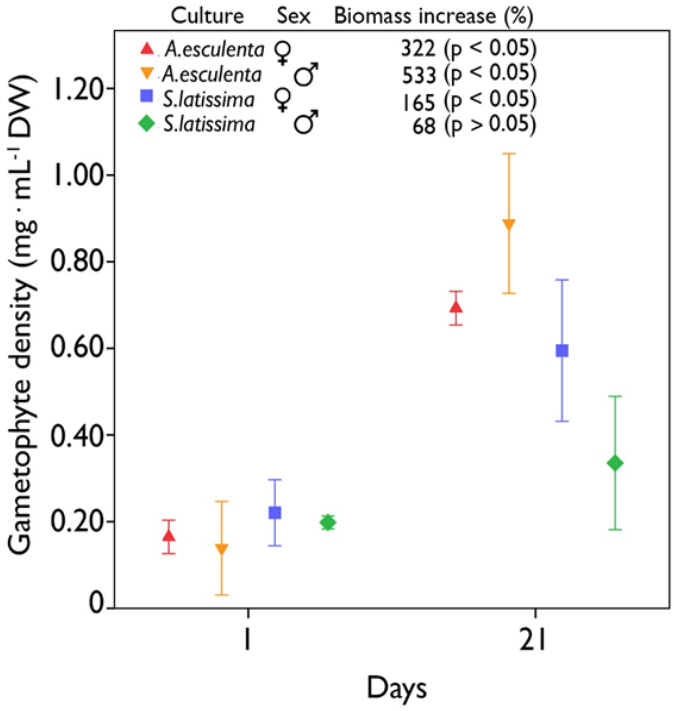


Figure 4-2. Gametophyte densities of a pool of six clonal male and female cultures of *S. latissima* and *A. esculenta* (mg · mL^{-1}DW) on day 1 and on day 21. Culture symbols, sex, and biomass increase (%) are depicted in the figure. Values are expressed as mean ± SD, n = 3

The effect of sex ratio on gametophyte reproduction of S. latissima. The male:female sex ratio had a significant effect on the reproductive success (sporophytes · mL^{-1}) of *S. latissima* gametophytes (WELCH ANOVA, $F_{4,55}$ = 7.6, $p < 0.05$; Table. S4-2). The female gametopyte density gradient showed an optimum in reproductive success (Fig. 4-3B), with optimal reproduction of 841 sporophytes · mL^{-1} observed at a female gameophyte densitiy of 0.013 mg · mL^{-1}. After this optimum the reproductive success rates declined continously to a minimum of 3 sporophytes · mL^{-1} at a female gametophyte density of 0.535 mg · mL^{-1}. Note that the positive trend between male biomass and reproductive success followed the male:female sex ratio rather than the male biomass alone (Fig. 4-3C). Overall, the lower the male:female ratio was, the lower the reproductive success (Fig. 4-3A), with a male:female ratio of 5:95 resulting in the overall lowest reproductive success rate that was observed.

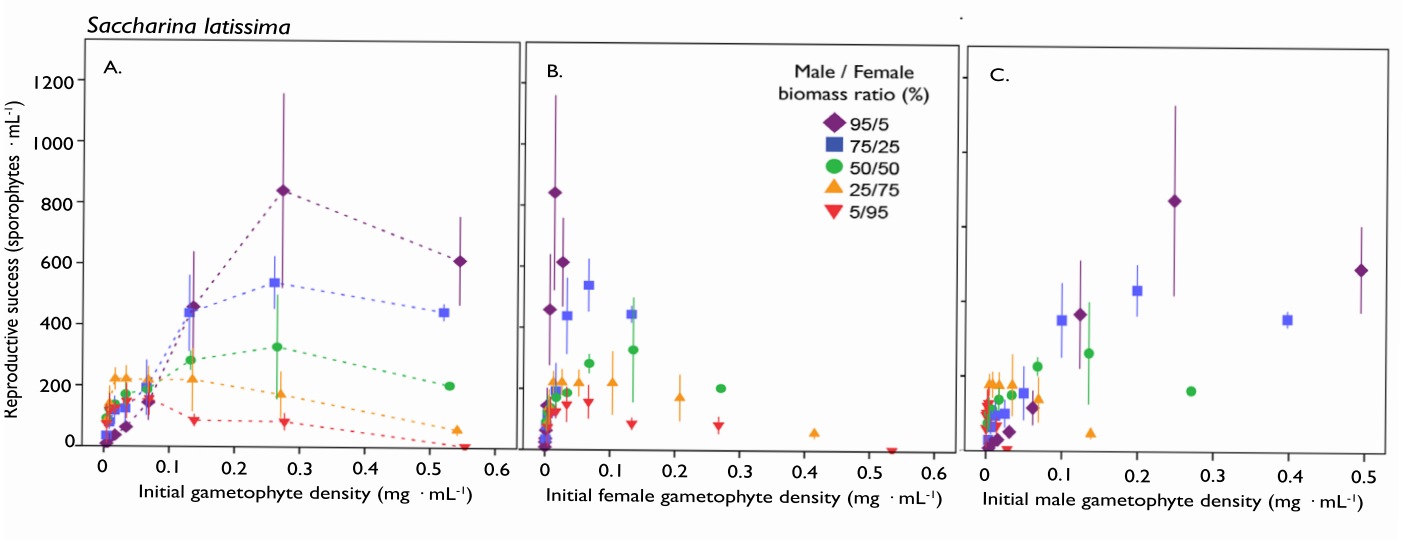


Figure 4-3 A-C. The initial gametophyte density of both sexes (x-axis$_a$; mg · mL^{-1}), the female gametophyte density (x-axis$_b$; mg · mL^{-1}) , and the male gametophyte density (x-axis$_c$; mg · mL^{-1}) versus the reproductive success (y-axis; sporophytes · mL^{-1}) of *S. latissima* gametophytes. The male:female ratios are described in the figure and symbols of these gradients are included in all three figures to allow for comparisons. The (*) is added to visually elaborate how the female gametophyte density (B) and male gametophyte density (C) are added up to form gametophyte density (A). Linear interpolation lines are added in A. for added readability. Values are expressed as mean ± SD, n = 3.

The effect of sex ratio on gametophyte reproduction of A. esculenta. The male:female sex ratio had a significant effect on the reproductive success (sporophytes · mL^{-1}) of *A. esculenta* gametophytes (WELCH ANOVA, $F_{4,57}$ = 7.5, $p < 0.05$; Table. S4-3). The reproductive success rates increased with decreasing male:female ratio's (Fig. 4-4A), with a reproductive optimum at a male:female ratio of 25:75 with a reproductive success rate of 856 sporophytes · mL^{-1}, after which reproduction showed a downward trend. Looking only at the female gametophyte density, the reproductive optimum was observed at 0.025 mg · mL^{-1} (Fig. 4-4B). After this optimum the reproducive success rates declined continuously to a minimum of 49 sporophtyes · mL^{-1} at a female gameotphyte density of 0.42 mg · mL^{-1}, independent of male gametophyte biomass (Fig. 4-4C).

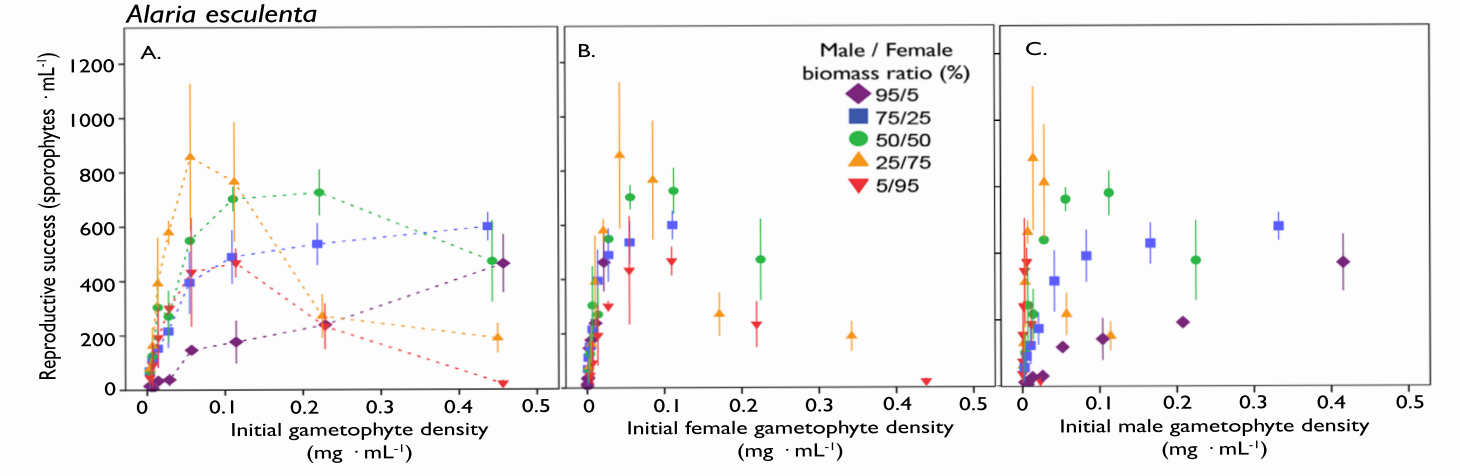


Figure 4-4 A-C. The influence of the gametophyte density of both sexes (x-axis$_a$; mg · mL^{-1}) and the initial female gametophyte density (x-axis$_b$; mg · mL^{-1}) , and the initial male gametophyte density (x-axis$_c$; mg · mL^{-1}) on the reproductive success (y-axis; sporophytes · mL^{-1}) of *A. esculenta* gametophytes. The male:female ratios are described in the figure and symbols of these gradients are depicted in all three figures to allow for comparisons. The (*) is added to visually elaborate how the female gametophyte density (B) and male gametophyte density (C) are added up to form gametophyte density (A). Values are expressed as mean ± SD, n = 3

The effects of female gametophyte density on the reproductive success (sporophytes · mL^{-1}) of both *S. latissima* and *A. esculenta* are grouped in Figure 4-5. A second order polynomial was fitted though the datapoints ($F_{2,234}$ = 141.9, R^2 = 0.54, n = 285, p < 0.0001) to predict the amount of sporophytes · mL^{-1}, independent of species, male biomass, or sex-ratio, that can be formed, depending on the female gametophyte density (mg · mL^{-1}), independent of the species considered. Peak reproductive success was calculated to be at 0.019 mg · mL^{-1} female gametophyte DW.

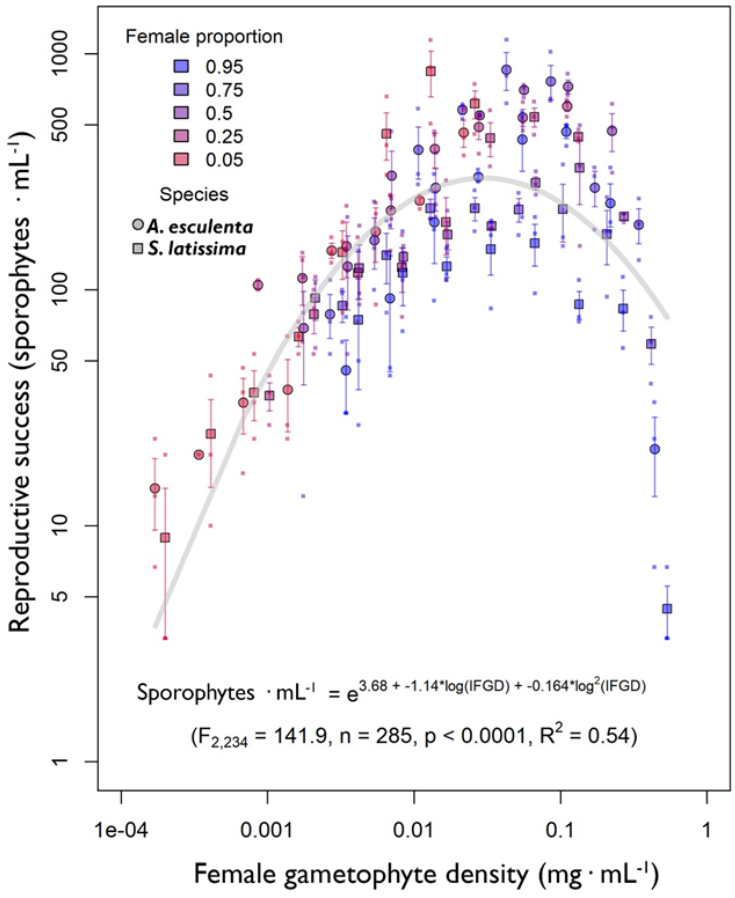


Figure 4-5.The female gametophyte density (x-axis, mg · mL^{-1}) of *S. latissima* (□) and *A. esculenta* (○) versus the reproductive success (y-axis; sporophytes · mL^{-1}) under different female gametophyte proportions. Both axis are log-log transformed and values are expressed as mean ± sd, n = 3. The raw values are also displayed using smaller coloured points.

Discussion

Our study into sex-ratio specific reproduction in multiannual delayed (MAD) *S. latissima* and *A. esculenta* gametophyte cultures, showed that in-culture selection can potentially take place due to different growth rates between the sexes. The calibration lines in figure 4-1 made it possible to visually compare sex specific gametophyte growth rates, by showing that the conversion between Chl-a and dry weight gametophyte biomass was indistinguishable between the sexes ($R^2 > 0.92$). Figure 4-2 strongly suggests that different growth rates between the sexes takes place, which is subsequently backed by other studies with more thorough datasets for *Laminaria digitata* (Destombe and Oppliger 2011). Note that the limited amount of replicates and short time span of only 21 days exemplifies the fact that the experiment initially was not set up to search for different growth rates between the sexes. In a separate experiment we showed that skewed sex ratio's, hypothetically resulting from the faster growth of a particular sex, could affect the reproductive yield of the cultures over time. Skewed male:female ratios turn out to be important biotic factors for the reproductive yield of MAD gametophyte cultures. In particular the amount of female gametophyte biomass appeared to be instrumental in the reproductive success rates obtained in both *S. latissima* and *A. esculenta* cultures. Clear reproductive optima were visible in both species, with optimal reproductive success at female gametophyte densities of 0.013 mg · mL^{-1} (*S. latissima*) and 0.025 mg · mL^{-1} (*A. esculenta*) respectively, independent of the amount of male biomass that is added. Increasing female biomass beyond these optima resulted in declining reproductive yields. This inverse correlation between gametophyte reproduction and culture density agrees with previous studies that used either zoospores (Reed 1990; Choi et al. 2005) or wildtype gametophyte cultures (Carney and Edwards 2010). Lastly, plotting female gametophyte density of both *S. latissima* and *A. esculenta* together lead to similarities between the two species ($R^2 = 0.54$; Fig. 4-5). These interspecific similarities between the reproductive success rate of a culture and female gametophyte density point towards a common driver influencing sex-ratio dependent reproduction within the family of the *Laminariaceae*.

What the driving force is behind the correlation between culture

density, in particular female gametophyte density, and the observed reproductive yields remains unclear. Putative density-associated nutrient deficiency is sometimes offered as an explanation [Bartsch et al. 2018; Reed 1991). However, Ebbing et al. 2020 showed that the interaction between gametophyte density and reproduction was not due to nutrient availability, but could also directly be correlated with the density of the culture itself. The logical next step was to assess whether male or female gametophyte biomass was instrumental to this apparent correlation. For illustration, Zhang et al. (2008) experimented with different ratios of delayed male and female *S. japonica* gametophytes, and had similar results as described in our study. They found decreasing reproductive success rates with increased female proportions, which was attributed to the decreased availability of male gametophytes in the culture. Decreased availability of male gametophyte biomass does, however, not explain our findings, since decreasing male biomass did not directly translate to decreased reproductive success, something that was especially apparent in *A. esculenta* cultures (Fig 4-4C). What is clear from both species is that the reproductive optimum was strongly female density-dependent, since optimal reproductive yields always hoovered around the same female densities, independent of male addition (Fig. 4-3B & Fig. 4-4B). The influence of female biomass is especially apparent in Fig. 4-3C, where the increase in *S. latissima* reproductive yields primarily follow the sex ratio that was used and not the absolute contribution of male gametophyte biomass. Although the reproductive trends are similar in both species, as is shown in Figure 4-5, the reproductive success rates are not completely interchangeable between the species. For example, the optimal female density in *A. esculenta* (0.025 mg $\cdot$ mL^{-1}) and *S. latissima* (0.013 mg $\cdot$ mL^{-1}) might seem similar at first glance, but the optimal density for *A. esculenta* is still almost double that of *S. latissima*. Another apparent difference between the species is the specific sex-ratio where optimal reproduction was achieved, with an optimal male:female ratio of 95:5 in *S. latissima*, while in *A. esculenta* the optimal male:female ratio was at 25:75. What the driving force is for these differences is not clear, but can be species specific, culture specific, or by factors that are not yet understood. The interspecific differences can also lie in characteristics that were simply not quantified during this experiment, like sex specific gametophyte cell counts,

the total egg production, sperm production, sperm activity, or gametophyte health.

The overlying similarity between the two species appears to revolve around how female biomass density is affecting the reproductive yield (Fig. 4-5). Whether the observed density dependent reproduction is affected by physical obstruction of the female gametophytes (*e.g.* obstructing sperm movement) or because of other (a)biotic factors (*e.g.* hormones) is not yet known. It is clear that more research is needed on the life cycle controls affecting delayed gametophytes, as it is becoming clear that delayed gametophytes behave differently than their non-delayed counterparts (Carney and Edwards 2010). These artificial culture environments allow for biotic factors to become important novel life cycle controls (Ebbing et al. 2021a). Our current study shows that female gametophyte biomass plays a central role in the observed inverse correlation between culture density and gametophyte reproduction, and that different growth rates between the sexes can change the contribution of female gametophytes over time. The fact that the sexes can grow at different rates (Destombe and Oppliger 2011; Ebbing et al. 2021b), thereby changing the sex ratios of a culture over time, altering the reproductive yields of cultures, has major ramifications for seaweed aquaculture. Nonetheless more studies are needed, especially those focussing on wildtype cultures, since the 6 strains of clonal gametophytes per gender that are used here may not represent the general output of the species.

Understanding the phenomenon of in-culture selection is essential in aquaculture of kelp, especially for farmers that aim to use the controlled reproductive method as their primary cultivation method (Ebbing et al., 2021a), since this method primarily uses MAD gametophytes as seed stock for their farms (Zhang et al., 2008). These farmers are dependent on large quantities of healthy sporophytes that can reliably be produced from their own gametophyte stock cultures (Redmond et al. 2014). Unreliable and especially underperforming gametophyte cultures can turn out to be damaging to a seaweed farmer, since lower reproductive success rates automatically translate to lower sporophyte counts to sow, adding unwanted insecurities during an already hectic planting season. A solution to this

problem is to grow out male and female gametophyte cultures separately, since it opens up the possibility to directly control the sex ratio prior to the induction of gametophyte reproduction, preventing unwanted in-culture selection, and opening up new ways to optimize the reproductive yields of MAD gametophyte cultures. Sex separation can be performed practically, by growing out male and female gametophyte lines within separate bioreactors (Ebbing et al. 2021b). More research is needed to understand how MAD gametophyte cultures change through time, and how the impact of this translates towards large scale seaweed industry.

Supplementary data

Tabel S4-1. Independent Samples T-test to test the difference between the biomasses on day 1 compared to the biomasses on day 21 for *S.latissima* and *A. esculenta* male and female gametophyte cultures.

Independent Sample Test

		t-test for Equality of Means						
							95% Confidence Interval of the Difference	
		t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
S.latissima Female biomass (mg · ml-1 DW)	Equal variances assumed	-3.598	4	**.023**	-.3744	.1040	-.6632	-.0855
	Equal variances not assumed	-3.598	2.832	**.040**	-.3744	.1040	-.7168	-.0319
S.latissima male biomass (mg · ml-1 DW)	Equal variances assumed	-1.536	4	**.199**	-.13715	.08931	-.38510	.11081
	Equal variances not assumed	-1.536	2.039	**.262**	-.13715	.08931	-.51449	.24020
A.esculenta female biomass (mg · ml-1 DW)	Equal variances assumed	-16.722	4	**.000**	-.52778	.03156	-.61541	-.44015
	Equal variances not assumed	-16.722	4.000	**.000**	-.52778	.03156	-.61542	-.44015
A.esculenta male biomass (mg · ml-1 DW)	Equal variances assumed	-6.513	4	**.003**	-.79271	.12172	-1.13065	-.45476
	Equal variances not assumed	-6.513	3.886	**.003**	-.79271	.12172	-1.13458	-.45083

*differences are significant at 0.05

Table S4-2. Robust test of variance for the effects of sex ratio on the reproductive success of *S.latissima* gametophytes (Fig. 4. 3; Welch ANOVA), after not passing the test of homogeneity of variances.

Robust Tests of Equality of Means

Reproductive success (sporophytes · ml-1)

	Statistic[a]	df1	df2	Sig.
Welch	7.601	4	55.047	.000

a. Asymptotically F distributed.

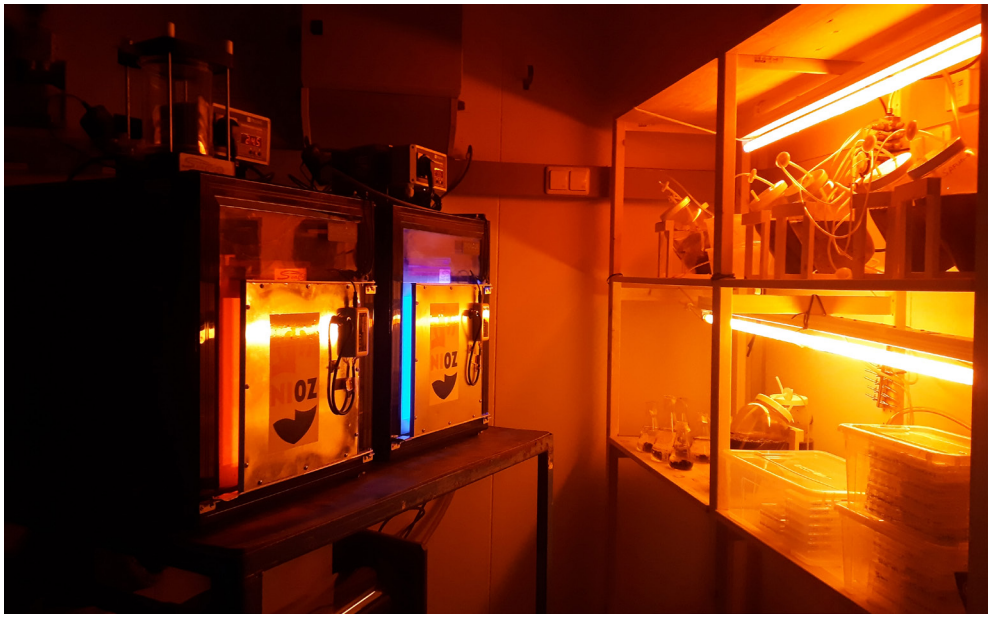
Table S4-3. Robust test of variance for the effects of sex ratio on the reproductive success of *A.esculenta* gametophytes (Fig. 4. 3; Welch ANOVA), after not passing the test of homogeneity of variances.

Robust Tests of Equality of Means

Reproductive success (sporophytes · ml-1)

	Statistic[a]	df1	df2	Sig.
Welch	7.539	4	56.785	.000

a. Asymptotically F distributed.



The SeaCoRe system (left) as alternative to the more conventional way of maintaining multiannual delayed gametophytes (right)

Chapter 5

THE SEACORE SYSTEM FOR LARGE SCALE KELP AQUACUTURE: A PLUG-AND-PLAY, COMPATIBLE, OPEN-SOURCE SYSTEM FOR THE PROPAGATION AND TRANSPORT OF CLONAL GAMETOPHYTE CULTURES.

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1. Abstract

The future of large-scale kelp aquaculture is standing at a crossroad, with the diverging paths being characterized by two fundamentally different cultivation methods that differ on how well gametophyte reproduction can be controlled. The cultivation method that does not directly control gametophyte reproduction is more widely utilized at the moment, but interest in better controlling gametophyte reproduction is growing steadily. Here we validate a bioreactor system that overcomes a number of implementation challenges for this controlled reproductive method, expanding the possibility of clonal gametophyte cultivation outside of expensive laboratory settings. The main goals of this system include *i)* the maintenance of clean gametophyte clonal cultures in non-sterile environments over prolonged periods of time, *ii)* the production of large numbers of juvenile sporophytes, and *iii)* effective transportation of gametophytes and sporophytes. The 'SeaCoRe system', consists out of three parts that correspond to these three challenges: (1) clone-reactors, (2) a clone-inducer and (3) a transporter. The validation of the system showed that delayed *S. latissima* and *A. esculenta* gametophytes can grow reliably for 75 days in the clone-reactors. Initial gametophyte densities of 0.4 mg DW and 0.6 mg DW gametophtyes · mL^{-1} were optimal for *S. latissima* and *A. esculenta*, resulting in reproductive successes of 604 and 422 sporophytes · mL^{-1}, respectively. Lastly, gametophyte transport was simulated, with high reproductive success still achieved within 19 days in ~20 °C environments. The SeaCoRe system helps unlock the full potential of large scale kelp cultivation using multiannual delayed clonal gametophyte cultures.

2. Introduction

Kelp aquaculture is on the cusp of becoming a large-scale offshore industry on a global scale (Bak & Infante 2020). The main driving force behind this sudden increase in interest is the promise of kelp as a sustainable source for food (van der Burg, 2021), feed (Carrier et al. 2017), bio energy (Davies et al. 1990), and as scalable carbon sink for the mitigation of climate change (Chung et al. 2013). Standing on its path towards industrially scaled offshore kelp cultivation lies a choice on how kelp should be cultivated. To this day there is no clear consensus on which cultivation method is best suited for large scale kelp aquaculture. Kelp aquaculture is still being practiced using two fundamentally different cultivation methods (Kerrison et al. 2019; Goecke et al. 2020), which are categorized here as the controlled- and uncontrolled reproductive methods (Ebbing et al., 2021). The Uncontrolled Reproductive Method (URM), is characterized by releasing substantial amounts of zoospores in large tanks that are filled with twine rope. This method is considered to be the traditional method and is used in large parts of the world (Shan et al. 2013). The released zoospores first need to attach to the twine rope after which they develop into gametophytes. These newly formed gametophytes then sexually reproduce and form young sporophytes, all the while still being attached to these twine ropes (Su et al. 2017). URM is categorized as 'uncontrolled' since farmers cannot precisely steer the reproductive success (sporophytes · mL^{-1}), or the relative reproductive success (sporophytes · mg^{-1} gametophyte DW) of these kelp gametophytes. Farmers can therefore not predictively quantify and control the amount of sporophytes on their coils or farm ropes. The second method is called the Controlled Reproductive Method (CRM), as it offers a more direct control on gametophyte reproduction because of the *in vitro* environment where gametophyte growth and reproduction takes place (Zhang et al. 2008). Gametophyte reproduction can be induced in a controlled setting and the subsequent cultures with juvenile sporophytes can then be attached to twine strings using techniques like paintbrush-seeding (Hwang et al 2006; Redmond et al., 2014; Umanzor 2021), or direct seeding (Forbord, 2020). However, using CRM as a primary cultivation method is still considered risky, more labour-intensive than URM, and showing a higher detachment from the seed strings when applied (Xu et al. 2009). These characteristics explain

for a large part why URM is still common practice in most parts of the world (Goecke et al. 2020).

Interest in using CRM as primary cultivation method is growing in the kelp industry because *i)* gametophytes can be kept in cultures for prolonged periods of time (Carney 2011; Barrento et al. 2016; Wade et al. 2020), *ii)* gametophytes can be cryopreserved (Visch et al. 2019), *iii)* gametophyte reproduction still has room to be optimized (Choi et al. 2005; Ratcliff et al. 2017), and *iv)* the crossing of distantly related unialgal male and female gametophyte clone cultures may evoke intraspecific hybrid vigour for future F1 hybrid cultivars (Shan et al. 2016; Zhao et al. 2016). Especially breeding can benefit from using CRM since the breeding possibilities that URM cultivation offers are limited, with long term degeneration in blade quality and productivity observed in their cultivation practices (Shan et al. 2016). This degeneration is caused by the inbreeding of limited numbers of sorus-bearing individuals that are continuously used in URM for multiple generations (Liu et al. 2014). The resulting reduced genetic diversity and narrowed germplasm bank decreases the variety's adaptability to changing environmental conditions. This reduced adaptability is made only more relevant under climate change, endangering the future performance of kelp with important economic traits (Bi et al. 2011; Liu et al. 2012). However, breeding using CRM can only be done by also fully understanding its limitations and by mastering the growth and reproduction of larger volumes of delayed gametophyte cultures (Zhang et al. 2008). For now this is still an understudied subject that has proven difficult to master. This includes taking into account the different growth rates between the sexes (Destombe and Oppliger 2011) or age related changes in fertility, especially since multiannual delayed gametophytes have shown to survive and able to grow vegetatively for decades (Carney and Edwards. 2006). These potential limitations need more research and ultimately necessitates innovation in a bioreactor system that is specifically designed to maintain and propagate sex specific multiannual delayed clonal cultures to facilitate CRM based cultivation.

Several different bioreactors have been designed for kelp aquaculture, covering both the production of sporophytes (Sato et al. 2017) as well as gametophytes (Zhang et al. 2019). In the case of gametophytes several different bioreactor designs were developed that successfully propagated

gametophyte biomass (Rorrer et al. 1995; Gao et al. 2005; Chen and Qi 2008) . However, these available bioreactor designs did not take into account the fact that gametophytes can delay their reproduction (Carney and Edwards 2006) grow vegetatively for prolonged periods of time (Westermeier et al. 2006), even up to years (Barrento et al. 2016; Ebbing et al., 2021). Delayed gametophytes result into aggregated clumps of gametophyte biomass, making it impossible to evenly expose all gametophyte biomass to comparable doses of light and nutrients, potentially compromising their growth and health. Zhang et al. (2019) published a well-designed bioreactor in order to address this issue, with an integrated four blade impeller that maintains and homogenizes gametophyte cultures from within the same bioreactor. The logical next step is to expand the singular bioreactor that only maintains gametophyte biomass, into a system of bioreactors that is not only designed to successfully maintain, and homogenise clonal cultures, but also induce sexual reproduction, and ultimately even addresses culture transportation. Looking into culture transportation is necessary, as this often overlooked segment in kelp aquaculture becomes increasingly important in an ever more globalized effort to establish large scale kelp aquaculture. Transporting cultures can have a wide variety of possibilities. From the transportation of sporophytes between more centralized kelp propagation companies and farmers, to the transportation of gametophyte cultures to farmers that intend to propagate their own cultures. What is most important in the transport bioreactor is that it is sturdy enough to handle rough transports, and simple enough to successfully use without any prior knowledge of kelp cultivation. Ultimately this system of bioreactors should form a closed loop system, that allows for clean gametophyte culture propagation and transportation, without the need of expensive laboratories.

Here we report on the design of such a continuous bioreactor system, the '**Sea**weed **Co**ntinuous bio**Re**actor' system, or SeaCoRe system (Fig. 5-1), and report on experiments that validate the effectiveness of its three parts: the clone-reactor, the clone-inducer, and the transporter. The main novelty of the SeaCoRe bioreactor system is that it is specifically designed and tailored for the propagation of multiannual delayed clonal gametophyte cultures. Overall, the SeaCoRe system reached TRL-5, which is defined as the validation of the system in its industrially relevant environment. The validation

is accompanied with the blueprints and dimensions of the SeaCoRe system, quantitatively measuring the kelp propagation process, from gametophyte clonal cultures to juvenile sporophyte, as a basis to be used in CRM-based kelp aquaculture.

3. Methods

3.1: Gametophyte culturing using the SeaCoRe system

3.1.1 Gametophyte culture and maintenance. Ripe sori of *Saccharina latissima* from Leknesund, Norway (61.212994, 4.896644°E) and *Alaria esculenta* from Fuhreholmen, Norway (61.043292, 4.868790°E) individuals were collected in the autumn and winter months of 2016. The extraction of zoospores was done in the Netherlands, using a standard extraction protocol (protocol of Ebbing et al. 2020, similar to earlier methods described by Redmond 2014 and Charrier 2018). Over time the zoospores developed into wildtype gametophyte cultures, with "wildtype" being characterized by a high genetic diversity and the jointly presence of both sexes in the culture. Male and female gametophytes were isolated from these wildtype cultures to create clean clonal cultures where the sexes are separated and the clonal lines genetically identical. The clonal male and clonal female cultures used here, consisted out of a mix of 6 individually bulked up clonal cultures, to increase the genetic diversity of the clonal cultures and deliberately making them more similar to wildtype cultures. We used gametophytes that delayed their sexual reproduction by more than a year, thereby transgressing seasonally induced reproduction, which are categorized here as multiannual delayed (MAD) gametophytes. Both MAD wildtype and clonal cultures derive from the same extraction and were simultaneously used in this experiment to assess potential differences in photosynthetic efficiency and growth rates between the two types of MAD cultures. The medium of all cultures were refreshed once every *~60 days* with new nutrient rich f/2 medium (Guillard and Ryther 1962), and grew vegetatively for approx. 2.5 years prior to the start of this experiment. The gametophyte cultures were not reused between the three bioreactor validation experiments. All experiments started with pristine stock cultures, so that there was no chance that the results of one bioreactor validation could influence the results of the next.

3.1.2 The SeaCoRe system. The SeaCoRe system consists of 4 bioreactors; two clone-reactors, a clone-inducer, and a transporter, whose culture content is interchangeable in a closed loop system using Luer-lock valve connectors (Fig. 5-1). The clone-reactors were specifically designed to maintain either male or female MAD gametophyte clonal cultures because separately growing out the sexes is central to the novelty introduced with this design. The clone-inducer was designed for the subsequent remerging of the two sexes, consisting out of stackable interlockable petri-dishes or well plates. Under a gradient of possible gametophyte densities (Fig. 5-3) in combination with light and temperature conditions (11°C; 30 µmol photons· m^{-2} · s^{-1} of white light) these cultures initiate gametogenesis and produce young sporophytes. Finally there is the transporter, a bioreactor that is specifically designed for the transportation of either gametophyte clonal cultures coming from the clone-reactors, or embryonic sporophytes that come from the clone-inducer. All these bioreactors fit in customized incubators (Polar-CE202; Fig. 5-5) and make the system small enough to easily fit in the back of a pick-up truck. Both incubators were temperature controlled, using external temperature regulators (H tronic TS 1000) in combination with air pumps (Aquaforte V-10) and LED panels (PAN-RGBCCT). The LED panels (LS LED; RGB + CCT 30 *30cm 18W) emitted multiple colours of light (*e.g.* white, red, green, or blue light), whose spectral distributions (Fig. S5-1) were analysed using a modular multispectral radiometer (TriOs Ramses ARC, Germany; Heuermann et al. 1999).

3.1.3 *The Clone-reactor.* The Clone-reactor is a vertical tube shaped bioreactor that includes three fluid valves, a grinder, and an airflow valve. This bioreactor was specifically designed for the prolonged vegetative growth of gametophyte clones without the need for sterile work environments. Gametophyte (vegetative) growth, medium refreshment and gametophyte homogenization can all be done from within the clone-reactor. Homogenization was achieved using a grinder that functions in a similar way as a French coffee press does, where a sieve/filter separates the coffee grounds from the liquid. The grinder consists of 4 small sieves and a rubber band that circumvents the edge (Fig. 5-1), making the grinding of gametophyte biomass possible. The sieves are included for easier transfer of seawater past the grinder, otherwise the grinding becomes too hard and manually straining. The grinder has a second purpose, separating the homogenized culture, that is ready for the induction of gametophyte reproduction, from the unhomogenized culture that can remain in the clone reactor for further vegetative growth.

The grinder is an essential part in the design, since its position changes the function of the clone-reactor (Fig. 5-1 & Fig. S5-2). *The stationary position* is the position where gametophytes are allowed to grow vegetatively by placing the grinder outside the culture. The gametophyte culture remains in motion using a filtered airlift system that is connected to valve-4. *The refreshment position* is used for the refreshment of the cultures by placing the grinder just under valve-2. This way medium can be discharged using valve-2 and refilled with new medium using valve-1 without the loss of gametophyte biomass. *The grinding position* is used to homogenize gametophyte biomass by pushing the grinder to the bottom of the reactor in a pumping motion, so that gametophytes become fragmented by passing past the rubber band of the grinder (Fig. S5-3). Grinded material can then be discharged using valve 2 without the possibility of discharging unhomogenized material that is kept back by the grinder.

3.1.4 *The clone-inducer*. The clone-inducer consists out of stackable petri dishes, with a valve on each petri dish (Fig. 5-1). Male and female gametophyte biomass, coming from the clone-reactors can be transferred to and merged in the clone-inducer. The clone-inducer is modular in design

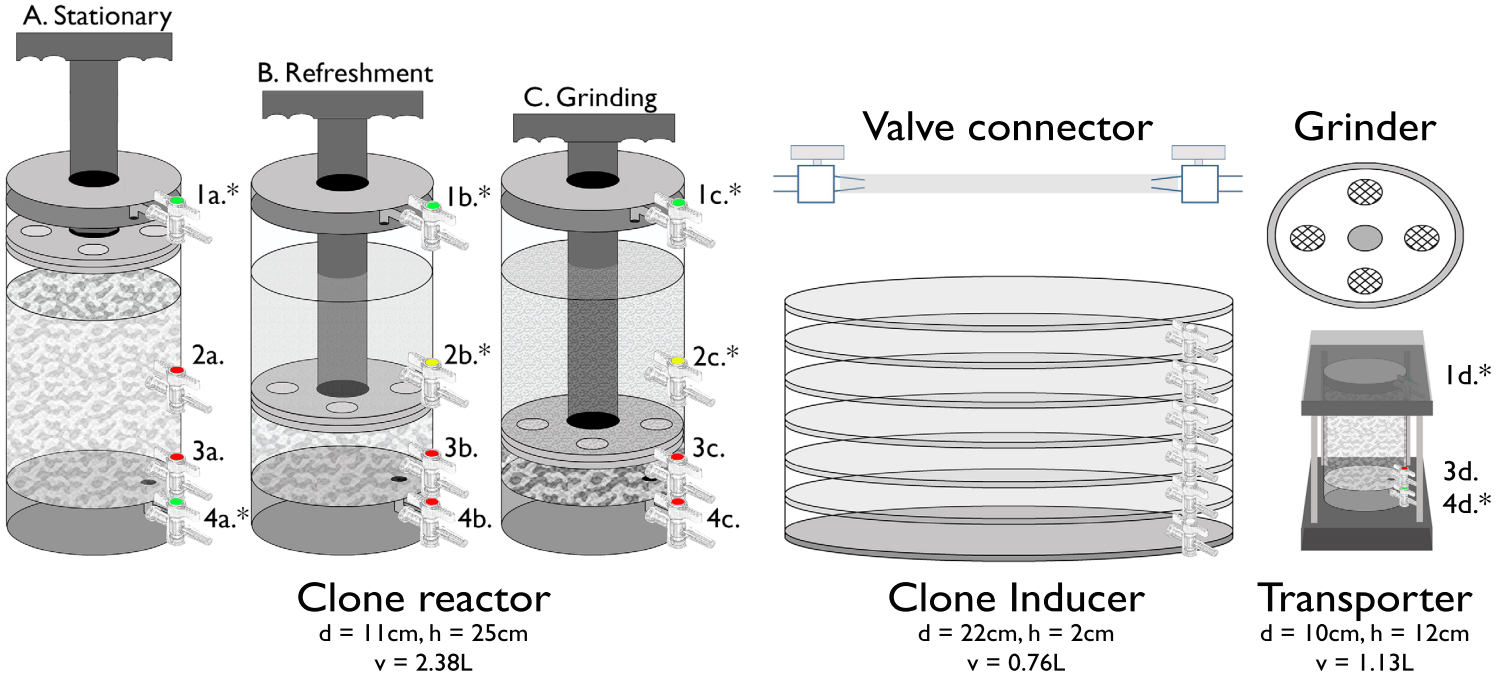

Figure 5-1. a schematic diagram of the SeaCoRe system, including the three positions in which the clone reactors can be used, a top view of the grinder, the stackable clone-inducer (6 well plates high), the transporter, and valve connector. Specifics on the diameter (d), height (h), and volume (v) of every bioreactor is included under the name. The numbered valves are used for: 1; air outflow and medium addition, 2; medium extraction and homogenized culture outflow, 3; unhomogenized culture outflow, and 4; air inflow. The valves can furthermore be opened (green *), functionally opened (yellow *), or closed (red). The stationary (a), refreshment (b), grinding (c) clone reactors all use their valves differently, with the transporter using the valves in a similar fashion as the stationary clone-reactor.

and can be stacked with as many plates as needed, by placing the large Petri dish like well plates on top of each other. The clone-inducer described here consists of 6 well plates uses well plates with a radius of 22 cm, resulting in a cumulative volume of 7600 mL. This is important to know for future extrapolations using data from the gametophyte reproduction experiment (Fig. 5-3). In order to make sure that all sides of the bioreactor receive similar quantities of light, a rotating disk can be included in the incubator, rotating the clone-inducer alongside the LED panel that is fitted outside of the incubator (Fig. 5-5). Temperatures for optimal reproductive success in *S. latissima* gametophytes that delayed their reproduction (Kinlan 2003), were previously reached using temperatures between 10 - 12°C, depending on the light intensity that was used (Ebbing et al. 2021). In this case we decided to use 30 µmol photons · m^{-2} · s^{-1} of white light in combination with 11°C, which resulted in near optimal reproductive success using *S. latissima* gametophytes (Lüning et al. 1980).

3.1.5.Transporter. The transporter is similar to the clone-reactor, utilizing two fluid valves, one airflow valve, but without a grinder, all encased in an exoskeleton (Fig. 5-1).This bioreactor is designed for the transportation of either embryonic sporophytes or gametophyte biomass. This bioreactor has an exoskeleton for sturdiness, protecting the cultures and the valves during transportation in a cooled polystyrene box. The exoskeleton is designed to tightly fit into the polystyrene box, and more importantly prevents any direct contact of the culture with the cooling elements inside the box, preventing unwanted excess cooling (Fig. S5-4).

3.2 Validation of SeaCoRe system

3.2.1 Clone-reactor - Gametophyte growth. The vegetative growth and photosynthetic efficiency of both wildtype and clonal cultures were assessed eight times within a timeframe of 75 days, in order to assess the suitability of the clone-reactor design for the maintenance of stock cultures. The cultures were grown vegetatively in the clone-reactors at 11°C, red light (5 µmol photons · m^{-2} · s^{-1}), using f/2 medium (Guillard and Ryther 1962). We used fluorometry measurements (Fast Ocean/Act2 FRRF, Chelsae Technologies Group Ltd) to estimate the Chl-a concentration [Chl-a], a proxy for phytoplankton biomass (Huot et al. 2007). These estimates were then

calibrated against freeze dried gametophyte biomasses of both *S. latissima* and *A. esculenta* wildtype and clonal cultures (Fig. S5-5). The maximum PS II photosynthetic efficiency (F_v/F_m), a proxy of cell viability in a dark-adapted state (Suggett et al. 2009), was furthermore measured using the same FRRF, whose maximum PSII photochemical efficiency typically decreases under stressful conditions, such as nutrient starvation or excessive light (Kolber et al. 1988; Suggest et el., 2009). We compared F_v/F_m changes from the starting value, as a sign of increased/decreased stress.

3.2.2 Clone-inducer - Reproductive success. In order to see at which initial gametophyte density the reproductive success (sporophytes · mL^{-1}) becomes optimal, a gametophyte biomass dilution gradient was used (~0.01- and ~1.5 mg · mL^{-1}), using a 1:1 mixture of male:female gametophyte clones. The used gradient of gametophyte densities was roughly based on the results from a previous study (Ebbing et al., 2020). The induction of gametophyte reproduction was done using 24-well plates (surface area = 1.9 cm^2), whose cylindrical design is identical to the clone-inducer and whose results can be extrapolated to the broader well-shape of the clone-inducer. The reproductive success was determined as the number of successfully formed young sporophytes (≥25 µm) per mL on day 21, not including oogonium formation, after initiation of the experiment.

3.2.3 *Transporter - Transport simulation.* Gametophyte culture transportation using the transporter was simulated by monitoring 28 vials containing either *S. latissima* or *A. esculenta* gametophyte cultures placed inside the transporter under transport conditions (Fig. S5-4). The transport conditions consisted of the placement of the bioreactor in a polystyrene box with additional cooling elements placed inside. The transporter was then filled with water so that the temperature between the 28 vials would follow the temperature of a regular culture transport. The temperature within a transporter was monitored during transport in the standard polystyrene box (L:W:H - 32:25:36cm). The photosynthetic efficiency (F_v/F_m) and reproductive success rate (sporophytes · mL^{-1}) of the gametophytes was monitored on a regular basis by taking out vials, in order to assess how long these cultures could be transported in between locations without compromising their reproductive success.

Statistical analysis. All statistical analysis was done using SPSS 20.0.0

statistical package (SPSS Inc. Chicago, USA). All data on the reproductive success (sporophytes · mL^{-1}) was normally distributed and analyzed for homogeneity using the Levene's test of variance. Factors that determined the reproductive success include the initial gametophyte density and transportation days of multiannual delayed gametophyte cultures. In case of unequal variances, a robust test of equality of means for unequal variances was applied (Welch t-test). A Games-Howell Nonparametric Post-Hoc comparison was subsequently applied to test for significant differences between the subgroups. If the data was found to be homogeneous, a one-way ANOVA was applied followed by the conservative Scheffe post-hoc test to determine which factor level was responsible for the specific treatment differences. All tests were run with a significance level of 0.05.

4. Results

4.1 *Vegetative growth in the clone reactors.* Our primary goal was to validate whether the clone reactors could be used for vegetative growth of gametophytes and to maintain stock cultures. The Chl-a concentrations increased in both wildtype cultures (*A. esculenta* & *S. latissima*) in a similar fashion, with overall biomass increasing 40% and 35% respectively (Fig. 5-2). The highest biomass increase was observed in the *S. latissima* female clonal culture with a biomass increase of 105% in 75 days. Two cultures showed no increase in their Chl-a concentrations and their growth of 0% are depicted in red. The absence of any vegetative growth corresponded with declining F_v/F_m values in both cultures.

Our secondary goal was to test the grinder and what the influence of the grinder was on the F_v/F_m of gametophyte cultures in the clone reactors. The multipurpose grinder worked as intended during culture refreshments as well as the homogenization of gametophyte biomass (Fig. S5-2 & Fig. S5-3). The grinding process also resulted in declining F_v/F_m values and lower Chl-a concentrations in most cultures. We decided to not homogenize the cultures for a prolonged period of time while still following the F_v/F_m and growth (grey background). Not using the grinder resulted in larger standard deviations, and at the end of the first month positive vegetative growth was visible again in all cultures apart from the *A. esculenta* female clone culture.

At the end of the first month culture grinding resumed, immediately resulting in lower F_v/F_m values in all cultures apart from the *S. latissima* wildtype. At the end of the experiment the cultures were refreshed on a weekly basis and grinded 4 times using the grinder of the clone reactors under non-sterile work conditions, during which no contamination was visible.

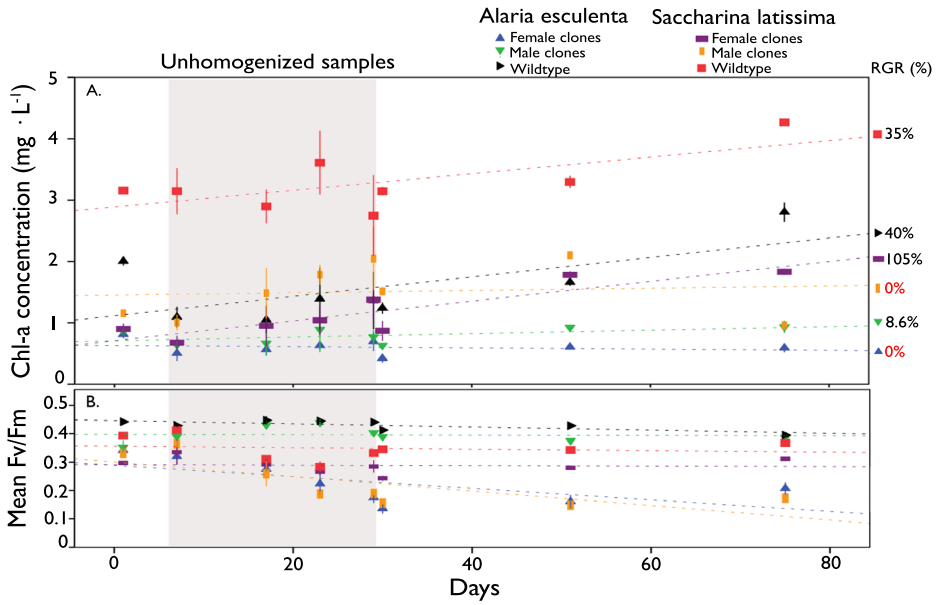

Figure 5-2; The Chl-a concentration (y-axis$_a$; mg · mL^{-1}) of MAD *S. latissima* and *A. esculenta* wildtype and clonal cultures followed over a period of 75 days (x-axis). The overall biomass increase at the end of the experiment is depicted as percentages (%) on the right side of the figure. The photosynthetic efficiency of the cultures was followed at the same time (y-axis$_b$; F_v/F_m), using the same cultures. All data is depicted as mean ± SD; n = 3

4.2 *Gametophyte reproduction.* Gametophytes initiated gametogenesis in all cultures with reproductive success in both *S. latissima* (Welch ANOVA $F_{7,6.4} = 13.3$, $p \leq 0.05$; Table. S5-1) as well as *A. esculenta* clonal cultures (Welch ANOVA $F_{7,\ 6.8} = 4.69$, $p \leq 0.05$; Table. S5-2) being significantly affected by the initial gametophyte density after 21 days (Fig. 5-3). In *S. latissima* cultures the optimal initial gametophyte density was observed at 0.46 mg gametophyte DW · mL^{-1} with 604 sporophytes · mL^{-1} (± 93 SD), while *A. esculenta* had no clear optimum with three similarly high initial gametophyte densities (0.3, 0.6, and 1.2 mg gametophyte DW · mL^{-1}),

with peak reproduction observed at an initial gametophyte density of 0.6 mg gametophyte DW · mL^{-1} with 422 sporophytes · mL^{-1} (± 148 SD).

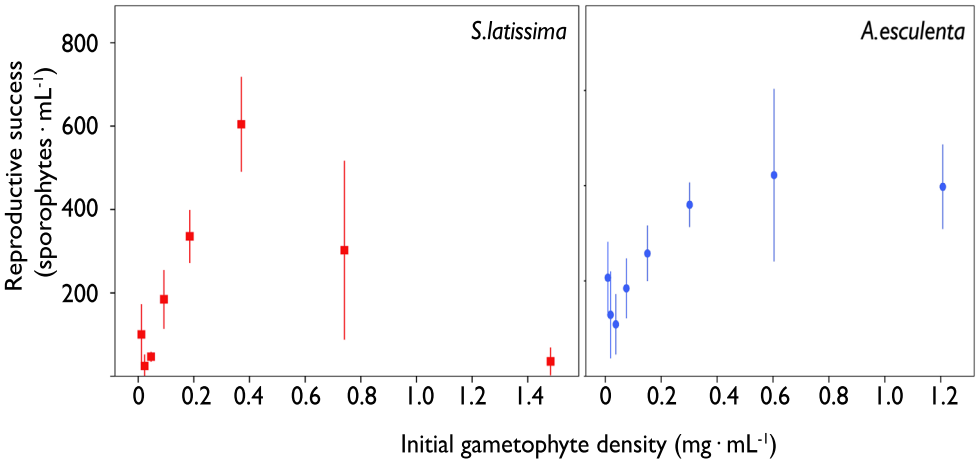

Figure 5-3; The influence of the initial gametophyte density (x-axis; mg DW · mL^{-1}) on the reproductive success of *S. latissima* and *A. esculenta* MAD gametophytes (y-axis; sporophytes · mL^{-1}), with the reproductive success (sporophytes · mL^{-1}) depicted as mean ±SD; n = 3

4.3 *Culture transport.* The transport simulation looked at culture temperature (°C ; Fig. 5-4A), F_v/F_m (Fig. 5-4B), and reproductive success (sporophytes · mL^{-1}; Fig. 5.4C), taking 31 days to complete (Fig. S5-7). Transport was successful in the first 19 to 23 days with no observed decline in F_v/F_m and reproductive success rates hoovering between ~200 to 400 sporophytes · mL^{-1}. The three cooling elements, included in the polystyrene boxes for short term cooled transport, reduced the temperatures in the first 2 to 3 days of transportation (highlighted in blue), with the lowest recorded temperatures being 5.5 °C (grey dashed line) and 4 °C (yellow pointed line) for respectively *S. latissima* and *A. esculenta*. Once, on day 20, the outside temperatures increased to more than 30 °C, thereby increasing the inside temperature of the cultures to around 25 °C (highlighted in orange). After this peak in temperature the reproduction declined significantly after day 27 in both *A. esculenta* (Welch ANOVA: $F_{13,26} = 552.9$, $p \leq 0.05$; Table. S5-3) and *S. latissima* (ANOVA: $F_{13,41} = 9.089$, $p \leq 0.05$; Table. S5-4). Lastly, strong fluctuations in reproductive success were observed in *S. latissima* cultures at the start of the experiment, coinciding with strong fluctuations in temperature of the same cultures.

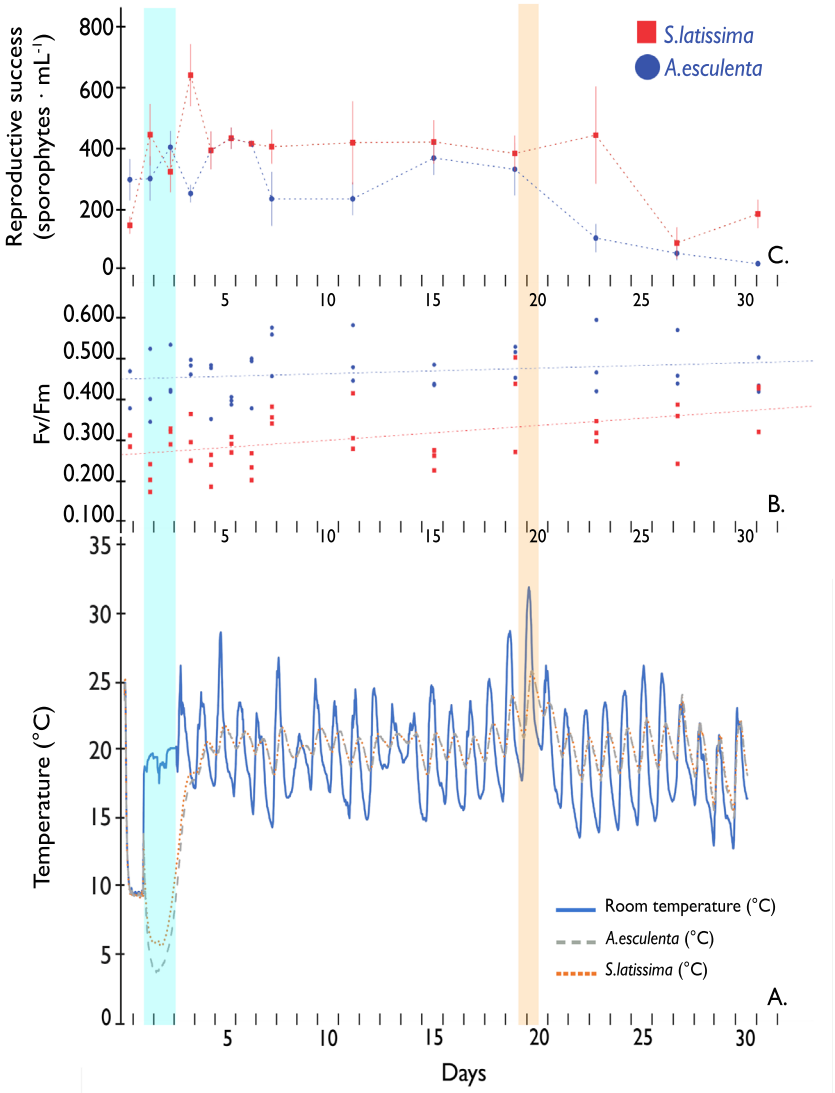

Figure 5-4 A-C. Transport simulation with the temperature, F_v/F_m, and reproductive success rates of *S. latissima* and *A. esculenta* MAD gametophytes followed through time (x-axis; days) with A: The temperature (y-axis[a]; °C) depicted as line graphs, B: F_v/F_m (y-axis[b]) depicted as scatter plots, and C: reproductive success (y-axis[c]; sporophytes · mL^{-1}) depicted as line graphs. The reproductive success was depicted as mean ± SD; n = 3. Blue and yellow bands are indicative for the lowest and highest temperatures that were measured.

5. Discussion

5.1 SeaCoRe-system validation. The fundamental elements of the system, including the vegetative growth, gametophyte reproduction, and culture transport were all successfully validated under relevant environments, thereby reaching technology readiness level – 5 (Tzinis 2021). The validation of the system can be subdivided into the functionality of the bioreactors and the performance of the gametophytes. The clone reactor performed as envisioned, with gametophyte cultures successfully being kept for 75 days. The cultures were refreshed on a weekly basis, and grinded frequently, using the three designed positions, under nonsterile environments (Fig. 5-1). The gametophytes also performed as envisioned, by slowly growing vegetatively, with the highest overall biomass increase observed in *S. latissima* female gametophyte cultures (105%). The clonal inducer was emulated successfully and gametophyte reproduction was successful under all initial gametophyte densities. Optimal initial gametophyte densities where reproduction was highest were observed at 0.4 and 0.6 mg gametophyte DW · mL^{-1} for *S. latissima* and *A. esculenta* respectively. The inverse correlation between initial gametophyte density and reproductive success is clearly visible in both species and agrees with previous well documented observations (Choi et al. 2005, Reed 1990; 1991, Ebbing et al. 2020). The simulated culture transport made clear that although these cultures cannot be transported indefinitely in closed polystyrene boxes, for the first 19 days of transportation it did not have any negative effect on the reproductive success of *S. latissima* and *A. esculenta* cultures (Fig. 5-4). That simulation searched for the limits of gametophyte culture transport, becoming an experiment that looked into the effects of high temperatures on gametophyte reproduction. This was especially informative considering that the overall ambient transport conditions of ~20 °C reached the upper limit of what these species normally can handle (Lee and Brinkhuis 1988; Fredersdorf et al. 2009). Regardless of the successful demonstration, there were also aspects of the system that still need closer examination. 1) The *S. latissima* male clonal culture and *A. esculenta* female clonal culture reacted strongly to the grinder, whose declining F_v/F_m directly coincided with the usage of the grinder. The grinder clearly functions the way it was meant to function, separating biomass when refreshing, and homogenizing biomass when the gametophyte clumps

grow too big (Fig. S5-2; Fig. S5-3). All other cultures recuperated well after grinding, raising the question whether the problem lies in the device or in the gametophyte cultures. Follow up experiments should both focus on the device and the health of gametophytes. On one hand studies are needed on whether grinders can be fit too tightly and thus become too damaging to gametophyte cultures. On the other hand emphasis should be placed on the recuperation period after grinding of gametophyte cultures, and whether resting periods without homogenization are needed in order to optimize the vegetative outgrowth. 2) The universal valve type used here (Luer lock) did not work optimally because of the small size of the valve and the bulky nature of gametophyte biomass. The universal interlockability of the valves make them ideal for opensource systems (Nie and Takeuchi 2020), however if gametophyte biomass cannot easily pass the valve, different interconnectable valves will be needed in following versions.

5.2 *Key characteristics.* The primary objective of the SeaCoRe project was to propagate kelp gametophyte cultures without the need of sterile work environments. An additional aim was using clonal cultures, whose culture characteristics complement our primary objective. Previous gametophyte bioreactor publications looked at single bioreactors, therefore needing wildtype cultures for the successful propagation of kelp sporophytes (Rorrer et al., 1995; Gao et al., 2005; Chen and Qi 2008). Utilizing sex specific clonal cultures is an essential element in our system, since clean starting cultures are an essential part of this system, which cannot be guaranteed by using wildtype gametophyte cultures. Clonal cultures generally start out cleaner from contaminations than their wildtype counterpart because there are reduced risks of microalgal, fungal, and bacterial infections. The main reason why they are cleaner is that clonal cultures start out from isolated gametophytes (Li et al. 1999), instead of mass extractions of zoospores. Moreover, separately growing out the two sexes also opens up unique opportunities like (1) adjustable growing conditions to compensate for the different growth rates between the sexes (Destombe and Oppliger 2011), (2) adjustable male/female ratios for optimal reproduction (Zhang et al. 2008), (3) adjustable genetic diversity through clonal inclusion or exclusion for breeding purposes (Martins et al. 2019), and (4) it paves the way to further optimize the vegetative growth of gametophytes (Zhang et al.

2019), without having to worry for any accidental sexual reproduction. This system is furthermore small enough to easy transport (Fig. 5-5), but at the same time scalable enough to meet the demands of an ever expanding kelp industry that has already more than doubled between 2000 and 2016 (FAO 2018). Lastly, the entire system, including all bioreactors and incubators, can be built for less than €2000 (Table. S5-2), making it accessible for small-scale seaweed farmers that do not have the resources for the normally expensive lab grade equipment needed for kelp cultivation (Wakamatsu and Miyata 2015).

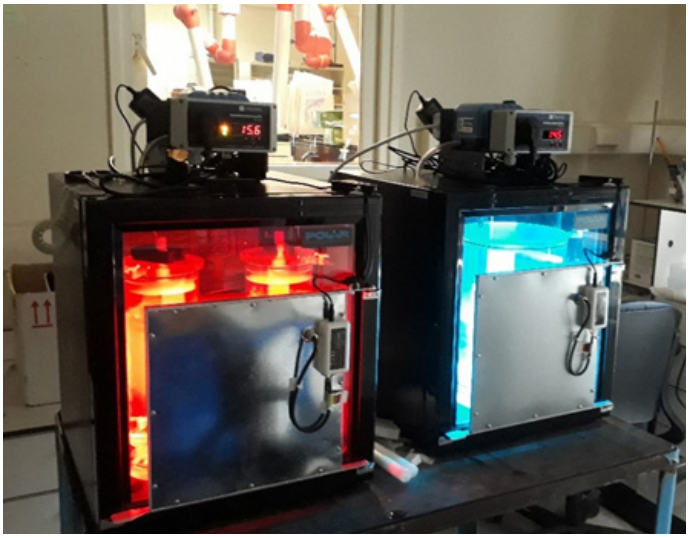

Figure 5-5; A demonstration of a fully functioning SeaCoRe system accompanied by transportable sized incubators.

5.3 *Future developments*. Although the essential parts of the SeaCoRe system were tested successfully, the system still has plenty of room for further improvement. In the end, only actually testing it on large scale farms will tell us whether the increased complexity of the SeaCoRe system can outperform the output of the more traditional propagation techniques. None the less, studies show there is still a lot of room for optimization. For example, the vegetative growth rates of the stock cultures, reached in the clone reactors, were intentionally achieved under stock-culture conditions, constituting of minimal light intensities of red light (5 µmol photons· m^{-2} · s^{-1}), conventional temperatures (11°C), and regular nutrient conditions (f/2 medium). Delving deeper in the different light, temperature and nutrient regimes, in search

for optimal growing conditions, are ripe for exploration. Evidence that the vegetative growth rates of these clonal cultures can be further optimized have already been shown by Rorrer et al., (1995) and Chen and Qi (2008). Zhang et al., (2019) even describes *S. japonica* gametophyte biomass growing up to 12.8 times the initial biomass in just 21 days, using higher light intensities (30 µmol photons · m^{-2} · s^{-1} of white light) and different nutrient conditions than described here. Adjustable airflows can help increase the vegetative growth of kelp gametophytes even more (Gao et al., 2005), although there are also indications that bubble-free bioreactors might be preferable due to the hydrodynamic stress coming from air bubbles (Chen and Qi 2008). Not only vegetative growth can be improved, gametophyte reproduction also has room to be further optimized (Ratcliff et al. 2017), with indications that different male:female ratios of a culture can result in altered reproductive success (Zhang et al., 2008). In order to further optimize we also need a better understanding of how regular stock culture conditions alters the photosynthetic efficiency of healthy dark adapted gametophytes (Fig. S5-6), so that our interpretation of what a healthy culture is, becomes more accurate. Quantifying the state of a gametophyte culture needs a broad spectrum of trackable parameters, with the F_v/F_m covering only part of it. Perhaps the biggest leap forward we can make is by extending this idea of system compatibility that is exemplified by this compatible system of bioreactors to the entire kelp production proces. For example, as of today it might not be economically sound for an individual kelp farmer to seed for itself, therefore always needing to co-op with more specialized organizations who maintain the gametophytes. A full-scale hatchery or direct seeding machine, that is plug and play compatible with the SeaCoRe system, is therefore something that needs to be incorporated in the future, to make sure a farmer can indeed function independently. The controlled reproductive method, as cultivation method, can be central to this process, since quantitatively being able to assess when, where, and how much gametophyte biomass is needed at your farm is key for the predictability and reliability needed for kelp farms that have the ambition to farm kelp on scales not seen before (Hooft and Slootweg 2021).

6. Acknowledgements

We would like to thank Hortimare B.V. for the usage of their wildtype and clonal cultures and for the work of Pieter Mulder and Jessica Schiller into the influence of light on Fv/Fm values of kelp gametophytes. We would also like to thank Matthias Schrama at Schrama-metaaltechniek for the construction of the bioreactor prototypes. The authors are also grateful for the help that the technical department of Royal NIOZ gave in the construction of the incubators. We furthermore want to thank Bas Oteman for his help in developing the SeaCoRe mobile app. Lastly, we would like to thank NWO for the Open-mind grant in 2017, making it possible for us to design, construct, and test the open source SeaCoRe system.

7. Supplementary data

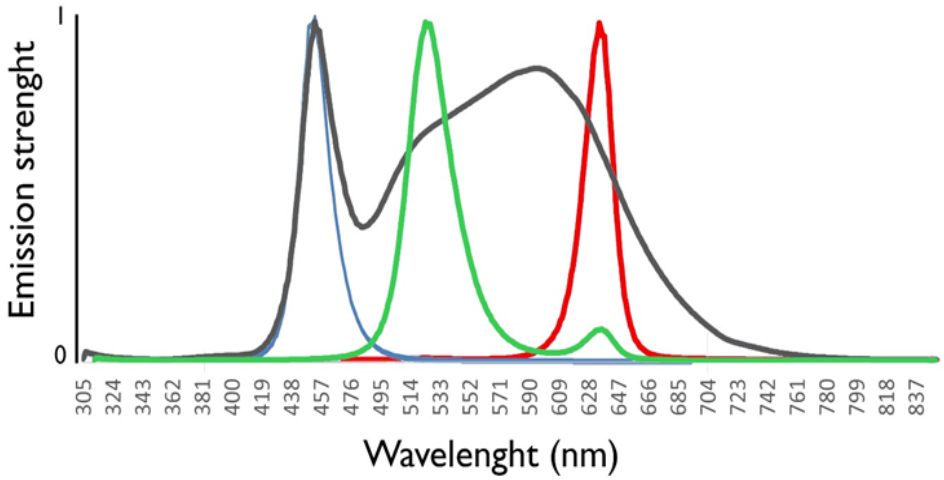

Figure S5-1. The spectral distribution of white (black line), blue (blue line), red (red line), and green (green line) L.E.D. light. Light was measured using a TriOs Ramses ARC at different wavelengths from 305nm until 840nm, with peak emission strength normalized to 1 for comparative reasons.

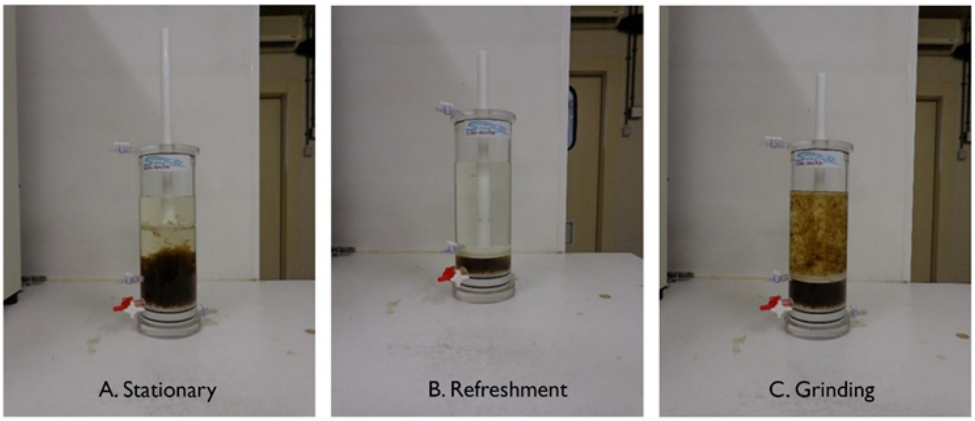

Figure S5-2. The three positions of the grinder in the clone-reactor. The stationary position is characterized by the grinder being placed above all gametophyte biomass. The refreshment position is characterized by the placement of the grinder under Valve 3 (See Fig. 1). The third position is characterised by the use of the grinder as a homogenizing tool for the gametophyte culture, by pumping the grinder until homogenized gametophytes start to pass the rubber band of the grinder.

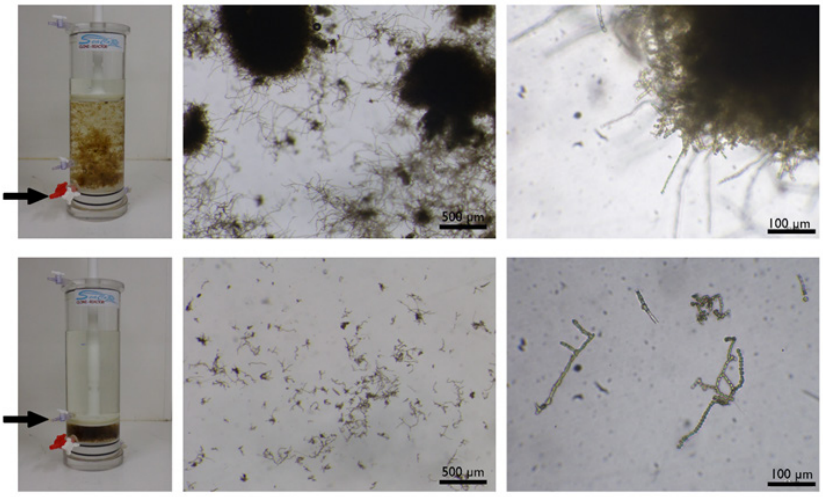

Figure S5-3. A microscopic representation of unhomogenised cultures above and homogenised cultures below. Culture samples for both representations are taken from the corresponding valves depicted on the left (black arrow).

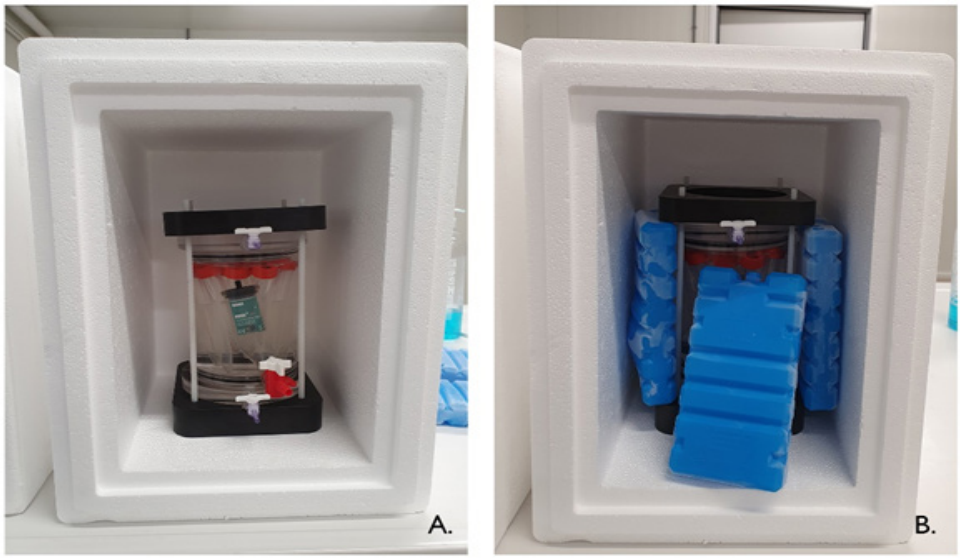

Figure S5-4. the experimental setup of the transport simulation, using a transporter bioreactor inside a tempex-box (L:B:H – 32:25:36cm). On the left (A.), a transport bioreactor is visible filled with the different gametophyte vials. On the right (B.) the cooling elements are included surrounding the transporter, keeping the cultures cool during the simulated transport experiment.

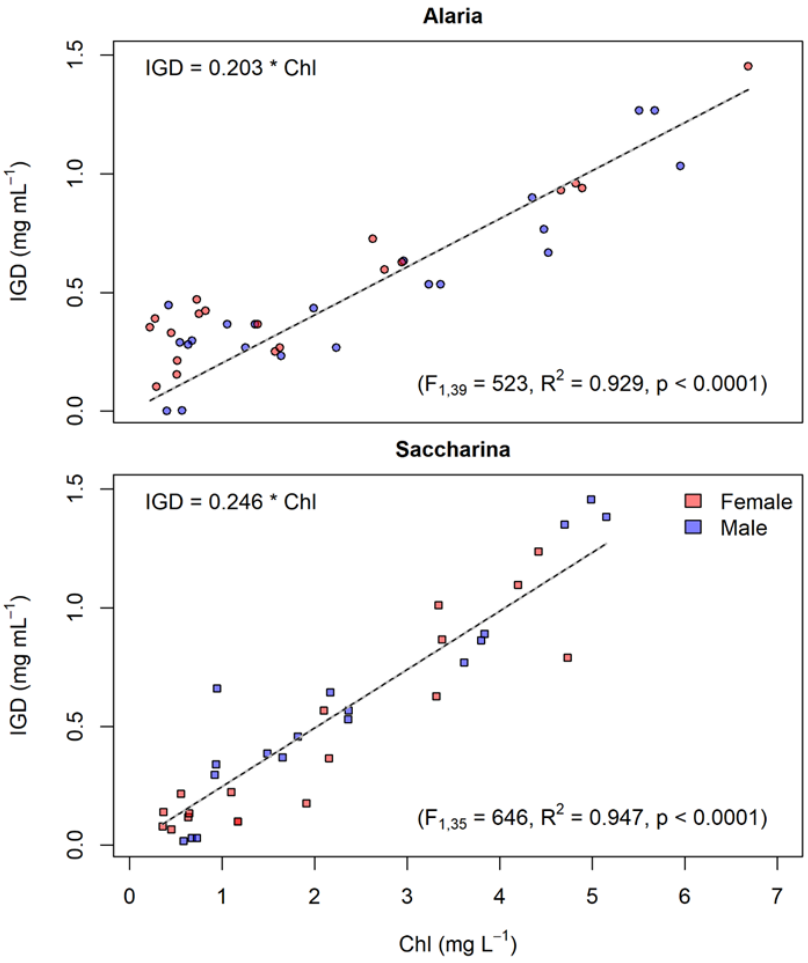

Figure S5-5. Calibration line between the chlorophyll - a concentration (mg Chl · L), and gametophyte dry weight per mL (mg DW · mL^{-1}) of *S.latissima* and *A.esculenta*. Gametophyte dry weights are extrapolations from 60mL cultures, whose [Chl] concentration were measured using a FRRF fluorometer.

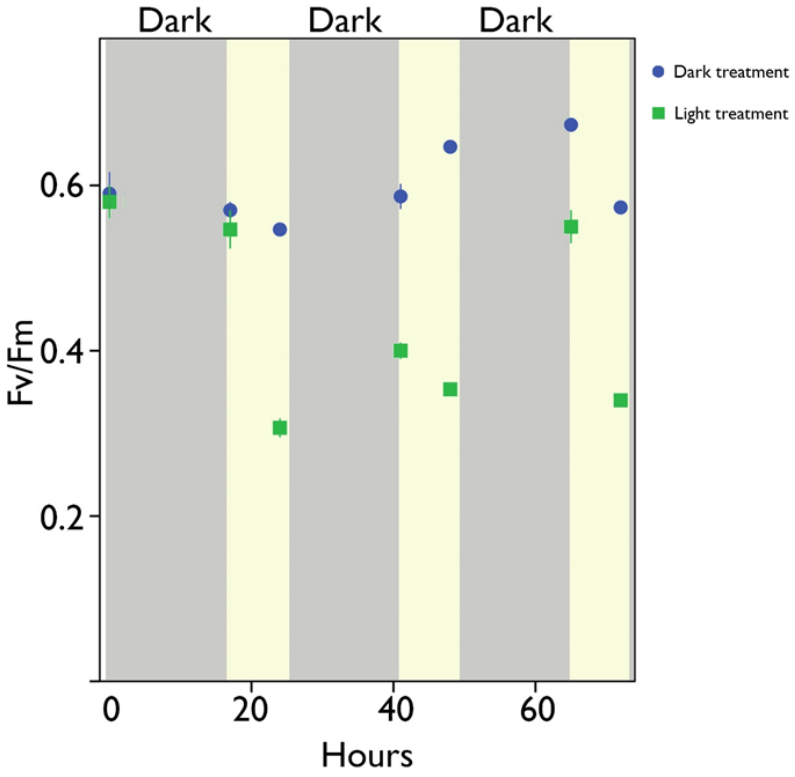

Figure S5-6. The Fv/Fm values of *S.latissima* multiannual delayed gametophytes (y-axis), through time (x-axis), under different light regimes (Legend), at a day/night cycle of 8:16. All cultures were dark adapted for minimally 25 minutes prior to measuring and the cultures receiving light treatment (squares) were measured at two specific moments each day. The first measurement was right at the start of the 8 hour long light period, while the second measurement was done at the end of this light period. Cultures that received a dark treatment (circles) were measured at the same time intervals, but were kept in the dark.

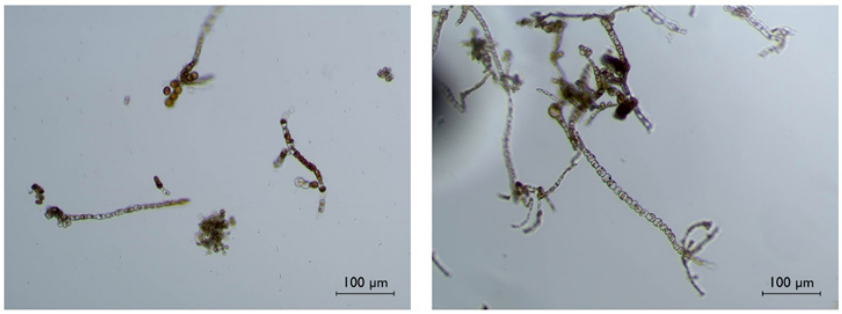

Figure S5-7. Photographs of *S.latissima* (left) and *A.esculenta* (right) gametophyte cultures after 31 days of transportation using the SeaCoRe transporter.

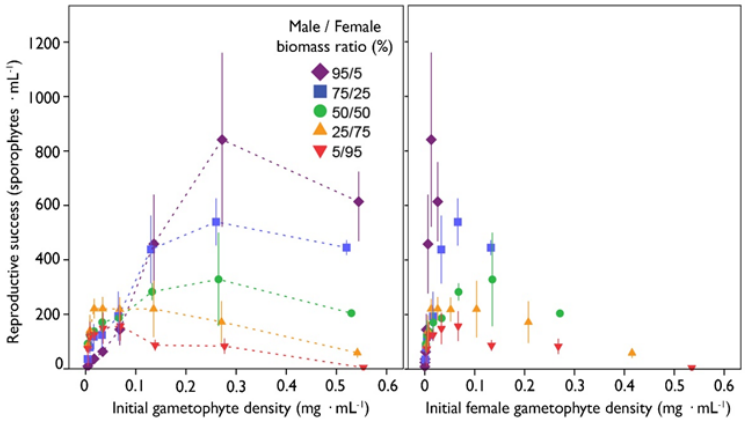

Figure S5-8. the reproductive success (y-axis) of *S.latissima* gametophyte clone cultures with varying male:female ratios (legend), along an initial gametophyte density gradient (x-axis$_1$; mg · mL^{-1}) and an initial female gametophyte density gradient (x-axis$_2$; mg · L^{-1})

Table S5-1. Robust test of variance for the effects of initial gametophyte density on the reproductive success of *A.esculenta* gametophytes, after not passing the test of homogeneity of variances.

Robust Tests of Equality of Means

Alaria reproductive succes (sporophytes * ml-1)

	Statistic[a]	df1	df2	Sig.
Welch	4.696	7	6.811	.031

a. Asymptotically F distributed.

Table S5-2. Robust test of variance for the effects of initial gametophyte density on the reproductive success of *S.latissima* gametophytes, after not passing the test of homogeneity of variances.

Robust Tests of Equality of Means

Saccharina reproductive succes (sporophytes * ml-1)

	Statistic[a]	df1	df2	Sig.
Welch	13.265	7	6.430	.002

a. Asymptotically F distributed.

Table S5-3. Games-Howell post hoc analysis for the influence of transport days on the reproductive success of gametophyte biomass (sporophytes * mL^{-1}) after we found significant differences using the Welch ANOVA. The mean difference is significant at $p < 0.05$.

Multiple Comparisons

Dependent Variable: Alaria Sporophytes * mL-1

					95% Confidence Interval	
(I) day (0)		Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Games howell	Day 1	-3.333	37.027	**1.000**	-161.28	154.62
	Day 2	-107.667	32.374	**.185**	-247.37	32.04
	Day 3	46.000	27.597	**.877**	-85.50	177.50
	Day 4	-97.000	34.322	**.336**	-243.60	49.60
	Day 5	-137.333*	28.562	**.040**	-269.00	-5.67
	Day 6	-119.333	25.639	**.082**	-254.68	16.02
	Day 7	63.667	41.579	**.929**	-116.25	243.58
	Day 11	63.667	32.378	**.758**	-76.05	203.39
	Day 15	-71.667	32.779	**.645**	-212.69	69.36
	Day 19	-34.333	40.812	**.999**	-210.37	141.70
	Day 23	193.667*	30.797	**.005**	58.38	328.95
	Day 27	244.333*	26.809	**.002**	112.08	376.59
	Day 31	278.333*	25.522	**.002**	142.51	414.16

* the mean difference is significant at the 0.05 level

Table S5-4. Scheffe post hoc analysis for the influence of transport days on the reproductive success of *S.latissima* gametophytes (sporophytes * mL^{-1}) after we found significant differences using ANOVA. The mean difference is significant at $p < 0.05$.

Multiple Comparisons

Dependent Variable: Saccharina Sporophyte *mL -1

					95% Confidence Interval	
(I) day (3)		Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Scheffe	Day 1	497.000	62.115	**.068**	-74.44	1068.44
	Day 2	197.000	84.008	**.595**	-309.41	703.41
	Day 3	319.667	71.908	**.155**	-151.58	790.92
	Day 4	249.000	70.007	**.281**	-227.04	725.04
	Day 5	208.667	63.210	**.377**	-338.05	755.39
	Day 6	226.667	59.995	**.333**	-412.08	865.42
	Day 7	237.000	68.391	**.307**	-246.94	720.94
	Day 11	223.667	99.580	**.636**	-404.49	851.82
	Day 15	221.000	72.954	**.383**	-249.31	691.31
	Day 19	259.000	68.850	**.254**	-222.28	740.28
	Day 23	198.667	110.966	**.811**	-542.34	939.67
	Day 27	555.667*	67.083	**.036**	62.10	1049.23
	Day 31	459.667	65.753	**.064**	-47.36	966.70

* the mean difference is significant at the 0.05 level

Table S5-5. Invoice of the building of SeaCoRe bioreactor prototypes by Schrama metaal techniek. The invoice is for the bioreactors only and the custom modification of the incubator is not included. Language of the invoice we kept Dutch for authenticity reasons.

schramametaaltechniek
vliegwiel 4
1792cs
oudeschild
texel, holland

SCHRAMA METAALTECHNIEK

FACTUURADRES

A.P.J.Ebbing PhD candidate
Department of Estuarine and Delta Systems
Royal Netherlands Institute for Sea Research
Korringaweg 7, 4401 NT Yerseke

factuurnummer:	2018113
crediteurnummer:	402044
project:	bioreactoren
datum :	17-12-2018

werk:	**aantal:**		
continu reactor	8	€	2.000,00
materiaal		€	1.145,20
totaal		€	3.145,20
btw 21%		€	660,49
totaal incl btw		**€**	**3.805,69**
draaien nieuwe deksel grote vat	1	€	100,00
materiaal		€	60,00
totaal		€	160,00
btw 21%		€	33,60
totaal incl btw		**€**	**193,60**
prototype grote reactor	1	€	400,00
materiaal		€	163,80
totaal		€	563,80
btw 21%		€	118,40
totaal incl btw		**€**	**682,20**
transport reactoren	2	€	700,00
materiaal		€	290,20
totaal		€	990,20
btw 21%		€	207,94
totaal incl btw		**€**	**1.198,14**
experimental reactors	3	€	266,70
materiaal		€	266,70
totaal		€	533,33
btw 21%		€	112,00
totaal incl btw		**€**	**645,33**
uitfrezen platen	4	€	50,00
totaal		€	50,00
btw 21%		€	10,50
totaal incl btw		€	60,50
transport kosten materialen divers		€	30,00
subtotaal ex 21% btw		€	5.472,53
Btw		€	1.149,23
totaal incl 21% Btw		**€**	**6.621,76**

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KVK 37140999 BTW 187266657BO1
schramametaaltechniek@hetnet.nl mob. 0031 06 16519360
betalingstermijn 14 dagen

The drying of kelp biomass on rock beds (Norway, Solund)

Chapter 6

SYNTHESIS

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Synthesis

The broader implications from the different published chapters of this thesis will be discussed here, starting with **(1)** the (a)biotic lifecycle controls of multiannual delayed (MAD) gametophytes of *S. latissima* and *A. esculenta*, **(2)** how the obtained results translate to the functioning of delayed gametophytes in their natural environment and **(3)** the practical implications of translating the fundamental scientific knowledge to the applicability for seaweed farmers. The synthesis will be finished on a **(4)** more philosophical note, by reflecting upon the potential of seaweed cultivation and weighing it in against the environmental implications of large scale seaweed aquaculture.

(1) The (a)biotic lifecycle controls of multiannual delayed gametophytes of S. latissima and A. esculenta (Laminariales).

Delayed gametophytes and in particular multiannual delayed gametophytes are an understudied subject (Carney and Edwards, 2006), while newly formed gametophytes have already been subject in studies for many decades (Harries et al., 1932). A presumed reason for this discrepancy is that delayed gametophytes and newly formed gametophytes are often thought of as one and the same thing. The assumption is that these gametophytes react to environmental conditions in similar ways, making it unnecessary and even time consuming to use delayed gametophyte cultures for fundamental research into the lifecycle of *Laminariales*. However, as demonstrated in Chapter 2, 3 & 4, assuming both types of gametophyte are one and the same thing greatly undervalues the differences that are clearly visible. First, their morphology is different, with newly formed gametophytes being single celled, while delayed gametophytes branch out by growing vegetatively (Fig. 6-1).

Second, the prolonged exposure to the artificial environments of delayed gametophytes also becomes a more and more significant element the longer they reside in these unnatural culture conditions. The artificial culture conditions change how delayed gametophytes react to lifecycle controls, especially compared to their newly formed counterparts (Carney and Edwards., 2010). Whether it is the constant low light intensities of stock

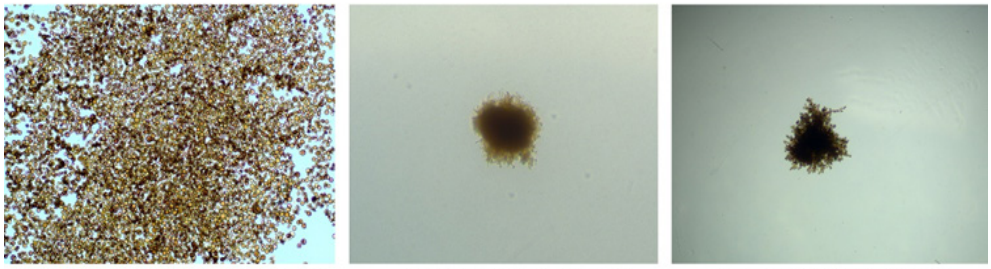

Figure 6-1. The different morphologies of newly formed gametophytes and delayed male and female gametophytes.

cultures (Chapter 2), the year-round stable temperatures of climate cabinets (Chapter 3), the unnaturally high culture densities due to prolonged periods of vegetative growth (Chapter 3 & 4), or the in-culture selection between the sexes because of different growth rates (Chapter 4). All factors had their own effects on how delayed gametophyte adapted to their artificial environment, and reacted to the (a)biotic factors that trigger the induction of sexual reproduction. Here we will summarize how delayed gametophytes react to changing (a)biotic factors (i.e. lifecycle controls) and how this resulted in varying degrees of success of sexual reproduction.

LIGHT

Light, temperature, culture density, and sex ratio all had optimal conditions for the induction of sexual reproduction in delayed gametophytes. In *S. latissima*, a clear optimum was observed for light intensity, with optimal photosynthetically usable radiations of between 14.2 µmol photons and 25.7 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}, independent of light quality (Fig. 2-3). The results obtained in Chapter 2 for new insights into a question that has been on the minds of many seaweed researchers in the last 5 decades*:*

> *Does light, as a lifecycle control, primarily function as a qualitative signal or as a quantitative signal for the reproduction of gametophytes?* The spectral composition of light is considered a major influencer of gametogenesis, with blue light acting as a major inducer of gametogenesis (Lüning and Dring 1972; Lüning and Dring 1975; Ratcliff et al. 2017). Chapter 2 shows that gametophyte reproduction was visible using all light qualities, with no tangible exception. Even using higher light intensities of red light resulted in high reproductive success rates and suggests

that not so much light quality but light intensity is an important lifecycle control. The combination of light intensity and light quality can be functionally integrated as the Photosynthetically Usable Radiation (PUR; Fig. 2-1), hence we concluded that light does not function as a qualitative signal, but functions as the quantitative interactive result of both light intensity and light quality, in the form of PUR, in gametophyte reproduction.

CULTURE DENSITY

Culture density also showed several optima when it came to the reproductive success of *S. latissima and A. esculenta* gametophytes. In Chapter 2 & 3 it is clear that the reproductive success in *S. latissima* was highest at a gametophyte density of between 0.01 and 0.02 mg · mL^{-1} DW. Chapter 4 & 5 also depict gradients of gametophyte biomass and here optimal reproduction was achieved at 0.28 – 0.39 mg · mL^{-1} DW (*S. latissima*) and 0.05 – 0.6 mg · mL^{-1} DW (*A. esculenta*). One possible big reason for the large discrepancies between optimal culture density and reproductive success are the different male:female ratios of the cultures, a biotic factor that greatly influences reproduction, and will be discussed further below. Nonetheless, the initial gametophyte density (IGD), the culture density at the start of gametophyte reproduction, turned out to be a strong determinant in all experiments done, and allowed us to delve deeper into the following question:

How does gametophyte culture density influence the reproduction of cultures that have grown vegetatively for prolonged periods of time? Reproduction decreased with increasing IGDs, after an initial optimum was reached, under all light intensities and light qualities in both *S. latissima* and *A. esculenta* cultures. These results are in agreement with data obtained by Choi et al. (2005) and Reed et al. (1990; 1991), where increasing spore densities of *Undaria pinnatifida* and *Macrocystis pyrifera* resulted in lower sporophyte counts. Nutrient addition had no significant influence on reproduction and the reproductive success (Fig. 2-4), suggesting that the decreased reproduction is not likely occurring via putative density-associated nutrient deficiency. However, thinking of culture density as a biotic lifecycle control is novel and the exact mode of action of the initial gametophyte density remains to be investigated. Whether the observed density dependent reproduction is controlled through physical obstruction, by pheromones, or is more similar to the

autoinducers found in quorum sensing bacterial communities, is not yet known. It might even be possible that density dependent reproduction is the result of interkingdom signaling between gametophytes and bacteria, a phenomenon already studied within diatom communities (Amin et al. 2012). Nonetheless, we can say that culture density has an inverse correlation, with declining reproductive success rates after an initial reproductive optimum is reached.

SEX RATIO

Before we can delve deeper into the optimal sex ratio for delayed gametophyte reproduction it is necessary to point out that there are fundamentally different cultures with whom we worked with. The first two chapters were done using so called 'wildtype' cultures of *S. latissima,* while the latter two chapters were done using 'clonal' cultures of *S. latissima* and *A. esculenta*. Wildtype cultures are genetically diverse cultures that are created directly from zoospore extractions, whose male:female ratio initially is presumed to be 50:50, but given time will change (Chapter 2 & 3). Alternatively clonal cultures can be used, whose sex ratio can be controlled through clonal inclusion/exclusion, resulting in the research of Chapter 4 & 5. Here we assessed, among others, the optimal sex ratio for sexual reproduction in both *S. latissima* and *A. esculenta*. The sex ratio was clearly important in both species, with the highest reproductive success rates observed in the cultures with higher male:female ratios (95:5 for *S .latissima* and 75:25 for *A. esculenta*). Our experiments show that especially female gametophyte densities affected the observed reproductive yields of the cultures the most, independent of the amount of male biomass that was included. *S. latissima* cultures showed a clear reproductive optimum (sporophytes · mL^{-1}) at 0.013 mg · mL^{-1} DW female gametophyte biomass, while the reproductive success of *A. esculenta* peaked at a density of 0.025 mg · mL^{-1} DW of female gametophyte biomass, after which the reproductive yield started to decline in both species (Fig. 4-3B & Fig. 4-4B). Zhang et al. (2008) also experimented with different ratios of male and female biomass and also observed decreasing reproductive rates with decreasing male:female ratios. They pointed out that this might be due to the decreased availability of male gametophyte biomass. However, that hypothesis does not explain our dataset since our results show that increased male biomass concentrations did not automatically translate to cultures with increased reproductive yields

(Fig. 4-3C & 4-4C). The strong influence of female biomass on reproductive yields, combined with the observation that male and female gametophytes can grow at different rates, resulted in us being able to answer the following question:

> *What is the influence of changing sex ratios on MAD gametophyte reproduction and what consequences does this have, over time, on aging MAD gametophyte cultures?* MAD gametophytes have minimally grown vegetatively for more than a year. During this period the selection towards individuals with higher growth rates causes changes to the frequencies of certain genotypes within the culture, like changing the sex-ratio of a culture. This phenomenon is called in-culture selection and has the potential to change a culture on a fundamental level. In-culture selection is already well-documented in microalgae cultures (Lakeman et al., 2009), but is a completely novel field in kelp gametophyte research. The data in Chapter 4 strongly suggests that in-culture selection has the potential to change the sex-ratio of a culture, given enough time to grow vegetatively (Fig. 4-2). In the case of faster growing female gametophytes it can have great implications for the 'shelf-life' of MAD gametophyte wildtype cultures, since a culture that becomes predominantly female will greatly be hampered in their reproductive fitness over time (Fig. 4-3 & 4-4), possible even resulting in sterile cultures the older the culture gets. In short we can say that the sex ratio of a culture can have significant effects on the reproductive fitness of a culture, and that changing sex ratios even have the potential to change into sterile MAD gametophyte cultures, given enough time.

TEMPERATURE

Although all the above described (a)biotic lifecycle controls had clear optima, in order to explain the influence of temperature as clearly as possible a small detour is needed into the interactive effects between (a) biotic lifecycle controls in MAD gametophyte reproduction. The interactive effects between different lifecycle controls on gametophyte reproduction have been increasingly of interest in recent studies (Martins et al., 2017), and here we also observed different interactive effects between the following (a) biotic lifecycle controls. How these (a)biotic factors precisely interacted can be read in the following Chapters:

- Light quality and light intensity (Chapter2)
- Light intensity and temperature (Chapter 3)
- Light intensity and culture density (Chapter 2 & Chapter 3)
- Sex ratio and culture density (Chapter 4)

The most interesting and substantial interaction that was observed was the interactive effect between light and temperature on the reproductive success of *S. latissima* gametophytes (Fig. 3-1), with high light intensities at low temperatures (<11.2°C) resulting in high reproductive yields, while high reproductive yields at low light intensities were only observed at higher temperatures (>12.6°C). These seemingly antagonistic effects were so stark that it prompted us to explore a novel hypothesis, while trying to answer the following question:

Are delayed gametophytes sensitive to temperature, and how does this interact with light intensity? Delayed gametophytes are clearly sensitive to temperature with the highest reproductive success of *S. latissima* observed at ~12°C (Fig. 3-1). Temperature strongly interacted with light intensity, having significant effects (ANCOVA, $F_{5,49} = 49$, $R^2 = 0.82$, $P \leq 0.001$; Table. S3-3) on the reproductive success of MAD *S. latissima* gametophytes (Fig. 3-3). The reproductive success of MAD *S. latissima* gametophytes in our experiment increased when the disparity between temperature and light intensity increased. Looking for a natural phenomenon that mirrors the increased disparity between temperatures and light gave us the seasonal lag hypothesis. The seasonal lag hypothesis is based on the yearly phenomenon of seasonally increasing and decreasing disparities between sea surface temperatures and solar radiation. Given the seasonal lag between solar radiation and sea surface temperature in natural systems (Fig. 3-4), the observed interactive conditions between temperature and light resemble those found during spring (i.e. increasing light intensity with low temperatures) and autumn (i.e. decreasing light intensity with higher temperatures). Multiannual delayed gametophytes appear to use the increasing disparity between solar radiation and sea surface temperature to reliably assess when spring and autumn starts, aligning their reproductive cycles with surrounding adult kelp that also might use this seasonal lag to initiate sporogenesis. So yes, delayed gametophytes are sensitive

to temperature and have shown a strong interactive effect with light when it comes to the induction of gametophyte reproduction.

AGE

All these lifecycle controls influenced the reproductive success of *S. latissima* and *A. esculenta* MAD gametophytes in different ways, showing both clear reproductive optima as well as clear interactive effects, acting both synergistically as well as antagonistically. However, when it comes to lifecycle controls it is important to note that understanding how to halt gametophyte reproduction is as important for our understanding of the *Laminariales* as the lifecycle controls to induce gametophyte reproduction. It is not only better to understand the lifecycle of the *Laminariales* on a fundamental level, but also from an applied perspective it can be very beneficial to know how to halt gametophyte reproduction, since the vegetative growth of the gametophyte is promoted when no reproduction takes place (Destombe and Oppliger 2011). Our studies show that high temperatures (>14.0°C; Fig. 3-1) and high IGDs (Fig. 2-3; Fig. 3-2; Fig. 4-3; Fig. 4-4; Fig. 5-3) strongly suppressed sexual reproduction. Understanding both sides of the action lever, the side that promotes the induction of gametophyte reproduction, as well as the side that promotes the vegetative growth of these gametophyte are essential in our journey to fully domesticate gametophytes that can actively delay their sexual reproduction for years. Bringing up the most influential of questions:

Can multiannual delayed gametophytes reliably sexually reproduce after more than a year of vegetative growth? The results in this thesis undoubtedly show that multiannual delayed gametophytes, from different cultures, from different locations, and from different families (*Laminariaceae* & *Alariaceae*), all successfully initiated reproduction after more than a year of vegetative growth. There were even cultures that successfully reproduced after remaining vegetative for more than 2.5 years (Chapter 3). In our experience we did not see any correlation between the age of the gametophytes and declining reproduction (an issue that is raised often in the kelp industry). It is clear to us the MAD gametophytes, in combination with the controlled reproductive method (Chapter 3), can be used as seed stock for large scale kelp aquaculture.

(2) The role of delayed gametophytes in their natural environment.

Our studies into the lifecycle controls of delayed gametophytes gave us novel insights into the role delayed gametophytes play in the lifecycle of kelp. The same data can also give us valuable insights into the potential role that delayed gametophytes might play in their natural environment, a subject that is strongly understudied. The primary culprit for the few amount of studies is that quantifying *in vivo* gametophyte presence in natural system remains notoriously difficult (Carney and Edwards 2006), resulting in the in-vivo asexual reproduction, growth, and increase of gametophyte biomass always being shrouded in mystery. Nonetheless, gametophytes are generally regarded to be the adaptive form for stressful environments (Kain 1964; Dayton 1985; Hoffmann et al. 1984; Hoffmann and Santelices 1991; Dieck 1993; Edwards 2000; Carney and Edwards 2006). Although our results point towards the same direction, they also guide us towards a new vantage point, giving us a different hypothesis of why gametophytes evolved the ability to delay their reproduction and grow vegetatively, seemingly in perpetuity. We agree that the gametophytes with which we worked indeed remained vegetative under conditions that can be deemed sub-optimal, like the very low/high light intensities (Chapter 2) or the very low/high temperatures (Chapter 3) we observed. These sub-optimal conditions closely resemble peak summer and peak winter conditions, which coincidently are also the periods when in-vivo kelp reproduction and growth is observed to occur the least (Parke 1948, Druehl 1965, Hsiao and Druehl 1973; Lüning 1982). However, one overlooked characteristic in these delayed gametophytes is the fact that the health of the gametophytes remained remarkably stable during these periods, not showing signs of stress (Fig. S2-3, Fig. 5-4). This suggests that delayed gametophytes might not have evolved to survive sub-optimal conditions, but instead have evolved to thrive in sub-optimal conditions, changing the entire vantage point of why gametophytes delay their reproduction and decide to grow vegetatively instead.

The essence of thriving is very different from the essence of surviving and gives a completely different twist to the perceived role that delayed gametophytes might play in the lifecycle of kelp. This leads us to form a new hypothesis in which we entertain the idea that delayed gametophytes

are not just the adaptive form for stressful environments, but that delayed gametophytes have evolved to become the most dominant part in the lifecycle of kelp. The hypothesis revolves around the idea that gametophytes have a small chance of finding each other in the ocean, and that only an insignificantly small percentage of newly formed gametophytes is actually able to find the other sex in the short period after being released as a zoospore. We hypothesize that gametophytes evolved to delay their reproduction, not as a last ditch effort to survive sub-optimal environments, but because it is a much more successful reproductive strategy compared to the chance encounter between two newly formed gametophytes. In other words, the reason why gametophytes delay their reproduction might be because it simply increases the chances of success in finding a suitable partner, and by doing so allowing for young sporophytes to develop and mature in the best conditions possible. There is a large chance that a newly formed gametophyte might end up in the wrong place at the wrong time, due to wrong currents, fierce competition, or just bad luck. This gametophyte will always have the ability to simply grow vegetatively and disperse through fracturing (Destombe and Oppliger 2011), settling somewhere else, try again, and again, until they found the right spot to settle and successfully reproduce. Moreover, if the right spot to settle is found it still has the ability to grow vegetatively, to fracture and further disperse, increasing its chances to produce viable offspring and ensuring its future lineage. If indeed this hypothetical reproductive strategy is their route to success, it is to no surprise that the gametophytes that are able do vegetatively grow in perpetuity become dominant long-term evolutionary drivers, possible resulting in the gametophytes we see today that have the ability to become very old (Edwards 2006; Barrento 2016), seem immortal, and maybe even are.

If delayed gametophytes are indeed such an important factor in the lifecycle of kelp, then our results also have implications on how climate change might influence the natural cycle of kelp forests. Since delayed gametophytes are highly sensitive to increasing temperatures it is not a long shot to imagine that the die-off of kelp forests is not only due to sporophytes being too sensitive to temperature increase (Assis et al. 2017), but because delayed gametophytes simply refuse to sexually reproduce (Chapter 2). The higher temperatures might incline delayed gametophytes to remain

vegetative, instead of putting their energy into the development of the next generation of kelp forests. This would greatly decrease the ability of an aging kelp forest to rejuvenate itself over time. If the climate change bottleneck in the lifecycle of kelp can indeed be found in the surrounding delayed gametophytes that collectively decide to remain vegetative, it gives insights into how kelp forest restoration needs to be done. For example, this can be done by allowing delayed gametophytes to reproduce under controlled "colder" *in vitro* conditions, through the controlled reproductive method (Chapter 5), and then by planting out the young kelp sporelings in the wild. This can be done for example by inoculating stones in rewilding programs. These results not only add to the fundamental understanding of gametogenesis on *Laminariales* like *S. latissima* and *A. esculenta*, it also adds to facilitate seaweed farming.

(3) The facilitation of the kelp farmer using the controlled reproductive method

As of today there are still two main bottlenecks that prevent large scale oceanic kelp aquaculture from becoming a reality. The first bottleneck is the technical aspect of ocean farming itself. Building kelp farms in the middle of the ocean have never been done before, especially not on the envisioned scales proposed by companies (Cascadia Seaweed, Ocean Rain Forest; Ocean harvest; Green wave). The challenges to build something easy enough to cultivate, sturdy enough to survive the harsh salty oceanic conditions, while also making it comfortable enough for kelp to thrive in is an engineering feat in itself. The second major bottleneck revolves around the knowledge transfer of kelp cultivation, and in particular the biotechnological understanding of kelp propagation (Kim et al. 2017). How can we spread the crucial baseline knowledge, that is needed to be able to cultivate kelp, as efficiently and successfully as possible? In order to tackle this second bottleneck, concerning the transfer of knowledge, there are generally two options we can choose from; (1) Increasing the baseline knowledge of aspiring seaweed farmer or (2) decreasing the baseline knowledge needed to successfully cultivate kelp.

The first option revolves around the education of aspiring kelp farmers,

so that their baseline knowledge is brought up to the standard needed to successfully cultivate kelp. This option is already gaining traction and multiple protocol handbooks have already been written that teach how kelp cultivation can be done (Redmond 2014, Charrier 2018). Although admirable, the major downside to this option is the time that is needed to educate aspiring kelp farmers. The reality is that this option of educating the farmer takes a lot of time, hampering the roll-out of kelp aquaculture around the world. The slow and inefficient transfer of knowledge becomes accentuated by the fact that many kelp scientists are not at the same time "farm savvy", and thus able to translate their scientifically outstanding fundamental knowledge of kelp propagation to the applicability of ocean farming. The second option is to lower the baseline knowledge that is needed to cultivate kelp by building plug and play equipment that makes kelp propagation as easy as possible, preferable using the controlled reproductive method.

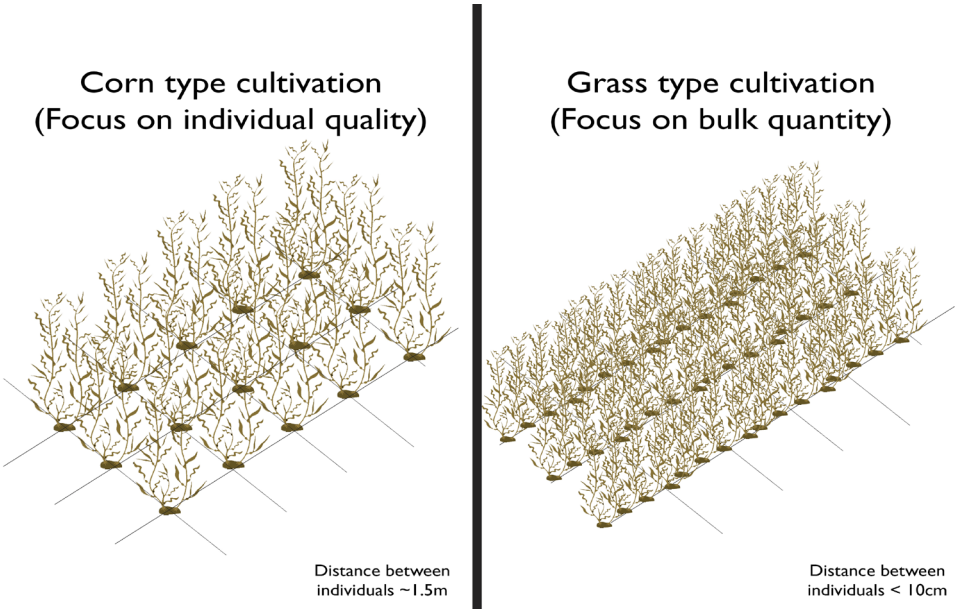

Figure 6-2. Corn type cultivation versus Grass type cultivation. Distances between individuals are based upon corn type designs and grass type designs that can be observed at kelp farmers.

The reason the controlled reproductive method has our preference is that it offers a more direct control on gametophyte reproduction because of the *in vitro* environment where gametophyte growth and reproduction takes place (Chapter 2 & 3). The controlled settings of gametophyte reproduction furthermore allows for subsequent cultures with juvenile sporophytes to be attached to seaweed lines through direct seeding (Kerrison et al., 2020; Forbord, 2020). To this day the controlled reproductive method remains a relatively labour intensive "specialized" method and in order for us to make this method a more feasible option for large scale kelp aquaculture, a simplification of the production process is needed. This in essence boils down to the following question:

> *Can we build a system that allows a starting kelp farmer without any fundamental understanding of the lifecycle of kelp or expensive laboratory facilities, to use the controlled reproductive method to cultivate their farm?* The results in Chapter 5 clearly show that the fundamental parts of such system are functioning and that such a system is a feasible option for both *S. latissima* as well as *A. esculenta* kelp farms. No laboratory equipment or sterile work environments were needed to maintain MAD gametophyte clonal cultures for 75 days. Gametophyte reproduction was furthermore successfully initiated with high numbers of sporophytes in both *S. latissima* as well as *A. esculenta*. The transport simulation showed that the cultures can ultimately be transported to farms in all corners of the earth using the transporter bioreactor. The best news is that there is still a lot of room for further optimization. For example, the slow vegetative growth rates of the stock cultures were intentionally kept low, with Zhang et al., (2019) already describing *S. japonica* gametophyte biomass growing up to 12.8 times the initial biomass in just 21 days. So yes, we have built a basic system that allows a farmer without any background in kelp cultivation to reliable propagate kelp gametophytes. However, there is still a lot of room for optimization in the system. Optimization that is still strongly needed in order to get the controlled reproductive method cost effective enough to cultivate kelp on scales not seen before.

(4) The implications of large scale kelp aquaculture

Now that we covered most discussion points around the domestication of kelp, let's hypothetically assume that kelp will be fully domesticated in the near future, making true large scale kelp aquaculture a tangible reality. How will this reality look like? What are the consequences? Who will become the main beneficiaries? Better understanding this new reality can be done by answering the following two questions;

(1) How big can large scale kelp aquaculture ultimately become?

(2) What are the consequences of these industrial scales of kelp production on their environment?

Thanks to thorough investigation by Campbell et al., 2019 we already have a good initial idea of the second question, on what some of the major implications of large scale kelp farming in European waters might entail (Table. 6-1).

Table 6-1. The environmental risks of kelp farming described by Campbell et al. (2019)

The environmental risks of kelp farming	
Absorption of light	Addition of noise
Absorption of nutrients	Release of dissolved and particular matter
Absorption of carbon	Habitat for diseases, parasites, and non-native species
Absorption of kinetic energy	Release of reproductive material
	Artificial habitat creation
	Addition of artificial material

Campbell et al. 2019 concluded that the sustainability of kelp farm developments would depend on the scale of the operation, the magnitude of the potential impacts, and the sensitivity of their surroundings. They graded and prioritized their concerns and their main points of concern were (i) the release of reproductive material, (ii) the facilitation of invasive diseases, parasites, and non-native species, (iii) the absorption of kinetic energy, and (iv) the addition of cultivation systems to otherwise pristine waters. Creating infertile hybrid sporophytes from isolated clonal diploid gametophyte cultures (Goecke et al., 2020) already has the ability to solve the first two main points of concern. The clonal gametophytes, being created from axenic starting cultures, minimizes the risk of introducing an invasive disease to an absolute minimum. It exemplifies why making infertile sporophytes from clonal cultures needs to become a major priority of research in the coming years. Their other two points of concern cannot be taken outside the context of their surroundings and can even become beneficial in many cases. For example, one can wonder how bad it is to absorb the kinetic energy of the ocean when this absorption happens in the middle of the ocean. Seaweed farms can even be used as forms of coastal protection by being part of novel storm surge protection strategies (Slobbe et al., 2012). Lastly, cultivation systems can be made more sustainable by making the farms sturdy enough to last for decades, or through the introduction of biodegradable ropes (Moreno et al., 2016; Kerrison et al., 2019). One can even raise the question whether the artificial environments in the middle of the ocean will have negative effects at all, opening up our discussion to the more positive sides of large scale kelp aquaculture.

Building seaweed farms in the middle of the ocean might not be how nature intended kelp to proliferate, but these artificial environments can become environmental hotspots, where organisms of all trophic levels can thrive within. Theuerkauf et al. (2021) conclusively showed that seaweed farms tend to have a positive impact on the biodiversity observed in their surroundings. What's more, the fact that kelp does not need any freshwater, arable land, or fertilizers to grow, makes them incredibly renewable and also a great scalable source for biological building blocks. As far as we know, there are no land-based alternatives that can tick all the boxes described in the introduction, while there are no ocean-based alternatives that can rival

the growth speeds and scale potential displayed by kelp. The potential to help the planet while also having the potential of becoming an economic commodity is a relatively unique characteristic of kelp, and this potential only grows following larger and larger seaweed farms, which in the end returns us to the first question, of how big large scale kelp aquaculture ultimately can become. This brings us to the last, unofficial chapter, taking us into the realm of forecasting and speculation, of what kelp might do in the decades to come.

(5) Kelp as a carbon sink

In order to assess how big kelp aquaculture ultimately can become we need to understand its final purpose. Recently kelp is receiving a lot of attention for it perceived potential as carbon sink . This sudden interest is explainable, since in order for us to limit global warming to 1.5°C or 2°C above preindustrial levels, we will need some form of negative emissions technologies described in most IPCC scenarios (IPCC 2018). People even suggest that kelp might be our only realistic scalable option we have at our disposal to combat and reverse human induced climate change, by acting as a carbon sink (Flannery 2017). Prof. Flannery's vision of kelp, as the low hanging fruit we still have at our disposal, is already shared with many initiatives and companies (*e.g*. Xprize carbon capture, Ocean rainforest, Arctic AS), but is it a realistic one?

Prof. Flannery's claim that kelp can be grown and sunk to the bottom of the ocean, and act as a carbon sink, is not an entirely otherworldly method of storing carbon. Proof that oceanic autotrophs have the ability to sequester large amounts of CO_2 and subsequently change the climate have been documented before. For example, the small freshwater fern *Azolla spp*., during the middle Eocene (~48.5Ma), successfully sequestered carbon in the arctic ocean (Speelman et al., 2009). Atmospheric CO_2 levels were reduced by 2000 ppm during this period (Pearson and Palmer 2000), transforming the world from a greenhouse to an icehouse state. The *Azolla* phase, that lasted approximately 800,000 years (Brinkhuis et al. 2006), contributed to this reduction in CO_2, validating the plausibility of carbon storage through the sinking of oceanic primary production, and in our case kelp. Which brings

us back to the question that remains:

How big of a farm do we need to help the world get rid of its excess atmospheric CO_2?

So we can answer an equally important question:

Will a farm that size still be considered to be the silver bullet we are searching for?

In order to progress this thinking exercise, we need to make assumptions that in real life might be considered a bit short-sighted. First, the hypothetical farm must work optimally (*e.g.* no nutrient deficiencies, no light deficiencies, no mechanical restrictions). Second, the consequences of the large scale dumping of kelp biomass on the deep ocean floor must be entirely negated. Third, we must assume a genus that is most suited for the task. In this case we assume that it is *Macrocystis spp.,* since they are the largest of the *Laminariales*. What's also important, the lifecycle of *M. pyrifera* is identical to what is researched in this thesis. Lastly, we need to assume the farm type we are going to use (Fig. 6-2), since this has great consequences on our biomass calculations. For our calculations we assume to use corn-type farms, mostly because it is the more conservative estimate (See Table. 6-2 column 3 & 4), but also because this specific set-up allows for easier extrapolations. Plus, the added benefit of using documented in-vivo weights of fully grown *Macrocystis spp.* sporophytes make the extrapolations more reliable, and in the end more realistic, than having to calculate biomasses for full lines of grass-type cultivated seaweeds.

We estimate, based on peer-reviewed biomass data of *Macrocystis spp*. (Table. 6-2), that a corn-type farm harbouring ~4444 kelp-plants per hectare (one sporophyte every 1.5m) can absorb ~12.4 tons of CO_2 per hectare, under optimal conditions, in one growth cycle. Extrapolating this potential to the yearly global emissions of ~40 gigatons of CO_2 results in a hypothetical farm roughly double the size of Russia (32,653,061 km^2) needed to offset current yearly human CO_2 emissions. To compare, using these calculations we need a swath of ocean the size of ~64% of all global agricultural land area, or ~6.78% of the surface of the earth (approximately 9.5% of the surface of the oceans). It immediately becomes clear that the enormous amount of kelp needed to offset human induced climate change

is not the silver bullet some people are hoping for. It primarily highlights the difficult road that still lies ahead of us, that there might be no silver bullet at all, and ultimately putting an uncomfortable spotlight on the gargantuan scale of the climate crisis.

Nonetheless, we should not underappreciate the potential that still resides in seaweed. Where we left behind our hunt-gatherer existence on land more than 10,000 years ago, we still pretty much just hunt and gather on the oceans. There is also a lot of ground to be won with regards to seaweed farm optimization, integrated multi-trophic aquacultures, and novel breeding practises. Seaweed already has a positive effect when cultivated on much smaller scales. For example, kelp remains an extremely renewable source for food (van der Burg, 2021), feed (Carrier et al. 2017), bioplastics (Lim et al., 2021), or as fertilizer (Gutierrez et al,. 2006). On intermediary scales kelp also has the added potential of deacidifying large patches of ocean (Hirsch et al., 2019), while not forgetting the intrinsic function of a kelp forest, by acting as a sanctuary for oceanic wildlife (Theuerkauf et al. 2021). Although kelp aquaculture does not directly appear to be the climate change silver bullet we hope for, it probably still remains as close as we can get to the low hanging fruit we all are searching for in the 21st century.

Table 6-2. Literature study and subsequent conversions to calculate the carbon sequestration potential of kelp cultivation. For these calculations some assumptions were necessary. These assumptions are based on data from peer-reviewed publications **(blue)**. In **(1.)** we make assumptions on the wet weight of Macrocystis spp.. In **(2).** we assume the dry weight / wet weight ratio of the plants in order to better calculate the carbon content of Macrocystis spp.,. Calculating the carbon content of these kelp individuals is based on **(3),** the carbon content of kelp DW. Lastly, **(4.)** is the assumption on the distance between the kelp individuals in order to calculate the amount of kelp individuals that can be grown per hectare of seaweed farm. All conversions **(green)** are depicted in the second column, and are calculated for corn-type farm set-ups (25.9Kg biomass per individual). To convert the carbon of the kelp's biomass to the CO_2 that is absorbed a conversion of 3.67 is applied because the atomic weight of Carbon = 12u, while $O_2 = 2 \cdot 16u$. Grass type cultivations are also included in the fourth column, but are considered imprecise. For comparison, peer-reviewed land based alternatives **(Yellow)** are included, including all the calculated tonnes of CO_2 the different methods can attain.

Assumptions	**Specifics**	**Corn type cultivation**	**Grass type cultivation**
1. WW of cultivated Macrocystis spp.	-	9.7 - 42kg · Individual^{-1} *(Tussenbroek 1993)*	12.4Kg · m^{-1} · year^{-1} *(Camus et al., 2018)*
	-	20.1kg · Individual^{-1} *(Druehl and Wheeler 1986)*	43.33Kg · m^{-1} · year^{-1} *(Zuñiga-Jara et al., 2016)*
	-	22kg · m^{2} *(North 1971)*	41.3Kg · m^{-1} · year^{-1} *(Correa et al., 2016)*
2. DW/WW ratio	10 to 15% *(Roesadji 2010)*	-	-
	12% *(Hart et al. 1976)*	-	-
3. Carbon content in DW (%)	20-33% *(Zimmerman 1986)*	-	-
4. Distance between plants on farm	-	1.5m *(Hoofdt 2021)*	-
Conversions	Calculations on corn type cultivation	Corn type cultivation	Grass type cultivation
Plants per hectare (one plant every 1.5m)	66.7 · 66.7	4444	na
Biomass (WW; Kg · hectare^{-1})	((25.9 + 20.1+22)/3) · *4444*	100,731Kg	124,000Kg - 433,000Kg*
Biomass (DW; Kg · hectare^{-1}) based on Roesadji (2010); Hart et al. (1976)	*100,731* · 0,125	12,591Kg	15,500Kg - 54,125Kg
Carbon (Kg · hectare^{-1}) average carbon content based on Zimmerman (1986)	*12,591* · 0,265	3337Kg	4108Kg - 14343Kg
CO_2 absorbed from atmosphere per hectare (based on molar mass ratios)	*3337* · 3.67	**12,25 tonnes CO_2 · hectare^{-1}**	**15 - 52.4 tonnes CO_2 · hectare^{-1}**
Hectares of kelp to absorb 1 $GtCO_2$	10^9 / *12,25* ton	81,632,653	19,101 - 66,692
Hectares needed to offset yearly emissions (40 gigatons CO_2)	40 · *8,1*10^6*	3,265,306,120 hectares	764,059 - 2,667,698 hectares
Comparative land based alternatives			
Yearly Carbon storage			
Borreal forest CO_2 absorbance per hectare	-	0.8 - 2.4 tonnes CO_2 / year *(Brown et al., 1996)*	
Temperate forest CO_2 absorbance per hectare	-	0.7 - 7.5 tonnes CO_2 / year *(Brown et al., 1996)*	
Tropical forest CO_2 absorbance per hectare	-	3.2 - 10 tonnes CO_2 / year *(Brown et al., 1996)*	
Above ground Carbon pool per hectare			
Borreal forest pool	-	21 - 24 tonnes · hectare^{-1}** *(IPCC 2006)*	
Temperate forest pool	-	61 - 155 tonnes · hectare^{-1}** *(IPCC 2006)*	
Subtropical forest	-	69 - 108 tonnes · hectare^{-1}** *(IPCC 2006)*	
Tropical forest pool	-	71 - 146 tonnes · hectare^{-1}** *(IPCC 2006)*	
Kelp cultivation estimates (CO_2 · hectare^{-1})		Corn type cultivation	Grass type cultivation
Conservative scenario based on literature	-	12.4 tonnes CO_2 · hectare^{-1}	15 - 52.4 tonnes CO_2 · hectare^{-1}
Doubling of biomass scenario based on breeding literature		24.8 tonnes CO_2 · hectare^{-1} (Liu et al., 2014)	30 - 104.8 tonnes CO_2 · hectare^{-1}

* yield extrapolation using a kelp farm with 10,000m of cultivation line

**averaged across continents for each ecological zone (*Keith et al. 2008*)

Can we quantify how much delayed gametophyte biomass is needed to farm 1 ha of seaweed farm? Extrapolating the relative reproductive success rates observed in this thesis (Fig. 2-4 & 3-2) to the sporophyte quantities needed for a corn type farm (Fig. 6-2; Table. 6-2) allows us to assess how much gametophyte biomass is needed to fill such a farm with viable kelp sporophytes. Looking at the more conservative relative reproductive success of ~18.000 sporophytes per gram of *S. latissima* gametophyte DW (Fig. 3-2), extrapolations can be made on the amount of gametophyte biomass needed to plant out one hectare of kelp. The 4444 plants needed to fill one hectare of farm will need around 0.25 grams DW of multiannual delayed gametophyte biomass. So yes, quantification is not only possible, it also shows the incredible output potential MAD gametophytes have, where 1 gram DW of MAD gametophytes can result in more than 50 tons of harvestable kelp biomass.

Akaike, H. (1969) ‘Fitting autoregressive models aikaike’, Annals of the institute of Statistical Mathematics, 21, pp. 243–247, doi: 10.1007/BF02532251

Alsuwaiyan, N. A., Mohring, M. B., Cambridge, M., Coleman, M. A., Kendrick, G. A., & Wernberg, T. (2019) 'A review of protocols for the experimental release of kelp (Laminariales) zoospores', Ecology and Evolution, 9(14), 8387–8398. https://doi.org/10.1002/ece3.5389

Amin, S. A., Parker, M. S. and Armbrust, E. V. (2012) ‘Interactions between Diatoms and Bacteria’, Microbiology and Molecular Biology Reviews, 76(3), pp. 667–684. doi: 10.1128/MMBR.00007-12.

Andrés, F., & Coupland, G. (2012) 'The genetic basis of flowering responses to seasonal cues', Nature Reviews Genetics, 13(9), pp. 627–639. https://doi.org/10.1038/nrg3291

Assis, Jorge, Miguel B. Araújo, and Ester A. Serrão (2018) ’Projected Climate Changes Threaten Ancient Refugia of Kelp Forests in the North Atlantic’. Global Change Biology 24, no. 1: e55–66. https://doi.org/10.1111/gcb.13818.

Augyte, S., Wikfors, G. H., Pitchford, S., Marty-rivera, M., Umanzor, S., Lindell, S., Yarish, C. (2020) 'The application of flowcytometry for kelp meiospore isolation', Algal Research, 46(1), https://doi.org/10.1016/j.algal.2020.101810

Bartsch, I., Wiencke, C., Bischof,K., Buchholz. C.M., Buck, B.H., Eggert, A., Feuerpfeil, P., Hanelt, D., Jacobsen, S., Karez, R., Karsten, U., Molis, M., Roleda, M.Y., Schubert, H ., Schumann, R., Valentin.K , Weinberger, F & Wiese, J (2008) ‘The genus Laminaria sensu lato : recent insights and developments’, European Journal of Phycology, 43(1), pp. 1–86. doi: 10.1080/09670260701711376.

Bak, U. G., Olavur, G. & Infante, J. (2020). 'Technical challenges for offshore cultivation of kelp species : lessons learned and future directions', Botanica marina, 63 (4), pp. 341 - 353. https://doi.org//10.1515/bot-2019-0005

Barrento, S., Camus, C., Sousa-pinto, I., & Buschmann, A. H. (2016) 'Germplasm banking of the giant kelp : Our biological insurance in a changing environment', Algal, 13(1), pp. 134–140. https://doi.org/10.1016/j.algal.2015.11.024

Bell, G. (1997) 'the evolution of the life cycle of brown seaweeds', Biological journal of the linnean society, 60, pp. 21 - 38. doi: 10.1111/j.1095-8312.1997.tb01481.x

Bi, Y., Hu, Y., & Zhou, Z. (2011) 'Genetic variation of Laminaria japonica (Phaeophyta) populations in China as revealed by RAPD markers', Acta Oceanologica Sinica, 30(2), pp. 103–112. https://doi.org/10.1007/s13131-011-0110-y

Blanchette, C. A. (1996) 'Seasonal patterns of disturbance influence recruitment of the sea palm, Postelsia palmaeformis'. J. Exp. Mar. Biol. Ecol. 197:1–14.

Bolton, J. J. and Levitt, G. J. (1985) 'Light and temperature requirements for growth and reproduction in gametophytes of Ecklonia maxima (Alariaceae: Laminariales)', Marine Biology, 87(2), pp. 131–135. doi: 10.1007/BF00539420

Brinkhuis H; Dickens, Gerald R, Matthew Huber, Thomas M Cronin, Jonaotaro Onodera, Kozo Takahashi, Francesca Sangiorgi, Han Van Konijnenburg-van Cittert, Jan W De Leeuw, and Jens Matthiessen. (2006) 'Episodic Fresh Surface Waters in the Eocene Arctic', 441, no. June 606–9. https://doi.org/10.1038/nature04692.

Broch, O. J., Alver, M. O., Bekkby, T., Gundersen, H., Forbord, S., Handå, A., ... & Hancke, K. (2019). The kelp cultivation potential in coastal and offshore regions of Norway. Frontiers in Marine Science, 5, 529.

van den Burg, S., Selnes, T., Alves, L., Giesbers, E., & Daniel, A. (2021) 'Prospects for upgrading by the European kelp sector'. Journal of Applied Phycology, 33(1), 557-566.

Campbell, Iona, Adrian Macleod, Christian Sahlmann, Luiza Neves, Jon Funderud, Margareth Øverland, Adam D Hughes, and Michele Stanley (2019). 'The Environmental Risks Associated With the Development of Seaweed Farming in Europe - Prioritizing Key Knowledge Gaps'. 6, no. March. https://doi.org/10.3389/fmars.2019.00107.

Carney, L. T. (2011) 'A multispecies laboratory assessment of rapid sporophyte recruitment from delayed kelp gametophytes', Journal of Phycology, 47 (2), pp.244 - 251, doi: 10.1111/j.1529-8817.2011.00957.x.

Carney, L. T. and Edwards, M. S. (2006) 'Cryptic Processes in the Sea : A Review of Delayed Development in the Microscopic Life Stages of Marine Macroalgae', Algae, 21(2), pp. 161–168, doi: 10.4490/algae.2006.21.2.161

Carney, L. T. and Edwards, M. S. (2010) 'Role of nutrient fluctuations and delayed development in gametophyte reproduction by Macrocystis pyrifera (phaeophyceae) in Southern California', Journal of Phycology, 46(5), pp. 987–996. doi: 10.1111/j.1529-8817.2010.00882.x.

Carrier, Tyler J, Stephen D Eddy, and Sarah Redmond (2017) 'Solar-Dried Kelp as Potential Feed in Sea Urchin Aquaculture', Aquaculture International, no. February. https://doi.org/10.1007/s10499-016-0033-x.

Chen, S., & Qi, H. (2008) 'Photolithotrophic cultivation of Laminaria japonica gametophyte cells in a silicone tubular membrane-aerated photobioreactor'. Plant Cell, Tissue and Organ Culture, 93(1), pp. 29–38. https://doi.org/10.1007/s11240-008-9339-3

Chung, I. K., Oak, J. H., Lee, J. A., Shin, J. A., Kim, J. G., & Park, K. (2013)' Installing kelp forests/seaweed beds for mitigation and adaptation against global warming: Korean Project Overview, Marine Science. 70, pp. 1038–1044. https://doi.org/10.1093/icesjms/fss206

Choi, H. G, Kim, Y. S, Lee. S. J, Park. E. J, and Nam. K (2005) 'Effects of daylength,

irradiance and settlement density on the growth and reproduction of Undaria pinnatifida gametophytes', Journal of Applied Phycology, 17(5), pp. 423–430. doi: 10.1007/s10811-005-0432-2.

Chopin, T., Buschmann, A. H., Halling, C., Troell, M., Kautsky, N., Neori, A., and Neefus, C. (2001) 'Integrating seaweeds into marine aquaculture systems: a key toward sustainability', Journal of Phycology, 37(6), 975-986.

Dayton, P. K., Currie, V. and Gerrodette, T. I. M. (1984) 'Patch Dynamics and Stability of Some California Kelp Communities, Ecological Society of America, 54(3), pp. 253–289, doi: 10.2307/1942498

Davies R.J.(1990)' Bio-energy: A case study of kelp industries, king island, Tasmania. Solar and wind technologies. 7(1). pp. 21-24. https://doi.org/10.1016/0741-983X(90)90006-N.

Destombe, C. and Oppliger, L. V. (2011) 'Male gametophyte fragmentation in laminaria digitata: A life history strategy to enhance reproductive success', Cahiers de Biologie Marine, 52(4), pp. 385–394.

Deysher, L. E., & Dean, T. A. (1986) 'Interactive effects of light and temperature on sporophyte production in the giant kelp Macrocystispyrifera', Marine Biology, 93, pp. 17–20. https://doi.org/10.1007/BF00428650

Dieck, I. T. (1993) 'Temperature tolerance and survival in darkness of kelp gametophytes (Laminariales, Phaeophyta) - Ecological and biogeographical implications', Marine Ecology Progress Series, 100(3), pp. 253–264. doi: 10.3354/meps100253.

Druehl, L. D. (1965) 'On the taxonomy and distribution of northeast Pacific species of Laminaria', Canadian Journal of Botany, 46(5), 539–547. https://doi.org/10.1139/b68-081

Donohoe, A. (2020) 'Seasonal Asymmetries in the Lag between Insolation and Surface Temperature', Journal of climate, 33, pp. 3921–3945. https://

doi.org/10.1175/JCLI-D-19-0329.1
Ebbing, A.P.J, Pierik, R., Bouma, T., Kromkamp, J. C., & Timmermans, K. (2020) 'How light and biomass density influence the reproduction of delayed Saccharina latissima gametophytes (Phaeophyceae)', Journal of Phycology, 56(3), pp. 709 -718, 56https://doi.org/10.1111/jpy.12976

Ebbing, A. P.J; Pierik, R.; Fivash, G. S.; van de Loosdrecht, N. C.; Bouma, T. J.; Kromkamp, J. C.; Timmermans, K. (2021a) 'The role of seasonality in reproduction of multiannual delayed gametophytes of Saccharina latissima'. Journal of phycology 2021. doi: 10.1111/jpy.13191.

Ebbing, A.; Fivash, G., Pierik, R., Bouma, T., Kromkamp, J. C., & Timmermans, K. (2021b), The SeaCoRe system for large scale kelp aquaculture: a plug-and-play, compatible, open-source system for the propagation and transport of clonal gametophyte cultures.[1]

Edwards, M. S. (2000) 'The role of alternate life-history stages of a marine macroalga: a seed bank analogue?', Ecology, 81:2404–15.

FAO (2015) 'The state of food insecurity in the world'. https://www.fao.org/publications/sofi/2015/en/

FAO (2020) 'The state of the world fisheries and aquaculture', https://www.fao.org/documents/card/en/c/ca9229en/

Flannery (2017) 'Can seaweed curb global warming', https://www.youtube.com/watch?v=tuvxXnQrRv8

Fredersdorf, J., Müller, R, Becker, S., Wiencke, C and Bischof, K (2009) 'Interactive effects of radiation, temperature and salinity on different life history stages of the Arctic kelp Alaria esculenta (Phaeophyceae)', Oecologia, 160(3), pp. 483–492. doi: 10.1007/s00442-009-1326-9.

Frenkel, J., Vyverman, W and Pohnert, G. (2014) 'Pheromone signaling during sexual reproduction in algae', Plant Journal, 79(4), pp. 632–644. doi: 10.1111/tpj.12496.

Forbord, S., Skjermo, J., & Arff, J. (2012) 'Development of Saccharina latissima (Phaeophyceae) kelp hatcheries with year-round production of zoospores and juvenile sporophytes on culture ropes for kelp aquaculture', J appl Phycol, 24, pp. 393–399, https://doi.org/10.1007/s10811-011-9784-y

Forbord d S, Steinhovden KB, Rød KK, Handå A, Skjermo J (2018) 'Cultivation protocol for Saccharina latissima. In: Charrier BTW, Reddy C (eds)', Protocols for Macroalgae Research, CRC Press, Boca Raton, pp 37–59

Forbord, S., Steinhovden, K.B., Solvang, T. et al. (2020) 'Effect of seeding methods and hatchery periods om sea cultivation of Saccharina latissima (Phaeophyceae): a Norwegian case study', J appl Phycol, 32, pp. 2201-2212 (2020). http://doi.org/10.1007/s10811-019-01936-0

Gao, J., Zhang, Y., Wang, H., & Qin, S. (2005) 'Suspension culture of gametophytes of transgenic kelp in a photobioreactor', Biotechnology Letters, 27(14), 1025–1028. https://doi.org/10.1007/s10529-005-8098-z

Gevaert, F., Creach, A., Davoult, D., Holl, C., Seuront, L and Lemoine, Y (2002). 'Photo-inhibition and seasonal photosynthetic performance of the seaweed Laminaria saccharina during a simulated tidal cycle: Chlorophyll fluorescence measurements and pigment analysis', Plant, Cell and Environment, 25(7), pp. 859–872. doi: 10.1046/j.1365-3040.2002.00869.x.

Guillard, R. R. L. and J. H. R. 1962 (1962). 'Studies of marine planktonic diatoms. i. Cyclotella nana Hustedt and Detonula Confervacea (cleve), Canadian Journal of Microbiology, 8(1140), pp. 229 - 239. doi: 10.1139/m62-029#.Xh8HTshKhPY

Gutierrez, A., Correa, T., Munoz, V., Santibanez, A., Marcos, R., Cáceres, C., & Buschmann, A. H. (2006). 'Farming of the giant kelp Macrocystis pyrifera in southern Chile for development of novel food products. In Eighteenth International Seaweed Symposium, pp. 33-41.

Goecke, F., Klemetsdal, G., & Ergon, Å. (2020) 'Cultivar Development of Kelps for Commercial Cultivation - Past Lessons and Future Prospects', Frontiers in marine science, https://doi.org/10.3389/fmars.2020.00110

Harries (1932) 'An Investigation by Cultural Methods of some of the Factors Influencing the Development of the Gametophytes and the Early Stages of the Sporophytes of Laminaria digitata , L . saccharina , and L . Cloustoni, Annals of Botany, 46(184), pp. 893–928. doi: 10.1139/43237797?seq=1

Hernandez-Carmona, G., Robledo, D. & Serviere-Zaragoza, E. (2001) 'Effect of nutrient availability on Macrocystis pyrifera recruitment and survival near its southern limit off Baja California'. Bot. Mar. 44:221–9

Heuermann, R., Reuter, R., & Willkomm, R. (1999) 'RAMSES: a modular multispectral radiometer for light measurements in the UV and VIS', Environmental Sensing and Applications, 3821, pp. 279–285. https://doi.org/10.1117/12.364189

Hirsh, H. K., Nickols, K. J., Takeshita, Y., Traiger, S. B., Mucciarone, D. A., Monismith, S., & Dunbar, R. B. (2020) 'Drivers of Biogeochemical Variability in a Central California Kelp Forest: Implications for Local Amelioration of Ocean Acidification'. Journal of Geophysical Research: Oceans, 125(11), e2020JC016320.

Hoffmann, A. J., Avila, M. & Santelices, B. (1984) 'Interactions of nitrate and phosphate on the development of microscopic stages of Lessonia nigrescens Bory (Phaeophyta)'. J. Exp. Mar. Biol. Ecol. 78:177–86

Hoffman, A. J. and Santelices, B. (1991) 'Banks of algal microscopic forms: hypotheses on their functioning and comparisons with seed banks', Marine ecology progress series, 79, pp. 185 - 194. doi: 10.3354/MEPS079185

Holdt, Susan Lovstad, and Stefan Kraan (2011) 'Bioactive Compounds in Seaweed: Functional Food Applications and Legislation', Journal of Applied Phycology, 23, no. 3 543–97. https://doi.org/10.1007/s10811-010-9632-5.\

Hooft, D., and Slootweg C (2021). ‘Kelp Blue’. available on http://kelp.blue/ (last accessed at 21 May 2021)

Hsiao, S. I. C. and Druehl, L. D. (1971) ‘Environmental control of gametogenesis in Laminaria saccharina . I. The effects of light and culture media’, Canadian Journal of Botany, 49(8), pp. 1503–1508. doi: 10.1139/b71-211.

Hsiao, S. I. C., & Druehl, L. D. (1973) 'Environmental control of gametogenesis in Laminaria saccharina. IV. in situ development of gametophytes and young sporophytes', Journal of phycology, 9 , pp. 160-164. http://doi.org/10.1111/j.1529-8817.1973.tb04073.x

Huot, Y., Babin, M., Bruyant, F., Grob, C., Twardowski, T.H and Claustre, H (2007) ‘Does chlorophyll a provide the best index of phytoplankton biomass for primary productivity studies ? Does chlorophyll a provide the best index of phytoplankton biomass for primary productivity studies ?’, Biogeosciences Discussions, 4(2), pp.707-745. doi: 10.1.1.668.3090&rep

Hwang E.K., Park C.S., Baek J.M (2006) ‘Artificial seed production and cultivation of the edible brown alga, Sargassum fulvellum (Turner) C. agarh: Developing a new species for seaweed cultivation in Korea’. Journal of applied phycology. 18: 251 - 257. Doi: 10.1007/s10811-006-9021-2

Jayathilake, Dinusha R M, and Mark John Costello (2020) ‘Policy Analysis A Modelled Global Distribution of the Kelp Biome’, Biological Conservation, 252, no. October: 108815. https://doi.org/10.1016/j.biocon.2020.108815.

IPCC (2018) ‘Global warming of 1.5°C. An IPCC Special Report on the impacts of global warming of 1.5°C above pre-industrial levels and related global greenhouse gas emission pathways, in the context of strengthening the global response to the threat of climate change, sustainable development, and efforts to eradicate poverty. V. Masson-Delmotte, P. Zhai, H. O. Pörtner, D. Roberts, J. Skea, P.R. Shukla, A. Pirani, W. Moufouma-Okia, C. Péan, R. Pidcock, S. Connors, J. B. R. Matthews, Y. Chen, X. Zhou, M. I. Gomis, E. Lonnoy, T. Maycock, M. Tignor, T. Waterfield (eds.). In Press.

Izquierdo, J. L., Pérez-Ruzafa, I. M. and Gallardo, T. (2002) 'Effect of temperature and photon fluence rate on gametophytes and young sporophytes of Laminaria ochroleuca Pylaie', Helgoland Marine Research, 55(4), pp. 285–292. doi: 10.1007/s10152-001-0087-6.

Jang K. Kim, Charles Yarish, Eun Kyoung Hwang, Miseon Park, Y. K. (2017) 'Seaweed aquaculture: cultivation technologies, challenges and its ecosystem services', Algae, 32(1), pp. 1-13. doi: 10.4490/algae.2017.32.3.3

Kinlan, B.P., Graham, H.G, Sala, E and Dayton Scripps, P.K (2003) 'Arrested development of giant kelp (macrocystis pyrifera, phaeophyceae) embryonic sporophytes: a mechanisms for delayed recruitment in perennial kelps?', Journal of phycology, 39 (1), pp. 47–57. doi: doi.org/10.1046/j.1529-8817.2003.02087.x

Kinoshita, Nana, Chikako Nagasato, and Taizo Motomura. 'Chemotactic movement in sperm of the oogamous brown algae, Saccharina japonica and Fucus distichus', Protoplasma, 254.1 (2017): 547-555.

Kim. J, Charles Yarish, Eun Kyoung Hwang, Miseon Park, Y. K. (2017) 'Seaweed aquaculture: cultivation technologies, challenges and its ecosystem services'. Algae. 32, pp.1-13. https://doi.org/10.4490/algae.2017.32.3.3

Klinger, T. (1984) 'Allocation of the blade surface area to meiospore production in the annual and perennial representatives of the genus Laminaria'. M.S. thesis, University of British Columbia, Vancouver, 96 pp.

Kennedy, L. (1982) 'The first agricultural revolution: property rights in their place', Agricultural History, 56(2), 379–390.

Kerrison, P. D., Stanley, M. S., & Hughes, A. D. (2018) 'Textile substrate seeding of Saccharina latissima sporophytes using a binder: An effective method for the aquaculture of kelp'. Algal Research, 33, pp. 352–357. https://doi.org/10.1016/j.algal.2018.06.005

Kerrison, P. D., Twigg, G., Stanley, M., De Smet, D., Buyle, G., Martínez Pina,

A., & Hughes, A. D. (2019) 'Twine selection is essential for successful hatchery cultivation of Saccharina latissima, seeded with either meiospores or juvenile sporophytes', Journal of Applied Phycology, 31(5), pp. 3051–3060. https://doi.org/10.1007/s10811-019-01793-x

Kinoshita, N., Nagasato, C., & Motomura, T. (2017). Phototaxis and chemotaxis of brown algal swarmers. Journal of plant research, 130(3).

Krumhansl, K. A., Okamoto, D. K., Rassweiler, A., Novak, M., Bolton, J. J., Cavanaugh, K. C., Byrnes, J. E. K. (2016) 'Global patterns of kelp forest change over the past half-century', Proceedings of the National Academy of Sciences of the United States of America, 113(48), 13785–13790. https://doi.org/10.1073/pnas.1606102113

Kudoh, H. (2019) 'Photoperiod–temperature phase lag: A universal environmental context of seasonal developmental plasticity', Development Growth and Differentiation, 61(1), pp. 5-11. https://doi.org/10.1111/dgd.12579

Kolber, Z., Zehr, J., & Falkowski, P. (1988) 'Effects of growth irradiance and nitrogen limitation on photosynthetic energy conversion in photosystem II'. Plant physiology, 88(3), pp. 923-929. https://doi.org/10.1104/pp.88.3.923
Ladah, L. B., Zertuche-Gonza´lez, J. A. & Herna´ndez-Carmona, G. (1999) ‘Giant kelp (Macrocystis pyrifera, Phaeophyceae) recruit- ment near its southern limit in Baja California after mass disappearance during ENSO 1997–1998’. J. Phycol. 35:1106– 12.

Lakeman, M.B., von Dassow, P., Cattolico, R.A. (2009) 'The strain concept in phytoplankton ecology'. Harmful Algae, 8, pp. 746–758. https://doi.org/10.1016/j.hal.2008.11.011

Lee, J.-A. and Brinkhuis, B. H. (1988) ‘Seasonal light and temperature interaction effects on development of Laminaria saccharina (Phaeophyta) gametophytes and juvenile sporophytes’, Journal of Phycology, 24 (1), pp. 181–191. doi: 10.1111/j.1529-8817.1988.tb04232.x.

Li, D.; Zhou, Z.; Liu, H.; Wu, C. A new method of Laminaria japonica strain selection and sporeling raising by the use of gametophyte clones, Hydrobiologia 1999, pp. 473–476. https://doi.org/10.1023/A:1017090130586

Lim, C, S Yusoff, C G Ng, P E Lim, and Y C Ching (2021) 'Journal of Environmental Chemical Engineering Bioplastic Made from Seaweed Polysaccharides with Green Production Methods'. 9, no. June. https://doi.org/10.1016/j.jece.2021.105895.

Liu, F., Sun, X., Wang, F., Wang, W., Liang, Z., Lin, Z., & Dong, Z. (2014) 'Breeding, economic traits evaluation, and commercial cultivation of a new Saccharina variety Huangguan No. 1.', Aquaculture International, 22(5), 1665–1675. https://doi.org/10.1007/s10499-014-9772-8

Lüning (1980) 'Critical levels of light and temperatures regulating the gametogenesis of three Laminaria species (Phaeophyceae)', Journal of Phycology, 16(1), pp. 1 - 15, doi.org/10.1111/j.1529-8817.1980.tb02992.x
Lüning, K. and Dring, M. J. (1972) 'Reproduction induced by blue light in female gametophytes of Laminaria saccharina', Planta, 104(3), pp. 252–256. doi: 10.1007/BF00387080.

Lüning, K. and Dring, M. J. (1975) 'Reproduction, growth and photosynthesis of gametophytes of Laminaria saccharina grown in blue and red light', Marine Biology, 29(3), pp. 195–200. doi: 10.1007/BF00391846.

Lüning, K. and Neushul, M. (1978) 'Light and temperature demands for growth and reproduction of laminarian gametophytes in southern and central California', Marine Biology, 45(4). pp. 297–309, doi: 10.1007/BF00391816.
Lüning (1980) 'Critical levels of light and temperatures regulating the gametogenesis of three Laminaria species (Phaeophyceae)', Journal of Phycology, 16(1), pp. 1 - 15, doi.org/10.1111/j.1529-8817.1980.tb02992.x

Lüning, K, Biologische Anstalt Helgoland, and D- Helgoland (1982) 'Seasonality in larger brown algae and its possible regulation by the environment', Synthetic and degradative processes in marine macrophytes (pp. 47-68). De Gruyter.

Lüning, K., and I. Tom Dieck (1989) ‘Environmental triggers in algal seasonality’ pp. 389-398.

Marner F J, Müller B, Jaenicke L. (1984) ‘Lamoxirene structural proof of the spermatozoid releasing and attracting pheromone of Laminariales’, Zeitschrift Für Naturforschung , 39 : 689-691.

Matsson, S., Christie, H., & Fieler, R. (2019) 'Variation in biomass and biofouling of kelp, Saccharina latissima, cultivated in the Arctic, Norway', Aquaculture, 506, pp. 445–452. https://doi.org/10.1016/j.aquaculture.2019.03.068

Martins, N., Tanttu, H., Pearson, G.A., Serrão, E.A and Bartsch, I (2017) ‘Interactions of daylength, temperature and nutrients affect thresholds for life stage transitions in the kelp Laminaria digitata (Phaeophyceae)’, Botanica Marina, 60(2), pp. 109–121. doi: 10.1515/bot-2016-0094.

McConnico, L. A. & Foster, M. S. (2005) ‘Population biology of the intertidal kelp, Alaria marginata Postels and Ruprecht: a non- fugitive annual’. J. Exp. Mar. Biol. Ecol. 324:61–75.

Morelissen, B., Dudley, B.D., Geange, S.W and Philips, E (2013) ‘Gametophyte reproduction and development of Undaria pinnatifida under varied nutrient and irradiance conditions’, Journal of Experimental Marine Biology and Ecology. Elsevier B.V., 448, pp. 197–206. doi: 10.1016/j.jembe.2013.07.009.

Morita, T., Kurashima, A. and Maegawa, M. (2003) ‘Temperature requirements for the growth of young sporophytes of Undaria pinnatifida and Undaria undarioides (Laminariales, Phaeophyceae)’, Phycological Research, 51(4), pp. 266–270. doi: 10.1111/j.1440-1835.2003.tb00194.x.

Müller (1971) ‘Sex Attractant in a Brown Alga: Chemical Structure’, Science, 27(2), pp. 743–755, doi: 10.1126/science.171.3976.1132

Müller and Jaenicke (1973) ‘Fucoserraten, the female seks attractant of fucus serratus l. (pheaophyta), Febs letters, 30 (1), pp. 137–139, doi: 10.1016/0014-

5793(73)80636-2

Müller, D. G., Gassmann, G. and Lüning, K. (1979) 'Isolation of a spermatozoid-releasing and -attracting substance from female gametophytes of Laminaria digitata [18]', Nature, pp. 430–431. doi: 10.1038/279430a0.

Murúa, P.; Patiño, D. J.; Müller, D. G.; Westermeier, R. (2021) 'Sexual compatibility in giant kelp gametophytes: inter-cultivar hybridization is average between parents but excels under harsher conditions'. Journal of Applied Phycology, 33, pp. 1-15, https://doi.org/10.1007/s10811-021-02506-z

Nagasato, C., Kawamoto, H., Tomioka, T., Tsuyuzaki, S., Kosugi, C., Kato, T., & Motomura, T. (2019) 'Quantification of laminarialean zoospores in seawater by real-time PCR', Phycological Research, 68(1), pp.57-62. https://doi.org/10.1111/pre.12384

Nahlah, A.; Mohring, M, B.; Cambridge, M.; Coleman, M, A.; Kendrick, G, A.; Wernberg, T. A 2019 'Review of Protocols for the Experimental Release of Kelp (Laminariales) Zoospores. , Ecology and Evolution, 14, pp.8387–98. https://doi.org/10.1002/ece3.5389.

Nie, M., & Takeuchi, S. (2020) 'Luer-lock valve: A pre-fabricated pneumatic valve for 3D printed microfluidic automation', Biomicrofluidics, 14(4). https://doi.org/10.1063/5.0020531

Orefice, I., Chandrasekaran, R., Smerilli, A., Corato, F., Caruso, T., Casillo, A., Corsaro, M.M, Dal Piaz, F., Ruban, A and Brunet, C (2016) 'Light-induced changes in the photosynthetic physiology and biochemistry in the diatom Skeletonema marinoi', Algal Research, 17, pp. 1–13. doi: 10.1016/j.algal.2016.04.013.

Park, J., Kim, J. K., Kong, J. A., Depuydt, S., Brown, M. T., & Han, T. (2017) 'Implications of rising temperatures for gametophyte performance of two kelp species from Arctic waters', Botanica Marina, 60(1), pp. 39–48. https://doi.org/10.1515/bot-2016-0103

Parke, M. (1948). Studies on british Laminariaceae. Marine Biological Association, XXVII, 651–709.

Pearson, Paul N, and Martin R Palmer (2000) ‘Atmospheric Carbon Dioxide Concentrations over the Past 60 Million Years’, Nature, 406(6797), 695-699

Peteiro, C., & Freire, Ó. (2009) 'Effect of outplanting time on commercial cultivation of kelp Laminaria saccharina at the southern limit in the Atlantic coast, N.W. Spain', Chinese Journal of Oceanology and Limnology, 27(1), pp. 54–60. https://doi.org/10.1007/s00343-009-0054-7

Peng, Y., & Li, W. (2013) 'A bacterial pathogen infecting gametophytes of Saccharina japonica (Laminariales, Phaeophyceae)', Chinese Journal of Oceanology and Limnology, 31(2), pp. 366–373. https://doi.org/10.1007/s00343-013-2136-9

Pohnert, G. and Boland, W. (2002) ‘The oxylipin chemistry of attraction and defense in brown algae and diatoms’, Natural Product Reports, 19(1), pp. 108–122. doi: 10.1039/a806888g.

Ratcliff, J. J., Soler-Vila, A., Hanniffy, D., Johnson, M.P and Edwards, M. D. (2017) ‘Optimisation of kelp (Laminaria digitata) gametophyte growth and gametogenesis: effects of photoperiod and culture media’, Journal of Applied Phycology. Journal of Applied Phycology, 29(4), pp. 1–10. doi: 10.1007/s10811-017-1070-1.

Reed, D. C. (1990) 'The Effects of Variable Settlement and Early Competition on Patterns of Kelp Recruitment’, Ecology, 71(2), pp. 776 - 787, doi: 10.2307/1940329

Reed, D. C., Neushul, M. and Ebeling, A. W. (1991) ‘Role of Settlement Density on Gametophyte Growth and Reproduction in the Kelps Pterygophora Californica and Macrocystis Pyrifera (Phaeophyceae)’, Journal of Phycology, 27(3), pp. 361–366. doi: 10.1111/j.0022-3646.1991.00361.x.

Reddy, C. R. K.; Jha, B.; Fujita, Y.; Ohno, M 2008 'Seaweed micropropagation techniques and their potentials: An overview', Journal of Applied Phycology 20(5),pp. 609–617, https://doi.org/10.1007/s10811-007-9205-4

Redmond, S., Kim, J., Yarish, C., Pietrak, M., & Bricknell, I. (2014a) 'Culture of Sargassum in Korea', 1st ed, NOAA Sea Grant. Maine. 12 pp.

Redmond, S; Green, L; Yarish, C; Kim, J; Neefus, C, New England Seaweed Culture Handbook (2014b). Seaweed Cultivation.

Rorrer, G. L., Modrell, J., Zhi, C., Yoo, H. D., Nagle, D. N., & Gerwick, W. H. (1995) 'Bioreactor seaweed cell culture for production of bioactive oxylipins, Journal of Applied Phycology, 7(2), pp. 187–198. https://doi.org/10.1007/BF00693067

Sato, Y., Yamaguchi, M., Hirano, T., Fukunishi, N., Abe, T., & Kawano, S. (2017) 'Effect of water velocity on Undaria pinnatifida and Saccharina japonica growth in a novel tank system designed for macroalgae cultivation', Journal of Applied Phycology, https://doi.org/10.1007/s10811-016-1013-2

Santelices, B., Aedo, D., & Hoffmann, A. (2002) 'Banks of microscopic forms and survival to darkness of propagules and microscopic stages of macroalgae', Revista Chilena de Historia, 75, pp 547 – 555

Schoenrock, K. M., McHugh, T. A., & Krueger-Hadfield, S. A. (2020) 'Revisiting the 'bank of microscopic forms' in macroalgal-dominated ecosystems', Journal of Phycology, 57(1), pp. 14-29. https://doi.org/10.1111/jpy.13092

Shan, T. F., Pang, S. J., & Gao, S. Q. (2013) 'Novel means for variety breeding and sporeling production in the brown seaweed Undaria pinnatifida (Phaeophyceae): Crossing female gametophytes from parthenosporophytes with male gametophyte clones'. Phycological Research, 61(2), 154–161. https://doi.org/10.1111/pre.12014

Shan, T. F., Pang, S. J., Li, J., & Gao, S. Q. (2016) 'Breeding of an elite cultivar

haibao no. 1 of Undaria pinnatifida (phaeophyceae) through gametophyte clone crossing and consecutive selection', Journal of Applied Phycology, 28(4), pp. 2419–2426. https://doi.org/10.1007/s10811-015-0748-5

Singh, S. P., & Singh, P. (2015) 'Effect of temperature and light on the growth of algae species: A review', Renewable and Sustainable Energy Reviews, 50, pp. 431–444. https://doi.org/10.1016/j.rser.2015.05.024

Slobbe, E. van, H. J. de Vriend, S. Aarninkhof, K. Lulofs, M. de Vries, and P. Dircke (2013) 'Building with Nature: In Search of Resilient Storm Surge Protection Strategies', Natural Hazards, 65, no. 1 947–66. https://doi.org/10.1007/s11069-012-0342-y.

Speelman E; Barke, J, H Brinkhuis, and A F L Otter. (2009) 'The Eocene Arctic Azolla Bloom : Environmental Conditions , Productivity and Carbon Drawdown, pp.155–70. https://doi.org/10.1111/j.1472-4669.2009.00195.x.

Su, Li, Shao Jun Pang, Ti Feng Shan, and Xia Li (2017) 'Large-Scale Hatchery of the Kelp Saccharina Japonica: A Case Study Experience at Lvshun in Northern China', Journal of Applied Phycology 29, no. 6 3003–13. https://doi.org/10.1007/s10811-017-1154-y.

Suggett, D. J. et al. (2009) 'Interpretation of fast repetition rate (FRR) fluorescence: Signatures of phytoplankton community structure versus physiological state', Marine Ecology Progress Series, 376(1), pp. 1–19. doi: 10.3354/meps07830.

Tatsumi, M. (2018) 'Dynamic resilience and stability of Ecklonia radiata : the importance of density-dependent ecosystem engineering feedbacks', Ph.D. dissertation, University of Tasmania, 144 pp.

Team R Core (2018) 'R: A Language and Environment for Statistical Computing', Vienna, Austria, 0. Available at: https://www.r-project.org/.

Theuerkauf, Seth J. (2021) 'Habitat value of bivalve shellfish and seaweed aquaculture for fish and invertebrates: Pathways, synthesis and next

steps, Reviews in Aquaculture

Tzinis, I (2021). NASA. 'Technology readiness level'. available on https://www.nasa.gov/directorates/heo/scan/engineering/technology/technology_readiness_level (last accessed on 07 Aug 2021)

Umanzor, S., Li, Y., & Yarish, C (2021) 'Effect of direct "seeding" binders and embryonic sporophyte sizes on the development of the sugar kelp, Saccharina Latissima. Journal of applied phycolgy. 32(6). pp. 4137 - 4143. https://doi.org/10.1007/s10811-020-02277-z

Valero, M., Guillemin, M. L., Destombe, C., Jacquemin, B., Gachon, C. M. M., Badis, Y., Buschmann. A., Camus, C., Faugeron, S. (2017) 'Perspectives on domestication research for sustainable seaweed aquaculture', Perspectives in Phycology, 4(1), pp. 33–46. https://doi.org/10.1127/pip/2017/0066

Visch, W.; Rad-Menéndez, C.; Nylund, G. M.; Pavia, H.; Ryan, M. J.; Day, J (2019) 'Underpinning the Development of Seaweed Biotechnology: Cryopreservation of Brown Algae (Saccharina latissima) Gametophytes', Biopreservation and Biobanking 17(5), pp. 1–9. https://doi.org/10.1089/bio.2018.0147

Vodouhè, R., & Dansi, A. (2012) 'The "bringing into cultivation" phase of the plant domestication process and its contributions to in Situ conservation of genetic resources in Benin', The Scientific World Journal, vol. 2012, https://doi.org/10.1100/2012/176939

Wade, R., Augyte, S., Harden, M., Nuzhdin, S., Yarish, C., & Alberto, F. (2020) 'Macroalgal germplasm banking for conservation, food security, and industry', PLoS Biology, 18(2), pp. 1-10. https://doi.org/10.1371/journal.pbio.3000641

Westermeier, R., Patin, D., Piel, M. I., Maier, I., & Mueller, D. G. (2006) 'A new approach to kelp mariculture in Chile : production of free-floating sporophyte seedlings from gametophyte cultures of Lessonia trabeculata and Macrocystis pyrifera', Aquaculture Research, 37, pp. 164–171, https://dx.doi.org/10.1111/j.1365-2109.2005.01414.x

Wiencke, C., Roleda, M.Y., Gruber, A., Clayton, M.N and Bischof, K (2006) 'Susceptibility of zoospores to UV radiation determines upper depth distribution limit of Arctic kelps: Evidence through field experiments', Journal of Ecology, 94(2), pp. 455–463. doi:10.1111/j.1365-2745.2006.01102.x.

Wu, C.; Li, D.; Liu, H. Mass culture of Undaria gametophyte clones and their use in sporeling culture. Hydrobiologia 2004, 512, 153–156, https://doi.org/10.1023/B:HYDR.0000020321.73728.21

Xu, Bo, Quan Sheng Zhang, Shan Cun Qu, Yi Zhou Cong, and Xue Xi Tang (2009) 'Introduction of a Seedling Production Method Using Vegetative Gametophytes to the Commercial Farming of Laminaria in China', Journal of Applied Phycology, 21, no. 2 171–78. https://doi.org/10.1007/s10811-008-9347-z.

Zeder, M. A. (2015) 'Core questions in domestication research', Proceedings of the National Academy of Sciences of the United States of America, 112(11), pp. 3191–3198. https://doi.org/10.1073/pnas.1501711112

Zhang, Q. S., Qu, S. C., Cong, Y. Z., Luo, S. J., & Tang, X. X. (2008) 'High throughput culture and gametogenesis induction of Laminaria japonica gametophyte clones', Journal of Applied Phycology, 20(2), pp. 205–211. https://doi.org/10.1007/s10811-007-9220-5

Zhang, J., Wang, X., Yao, J., Li, Q., Liu, F., Yotsukura, N., Duan, D. (2017) 'Effect of domestication on the genetic diversity and structure of Saccharina japonica populations in China', Scientific Reports, 7(1),pp. 1-11. https://doi.org/10.1038/srep42158

Zhao, X. B., Pang, S.J., Liu, F., Shan, T.F., Li, J., Gao, S.Q and Kim, H.G (2016) 'Intraspecific crossing of Saccharina japonica using distantly related unialgal gametophytes benefits kelp farming by improving blade quality and productivity at Sanggou Bay, China', Journal of Applied Phycology, 28(1), pp. 449–455. doi: 10.1007/s10811-015-0597-2.