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Enhancing the restoration success of *Laminaria ochroleuca* through microbiome manipulation

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Summary

Laminaria ochroleuca, a kelp species currently suffering the effects of global climate change, has been found to shift northwards due to current temperature alleviations. This thesis addresses the possible influence of microbiome manipulations on heat stress mediation of these seaweeds. It is hereby part of the RestoreSeas project which aims at enhancing restoration success of not only seaweeds but also seagrass and deep sea corals. It was hypothesized that inoculation of bacteria from adult individuals after axenization of gametophytes would induce gametogenesis, defined as the formation of sporophytes and used as a proxy for recruitment success. Microbiome composition of these adults were expected to depend on the thallus region (holdfast, meristem and blade) which would generate a different response as to the enhancement of recruitment success given their differential functional requirements.

For this purpose, adult *L. ochroleuca* individuals were sampled from France, UK, Portugal and Morocco. Per individual and location (France vs UK), swabs were taken from holdfast, meristem and blades and their microbiome composition was sequenced to gain insight with regards to the latitudinal effect on microbiome composition and the effect of thallus region. Zoospores were isolated from these adults and their growth and recruitment success was monitored under several culture conditions. Gametophytes were eventually sterilized using an antibiotic mix. Bacterial inoculation treatments were then made per thallus regions and contained bacteria from locations Morocco, England and France in equal amounts. Recruitment success was quantified in sporophyte counts after bacterial inoculation. These sporophyte counts were compared with the ones resulting from a control treatment containing environmental bacteria from seawater from the North Sea. Finally, gametophyte health and growth was quantified in comparison to control treatments (without bacterial inocula) during heatwave administrations with maximum temperatures of 16 °C, 18°C and 22 °C.

The key findings of this thesis are that microbiome composition of adult *L. ochroleuca* depends on location and thallus region. Inoculation treatments administered after bacterial isolation from adult tissue samples harbour a low diversity due to possible methodology bias, however bacterial isolates still show a significantly positive effect on recruitment success after axenization. This is however not significantly different per thallus inocula treatment. During heatwave administration, the introduction of bacterial inocula after axenization resulted in a seemingly positive trend towards promoting health of gametophytes. These results suggest that bacterial inoculation and more specifically the functions they provide enhance recruitment success and show promising effects for future implementations.

Introduction

Seaweeds are the dominant primary producers of coastal ecosystems around the world. They are often referred to as ecosystem engineers (Miller et al., 2018; Teagle et al., 2017) because of their pivotal contribution to nutrient cycling, carbon storage, stabilization of sediments (Krause-Jensen & Duarte, 2016) primary productivity, light level alteration (Wernberg et al., 2005), waterflow (Rosman et al., 2007) and protection against physical disturbances such as storms (Connell, 2003). Some marine organisms exclusively depend on the nursery grounds, protection and food that seaweed dominated ecosystems provide. Larger seaweed species such as kelp (brown algae belonging to the order *Laminariales*) can grow to significant heights and thus form gigantic kelp forests and canopies. Their sizes are essential for harbouring shoreline fish species, crustaceans and invertebrates while their blades, meristem and holdfast are host to many micro-organisms. (Lemay et al., 2021). Therefore, they highly contribute to the wide diversity in coastal marine ecosystems (Smale et al., 2015).

Seaweed and kelp cultivation is commercially important due to their significance in food production (e.g. kombu), colloids for the food industry (alginates used in candy, processed meat, food supplements…) (Saha, Barboza, et al., 2020) use in health care products (tooth paste, cosmetics, nutraceuticals) and bioplastics. Volume-wise commercial seaweed cultivation is currently the largest sector in aquaculture (J. Li, Majzoub, et al., 2022). However, there are many factors that could interfere with their growth, reproduction and defence against diseases. Not only poor or substandard culture conditions but also external stressors can make seaweed cultivation extremely difficult for commercial applications or in restoration efforts (Eriksson et al., 2002; Gorman & Connell, 2009; J. Li, Majzoub, et al., 2022; Österblom et al., 2007). External stressors range from direct anthropogenic disturbances such as eutrophication, fisheries, pollution and habitat alteration (Connell & Irving, 2008; Gorman & Connell, 2009; Österblom et al., 2007; Smale et al., 2015) to indirect anthropogenic disturbances such as climate change induced heat waves (Dayton & Tegner, 1984; Provost et al., 2017; Wernberg et al., 2013), acidification (Provost et al., 2017; Qiu et al., 2019) and storms intensifications (Dayton & Tegner, 1984; Wernberg et al., 2010). These effects could result in lack of productivity or a complete loss of the seaweed populations (Li et al., 2022).

Kelp canopy growth and threats

Kelp grows along rocky shores from the upper subtidal to several tens of meters below the surface in temperate cold seas. Their obligate heteromorphic life cycle consists of a macroscopic phase, with sporophytes growing up to 50 meters in hight, and a microscopic phase that begins after sporulation. Zoospores grow into male and female gametophytes with respective formation of antheridia and oogonia. They fertilize each other forming zygotes that become new microscopic and consequently macroscopic sporophytes (Lüning, 1980)(Figure 1). The microscopic stages of kelps lifecycle are very sensitive to temperature changes, physical disturbances and light (Biskup et al., 2014; Izquierdo et al., 2002). Warming can therefore have a deteriorating effect on kelp canopies and these species' growth and health (Qiu et al., 2019).

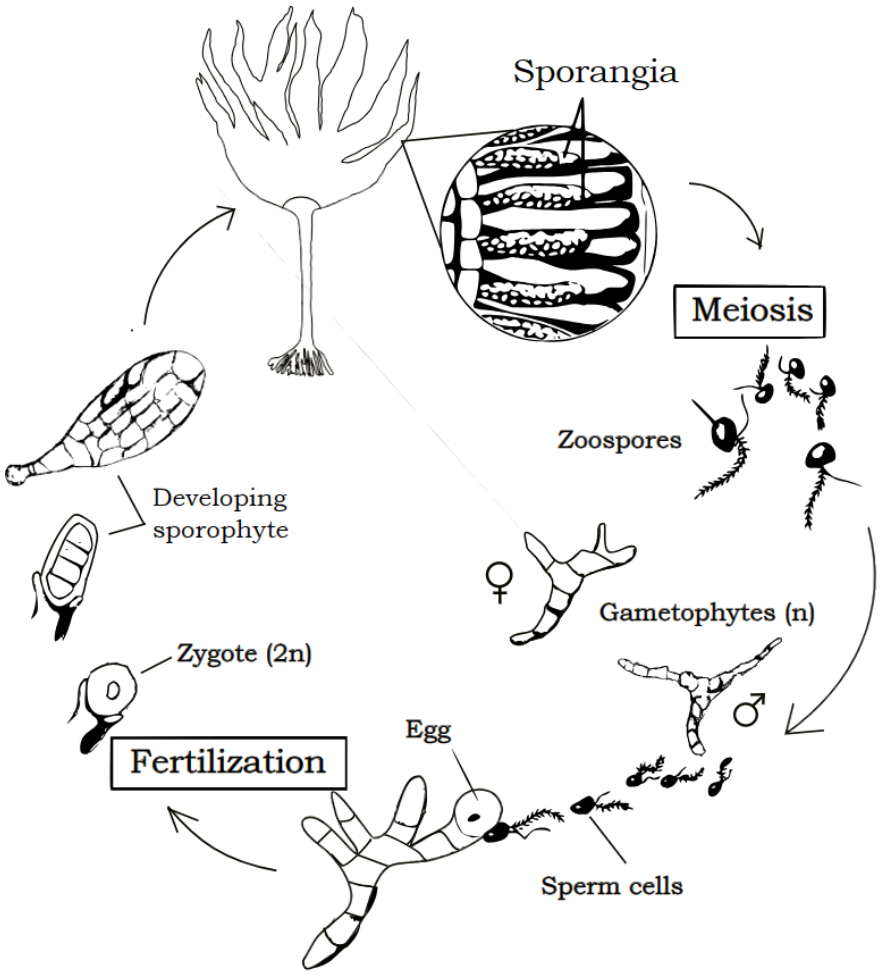


Figure 1: Life cycle of the genus Laminaria, adapted from figure by Pearson Benjamin Cummings (Campbell et al., 2008).

The effect that global temperature has on the aforementioned life stages could be expected to vary regionally. However, a general decrease of recruitment success has been observed in kelp taxa from different regions across the eastern Pacific when exposed to higher temperatures. Sporophyte counts were compared for temperatures 12 and 18 degrees and it was observed that when recruitment failure occurred, it was at 18 degrees for all regions (Muth et al., 2019). However, not only recruitment but also growth and stability of adult kelp can be affected by high temperatures, especially when exposed for a longer time, for example during heat waves. Bull kelp (*Durvillaea poha*) was found locally extinct after a marine heatwave in the summer of 2017 in New Zealand (Thomsen et al., 2019). Furthermore, long-lasting heat waves such as El Niño episodes have been mentioned to deprive kelp species of available nitrate as nutrient concentrations correlate negatively with temperature (Dayton et al., 1992; Dayton & Tegner, 1984; G. A. Jackson, 1977; Zimmerman & Kremer, 1984). Dominant kelp species, such as *Macrocystis pyrifera*, can initially maintain their growth in these nutrient-limited conditions because of internal nitrogen reserves. However, when the heat endures for more than a month, there would be extreme nitrogen deprivation followed by a weakening of their tissues and canopy declines as a result (Dayton & Tegner, 1984).

Some kelp species can adjust their metabolic processes to reduce sensitivity to temperature changes. These metabolic alterations have however been observed to reduce the capacity to respond to other disturbances (Wernberg et al., 2010). When exposed too long, this could

mean that kelp species lose their overall strength in the ecosystem. In other words, kelp forests that are situated in their distribution's low-latitude margins will have to adapt in order to overcome these pressures (Provost et al., 2017) or will be forced to migrate northwards. Higher ocean temperature has already led to a redistribution of kelp species from temperate to northern regions (Smale et al., 2015).

Another direct stressor currently threatening kelp canopies is eutrophication which causes local acidification of coastal waters due to enhanced growth of algae followed by anaerobic microbial breakdown which create anoxic conditions and produce acidic compounds. This causes reduction of fecundity, germination and reproductive success in early-stage kelp individuals such as *Saccharina japonica* (Xu et al., 2015, 2019) and promotes the growth of less complex and less productive inhabitants (Qiu et al., 2019) such as turf-forming algae which have a faster uptake of nutrients and carbon in polluted waters (Connell et al., 2013; Gorman & Connell, 2009; Provost et al., 2017; Wernberg et al., 2010). This has a negative effect on kelp growth, especially in the presence of consumers (Provost et al., 2017).

Mesocosm experiments with seagrass (*Zostera marina*) and seaweed canopies *(Fucus vesicolosus, Ecklonia radiata*) (Provost et al., 2017; Qiu et al., 2019; Saha et al., 2020), have noted that acidification and temperature change cause indirect negative effects because of changes in key consumers such as urchins. These studies showed that seaweed growth is reduced (J. Li, Majzoub, et al., 2022; Provost et al., 2017; Qiu et al., 2019) and growth of the consumers increases as well as their consumptive rates. This leads to a possible shift towards grazer dominated habitats such as sea urchin barrens (Wernberg et al., 2010). Changes in nitrate concentrations and salinity have also been investigated as a dual stressor with temperature change. Salinity has shown an impact on response to temperature changes, however, results suggested that temperature is the main factor that impact recruitment in kelp species (Lind & Konar, 2017; Muth et al., 2019).

Restoration efforts to minimize canopy decline

To recover from large disturbances resulting in the loss of entire kelp canopies, new recruits must establish that are adapted to canopy-free conditions. Toohey & Kendrick (2007) observed that recovery of *Ecklonia radiata* is not guaranteed and depends on different factors such as photo acclimation capacity of the new recruits, the timing of the canopy loss and the presence of competitors. Restoration of kelp forests after it has been disturbed is therefore not trivial and requires a multifaceted approach (Toohey & Kendrick, 2007).

Previous restoration efforts have ranged from salvaging pre-existing juveniles to transplanting donor plants into degraded reefs (Alsuwaiyan et al., 2022). Next to this, removal of algal turfs and mitigating populations of grazers have also been proven effective in restoration of kelp canopies (Gorman & Connell, 2009; Steneck et al., 2013).

Transplanting donor plants however provides its own challenges. In theory, kelp gametophytes from spores are easy to release in the laboratory (Alsuwaiyan et al., 2022), but it is very challenging to maintain their growth once planted in situ since they have been reared under laboratory conditions. Seedlings must survive stressors of the environment such as storms and waves when they are planted. This creates difficulties, especially when the kelp is planted into reefs with suboptimal conditions (Vanderklift et al., 2020). However, there is a way to up-scale degraded areas with low costs, using the "green gravel" approach for adhesion to the

substrate. Several types of substrates can be used (Alsuwaiyan et al., 2022). Increasing survival of introduced kelp sporophytes is an essential step in the restoration efforts that warrant further research if we are to restore kelp forests efficiently.

Kelp as a symbiont

Kelp restoration approaches have thus far often evolved around the kelp species themselves and their interactions with other seaweeds and grazers. However, seaweeds have a close association with the bacteria living on their surface. Some provide important defence systems against diseases, others help with their growth and improve their survival under environmentally stressful conditions (Li et al., 2022). Insight into this relationship and the potential of manipulating the associated bacteria for stress mitigation might enhance restoration strategies.

Host associated microorganisms are usually referred to as the microbiome of the host. The microbiome has been widely studied and proven important for an organism's health and metabolism (Hitch et al.,2022). It is assumed that a microbiome with maintained diversity is better for its host because of the functions particular taxa contribute.

Aquatic autotrophic organisms especially rely on their symbionts because of the contribution to their primary productivity or the supplementation of their nutrients and help in food digestion. The micro-organisms in turn receive protection from the host and are supplemented with inorganic nutrients (Gordon & Leggat, 2010). The uptake of certain nutrients and the supply of resources usually not accessible for the host is one of the many advantages phototrophs in particular have from this symbiosis (Stock et al., 2021).

Host-bacterial associations have been found to be under influence of anthropogenic disturbances however. The host's associated microbiota can be directly influenced by changing temperature (Stock et al., 2021). Alpha diversity may change, which can result in a decrease or loss of certain indispensable bacteria or an increase of opportunistic pathogens. Temperature can also indirectly affect microbiome composition because of a change in the phenotype of the host (tadpoles: (Kohl & Yahn, 2016), mussels: (Li et al., 2018), amphibians: (Fontaine et al., 2018)). The composition of associated micro-organisms in algae has also been found to alter due to anthropogenic disturbances. (Stratil et al., 2013; Webster et al., 2011) The microbiome of *Ecklonia radiata,* for example*,* was altered due to ocean warming (decrease of *Bacteroidetes* and *Alphaproteobacteria*) and acidification (increase of *Aquimarina*), which reduced the photosynthetic efficiency and resulted in a higher sensitivity to pathogens (Qiu et al., 2019).

Microbiome manipulations

It can be expected that manipulating the composition of seaweed microbiota can be beneficial for restoration strategies, although there is a significant knowledge gap in that aspect. Hitch et al. (2022) mentions the different ways a person's gut microbiome can be improved such as the use of faecal microbiota transplantations, faecal filtrate transplants, a change in diet, fermented foods, prebiotics and probiotics. These are all aimed at enhancing taxa with beneficial functions to protect and nourish the gut. Gut microbiome transplantations have also been performed on

Daphnia magna to investigate the response to toxic cyanobacteria exposure. The gut microbiota of *Daphnia magna* that were tolerant to cyanobacteria exposure were reciprocally transplanted with susceptible individuals. It was observed that all recipients then showed a similar response when exposed to toxic cyanobacteria. This suggests that the gut microbiota plays a crucial role in response to cyanobacteria and that gut transplants can prove very efficient in improving a genotype's fitness (Macke et al., 2017).

Aside from gut microbiome enhancements in invertebrates and humans, changing the microbiome composition in early life stages of autotrophs could have long-lasting impacts on the adult stages and fitness as well, as has been observed in terrestrial plants and corals (Barret et al., 2015; Damjanovic et al., 2019; Walsh et al., 2021) A study on *S. japonica* (Han et al., 2021) shows that microbiota significantly differ between life stages and that the juvenile life stage has the highest abundance of metabolic functions in their microbiome. This emphasizes the significance of preserving a diverse microbiota composition in the nursery stage for disease prevention in commercially cultivated seaweeds. The effects of microbiome manipulations on early life stages has however not been extensively researched on kelp species (Davis et al., 2023).

Microbiome manipulations can entail transplantations as mentioned before, but adding to the diversity of the microbiome could also be a viable option for enhancement of restoration success. Herrmann et al., (2022) and Schütz et al., (2018) observed a 10-30% increase in crop yield by using Plant Growth-Promoting Microorganisms (PGPM's) (Li et al., 2022). The question remains if knowledge about microbiome manipulations in the gut microbiome of invertebrates and humans and probiotics on terrestrial plants are comparable to what can be done for marine autotrophs such as kelp. Therefore it is important to start by highlighting the differences between terrestrial and freshwater aquatic systems on one hand and marine ecosystems on the other. According to a paper by Dittami et al. (2021) there are some fundamental differences between terrestrial and aquatic systems in general. Water has a higher chemical connectivity and signalling between organisms and carbon fluxes seem to be more rapid. Since dispersal barriers are therefore lower, there can be a faster microbial community shift (Burgess et al., 2016; Houwenhuyse et al., 2021; Kinlan & Gaines, 2003; Martin-Platero et al., 2018). Secondly, there appears to be a higher phylogenetic diversity in aquatic ecosystems, especially when it comes to marine pathogens (Dittami et al., 2021). It can be concluded that microbiome composition of aquatic organisms in general are thus more susceptible to temporal and spatial effects than in terrestrial hosts. Aquatic (marine and freshwater) microbiome composition can therefore be seen as a "snapshot" in time and space which is less the case for terrestrial plants and animals. There are some significant differences between saltwater and freshwater however, since salinity is a major controlling factor of the composition of microbiota in aquatic systems (Wu et al., 2006) which also translates to changes in the gut microbiome of Atlantic salmon (Rudi et al., 2018) Restoration strategies coming from terrestrial and freshwater systems are therefore to be taken with some necessary caution, as these systems have some considerable differences. Microbiome manipulations or diversity enhancement are thus deemed to be an interesting tool for strengthening of an organisms fitness, but applications in marine ecosystems and kelp in particular are still underrepresented in current literature (Hitch et al., 2022).

Microbiome enhancement in Laminaria ochroleuca

Many opportunities arise for using microbiome manipulations based on typically researched seaweed species such as *Ecklonia radiata, Macrocystis poryfira, Dellisea pulchra* and *Fucus vesiculosus*. However, microbiome manipulation with regard to restoration approaches has not been investigated for most kelp species.

One of these underrepresented kelp species is *Laminaria ochroleuca*, a species originally from warm-temperate regions distributed from Morocco to Southern England but its range is currently shifting from southern coasts to more northern coasts (Smale et al., 2015; Blight & Thompson, 2008; Wright & Foggo, 2021). Loss of *Laminaria ochrolecua* in its original habitat is however unacceptable. It is the only species of the genus *Laminaria* able to live in warm and temperate regions in the northern hemisphere (Dieck, 1992; Izquierdo et al., 2002). Research on microbial composition of *L. ochroleuca* is not very extensive. However its microbiome appears to contain Actinobacteria which are said to have antimicrobial activities and could even aid in the struggle against cancer. These actinobacterial strains were found residing on the macroalgal species and were associated with the genera *Rhodococcus, Nocardiopsis, Microbispora, Microbacterium, Isoptericola* and *Nonomuraeae* (Girão et al., 2019).

This thesis investigated the potential to enhance the restoration success of *Laminaria ochroleuca* through the use of microbiome manipulations. It is part of the RestoreSeas Project, a European Biodiversa+ project focussed on restoring seagrass, seaweed and deep-sea corals. Partners include Ghent university (BE) and the Centre of Marine Sciences (CCMAR: university of Algarve; PT). The aim of this thesis is to design potential probiotics composed of bacteria that have advantageous microbial traits to protect juvenile *L. ochroleuca* sporophytes against the effects of climate change in the form of heat stress. For the purpose of this thesis, *L. ochroleuca* is sampled in several locations: 1) Normandy in Northern France; 2) Plymouth in Southern UK; 3) Faro in Portugal and 4) Morocco. Their bacterial diversity is assessed using 16S rRNA gene amplicon sequencing. Bacteria are isolated from these individuals and their genomes are sequenced. The effect of the isolates on growth and development of the seaweed is assessed under various conditions.

We characterise the bacterial community on different thallus regions for each *L. ochroleuca* collected specimen: holdfast, meristem, blade. It is hypothesized that the bacterial community will differ between the thallus regions (holdfast, meristem, blade) and that this is connected to their function. We base our assumptions on various earlier studies. In *Laminaria digitata,* there was a clear difference in microbial diversity between holdfast, blade and meristem. Although holdfast and blade tissues were more distinct while meristem and stipe shared more similarities (Ihua et al., 2020). Lemay et al. (2021) reached similar conclusions in *Laminaria setchelli* but attributed the difference in diversity to age of the tissue structures. It is said that the holdfast and the blade tips are older thus harbour a greater richness in microbiota compared to the younger meristem regions (Lemay et al., 2021). Aside from tissue age, functional requirements also differ between thallus regions as stated in the “Competitive Lottery hypothesis”, described by Burke et al (2011). This theory states that bacterial recruitment and thus microbiome composition is dependent on both stochastic factors as well as deterministic aspects, such as the functional requirements each thallus region requires. These functional requirements are therefore also expected to differ. Holdfast tissue, for example is expected to be characterized by bacteria that aid in the uptake of phosphorus, carbon and nitrogen from the substrate (Ranjan et al., 2015). Subsequently, given the photosynthetic capacity, aerobic nitrogen-fixing

bacteria are expected on the blades (Weigel & Pfister, 2021). Lastly, since the meristem is the active growthregion in the seaweed (Lemay et al., 2021), bacterial functionalities are expected to promote cell division and production of growth factors. Given these differences, the thallus inocula treatments are expected to vary in their effect on recruitment of juveniles during heat stress exposure.

It is to be expected that microbiome diversity will also shift across latitudinal gradients. Proof has already been provided that there is regional structuring across spatial scales of hundred kilometres and even variation between individuals ten meters apart of *Laminaria hyperborea* and *Saccharina latissima* (King et al., 2022). It is therefore expected that the diversity of the microbiome will vary between the sampled populations. Moreover, it is hypothesized that this difference between northern and southern populations can be partially explained by the presence of heat stress mediating bacteria in southern populations.

One of the major challenges of this research is selecting for the bacteria that we want to isolate and use for the inocula treatments. This is complicated by large temporal and spatial variation that can be expected to occur within the microbiome (King et al., 2022). Because as said before, marine ecosystems have lower dispersal barriers so seaweeds and their microbiome can act as vectors for spreading some very spatially restricted taxa of microorganisms. They can also extend their metabolic abilities which strengthens their flexibility in changing environments (Dittami et al., 2021). Since the microbiome is a “snapshot” dependent on the environmental conditions (Stock et al., 2021), it is not only difficult to predict which bacteria will be available but also what function they will provide and if the interaction with other bacteria is not antagonistic (Ludington, 2022). Studies on *Laminaria digitata* (Izquierdo et al., 2002), *Laminaria hyperborea* (Bengtsson et al., 2012) and other green and red seaweed species (del Olmo et al., 2018; Holmström & Kjelleberg, 2006; Singh & Reddy, 2014) can give some insight into which bacteria could be found and which function they most likely will have.

The most likely genera found, based on an abundance study on *Laminaria digitata* (Izquierdo et al., 2002) are *Blastopirellula, Granulosicoccus*, *Psychromonas, Roseobacter, Aquimarina, Bacillus, Psychrobacter, Vibrio*, … and functions range from formation of biofilms (*Psychromonas, Planctomycetes*), polymer degradation and catalase production (*Pseudoalteromonas*), nitrogen uptake (*Azotobacter, Rhizobium, Agrobacterium*) and vitamin B12 production (*Ectocarpus*) to auxin production (*Exiguobacterium*), morphogenesis-induction (*Marinomonas, Bacillus, Cytophaga, Caulobacter*), antibacterial activity (*Actinobacteria*), enhancing cell division (*Roseobacter*) and enlargement and stretching of algal cells (*Maribacter*) (Izquierdo et al., 2002; del Olmo et al., 2018; Holmström & Kjelleberg, 2006; Singh & Reddy, 2014;Bengtsson et al., 2012). We will focus on functions that might provide protection against global temperature change and specifically enhance growth and recruitment success. It is hypothesized that isolated bacteria from the adult individuals will enhance growth and recruitment success of *L. ochroleuca* during heat stress and that their origin (being thallus region and location) will show differential effects due to functional and composition differences.

Major challenge in this research

It is important to take into account that the microbiome can also consist of disadvantageous bacteria, bacteria that have interactions with each other (Ludington, 2022) which could therefore alter functionality and bacteria that have a defensive function but provide negative

side effects (G. Wang et al., 2019). Since the aim is to test which specific taxa are truly beneficial for protection against temperature change, the seaweed juveniles are made axenic before administering the bacterial inocula. This is not without health costs and a right amount of care is to be taken (Jones et al., 1973) as administering antibiotics to *Ulva sp*. has been found to change its crucial morphology (Spoerner et al, 2012). Methods for sterilizing were investigated by Jones et al. (1973) where four classes of micro-algae were exposed to a mix of four different antibiotics. Since every antibiotic has a specific way of working, a mixture appeared to be more effective than a single antibiotic. Since the sterilisation success and effects of it differed for every class of micro-algae and was dependent on which antibiotics were used, it is to be expected that different types of antibiotics will provide different results. Therefore, different types of antibiotics will be tested for their effect on bleaching and biomass growth on *Laminaria ochroleuca.*

Objectives

In summary, the main objective of this thesis is to test the potential for microbial manipulations on the kelp species *Laminaria ochroleuca* to enhance its restoration success during temperature alleviations. To achieve this, several research questions are posed.

First, we assess if thallus region (blade, meristem and holdfast) and location (France and England) have an effect on the bacterial community composition of kelp individuals from the East Atlantic Ocean's coast. To achieve this, the microbial composition of thallus regions for locations France and England will be characterised using 16S amplicon sequencing. We expect that more heat stress mediating bacteria will be found on southern individuals in comparison to northern individuals and that thallus regions have differential functional requirements, which translates to different microbiome compositions (Burke et al., 2011, Morrissey et al., 2019).

Secondly, given the potential of certain bacteria inhibiting growth of beneficial bacteria on adult offspring, it is assessed if the gametophytes can be sterilized without affecting their normal growth and health. For this purpose, several antibiotics are compared for their acute and long-term effect on the growth and photosynthetic yield of the gametophytes. It is expected that complete sterilization will be difficult to obtain as antibiotics might also kill bacteria that are crucial for normal morphogenesis (Jones, 1973, Spoerner et al., 2012).

Thirdly, we investigate if bacteria from the adults can have a positive influence on recruitment success of sterilized gametophytes after inoculation and if this effect depends on the thallus regions the bacteria are isolated from. Therefore, normal gametogenesis is assessed and induced by tweaking the culture conditions after axenization and eventually, three types of bacterial inocula treatments are provided: from the holdfast, the blade and the meristem. It is expected that they will positively affect gametogenesis, in comparison to control treatments, given their functional contribution to the adults and that these effects might differ between treatments given the expected differential requirements of thallus regions (Burke et al., 2011, Morrissey et al., 2019).

Finally, if gametogenesis is induced by bacterial inoculation, it is studied if this is also the case during heat wave administrations. Therefore, the thallus treatments are used prior to administration of 16°C, 18°C and 22°C heatwaves. The results from this will then provide possible implementations of future restoration strategies and knowledge on probiotic treatments for cultivation of kelp.

Materials and methods

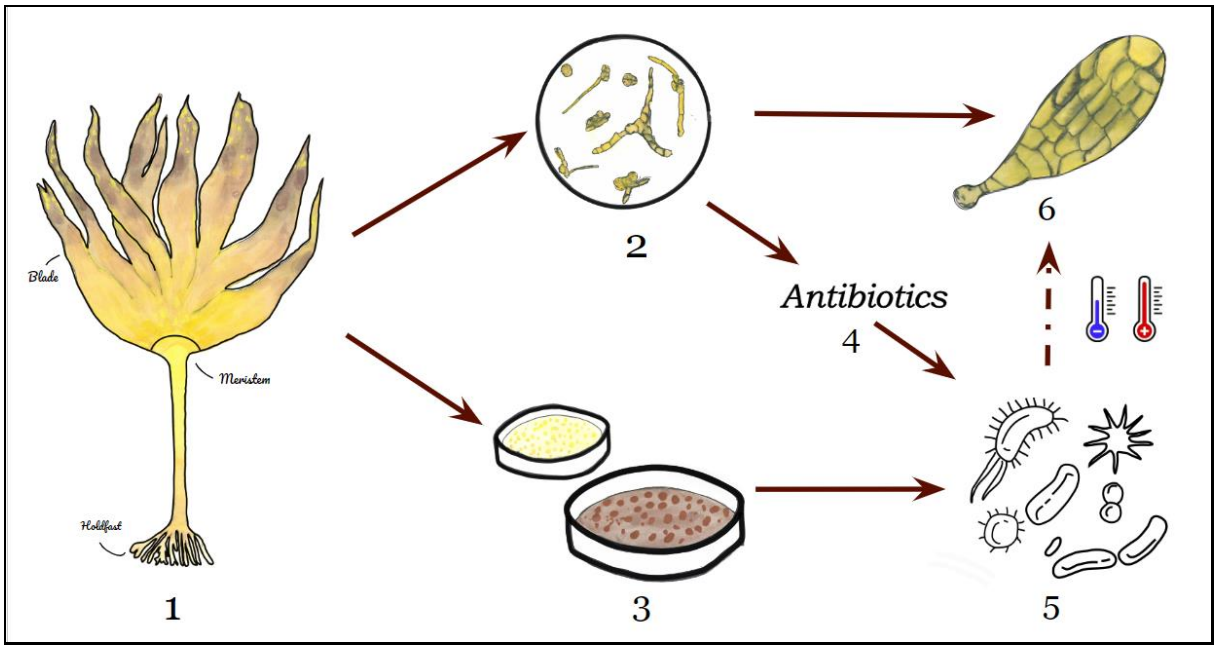


Figure 2: Flowchart for schematic representation of the methods. 1: Adult individuals swabbed and sampled from holdfast, meristem and blade, sori sampled and allowed to sporulate under laboratory conditions; 2: Gametophyte growth monitored under microscope; 3: Isolation of bacteria from holdfast, meristem and blade, grown on marine agar and kelp agar; 4: Administration of antibiotics for axenization; 5: Inoculation with bacterial isolates; 6: Monitoring of gametogenesis (transitioning to sporophytes) with inoculated bacteria under heat stress or normal laboratory conditions.

Collection of adult individuals

To initiate the workflow as schematically represented in Figure 2, individuals were sampled in Normandy, France (W. Stock, E. Gouwy and J. Knoop) , Morocco (S. Kaidi, Z. Belattmania, B. Sabour)), Faro, Portugal (F. Petrucci, A. Engelen)) and England, united Kingdom (J. Knoop). Rocky shores were visited at low tide to search for adult *L. ochroleuca* with clearly visible sori that had not released yet (with smoother/cleaner leaves, preferably no pronounced tissue lysis and with clear dark brown sori without epiphyte growth). For example, in Normandy, eleven individuals were sampled from cap Lévi and processed at the site. Swabs were taken from holdfast, meristem and blades for 30 seconds and stored in microcentrifuge tubes with RNAlater stabilization solution (Thermofisher, 1.4 mL). The swabs from the blades were taken below raffled tissue. Tissue samples of roughly the same size from holdfast, meristem (the moonshape and a bit of the stem), blades (without spores, just under already sporulated parts) and sori were taken and conserved in zip lock bags with locally collected seawater and transported back to the lab in chilled conditions to prevent tissue degradation.

Preparation of gametophytes

Monitoring of gametophyte growth towards new individuals initiated with cleaning of tissue samples from sori and allowing them to sporulate. The cleanest sori were selected and brushed in 0.5 % KI to disinfect the tissue. Sterilized seawater was used to wash the sori after the KI treatment. This was repeated two times. Material was processed with heat sterilized tweezers

and wiped down with paper towels in between steps. Finally, the sori were stored in paper towels drenched in sterilized seawater to stimulate spore release (samples were labelled from A to K) during preservation overnight. Spores were released when the sori were transferred to 15 °C sea water the next day. The sori were removed after one hour. Sporulation was observed by a small “cloud” formation. The spores were kept in sterilized seawater with (½) Provasoli-Enriched Seawater (PES) (10mL/l) and Germanium oxide (1mL/l) to remove diatoms. Diatoms’ main cell wall component is silica, Germanium oxide replaced this silica with Germanium during administration, successfully killing the diatoms. The spores were incubated at 16°C in a Lovibond incubator with red light to inhibit fertilization of gametophytes and refreshed every month with ½ PES medium. Individual gametophytes were isolated for long term stock maintenance.

The extraction and isolation of bacterial strains from adult samples

Bacterial isolation for later probiotic treatments of the new individuals was restricted to the kelp specimens for which a successful spore release had been observed (e.g. for England these were individuals A, B, C, F, K, M, N and O). The tissue samples from blade, meristem and holdfast were rinsed with sterile seawater. A piece of similar size was taken for each and loosely attached bacteria were removed by shaking the piece in sterile seawater. The samples were then vortexed in a tube that contained glass beads and seawater, to dislodge the remaining bacteria from the algal tissue. A volume of 50 microliter was used from this aforementioned tube to isolate bacteria. The volume was spread on nutrient rich agar (BD Difco) for fast growing bacteria and a petri dish with kelp agar (100 grams of blended Saccharina japonica/L with fragments >1 mm removed in 1% agar (Sigma-aldrich). Plates were incubated at 18 °C with indirect light at a 12:2 light regime. From each agar type, two bacterial colonies were picked and streaked on marine agar after two to three days of growth, this is depicted in Figure 3. For the Normandy region, the bacteria from eight different individuals and on three different thallus regions were isolated on two types of agar. From the agar plates, a pair of colonies were handpicked for isolation purposes. This therefore entailed handpicking a total of 96 colonies from this region. In addition, supplementary strains were handpicked which showed distinct morphologies. The isolates were streaked on marine agar and the process was repeated after three days to keep them monoclonal. Bacterial isolates, which were deemed monoclonal based on morphological assessments, were cryo-preserved in pre-autoclaved 2 mL microcentrifuge tubes filled with 30 % glycerol (Sigma-aldrich) in marine broth (Millipore) at -20°C. The viability of cryopreserved bacteria were checked by random plating of 15 cryopreserved isolates onto marine agar. Out of the 15 isolates that were plated, all but one thrived, resulting in confirmation of 14 successful cultivations.

Nomenclature of bacterial isolates

Every isolated bacteria was named according to its location, individual, origin on the individual, type of agar on which it grew and number of isolate. Locations England, Morocco, Normandy and Faro were abbreviated to ENG, MAR, NOR and FAR respectively. Thallus regions were given a consecutive number where holdfast equals 1, meristem equals 2 and blade equals 3. Marine agar was referred to as M, kelp agar was referred to as S. According to this, a bacteria originally from England isolated from the blade of individual A, manually selected from marine agar and cultured on a marine agar plate (number 1) was named ENG A3 M1.

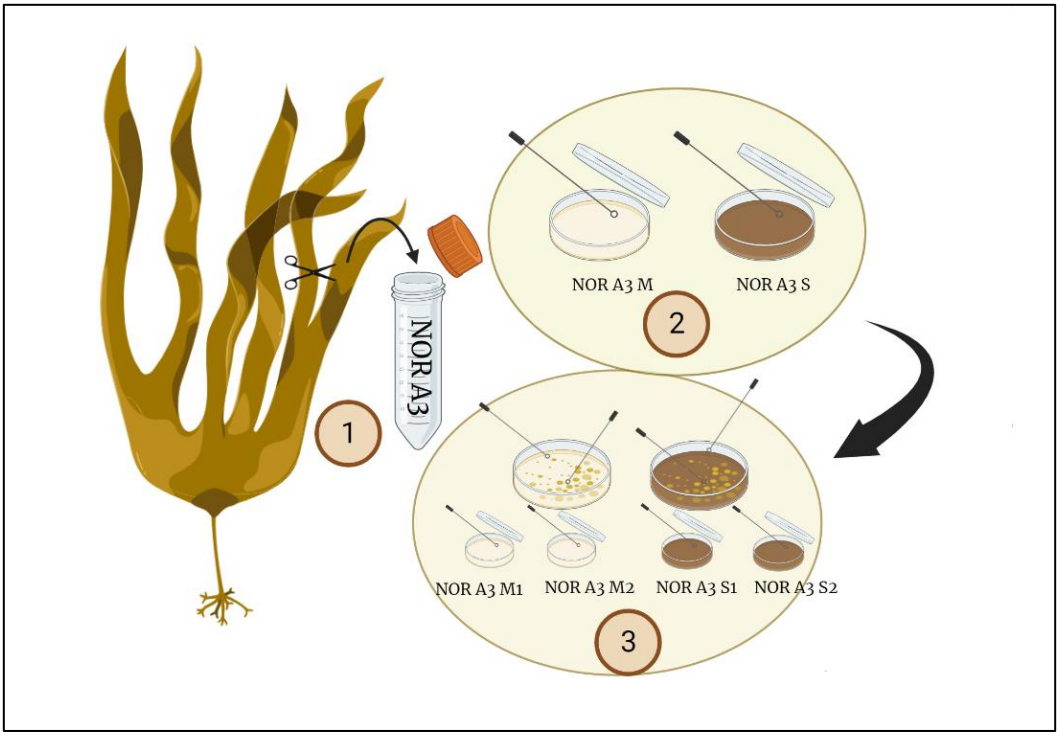


Figure 3: Schematic representation of bacterial isolation (created in BioRender).

Full-length 16S rRNA gene high throughput amplicon sequencing of swabs

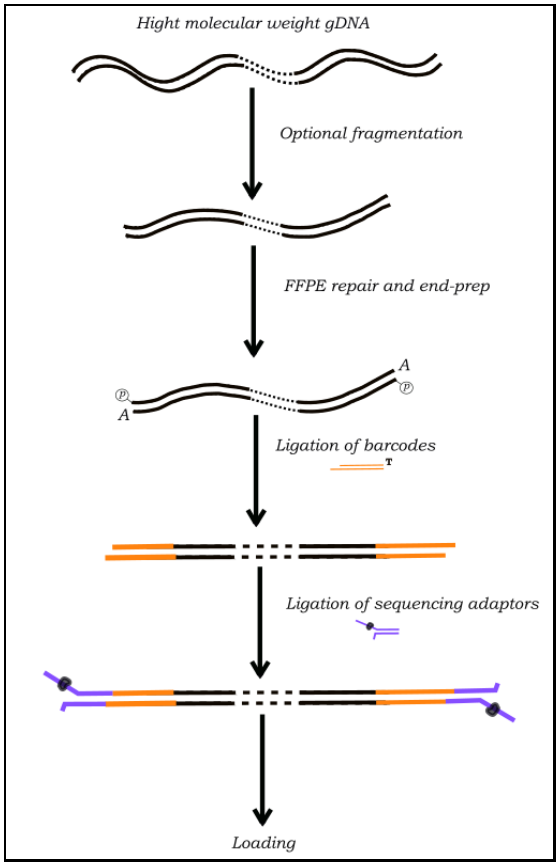


Figure 4: Schematic representation of barcoding and sequencing of 16S rRNA. Adapted from the Native Barcoding Expansion and Ligation Sequencing Kit protocol (Oxford Nanopore Technology).

As it is the intention to obtain an idea of the overall biodiversity of the microbiome of holdfast, meristem and blades for the different locations of sampling, a 16S rRNA gene high throughput amplicon sequencing was performed on the swabs collected at the sites (Normandy, Plymouth, Pharo and Morocco). For this, the Native Barcoding Expansion 1-12 (EXP-NBD104) and 13/24 (EXP-NBD114) and the Ligation Sequencing Kit (SQK-LSK109) was used. First, DNA extraction was performed on these swabs using the Qiagen PowerSoil kit which included a negative extraction control and a positive control (ATCC microbial standard MSA-1002). After DNA extraction, length, quantity and purity of 1ug of high molecular DNA was checked according to the DNA/RNA QC protocol, using agarose gel electrophoresis and a PCR was done using primers: 1) 27F_BCtail-FW (with sequence TTTCTGTTGGTGCTGATATTGC_AGAGTTTGATCMTGGCTCAG) and 1492R_BCtail-RV (with sequence ACTTGCCTGTCGCTCTATCTTC_CGGTTACCTTGTTACGACTT). These primers both contained a 5' extension which allows for barcoding by PCR. The PCR was performed with the Phire Tissue direct PCR Master Mix from Thermo Fisher with a following cycle: 1) Three minutes at 98 degrees 2) 30 eight-second cycles 98 degrees, 3) an eight second cycle at 60 degrees and 4) a 30 second cycle at 72 degrees. The final extension was done at 72 degrees for three minutes. Then an Oxford Nanopore Sequencing was performed which included a DNA end repair using the NEBNext FFPE DNA Repair Mix and NEBNext ultra II ENd repair/ dA-tailing Module reagents and attachment of adapters (Figure 4). Native barcodes and sequencing adapters (Adapter Mix II Expansion use) supplied in the kit were ligated to the DNA ends, this created a DNA library which could be primed and loaded onto a SpotON flow cell using the Flow Cell Priming Kit (EXP-FLP002), Loading Breads and a Sequencing Buffer. Using the MinKNOW software, a sequencing run was initiated to collect data and transfer it to base-called reads. The EPI2ME software was subsequently applied to select the barcoding workflow.

Visualisation and analysis of the nanopore data

Statistical analysis was performed in R version 4.2.1. The reads from the full-length 16S rRNA sequencing were assigned their genus by using Kraken2 in combination with the SILVA 16S database (138.1). SILVA contains quality checked databases for small (e.g. 16S) and large (e.g. 23S) rRNA sequences (Quast et al., 2013). After taxonomy allocation, the reads assigned to mitochondria, the reads without a phylum and the chloroplast reads were removed from the data. The taxa with the fewest reads were removed from the data and the data was then rarefied to 23723 reads which was defined as the threshold for the other taxa.

Bacterial diversity was calculated using the Phyloseq (McMurdie & Holmes, 2013) Visualisation was done using ggplot2 in R. To analyse differences in community composition between locations and thallus region, a non-metric multidimensional scaling (NMDS) was performed based on the amount of OTU's (and their genus name) per location and thallus region. Only the most abundant taxa were displayed, taxa that had a lower amount of reads were placed in an "other" category. A permutational analysis of variance (PERMANOVA) with 9999 permutations was used to quantify the differences and detect significant effects of thallus region, location and their interaction on bacterial community composition. Homogeneity of variances was ascertained using a *betadisper* function. Both the NMDS and PERMANOVA were assessed using the Bray-Curtis dissimilarity.

Antibiotics

Selection of suitable antibiotics and their usable concentrations

To deduce a working protocol for axenization of the gametophytes that will allow us to infer the role of bacteria during gametogenesis and the development of the sporophyte, seven antibiotics (Streptomycin sulphate salt (Sigma-Aldrich), Kanamycin Sulphate (Gibco™), Ampicillin sodium salt (Gibco), Penicillin (Sigma-Aldrich), Rifampicin (Roth), Erythromycin (Sigma-Aldrich) and Chloramphenicol (Sigma-Aldrich)) were considered for curing *Laminaria* of bacteria. Their bacteriostatic effect and toxicity on *Laminaria* gametophytes was assessed for the concentrations 10, 50, 100 and 500 µg/mL. Erythromycin, Chloramphenicol and Rifampicin were dissolved in ethanol (10mg/mL) due to low solubility in seawater. A VWR 24 well plate was filled with these antibiotic concentrations and *Laminaria* gametophyte culture in a final volume of 2 mL ½ PES medium. In addition, a 2 µg/mL concentration of yeast extract (Sigma-Aldrich) was added to each well to ascertain viability of bacteria despite antibiotic treatments. In case of non-axenic cultures, the yeast extract would enhance to a visible biofilm in the wells, clearly making non-axenic conditions visible.

The *Laminaria* gametophytes cultures used in this experiment were previously under red light incubation in 16 °C but were now harvested and their medium was refreshed. Cultures were concentrated and centrifuged for three minutes at room temperature (speed 3430, RCF: 2499, Sigma 4k15 laboratory centrifuge). The experiment was run in duplicate and incubated in 16 °C at a 16:8 light cycle. An imaging-Pulse Amplitude Modulation (imaging-PAM) fluorometer (WALZ IMAG- K4) was used to assess photosynthetic yield as a proxy for the seaweed health. This method allowed the observation of changes or differences in fluorescence originating from photosystem II. The yield of photosystem II (Y(II)) is calculated as (Fm-F0)/Fm, where F0 represents the minimal fluorescence and Fm corresponds to a maximal fluorescence (Consalvey et al., 2005). Prior to administration of the pulses, the sample plates were incubated in the dark for at least 15 minutes to ensure reaction centres of the photosystems were available. Pulse Amplitude Modulation (PAM) was used to measure the yield of photosynthesis on both the day of antibiotic treatment and on day eight post-treatment. Yield ranges that are closer to zero can be attributed to the direct toxic effects of antibiotics. Morphological changes and mortality was also assessed using microscopic observations.

A visual analysis on these concentrations was performed in R version 4.2.1. The photosynthetic yield values (YII), as measures with the PAM fluorometer, were visualised using ggplot2 (Wickham, 2009). This was done for day 0 and day 8 to get a comparison for direct effects and long term effects. A ggplot was also coded to get the yield differences between day 8 and day 1. However, deciding which antibiotics to use and at which concentrations was dependent on microscopic observations regarding the biomass growth rather than the PAM results.

Composing a broad activity spectrum antibiotic mix

As none of the individual antibiotics proved to be sufficient to completely cure the algal cultures, it was decided to use a mixture of Ampicillin (A), Rifampicin (R) and Kanamycin (K) with concentrations of respectively 500µg/mL and 100µg/mL for the latter two. These antibiotics were selected at these concentrations based on the antibiotic mode of action together with the

observation of the previous experiment. A 24 well plate (VWR) was filled with either a combination of the three antibiotics (AKR treatment) or a combination of only Ampicillin and Kanamycin (AK treatment) to test the synergetic effect of the antibiotics on the axenization and health of gametophytes. Rifampicin was not tested separately. The gametophytes were prepared from the stock as previously stated. Two cultures from the ENG stocks (individual E and K) and two cultures from the NOR stocks (individual H and A) were selected to cover some of the available gametophyte diversity. Antibiotics were prepared as previously stated with the exception of Rifampicin which was not dissolved in ethanol in the stock solution. The wells of the 24 well-plate were filled with 500 µl of each antibiotic concentration and 200 µl gametophyte solution. Both AKR and AK treatment were supplemented with ½ PES medium up to 2mL. The health and development of the cultures was monitored microscopically after one week. To confirm axenicity, A volume of 5 µl from each well was drop plated on marine agar. Both the well plate and the outplated bacteria were incubated at 16 °C, under a 16/8 light regime, provided by led lights. A DNA extraction and gel electrophoresis was also performed on still growing bacteria followed by full-length 16S rRNA gene high throughput amplicon sequencing.

As fungal growth was observed in some of the cultures after antibiotic treatments of this experiment (see results) the antifungal Nystatin (Sigma-Aldrich) was later added to the mix and tested on a new 12-well plate with individuals NOR H, NOR B and ENG E. Different concentrations (5µg & 50 µg) of Nystatin were added to the AKR treatment mix (same concentrations as mentioned before) to assess which concentration is the most advantageous for avoidance of fungal growth while maintaining healthy growth of the gametophytes. The experiment thus consisted of three individuals with three treatments (AKR, AKR + 5 µg/mL Nys, AKR + 50 µg/mL Nys) and a control treatment (no antibiotics). Incubation of the experimental well plate with the antibiotic and nystatin mix was in a 16 °C room with a 16:8 light cycle.

Inducing gametogenesis

Inducing gametogenesis during axenization

As it was the intention to sterilize the gametophytes prior to heatwave administration, this experiment was aimed towards testing if gametogenesis actually occurs in the presence of antibiotics. The antibiotic mix designed based on the previous experiments (500 µg/mL Ampicillin , 100 µg/mL Kanamycin, 10 µg/mL Rifampicin and 5 µg/mL Nystatin) was added to gametophytes of locations Normandy (individual B, G and H) and England (individual E, K and O). The experiment was performed in a VWR 12 well-plate with culture medium (½ PES) and two antibiotic treatments and two control treatments were created.

Since gametogenesis was low in initial tests (supplementary material) before, a change of culture conditions was deemed beneficial to actively enhance it. The plates were now maintained at 18°C with a 16:8 light cycle with additional blue light (provided by an RGB led strip-band) to further enhance gametogenesis. Culture medium was refreshed every week and the antibiotic treatment was refreshed in week one and two and completely removed in week three. Every well with antibiotics was plated out on Difco Marine agar to inspect the presence of surviving bacteria. Health of the gametophytes/sporophytes was quantified through PAM fluorescence and observed microscopically (Zeiss observer A1 AX10 with an attached

Olympus CellSens entry 2.3 camera). Any irregularities were photographed using the CellSens programme.

Density dependence of gametogenesis

To test the gametophyte density effect on the onset of gametogenesis, a VWR 12 well-plate was filled with four different dilutions (1mL, 2mL, 3mL, 4mL) of individual ENG K, ENG O and NOR H gametophytes. Sporophyte growth was monitored weekly through a microscope for a duration of three weeks.

Administration of bacterial inocula to induce gametogenesis

Since gametogenesis was not frequently observed in the previous experiments at 18 °C, the lower 16 °C temperature was preferred for further experimentation. Axenic (NOR H, NOR G) or partly axenic (ENG K, ENG E) cultures from the 'induction of gametogenesis during axenization experiment were reused and the stock cultures (NOR G, NOR D, ENG E, ENG L) that were not axenic, were collected to test the effect that a mix of various isolated bacteria have on gametogenesis. The (partly) axenic and non-axenic individuals were compared to identify the effect of the prolonged exposure to blue/white light and antibiotic treatments as opposed to the longer suppression of fertility transition under red light incubation without sterilization. Density of the cultures could not be equalized due to a malfunctioning of the PAM fluorometer. Microscopic observations were therefore opted for further analysis and sporophyte presence was counted according to a standardized method where the number of sporophytes in five different locations in the well was counted. These five positions were in the left upper corner, the right upper corner, right lower corner, left lower corner and in the middle. This meant that approximately 25 percent of the total surface was counted.

Four bacterial mixtures were prepared: three consisting of bacterial isolates from one thallus region and one natural inoculum from seawater collected at 51°13'53.6"N 2°57'04.4"E (Spuikom Oostende) by Peter Chaerle. The four bacterial treatments were thus: 1) holdfast inocula (Ho): 2) meristem inocula (M); 3) blade inocula(Bl) and 4) natural seawater inocula (C) . Ho, M and Bl each consisted of six bacteria, selected based on genomic data availability. DNA of these bacteria where extracted as explained later and the number of reads and its growth in marine broth over the course of two consecutive days were used as a selection method. Therefore only bacteria that grew sufficiently and quicky enough were used for inocula treatment. A volume of 100 microliter of each bacteria overnight culture in marine broth (incubated shaking at room temperature) was pipetted into a 48 well plate (VWR). Optic densities at 600 nm were measured by a Cytation3 Imaging reader (BioTek) and the second lowest optic density (OD) was used as a proxy for dilution of the other bacteria with ½ PES medium to get roughly the same bacterial concentrations in every final probiotic. The lowest bacterial optic density measurement was equal to the OD of the blank. Bacteria were pooled after which they were centrifuged (3000 g, 10 min; Sigma 4k15 laboratory centrifuge) to pellet. The supernatant was discarded and the pellet was washed with ½ PES medium a second time with a centrifugation step and supernatant disposal. The pellet was then diluted in 18 to 20 mL of ½ PES medium.

For the seawater probiotics, a bottle of seawater from the Belgian north sea was filtered two times. First through a 5 um filter (GLA 25mm PALL Life Sciences) to filter out larger organisms and particles. A second filtration step involved the previously filtered water passing through a 0.1 um filter (PALL corporation, 25 mm) to collect the bacteria. The bacteria captured on the 0.1 um filters were resuspended in 20 mL of ½ PES medium and vortexed. 24-well plates were filled with the individuals and the different treatments via randomisation. The wells were filled with 1 mL of probiotics and 1 mL of gametophytes and put in a 16:8 light cycle in 16 °C. The well-plates were refreshed weekly.

Bacterial diversity of the bacterial inocula mix for each treatment was assessed using Sanger Sequencing. The available KO functions provided by these bacteria are also identified using Kyoto Encyclopedia of Genes and Genomes (KEGG) to get an idea of the presence of useful functions according to literature (see discussion). A visual inspection was conducted using ggplot2 to get an idea of the general distribution of the sporophyte counts and the effect of the different inocula treatments over time. Analysis on this data was performed using ordinal logistic regression as it was noted that the distribution of sporophyte counts did not fit within the assumptions of a linear distribution. Therefore, counts were put into categories depending on their mean. The sporophyte counts following the standardized counting method as mentioned earlier (day 14, day 21 and day 30) was calculated per day. Reference levels for the estimates in these models were manually selected to ensure that each treatment Ho, Bl and M was compared to the seawater inocula. P-values were calculated separately as the polr() function used in this instant did not provide them. They were however interpreted as in any other model.

Temperature control experiment

To analyse the temperature effect, a control experiment was set up with gametophytes from two individuals (NOR H and ENG E) in three different temperatures (13 °C, 16°C, 18°C). Because gametophytes clustered strongly, they were fragmented with a kitchen blender . Gametophytes were transferred to ½ PES medium with ¼ Ge2O2 in 50 mL Erlenmeyer flasks. Light intensity was aimed towards 40 µmmol photons /m² s² for all three temperatures. Three duplicates were created per treatment and put under aeration for ultimate gametogenesis conditions. The flasks were sealed off using parafilm. Medium was refreshed every week and 1 mL of culture from the flasks was put into a 24 well-plate to be observed under a Zeiss (Zeiss observer A1 AX10) microscope for sporophyte counts. Counting of sporophytes was done by consistently choosing the same five positions in the well and counting the sporophytes observed in these positions, as described in the ‘inocula administration experiment’. As gametogenesis was not present as quickly as expected, 50 mL plastic cell culture flasks were provided for each individual’s gametophytes at each temperature without aeration to provide adherable substance for faster sporophyte growth. Culture flasks contained either ½ PES medium with ¼ Ge2O2 or Tropic Marin® culture medium supplemented with ½ PES & Ge2O2

No statistical analysis was performed on these results as aeration seemed to provoke significant disruption of gametophyte growth and not enough sporophytes were counted for meaningful results when comparing artificial and natural seawater conditions.

Inducing gametogenesis during heatwave treatments by bacterial inoculation

The goal of this experiment was to deduce if bacterial inocula from the sampled sporophytes could increase the occurrence of gametogenesis and consecutive sporophyte development during heat stress. Gametophyte cultures came from either the red light incubated stock or from the 16°C temperature control experiment and from individual NOR H and ENG K. Each gametophyte stock was treated with 500 ul/mL Ampicillin, 100 ul/mL Kanamycin, 10 µl Rifampicin and 5 ul/mL Nystatin for one week in 200 mL culture flasks. Medium was then completely refreshed and this was followed by bacterial treatments as described in the 'bacterial inocula administration' experiment. The experiment was done in duplicates that were randomized. Three 24 well plates per duplicate were prepared with nearly equalized gametophyte density through PAM fluorescence measurements with minimal fluorescence as a proxy for biomass. A control treatment (no bacteria added) was included. The six 24 well plates were incubated for three days on the 16°C baseline temperature on a 16:8 light cycle of cool white light.

After bacterial inoculum administration, heat stress treatment was administered with three different maximum temperatures: 16°C, 18°C and 22°C. Temperature was increased with one degree every day until the maximum temperature was reached as schematically represented in Figure 5. Gametophytes then remained at this temperature for a week until temperature was decreased again for 1 °C each day. The duration of this experiment was thus set at two weeks.

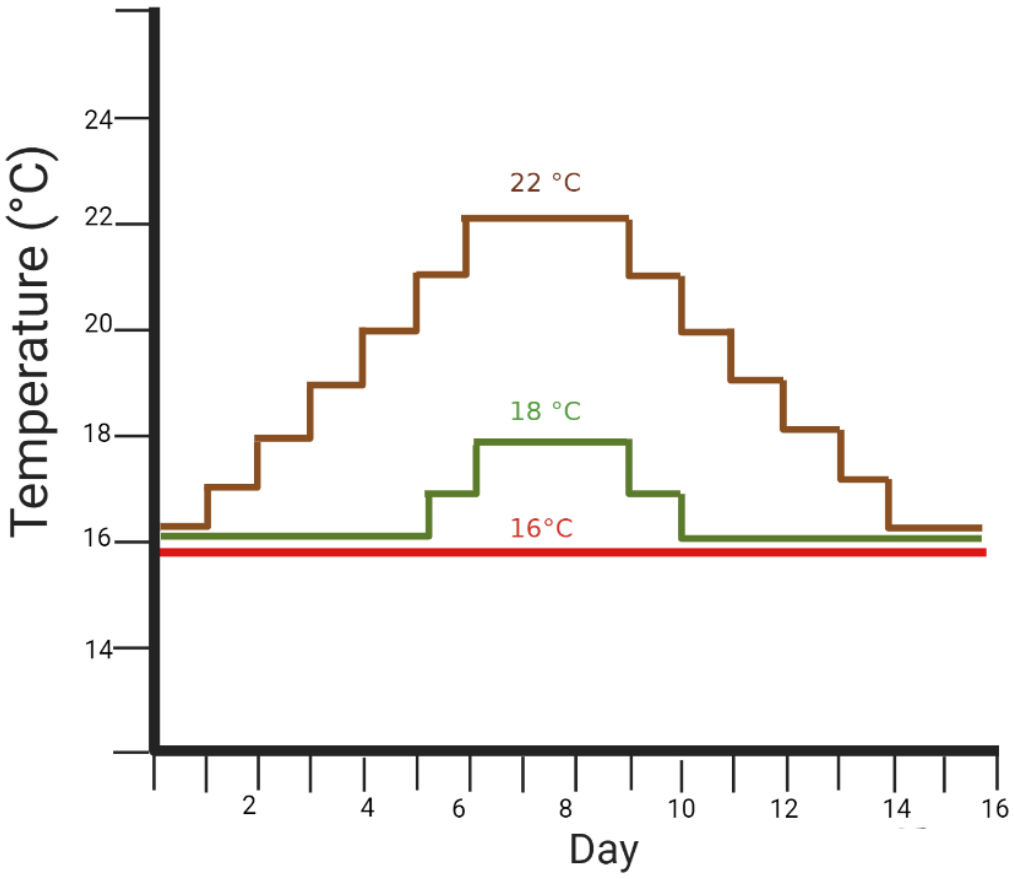


Figure 5: Schematic representation of heatwave experiments.

Statistical analysis on this data was done using ggplot2 visualisation for an overall idea of the treatment effects on the 16, 18 and 22 degree heatwaves. However, not the sporophyte counts but rather the photosynthetic yield provided by the PAM fluorescence meter was used for this, as gametogenesis was not observed frequently enough. A binomial logistic regression was nonetheless performed on the sporophyte counts after the heatwave treatments (day 25).

DNA extraction

High molecular weight DNA extraction

High molecular weight DNA of the bacteria isolated from the *Laminaria* individuals was extracted. A volume of 200 µl of bacteria (either from the cryo preserved stock or a streak from agar plates was used to inoculate 8 mL of marine broth (Difco). Bacteria were grown overnight on a shaker to ensure sufficient bacterial biomass for DNA extraction. The day after, the Wizard HMW DNA Extraction (Promega) kit protocol was followed to get high molecular weight of DNA from the isolated bacteria. One mL of the broth was centrifuged (13000rpm) to pellet the bacteria and processed further as indicated by the protocol.

Every bacteria that was extracted was also plated out on marine agar to make sure it was free of contaminants. If it was noticed that there was contamination with another bacteria, this extraction was repeated with a new inoculant from the cryo-preserved material.
DNA extractions were followed by gel electrophoresis to see if there was enough high molecular weight. Because of a very high amount of low molecular weight (see results) the use of kit was thus revised and the protocol from the Monarch HMW DNA Extraction Kit for Tissue (NE#T3060S/L) from New England Biolabs Inc. was then preferred.

Protocol Qiagen DNeasy Powerlyzer Microbial kit and Sanger Sequencing

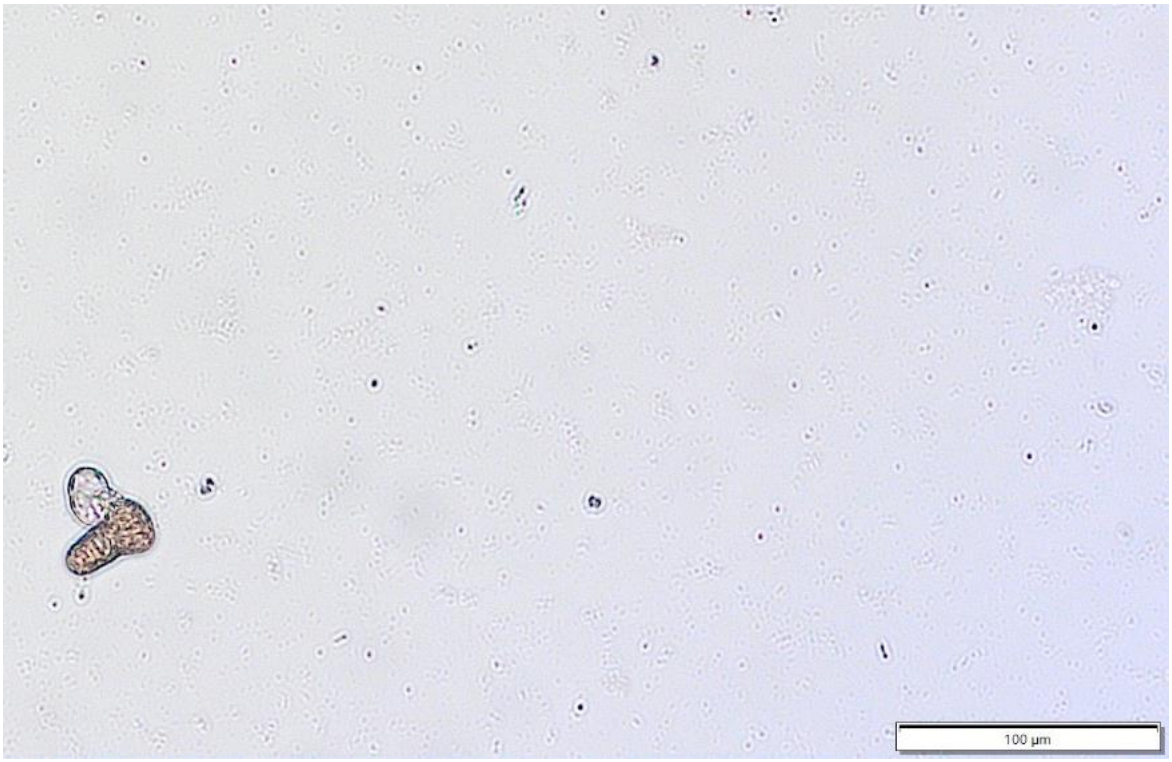
The bacteria that still persisted in the antibiotic treatment during the final axenization experiment were identified based on their 16S rRNA gene sequence. Isolates from the antibiotic treated gametophyte cultures were picked for 16S rRNA gene Sanger sequencing. The DNeasy Powerlyzer Microbial kit protocol was followed on a smear from a colony. Bacterial cell lyses was enhanced by bead beating. A 300 µl powerbeat solution and a 50 µl SL solution (a lysis buffer) was added. A Retsch beadbeater was used at a frequency of 30 beats per second and the mix was centrifuged at 10 000g for 30 seconds at room temperature. The Quick-Start Protocol from Quagen was then further ensued. The 16S rRNA gene was amplified using primer set 27F and 1492R as described in the protocol and negative controls were included.

Results

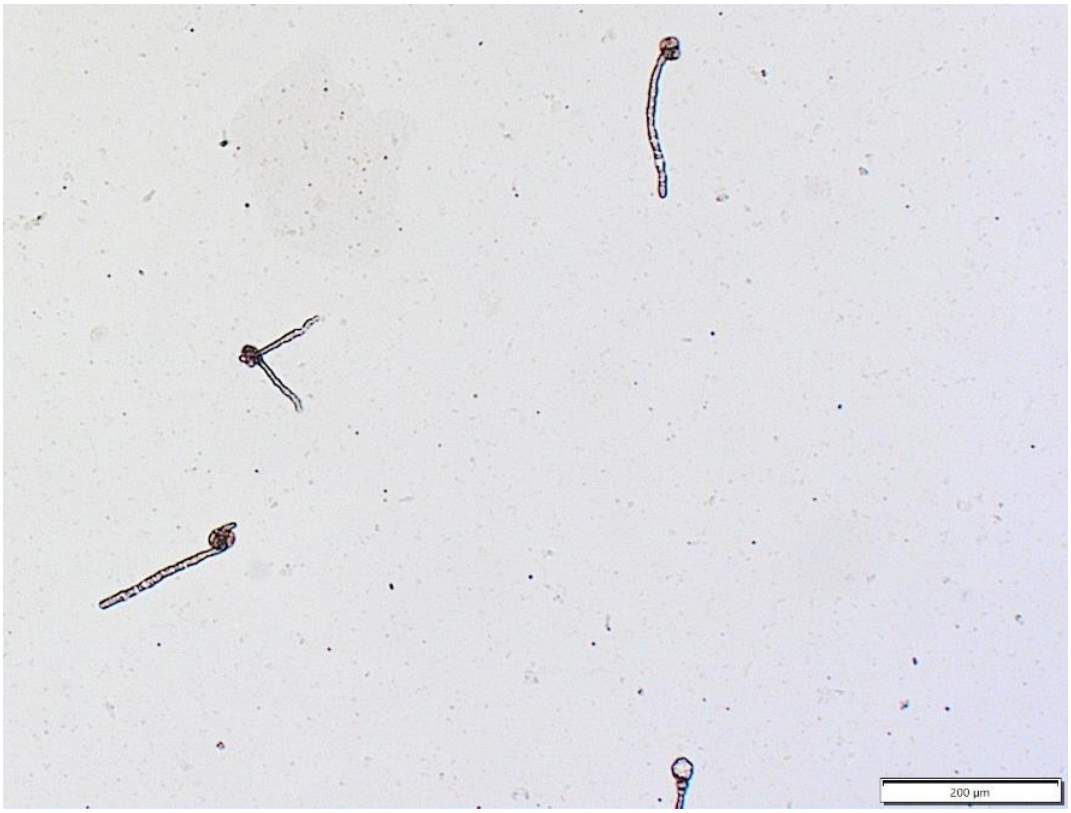
Growth of gametophytes

During the course of this research, growth of gametophytes was monitored several times to deduct their life stage, overall health and to get an idea of the experimentation effects. Therefore, some insight into the life cycle of *L. ochroleuca* is necessary. An overview of all life phases according to Lüning (1980) is hereby provided in the form of microscopic photos taken by an attached Olympus camera and Cellsens programme for future comparison during experimentation.

Primary cell and gametophyte growth towards vegetative phase (29/09/2023)

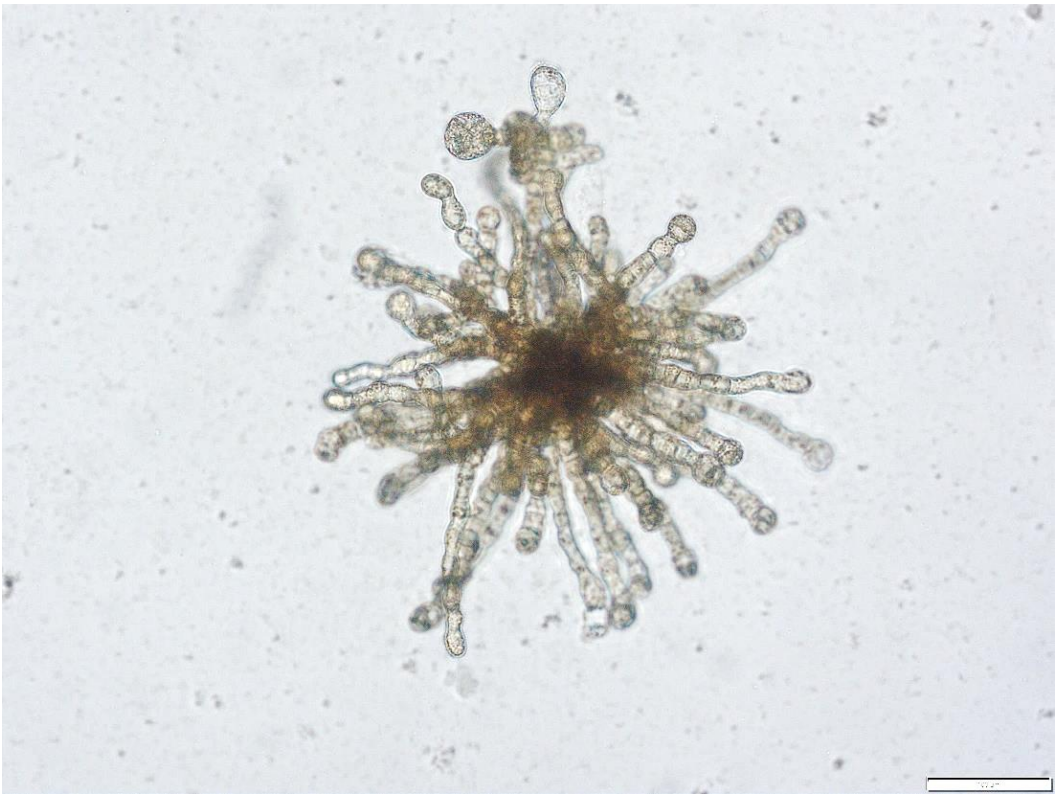


Vegetative phase: growth of gametophyte filaments (29/09/2023)

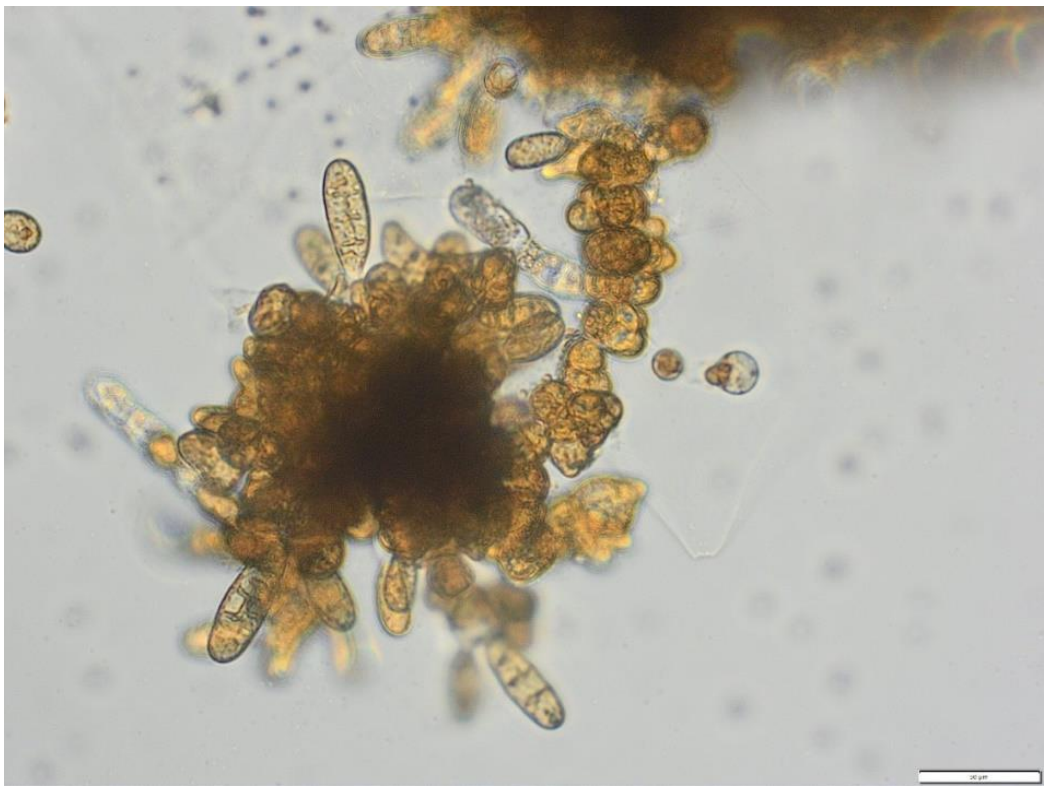


Growth of filaments, formation of female (upper) and male (lower) gametophytes (19/12/2023)

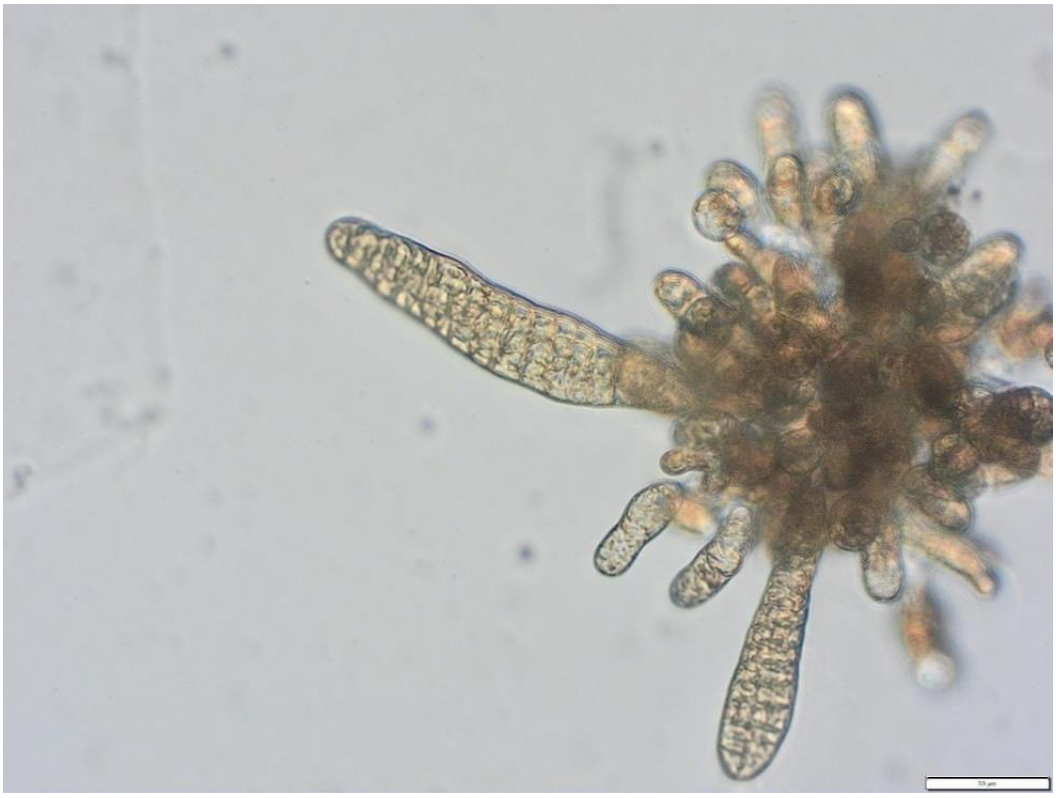
Fertile female gametophytes carrying oogonia (23/02/2023)



Fertilized female gametophytes: onset of gametogenesis (17/03/2023)



Microscopic sporophyte growth (20/03/2023) – a few days old



Further sporophyte growth (19/12/2023) – approximately 2 weeks old

Full-length 16S rRNA gene high throughput amplicon sequencing of swabs

To gain insight into the bacterial taxa present on each swabbed region on the adult individuals, we visualised the microbiome composition of the blade, holdfast and meristem. Figure 6 shows the abundance of the most common bacterial orders for England and France on blade, holdfast and meristem tissue. This represents the main biodiversity of both locations and all three thallus-regions and suggests that differences in both thallus region and location affects composition.

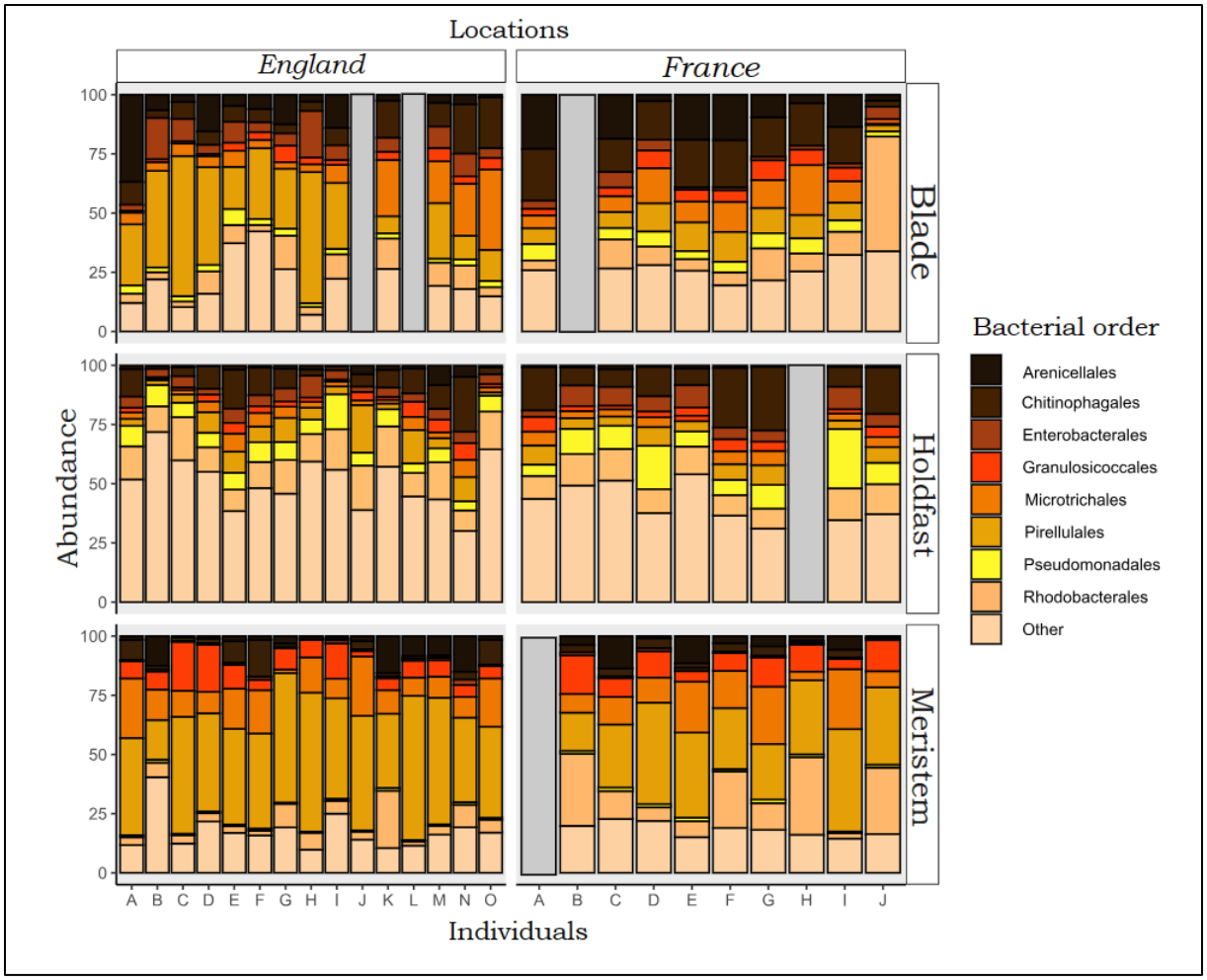


Figure 6: Microbiome composition visualised per location (x-axis) and per treatment (y-axis).

Microbiome composition on holdfast, blade and meristem are significantly different (PERMANOVA, Table 1). Nonmetric multidimensional scaling shows clustering of thallus-regions (Figure 7) and Permutational multivariate analysis of variance show significant effects of thallus-region ($p = 0.001$), location ($p=0.015$) and their interaction ($p=0.017$) on community composition. These results suggest that bacterial community composition of the same thallus region varies between England and France.

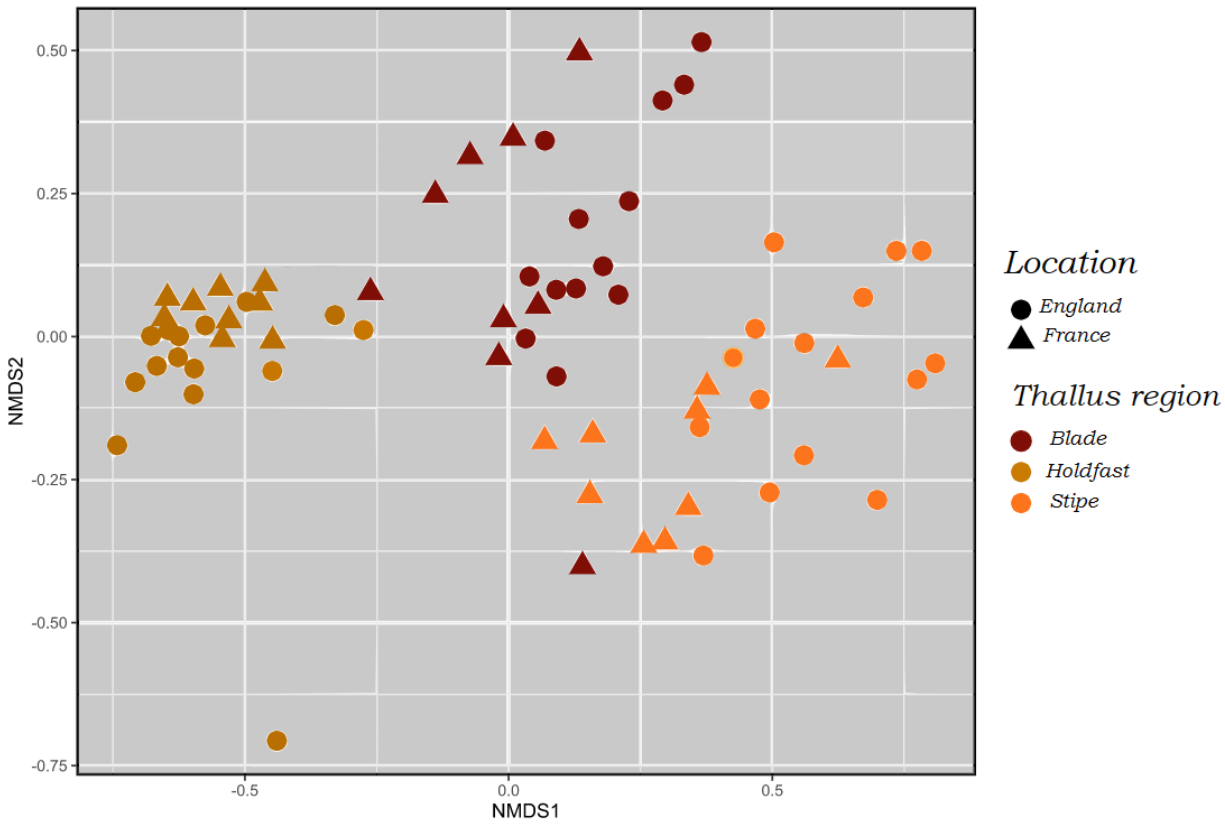


Figure 7: Non-metric multidimensional scaling shows clustering of thallus regions.

Table 1: Results of permutational multivariate analysis of variance on microbiome composition.

Effect	Df	Sum of Sqs	R^2	F	Pr(>F)
Thallus region	2	3.4455	0.33327	17.495	0.001***
Location	1	0.2559	0.02475	2.7499	0.015*
Thallus region: Location	2	0.4018	0.03887	2.1589	0.017*

Antibiotics

Selection of suitable antibiotics and their usable concentrations

A total of seven different antibiotics were investigated for their acute and long term effects on biomass growth by using a PAM fluorometer. The medium and ethanol treatment served as a control. The photosynthetic yield changes after eight days can be found in the supplementary data. They were not used to determine the utilized antibiotics and concentrations as they were deemed unreliable. Long term health changes were however also monitored using microscopic observations (Table 2).

Table 2 : Effects of different concentrations of antibiotics 8 days after administration

Antibiotic	*Concentration*	*Observations on day 8*
Ampicillin	500 µg/mL	no immediate changes
	100 µg/mL	no immediate changes
	50 µg/mL	no immediate changes
	10 µg/mL	no immediate changes
Chloramphenicol	500 µg/mL	some biomass loss and diminished growth
	100 µg/mL	better growth, less biomass loss
	50 µg/mL	in bad condition
	10 µg/mL	no immediate changes
Erythromycin	500 µg/mL	diminished growth, biomass loss
	100 µg/mL	biomass loss but better
	50 µg/mL	no immediate changes
	10 µg/mL	no immediate changes
Kanamycin	500 µg/mL	bad state but controversial results from the PAM, duplicate showed a lot of biomass loss
	100 µg/mL	better growth, but still biomass loss
	50 µg/mL	no immediate changes
	10 µg/mL	no immediate changes
Penicillin	500 µg/mL	some biomass loss
	100 µg/mL	some biomass loss
	50 µg/mL	no immediate changes
	10 µg/mL	no immediate changes
Rifampicin	500 µg/mL	no immediate changes
	100 µg/mL	no immediate changes
	50 µg/mL	no immediate changes
	10 µg/mL	no immediate changes
Streptomycin	500 µg/mL	some biomass loss
	100 µg/mL	better growth
	50 µg/mL	no immediate changes
	10 µg/mL	no immediate changes
Ethanol control		a lot of bacteria visible, biofilm formation so not a lot of biomass

Summarized, these results depict that Ampicillin, Rifampicin and Kanamycin showed the least amount of biomass loss, with the strongest effects observed for concentrations 500 µg/mL, 100 µg/mL and 50 or 10 µg/mL respectively.

A broad activity spectrum antibiotic mix

To further evaluate the effects of the antibiotic mix on the growth and health of gametophytes for future experimentation, a 24 well-plate was prepared with a mix of either Ampicillin and Kanamycin or Ampicillin, Kanamycin and Rifampicin. As Rifampicin strongly colours the medium, it was tested if a mix with only the other two antibiotics was suitable without Rifampicin for axenization.

Table 3: Observations of the effects of a mix of selected antibiotics on 4 different individuals.

Treatment	*Observations*
AK NOR H	healthy gametophytes and growth
plate	axenic
AKR NOR H	no growth, mycelium present and biomass loss
plate	axenic
AK ENG E	healthy gametophytes and growth
plate	bacteria present: big orange colony
AKR ENG E	no growth, mycelium present
plate	axenic
AK NOR A	healthy gametophytes and a lot of growth
plate	bacteria present
AKR NOR A	no growth, a lot of biomass loss
plate	axenic
AK ENG K	healthy gametophytes and growth
plate	bacteria present
AKR ENG K	relatively healthy
plate	bacteria present

Only in the combination of all three antibiotics, complete sterility was approached (Table 3). AKR ENG K was not completely axenic since there was still growth on the marine agar plates. The same was noted for the combinations without rifampicin on NOR A, ENG E and ENG K.

Fungal growth was observed in antibiotic treatment AKR NOR H and AK ENG E. Therefore, Nystatin (Sigma-Aldrich) was added to the antibiotic mix and tested on a new plate with individuals NOR H, NOR B and ENG E. Different concentrations (5 & 50 µg/mL) of Nystatin were mixed with the three antibiotics. The lowest concentration (5 µg) already showed inhibition of fungal growth so this was opted for further experimentation.

Inducing gametogenesis

Inducing gametogenesis during axenization

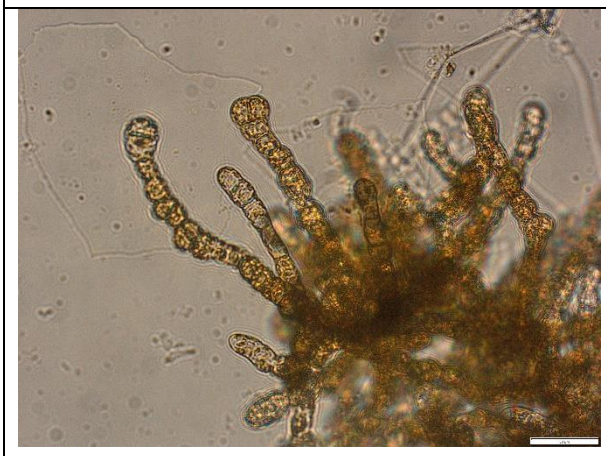
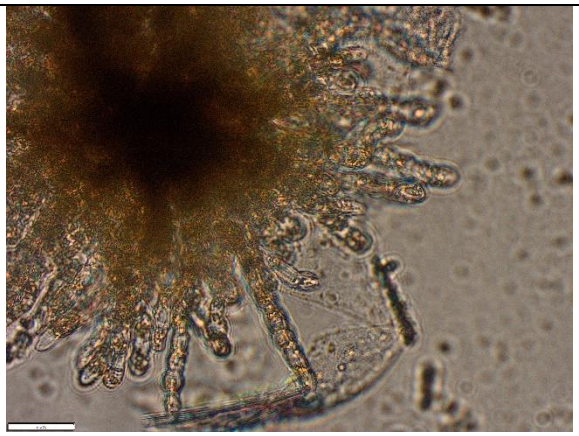
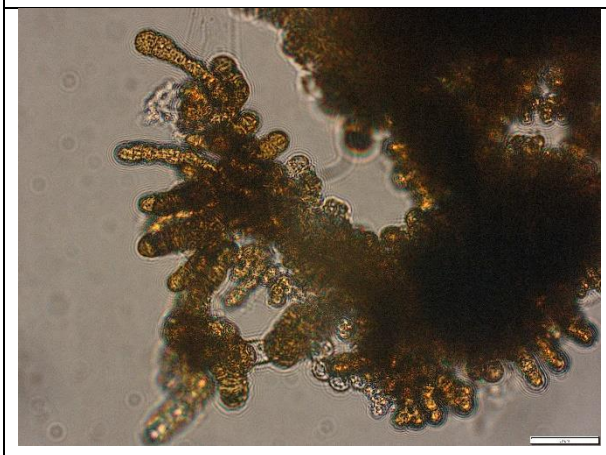
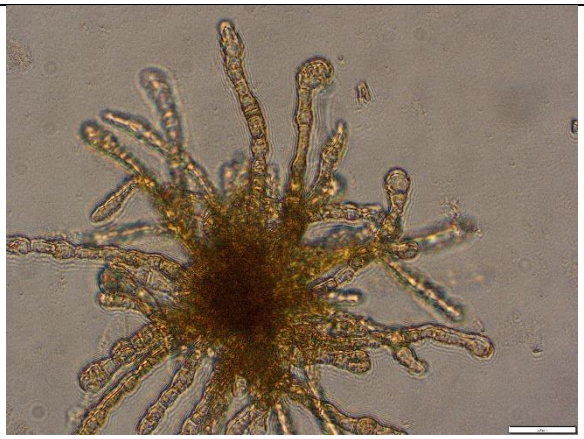
To enhance sporophyte growth during gametogenesis, culture conditions were changed to a temperature of 18°C, additional blue light was provided, and the medium was refreshed weekly. To investigate the potential impact of antibiotics on gametogenesis, a second round of antibiotic treatment was administered. Duplicate wells with antibiotics were compared to control treatments for this purpose. Effects were monitored weekly by microscopy for a duration of four weeks post treatment (Table 4) and were photographed for comparison (Table 5). As in the previous experiment, the axenization was reviewed by plating each well following every antibiotic treatment. Remaining bacterial growth was thus sampled and grown into marine broth to perform a DNA extraction for 16S nanopore sequencing to gain insight into these antibiotic-resistant bacteria. The resulting taxa can be found in the section covering the DNA extraction. These observations show an effect of antibiotic treatments being the “strange cell growth” (Table 5) . This is defined as small clusters of cells without pronounced cell elongation. This type of cell growth was nearly exclusively found after antibiotic treatment, suggesting that the antibiotics might have a negative effect on normal cell growth, possible due to a lack of beneficial bacteria aiding in normal cell growth.

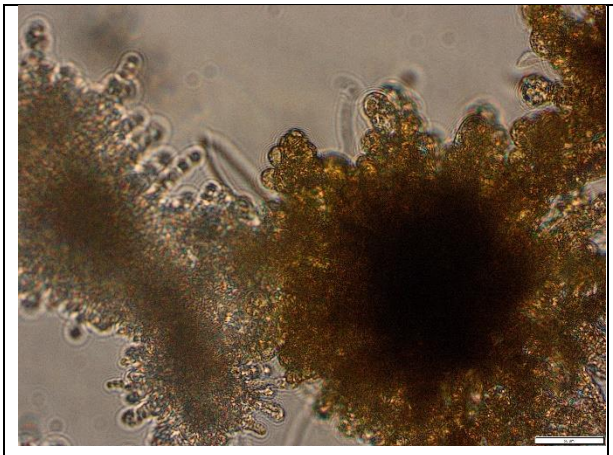
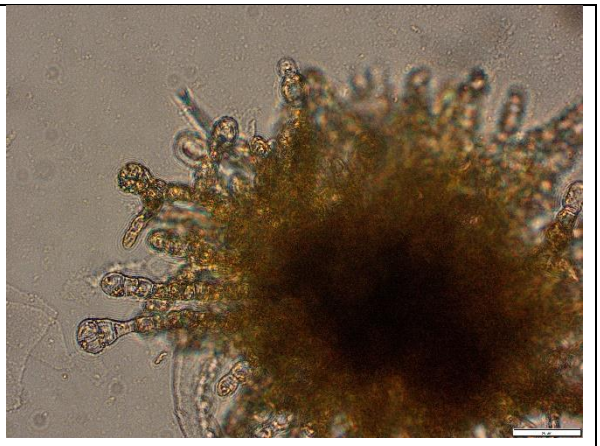
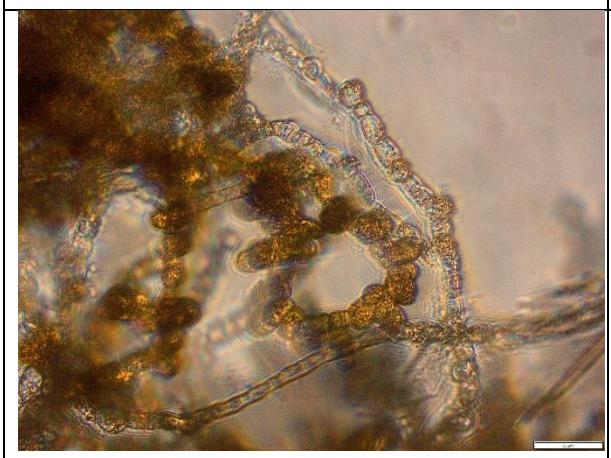
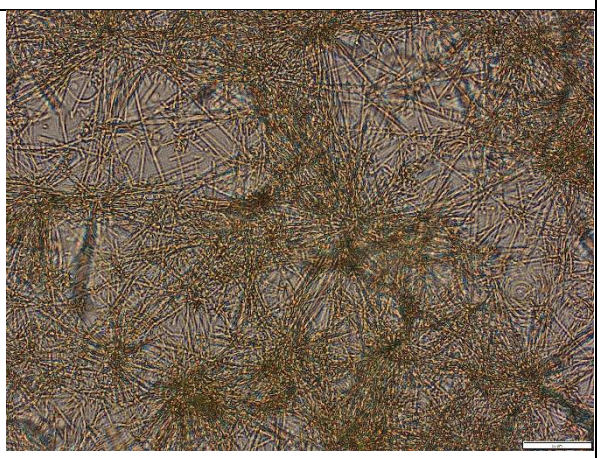
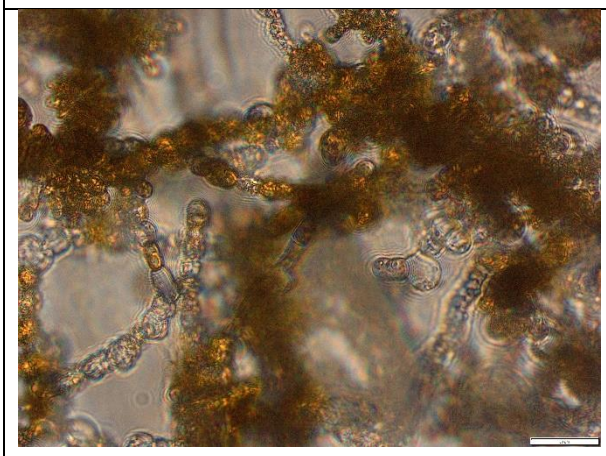
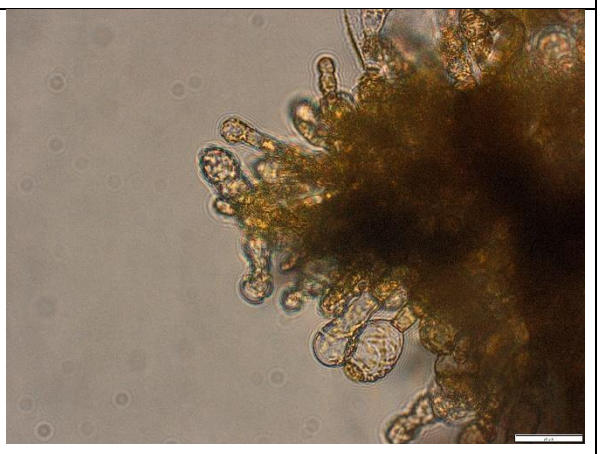
Table 4: Weekly observations of antibiotic effects on gametogenesis of 4 individuals from England and 4 individuals from Normandy where AB = antibiotic treatment, C = control treatment.

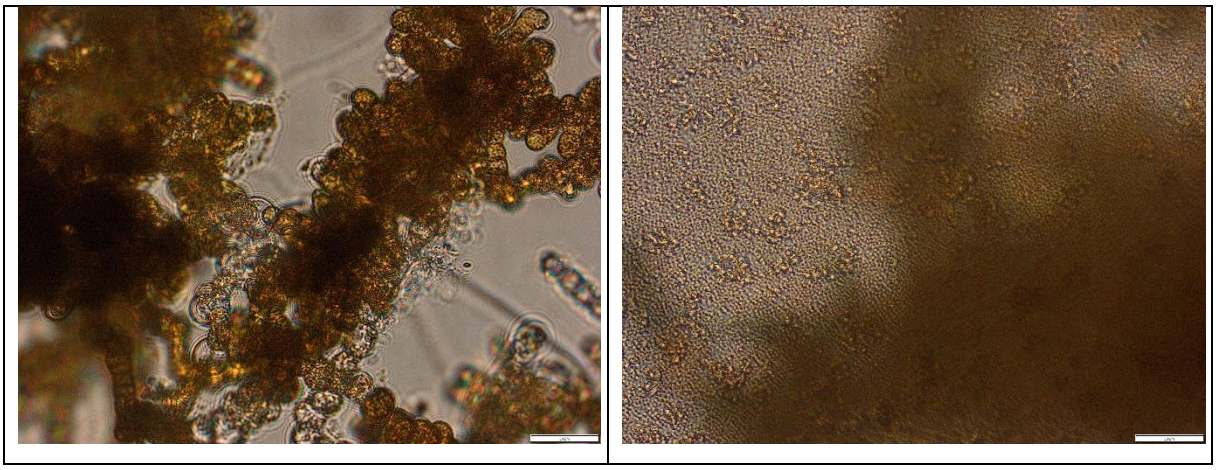
INDIVIDUAL	DAY 0	DAY 7	DAY 14	DAY 21
NOR B AB	Oogonia present, almost no biomass loss	healthy gametophytes, visible biomass growth	no sporophytes present, otherwise healthy gametophytes	no sporophytes, normal growth
NOR B C	Oogonia present, salt kristals (geen idee wat ik hiermee bedoelde)	healthy gametophytes, visible biomass growth	no sporophytes present, otherwise healthy gametophytes	no sporophytes, visible biomass growth
NOR G AB	big clusters of gametophytes and various bloated filaments	No pronounced cell elongation bloated cells, some healthy gametophytes	No pronounced cell elongation	no sporophytes, no pronounced cell elongation , no sporophytes
NOR G C	Oogonia present, big clusters, various bloated cells	healthy gametophytes, normal growth but clustered	normal cell elongation and growth, healthy looking gametophytes	no sporophytes, normal growth
NOR H AB	Healthy gametophytes and a lot of clustering, oogonia present	healthy gametophytes, biomass growth	normal growth, healthy looking gametophytes	no sporophytes, approaching normal growth, healthy looking gametophytes, some celldeath and irregular growth
NOR H C	Healthy gametophytes, oogonia present	healthy gametophytes, oogonia present	normal growth, healthy looking gametophytes	no sporophytes, normal growth,
ENG O AB	biomass loss, traces of other algae	Healthy gametophytes, no sporophytes, big clusters of gametophytes, traces of other algae around gametophytes, oogonia present	Healthy gametophytes, no sporophytes	no sporophytes present, a lot of debris from pipetting present, some moving structures
ENG O C	healthy gametophytes, traces of trichodesmium, originally thought to be salt cristals	A lot of trichodesmium growth, gametophytes hard to spot because of overgrowth	Trichodesmia has overgrown gametophytes	no sporophytes, Trichodesmia completely overgrowing
ENG E AB	biomass loss, strange cellgrowth	Some healthy some bloated cells oogonia present, small amount of biomass loss,	Bloated cells, biomass loss	no sporophytes a lot of debris present from pipetting, no sporophytes, cell death and bloated/strange cell growth

ENG E C	healthy gametophytes but also biomass loss	healthy gametophytes, a lot of visible growth, oogonia present	Cyanobacteria present	no sporophytes, diatoms and cyanobacteria present, some traces of trichodesmium, very dense in biomass
ENG K AB	many filaments, healthy gametophytes	healthy gametophytes, oogonia present	no sporophytes present	no sporophytes, strange cell growth
ENG K C	oogonia present, mix of cell aggregation, strange cell growth and healthy gametophytes	sporophytes present on sparse gametophyte clusters, red algae present cyanobacteria present	sporophytes not visible anymore due to overgrown cyanobacteria	no sporophytes cyanobacteria overgrown, gametophytes not visible

Table 5: Final observations of gametophytes under antibiotic treatments (AB) in comparison to control treatment (C).

NOR B AB	NOR B C
	
NOR G AB	**NOR G C**
	
NOR H AB	**NOR H C**

	
ENG O AB	ENG O C
	
ENG E AB	ENG E C
	
ENG K AB	ENG K C



Density dependence of gametogenesis

To test if gametogenesis is density dependent, a VWR 12 well-plate was filled with four different concentrations (1mL, 2mL, 3mL, 4mL) of individual ENG K, ENG O, NOR H. Sporophyte growth was monitored through a microscope for three weeks (Table 6). This does not show an immediate density effect, except for ENG K, however this could be due to individual effects, since ENG K also showed gametogenesis in the previous experiment.

Table 6: Weekly observations of density effects on gametogenesis

	Day 7	Day 14	Day 25
Concentrations		**ENG K**	
1 mL	Sporophytes present	sporophytes present	sporphytes present but overgrown?
2 mL	no sporophytes yet, but eggs present	no sporophytes, cyanobacteria present	no sporophytes, more cyanobacteria present
3 mL	no sporophytes yet, but eggs present	cell death	red algae present
4 mL	no sporophytes yet, but eggs present	cell death	red algae present
		ENG O	
1 mL	trichodesmium growth, sporophytes present	more trichodesmium growth	more trichodesmium growth
2 mL	biomass loss	trichodesmium growth and biomass loss	more trichodesmium growth
3 mL	more biomass loss	trichodesmium growth and biomass loss	more trichodesmium growth
4 mL	healthier gametophytes	trichodesmium growth and biomass loss	more trichodesmium growth
		NOR H	
1 mL	healthy gametophytes, eggs present, no sporophytes	no sporophytes present, gametophytes healthy	no sporophytes present, gametophytes healthy
2 mL	no sporophytes present, gametophytes healthy	no sporophytes present, gametophytes healthy	no sporophytes present, gametophytes healthy
3 mL	no sporophytes present, gametophytes healthy	no sporophytes present, gametophytes healthy	no sporophytes present, gametophytes healthy
4 mL	no sporophytes present, gametophytes healthy	no sporophytes present, gametophytes healthy	no sporophytes present, gametophytes healthy

Administration of bacterial inocula to induce gametogenesis

Axenic (NOR H, NOR G) or partly axenic (ENG K, ENG E) cultures from the 'induction of gametogenesis during axenization' experiment were reused and the stock cultures (NOR G, NOR D, ENG E, ENG L) that were not axenic were collected to test the effect of the bacterial mixture on gametogenesis. The (partly) axenic and non-axenic individuals were compared to identify the effect of the prolonged red-light incubation versus exposure to blue/white light and antibiotic treatments. The taxa of the bacterial inocula treatments were identified using Sanger Sequencing (Sofie Peeters). Note that each bacteria mix had an equal amount of bacteria (Table 7) from England and France, but also from Morocco (MAR), from which the gametophytes are not used for experimentation.

Table 7: Identified taxa from bacterial inocula per treatment using Sanger Sequencing (Sofie Peeters

Probiotica	Location	Individual	Thallus_region	Agar	Genus
Bl	ENG	C	3	S1	Psychromonas
Bl	ENG	F	3	M1	Vibrio
Bl	NOR	G	3	M2	Vibrio
Bl	NOR	C	3	M1	Vibrio
Bl	MAR	B	3	M3	Vibrio
Bl	MAR	D	3	S1	Pseudoalteromonas
M	ENG	B	2	M2	Pseudoalteromonas
M	ENG	F	2	S2	Vibrio
M	NOR	G	2	M1	Pseudoalteromonas
M	NOR	F	2	M1	Pseudoalteromonas
M	MAR	B	2	S4	Psychromonas
M	MAR	A	2	M2	Vibrio
Ho	ENG	A	1	S2	Vibrio
Ho	ENG	B	1	S2	Vibrio
Ho	NOR	D	1	M1	Vibrio
Ho	NOR	E	1	M1	Pseudoalteromonas
Ho	MAR	D	1	S1	Vibrio
Ho	MAR	E	1	S1	Pseudoalteromonas

The available KO functions provided by these bacteria are also identified using Kyoto Encyclopedia of Genes and Genomes (KEGG, Table 8) to get an idea of the presence of useful functions according to literature (see discussion).

Table: 8: Identified functions (KEGG) of the bacteria used as inocula

	Vibrio	*Psychromonas*	*Pseudoalteromonas*
Nitrogen metabolism			
Dissimilatory nitrate reduction	yes	no	no
Sulfur metabolism			
DMSP	no	no	no
Assimilatory sulfate reduction	yes	yes	no
Lipid metabolism			
Fatty acid metabolism	yes	yes	yes

Metabolism of vitamins			
Thiamine biosynthesis	yes	yes	no
Riboflavin biosynthesis	yes	yes	yes
Biotin biosynthesis	yes	yes	no
Vitamin b6 biosynthesis	yes	yes	yes
Starch and sucrose metabolism			
Glycogen biosynthesis	no	yes	no
Glycogen degradation	yes	no	yes
Quorum sensing			
Biofilm formation	no-almost	no	no
N-Acyl-L-homoserine	yes	no	no
Symbiosis for bacteria			
Glycine breakdown	no	no	no
Alanine breakdown	yes	yes	no
Aspartate breakdown	yes	no	no
Glutamate metabolism	yes	yes	no
ABC transporters			
Iron	yes	yes	yes
Thiamin	yes	yes	no
Phospholipids	yes	yes	yes
Glucose/mannose	yes	no	no
Histidine	no	no	no
Aspartate	no	no	no
Glutamate	no	no	no
Biotin	no	no	no
Riboflavin	no	no	no
Vitamin B12	yes	no	no
Biosynthesis essential amino acids			
Valine	yes	yes	yes- but not all
Leucine	yes	yes	yes
Isoleucine	yes	yes	yes-but not all
Phenylalanine	yes	no	no
Tryptophan	yes	no	no
Antibiotics			
Streptomycin	no	no	no
Vancomycin	no	no	no
Ansamysin	no	no	no

Sporophyte counts can be found in the supplementary material. These counts show no direct effect between probiotic treatments, yet there is a lower number of sporophytes in the control treatments in comparison to the thallus inocula treatments. Sporophyte counts over time (visualisation: supplementary) differed per treatment (Figure 8).

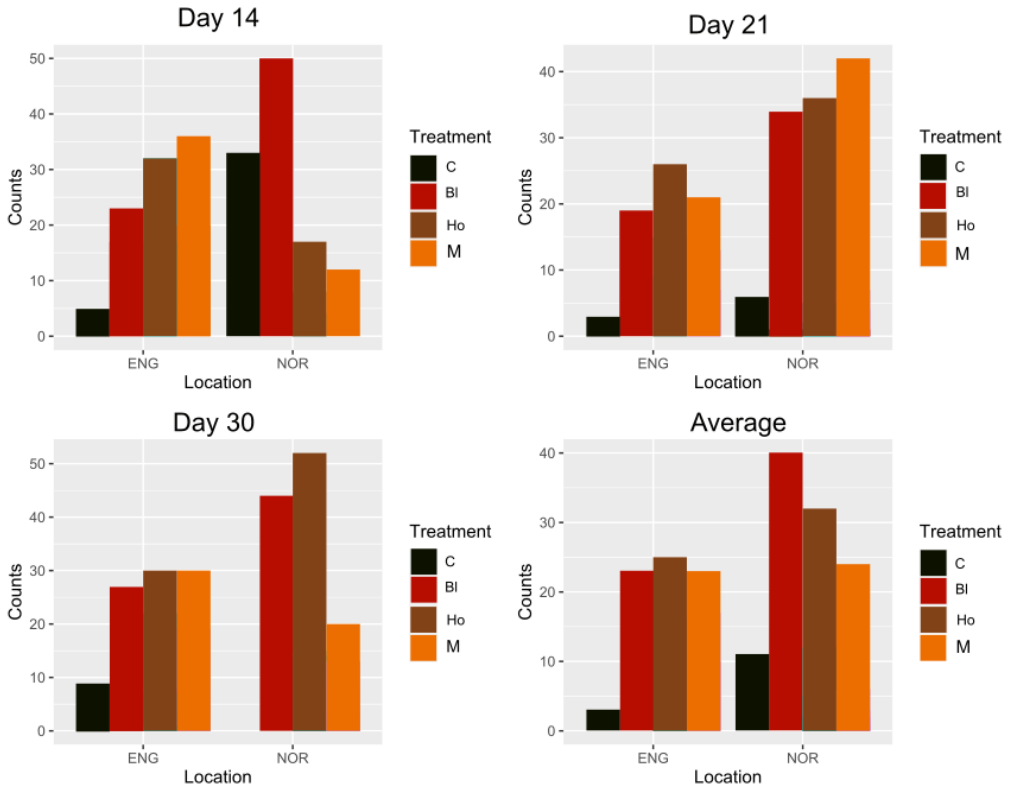


Figure 8: Sporophyte counts per counted day post treatment and treatments (C = control, Bl = Blade, Ho = Holdfast, M = Meristem). Counts were averaged in panel 4.

It seemed that location had an effect on the sporophyte counts, however it was microscopically observed that rate of gametogenesis differed between the origins (axenic = AX or from red-light incubation = RL) of the gametophytes. An ordinal logistic regression model was performed on the additive origin (AX or RL) and treatment effect on sporophyte counts. This was compared with the additive location (England or France) and treatment effect on sporophyte counts. Aikeke's information criterion (AIC) was used to assess the best fit for the data of all separate days and generally showed a better fit for the origin and treatment model. An origin and treatment interaction effect was also modelled and compared to the additive one. Aikeke's information criterion however showed a better fit for the additive model. In the additive location and treatment model, no significant location effect on the sporophyte counts was found (supplementary material).

The models showed differences per origin and per day, affected by the treatments (Table 9). Ordinal logistic regression on day 30 showed that all thallus inocula treatments had a significantly bigger positive effect on gametogenesis than seawater inocula (Blade: p= 0.00118, Holdfast: p=0.00034, Meristem: p=0.00333). Red light incubation had a significantly negative effect on gametogenesis in comparison to the (partly) axenic gametophytes. Day 14 showed no significant effect of treatments on gametogenesis but also showed a significant negative effect of red light incubation (p=0.00156). Day 21 only showed a significantly positive effect of Blade inocula (p = 0.04336) and meristem inocula (p =0.0216), a significantly negative effect of origin (p =0.00006) and a marginally significant effect of holdfast inocula (p =0.09096).

Table 9: Results of the ordinal logistic regression models of Day 14, Day 21 and Day 30.

	Day 14				Day 21				Day 30			
Effect	E	SE	T	P	E	SE	T	P	E	SE	T	P
Blade	0.16124	0.89946	0.17926	0.85773	1.70132	0.84216	2.02019	0.04336*	3.877332	1.1952932	3.243834	0.00118**
Holdfast	0.74786	0.86181	0.86778	0.38551	1.39293	0.82404	1.69038	0.09096	4.345373	1.2131995	3.581747	0.00034***
Meristem	-0.28630	0.93480	-0.30627	0.75940	1.93493	0.84223	2.29740	0.02160*	3.494627	1.1903997	2.935675	0.00333**
Origin	-2.59507	0.82036	-3.16332	0.00156**	-2.37342	0.59036	-4.02027	0.00006****	-1.748928	0.5755814	-3.038541	0.00238**
0|1	0.31796	0.64267	0.49475	0.62077	0.23389	0.64410	0.36312	0.71651	2.12629	1.0545134	2.016371	0.04376*
1|2	0.53002	0.64513	0.82157	0.41132	1.64769	0.69188	2.38148	0.01724*	3.509424	1.0991604	3.192823	0.00141**

Post-hoc comparisons however showed no significant differences between the thallus inocula effects on gametogenesis on day 30 (Bl – Ho: p = 0.899; Bl-M: p= 0.9437; Ho-M: p = 0.6076).

However, it is important to note that many of the sporophytes had died on day 30. This was not accounted for in the sporophyte counts as this experiment was aimed at investigating the effect on recruitment success and there might have been a number of external factors influencing death of these sporophytes, such as lack of sufficient nutrients for sustained growth, light intensity which could have been too high for further growth.

Temperature control experiment:

This experiment aimed to gain insight into the temperature effect on gametogenesis of the gametophytes. As the aerated culture flasks showed significant disruption of gametogenesis and the gametophyte health, the sporophytes counts of these flasks are not shown here but can be found in the supplementary material. Results of the cell culture flasks are displayed in Table 10. No big difference between seawater treatments were observed in this data, however what can be deducted from these results is that the English gametophytes (ENG) have higher recruitment success in the colder 12 degrees, while the French (NOR) gametophytes have higher recruitment success in the warmer 18 degrees in comparison to the English.

Table 10: Sporophyte counts for natural (C) and artificial (C+ASW) seawater and for three different temperatures.

Location	Individual	Temperature	Duplicate	Day 14	Day 24	Day 32
ENG	E	18	C + ASW	0	0	0
ENG	E	18	C	0	0	0
ENG	E	16	C + ASW	0	Dead	0
ENG	E	16	C	0	Dead	0
ENG	E	12	C + ASW	1	4	3
ENG	E	12	C	1	7	0
NOR	H	18	C + ASW	0	0	4
NOR	H	18	C	0	0	5
NOR	H	16	C + ASW	0	0	0
NOR	H	16	C	0	0	0
NOR	H	12	C + ASW	0	0	8
NOR	H	12	C	0	0	1

Inducing gametogenesis during heatwave treatments by bacterial inoculation:

The goal of this experiment was to deduce if bacterial inocula could increase the occurrence of gametogenesis and consecutive sporophyte development during heat stress. Treatments Ho, Bl and M were compared with a control treatment (no added bacterial inocula) on three

different heatwave stressors with maximal temperatures 16, 18 and 22 °C. Health, growth and gametogenesis were monitored using sporophyte counts (supplementary material) and PAM fluorescence measurement. A binomial regression model analysis was performed on the treatment effect on gametogenesis but showed no significant results (supplementary). The treatment effect was also visualised, which shows statistical analysis was not worthwhile (supplementary).

The photosynthetic yield of photosystem II was measured using the PAM fluorometer per heatwave treatment and per treatment along the different timepoints. This was done at day 0, which represented the day before heatwave administration but when bacterial inocula were already added (day 3 after inocula administering); day 7 which represented the gradual increase of temperatures and the start of the heatwave treatments; day 14 which represented the gradual decline of heatwave temperature towards the baseline 16 °C and day 25 which represented the recovery phase. The boxplots in Figures 9, 10 and 11 show this timeline and the corresponding yields measured. The 16 °C treatment serves as a control. Nevertheless, the control treatment in 16 °C showed a different trend to the ones exposed to thallus inocula. A gradual decline in yield is observed, which is resumed in the next time point. This is different for the blade, meristem and holdfast inocula treatments, in which gradual increase in yield is seen during the heatwave and a significant increase after heatwave administration. The same differences between control and thallus inocula treatments can be observed in the 18 °C heatwave treatment. For the 22 °C heatwave however, it can be observed that yield decreases for the control treatment, which is not the case for the thallus inocula treatments.

16 degrees

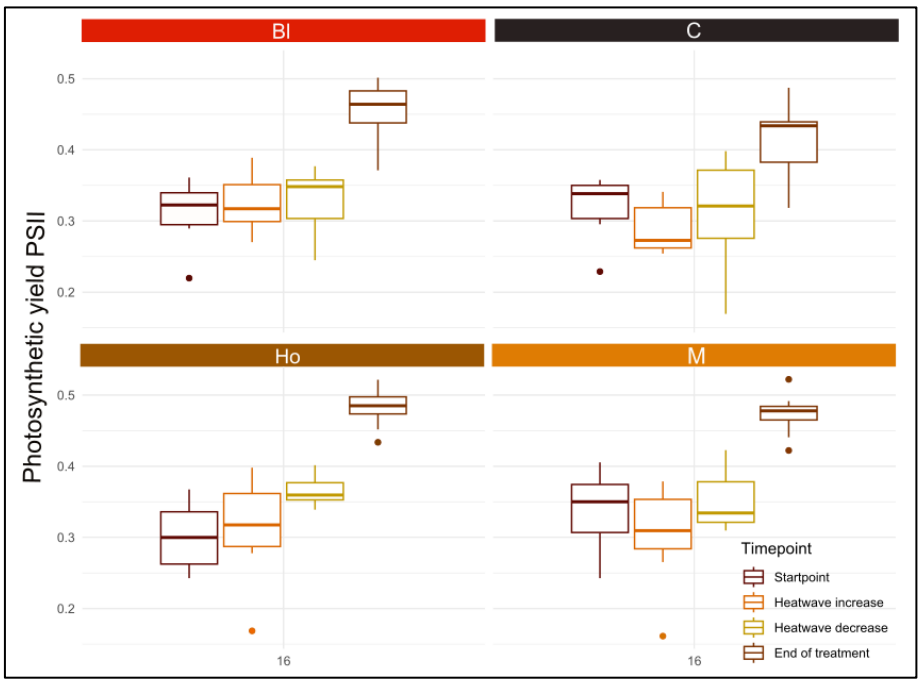


Figure 9: Boxplots concerning the effect of thallus inocula treatment on gametogenesis during a 16 degrees heatwave.

18 degrees

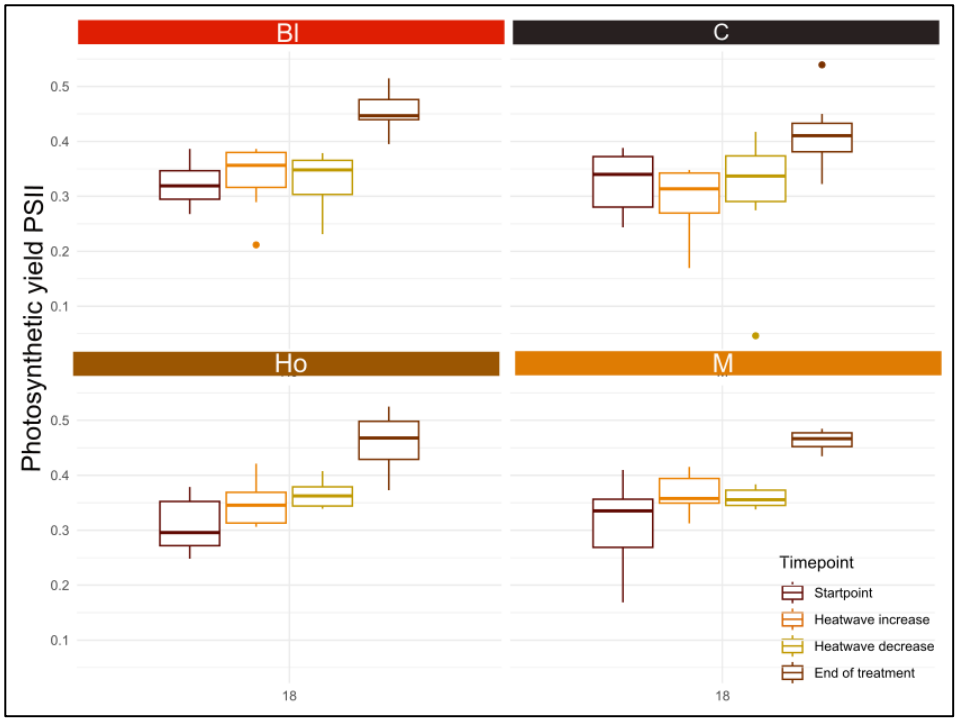


Figure 10: Boxplots concerning the effect of thallus inocula treatment on gametogenesis during a 18 degrees heatwave.

22 degrees

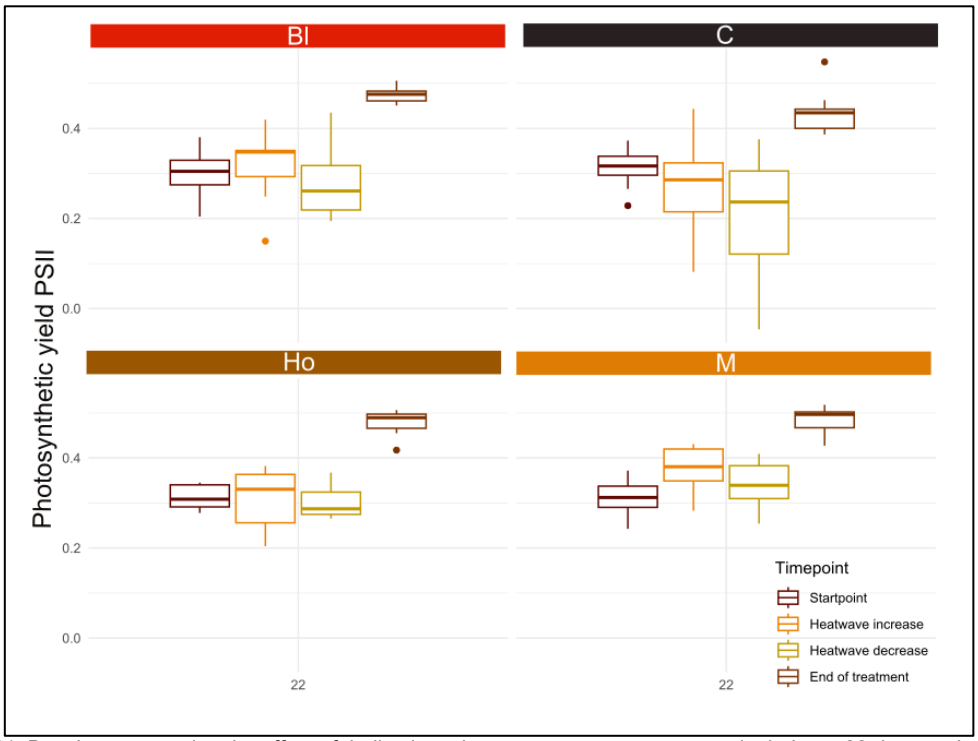


Figure 11: Boxplots concerning the effect of thallus inocula treatment on gametogenesis during a 22 degrees heatwave.

DNA extraction

Protocol Qiagen DNeasy Powerlyzer Microbial kit + sanger sequencing

Table 10 shows the 16S Sanger Sequencing results, identified by using Basic Local Alignment Search Tool (BLAST, Sofie D'Hondt).

Table 10 The identified bacteria that still grew after antibiotic treatments.

Individual sampled	***bacterial taxa***
ENG O	*Lacinutrix sp.*
ENG E	*Maribacter sp.*
ENG K1	*Proteobacter/Maribacter*
ENG K2	*Zobelia sp.*
ENG O	*Zobelia russellii*
NOR B	*Alteromonas sp.*
NOR H	*Alteromonas sp.*

Discussion

Effect of thallus region and location on bacterial community composition

Bacterial community composition differs between thallus regions

The results from the 16S rRNA gene ONT sequencing analysis show that meristem, holdfast and blade swabs from the adult individuals sampled in England and France harbour significantly different bacterial communities. The blade microbiome is dominated by Proteobacteria (Rhodobacterales, Arenicellales), Planctomycetes (Pirellulales), and Actinomycetota (Microtrichales). The holdfast is also dominated by Proteobacteria (Rhodobacterales, pseudomonadales) and to a lesser extent by Bacteroidota (Chitinophagales) while a substantial part of the community was assigned to less prevalent groups. The meristem microbiome shows the highest abundances for Planctomycetes, Actinomycetota (Microtrichales) and Gammaproteobacteria (Granulosicoccales).

Our results are largely in line with a study on *L. digitata* (Ihua et al., 2020). This study, among others (Morrissey et al., 2019) mentions the importance of functional diversity between different niches (here: thallus regions) on the host. "The Competitive Lottery Hypothesis" suggested by Burke et al (2011) states that bacterial recruitment, driven by chance attachments, more likely depends on functional organization rather than taxonomic composition (Burke et al., 2011; Morrissey et al., 2019). This has been observed in *Ulva sp*.,

where there is a higher similarity of bacterial community composition based on the metabolic functions rather than random processes. The differences in bacterial composition observed in this thesis are thus expected to be based predominantly on the functional needs of each separate thallus (Burke et al., 2011). These functional needs have been found to differ significantly from apex to base for *Caulerpa,* a siphonous algae species (Ranjan et al., 2015). A differential functional diversity was therefore hypothesized on *L. ochroleuca* as well. Holdfast tissue, for example was expected to be characterized by bacteria that aid in the uptake of phosphorus, carbon and nitrogen from the substrate (Ranjan et al., 2015). Subsequently, given the photosynthetic capacity of kelp, aerobic nitrogen-fixing bacteria were expected on the blades (Weigel & Pfister, 2021). Lastly, since the meristem is the active growth region in the seaweed (Lemay et al., 2021), bacterial functionalities were expected to promote cell division and production of growth factors. The orders from Figure 6 and their associated functions described in literature are hereby evaluated in an attempt to gain more insight into this functional diversity and the effect it has on microbiome composition.

Bacteroidetes are known to degrade biopolymers, which creates an aerobic environment on the seaweed's surface biofilm, thereby allowing colonizing bacteria growth (Dang et al., 2011). Chitinophagales, more specifically, convert chitin to N-acetylglucosamine which in turn produces poly-N-acetylglucosamine (PGA). This is often taken up by other bacteria such as *Saccharimonadia* (Fujii et al., 2022) and results in possible biofilm formation (Izano et al., 2007). Chitinophagales also possess a subunit of nitric oxide reductase, important for the denitrification pathway which adds to the regulation of nitrogen availability (Betlach And & Tiedje, 1981). Finally, Chitinophagales possess several ATP-binding cassette (ABC) transporters responsible for releasing phospholipids (Fujii et al., 2022). In our data, these bacteria have been found most abundant in the holdfast and blade microbiome. The fact that the Chitinophagales are less abundantly found in the meristem suggests that these bacteria lack functionalities towards active growth of *L. ochroleuca*. Enterobacterales are generally capable of nitrogen fixation (Mehnaz, 2013), which could explain why they are mostly found on the blade and holdfast, since these regions have been mentioned to require available nitrogen (Ranjan et al., 2015; Weigel & Pfister, 2021).

Granluosicoccales and especially the genus *Granulosicoccus* is commonly found in association with marine macroalgae. It harbours genes that are usually involved in sulphur cycling, such as those encoding dimethyl sulfoniopropionate (DMSP) demethylase (Kang et al., 2018) and those encoding for sulphide:quinone oxidoreductase. DMSP (Bullock et al., 2017) in its turn attracts Roseovarius *sp*. which promote cell division and growth through secretion of morphogens (Kessler et al., 2018; Spoerner et al., 2012). *Granulosicoccus* also pioneer the colonization of *L. hyperborea surfaces* (Bengtsson et al., 2012) they have a rapid attachment strategy and high growth rate at early biofilm formation. These findings explain why they are most abundant in the active growth region (Kang et al., 2018). .

Pseudomonas are also prominent biofilm formers because of their production of alginate, an important structural component in seaweeds providing resilience against environmental pressures (Dharshini et al., 2021). This affects kelp propagule fitness which is crucial in a kelp's recruitment success (Morris et al., 2016). Some *Pseudomonas* have even been found to form antibiotics (Drenkard & Ausubel, 2002; Harmsen et al., 2010). Given these functions, the *Pseudomonas* found in blade and holdfast microbiome are expected to be beneficial for protection against external stressors (Singh & Reddy, 2014). *Pseudomonas, Vibrios and*

Rhodobacter also produce N-acylhomoserine lactone (AHL), which are signalling molecules. These AHL's are autoinducers for bacterial co-operative behaviour resulting from a simultaneous coordination of single cells, a process called quorum sensing. This regulates certain gene expressions in bacteria (Williams et al, 2007). Joint et al. (2007) has found that zoospores from *Ulva* sp. can actually detect the signalling molecules produced by these aforementioned bacteria and this appears to have a positive effect on zoospore settlement.

Pirellulales in their turn have been found to relate to anaerobic oxidation of ammonia in marine sponges (Jackson et al., 2013). They are also mentioned to play a role in organic mineralization and the sulphur cycle in seagrass meadows (Jiang et al., 2015). Pirellulales have a very high abundance in the meristem of our samples and less on the holdfast and blade. The same is observed for Microtrichales which are said to be key for carbon mineralization (Miksch et al., 2021). Once again, this suggests that the location of these bacteria is linked to the functional requirements of the active growth region.

Bacterial community composition differs between locations

Not only thallus region, but also location significantly affects microbial composition. This can be seen in Figure 6. Examples here involve the different abundances of Pirellulales and Rhodobacterales between England and France. This raises the question whether the distribution of these bacteria have a certain temperature dependency. Serebryakova et al (2018) have described the shifts of bacterial community composition in *Sargassum muticum* and have found a higher abundance of Planctomycetes, amongst which an unidentified Pirellulales, increased in abundance during the summer in their more northern individuals. For Rhodobactaraceae, a decline was found during the summer. As this controverses what our data suggests, there might also be other factors influencing the differing abundances of Pirellulales and Rhodobacterales (Serebryakova et al., 2018). It has been found that microbial communities have a geographic distribution that highly depends on temperature, light and its chemical environment (Gusareva et al., 2019; Rusch et al., 2007). In contrast, microbial communities associated to a host appear to have a distribution highly dependent on the selective forces from their host (Wood et al., 2022), such as the Pirellulales associated with *Sargassum* (Serebryakova et al., 2018). This host-characteristic dependency of microbiome composition has been found in humans, at which certain intestinal enterotype presence was correlated with age and body mass index of the host (Arumugam et al., 2011) as well as in mice, which have variability in skin microbiome depending on their health (Srinivas et al., 2013). Marzinelli, et al. (2015) found that geography is not even the most important factor for bacteria variation across continental scales for *Ecklonia radiata*. Therefore, it is difficult to predict whether the significant differences in microbial community between England and France is a proxy for the different environmental conditions or rather the response to host-characteristics as a consequence of these environmental conditions.

Axenization of the host

Incomplete axenization and implementations

Our results after administration of the chosen antibiotic mix show an incomplete axenization, expect for some of the cultures (NOR H and partly on NOR A). In the "Inducing gametogenesis during axenization" experiment, control treatments and antibiotic treatments were compared

with each other. The irregular cell growth from the results with no pronounced cell elongation was then exclusively found in antibiotic treatments. This irregular growth has previously been observed in *L. digitata* and *L. hyperborean* (Fries, 1980) and was then termed “callus-growth”. It has been found to depend on the season of sampling. This could be due to the difference in nutrient storage in the Laminarians (Fries, 1980) although callus growth was more rapidly observed for the individuals of Normandy than in England, no such reasoning can explain the difference here, since the adult individuals were sampled at the same approximate life stage and thus had the same nutrient storage. Callus-growth has also been found in *Ulva* sp. when made axenic (Wichard, 2023) and was then allocated to the absence of important seaweed morphogenic bacteria such as *Roseobacter* and *Maribacter*, which could explain the results here as well.

The antibiotic mix used here were not enough to keep growth of cyanobacteria (or microalgae) at bay. It is possible that they were not specific enough. Guo et al (2016) described the sensitivity of chlorophytes, cyanobacteria and diatoms to three major-use antibiotic exposures (Tylosin, Lincomycin, Trimethoprim). They found that cyanobacteria were in fact the most sensitive to Lincomycin and diatoms and chlorophytes to Trimethoprim (Guo et al, 2016). In Le et al. (2023), a list of antibiotics and their toxicity for several cyanobacteria and microalgae are given. Erythromycin, for example, is a renowned suppressor of photosynthesis in *Selenastrum capricornutum* (Liu et al., 2011) and *Microcystis aeruginosa* (Deng et al., 2014). Additionally, two or more antibiotics together usually show the highest effect on cyanobacteria and algae (Le et al., 2023). Ampicillin, for example, has the biggest effect on *M. aeruginosa* when combined with Spiramycin (Wang et al., 2018) while it depends on the species it’s dealing with and the effect it will have, this can vary from inhibition of growth to photochemical stress (Baselga-Cervera et al., 2019). Therefore, it is possible that the antibiotic mix used in these experiments were not the right ones for the specific species of cyanobacteria that was dealt with and more research is needed regarding the correct combination of antibiotics and their concentrations, especially to keep them safe from callus-growth and cyanobacteria.

Inducing gametogenesis

Effect of gametophyte aggregation on gametogenesis

During experimentation, we observed that sporophyte growth seemed to exclusively occur on sparsely distributed gametophytes. It was briefly believed that high density of the gametophytes had negative effects on gametogenesis due to a lack of nutrient availability and oxygen. This was ruled out in the “Density dependence of gametogenesis” experiment as no general effect nor abundant gametogenesis was observed before cultures were overgrown with cyanobacteria. ENG K however did show a quicker gametogenesis in the lowest gametophyte concentration but this could be due to individual effects since ENG K showed rapid gametogenesis in comparison to the other individuals during the “Inducing gametogenesis during axenization” experiment. Another possible factor influencing the results in ENG K could be the differential nutrient supplementation in the lowest concentration since more medium was added to dilute the gametophyte medium to 4 mL. Similar observations were made by Reed et al as they have found that lower gametogenesis at higher densities was most likely due to nutrient limitation. (Reed et al., 1991). In contrast, kelp recruitment has been mentioned to be enhanced by proximity of settled zoospores (Muth, 2012). Thus given

current scientific evidence, gametophyte aggregation has no negative affect on gametogenesis if enough nutrients are provided, on the contrary, it provides gametogenesis enhancement.

Optima for gametogenesis in mature gametophytes

The factors most highly influencing growth towards *Laminaria's* different life phases are nutrient availability, light intensity, temperature and photoperiod length (Haxo & Blinks, 1949; Lewis et al., 2013; Lüning, 1980; Lüning & Dring, 1975; Lüning & tom Dieck, 1989; Martins et al., 2017; Ratcliff et al., 2017) Several choices have been made for optimal growth in laboratory conditions, involving culture medium, artificial light and the day:night cycle, incubation temperature and frequency of medium renewal. These choices are now evaluated.

❖ *Culture medium*

Growth and maintenance of the gametophytes was optimized using sterilized seawater that was supplemented with G_2O_2 and Provasoli Enriched Seawater (PES). The gametophytes were kept under a red light regime in 15 °C for maintenance during their vegetative filamentous state. Transition to fertile gametophytes was induced using exposure to white light. PES is commercially available and is mentioned to positively affect the growth of gametophytes (Ratcliff et al., 2017). The same medium was used throughout the entire research duration, and demonstrated efficacy. However, the rate of gametogenesis induction after red light incubation was less than ideal. One possible explanation for this is that Provasoli Enriched Seawater is not the optimal medium for gametogenesis. Another commonly used commercially available medium, f/2, has indeed provided more successful transition to fertile gametophytes for *Laminaria digitata* under blue light (Ratcliff et al., 2017). The differential composition between these media is the probable explanation, since PES contains Boron, which is absent in f/2. *Laminaria* species' growth is said to be enhanced by Iron (Lewis et al., 2013), but adding Boron has been observed to inhibit gametogenesis in *S.longissima* and *S.japonica* (Ratcliff et al., 2017)

❖ *Light quality and photoperiod length*

Light conditions are essential for photosynthesis of these gametophytes but also for proper transitioning between life stages. Fertility induction can then only happen with enough formation of photosynthate in the prior life stage which in its turn depends on the photoperiod length (Gao et al., 2013; Hoffmann & Santelices, 1982; Ratcliff et al., 2017). Presence of light and photoperiod length can also impact gametogenesis indirectly since there appears to be a positive relationship between the size of the gametophytes before fertility and the rate of gametogenesis in *S.latissima* (Ratcliff et al., 2017). The growth of gametophytes is said to be uncorrelated with the light quality for brown algae (Haxo & Blinks, 1949; Lüning, 1980).

A factor influencing the slow transition to gametogenesis in this research could thus be the use of white light, instead of direct blue light. The optimal wavelengths for gametogenesis induction in *S. latissima, L. digitata* and *L. hyperborea* are said to be around 400-500nm (Lüning, 1980; Lüning & Dring, 1972, 1975). Thus, violet or blue light is needed for fast induction of gametogenesis. As white light was mostly used in the laboratory for seaweed growth, the wavelengths were not specifically altered for gametogenesis as literature demands. However,

when it was observed that gametogenesis was lower than expected, additional blue light was added. It is unclear however if this was the main cause of faster gametogenesis.

❖ *Temperature*

Thermal sensitivity has been found to play a major part in seaweed growth as well (Andrews et al., 2014). It even seems to have combined effects with photoperiod length since some kelp species only show gametogenesis on short days combined with low temperatures (Lüning & tom Dieck, 1989; Martins et al., 2017). The gametophytes from England and France, are used to different temperature ranges. This temperature difference is around 1 °C according to Copernicus.eu and shows an average of 18 °C in August and 7.5 °C in March on the French coast while England ranges from 17 °C in 8.5 °C in March on the Southern-English coast.

Interesting to note is that gametophytes also show a separate response to temperature in the lab. The results of the temperature control experiment showed a higher sporophyte count for England in the colder 12 degrees in comparison to France, while France showed a higher sporophyte count for the warmer 18 degrees. Even though these results have not been submitted to statistical analysis, it opens the discussion regarding local adaptation in the parental individuals of the gametophytes in this research. It suggests that there are adaptive traits toward temperature optima and more importantly that these traits could either be genetically inherited or acquired due to vertical transmission of the microbiome. The latter is plausible given the fact that the individuals used for this experiment were never made axenic but came directly from the 15 °C red light incubator. However it has been mentioned to be less significant in aquatic ecosystems due to the bigger effect of other environmental factors affecting microbiome composition and the highly different environments of each life stage (Lemay et al., 2018).

Alsuwaiyan et al (2021) already discussed how genetic variation in *Ecklonia radiata* explains a differential response to marine heatwaves, and Pang et al (2007) mentions that thermal tolerance is a genetically inherited trait in *Laminaria japonica*. This implies that there is indeed a genetic response to temperature and that the recruitment success of the gametophytes is linked to the original location of their parent. The individuals in our experimentation have reduced genetic diversity because they result from self-fertilization but possibilities exist to use outcrossing experiments for the fitness increase during future temperature changes, as has been done by Liesner et al. (2022). Attempts were made to outbreed Arctic *Laminaria digitata* populations with temperate ones. This was done to investigate the potential effect of enhancing genetic diversity on heat stress defence. The results showed a significantly higher heat tolerance for outbred populations than for inbred Arctic individuals. Supporting genetic diversity of populations can prove very important for future conservation and management strategies (Liesner et al., 2022).

❖ *Other factors*

Ratcliff et al. (2017) also mentions the importance of agitating culture flasks with gametophytes to enable gas exchange and to interrupt formation of a so-called boundary layer that could form around the gametophytes. It could therefore be expected that the aeration in the

temperature control experiment shows an improved gametogenesis rate, however, more research is needed for the proper methodology. These findings highlight that not one but many environmental factors play a role and that it is extremely difficult, given the seasonal and latitudinal variations, to create circumstances ideal for gametogenesis of all individuals under study here.

Effect of bacterial inocula on gametogenesis

All thallus inocula significantly and positively affected gametogenesis in comparison to the control treatment. Given the fact that these thallus inocula contained equal amounts of bacteria from Morocco, France and England, it suggests that bacterial inoculation by bacteria from adults across latitudinal regions is beneficial for gametogenesis or sporophyte recruitment. However, red light incubation negatively affected the gametogenesis. This can be expected since, as mentioned before, light exposure of the right wavelength is needed to transition from the vegetative phase to fertile gametophytes (Lüning, 1980; Lüning & Dring, 1975; Lüning & tom Dieck, 1989; Ratcliff et al., 2017). The lack of significant effects of location could be allocated to the use of a bacterial mix rather than a separate inoculum for each location.

Our results also showed low levels of bacterial diversity in inocula treatments (*Vibrio, Pseudomonas* and *Pseudoalteromonas*). This could be due to the selection of bacterial isolates which was nudged towards easily separated colonies, although colours of these colonies were variable. However, the bacteria used for the treatments still appeared to provide advantageous functions that enhanced gametogenesis.

The most important bacterial functions relevant for seaweed symbiosis and sporophyte recruitment appear to be among others biofilm formation, the presence of ABC-transporters, nitrogen fixation, carbon mineralization(Betlach And & Tiedje, 1981; Bullock et al., 2017; Dang et al., 2011; Dharshini et al., 2021; Fujii et al., 2022; Lemay et al., 2021c; Ranjan et al., 2015; Weigel & Pfister, 2021; Williams, 2007). To get an idea of the available functions in the bacteria used for bacterial inoculation, the Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used and the sequences of the present *Vibrio's*, *Pseudomona's and Pseudoalteromonas* were investigated for these previously mentioned functions. The results show that the *Vibrio's* most likely and exclusively contributed to nitrate reduction, nitrogen availability, quorum sensing, glucose/mannose transport, vitamin B12 membrane transport and Phenylalanine and Tryptophan biosynthesis. *Psychromonas* only provide glycogen synthesis and *Pseudoalteromonas* seem to have no function that are exclusive to the genus, however all three genera provide fatty acids, Riboflavin and vitamin B6 biosynthesis and Iron and Phospholipid membrane transport.

The blade and holdfast treatment are mostly characterized by *Vibrio*'s, while the meristem treatment were dominated by *Pseudoalteromonas*. It could thus be expected that, because of the previously mentioned functions, the blade and holdfast treatment would affect gametogenesis the most. *Vibrio*'s provide the most functions related to seaweed growth (nutrients, vitamins, biofilm, amino-acids, lipids) and visualisation of the data (Figure 8) shows that on average, the blade and holdfast treatment shows the biggest effect on gametogenesis on the individuals originally from France (Figure 8). A post-hoc comparison however (supplementary) show that there is no significant difference between thallus inocula effects on gametogenesis.

Important to note is that in the nearly axenic cultures, bacteria were still growing on the streaked agar plates. These bacteria were sequenced and contained *Alteromonas, Maribacter, Lacinutrix sp., Proteobacter* and *Zobelia sp*. The bacteria *Alteromonas* and *Maribacter* have been found to play a major role in marine carbon cycling (Bengtsson et al., 2011; Selvarajan et al., 2019). Some *Alteromonas* have metal-resistance and capabilities of binding Cu^{2+} and Zn^{2+} cations, which is not particularly of impact here, however it has interesting implications for future inocula administration(Selvarajan et al., 2019). The role of *Maribacter* in morphogenesis of *Ulva sp.* has been discussed before, and might provide this functionality for *L. ochroleuca* as well. The Flavobacter *Lacinutrix sp*. degrade algal polysaccharides (Saha, Ferguson, et al., 2020) however, they were only present on NOR B which was not used in this experiment. Zobelia species are not discussed a lot in the literature, besides their carrageenan degrading activity in red algae (Mystkowska et al., 2018). These supplementary bacteria could provide a higher sporophyte count. The bacteria under possible influence of these additional bacteria are highlighted in the data of the supplementary. *Maribacter* (ENG E) does not seem to provide additional recruitment success in general, except for some individuals also exposed to meristem and blade inocula. ENG K, supplemented by *Zobelia* and *Maribacter/Proteobacter sp* does slightly better, NOR H however has additional *Alteromonas* and shows an overall positive trend towards gametogenesis. This means that the bacteria added could in fact provide additional positive effects.

A second important note is that the control treatment contained bacteria from a different environment as the adults which could explain the lack of response to the inocula. Next to this, the control treatments were also overgrown by diatoms due to an inefficient filtration step. A third and final remark on these results is that, during the final counting at day 30, it was noted that a lot of the sporophytes lost their colour and were therefore considered dead. However, since it is not sporophyte survival, rather recruitment success that was investigated here, this was dismissed. Additionally, sporophyte death could also have been a result of too much light or too little nutrient availability, since the dead ones were also quite large and developed (Gao et al, 2013).

Effect of bacterial inocula on recruitment during heat stress

Bacterial inocula experiments have been done before and have shown positive effects (Malfatti et al., 2023). However, when an external stressor is involved, positive results are not as easily obtained (Delva et al., 2023). Therefore, the final experiment in this thesis was aimed at enhancing the heat tolerance of *L. ochroleuca* that was inoculated with bacteria from adult individuals. The sporophyte counts were too low to perform statistical analysis. However, the photosynthetic yield results from the PAM fluorescence show interesting trends.

The 16 °C treatment is a control for all inocula treatments. Nevertheless, the control treatment in 16 °C shows a different trend to the ones exposed to thallus inocula. A gradual decline in yield is observed, which is resumed in the next datapoint. This is different for the blade, meristem and holdfast inocula treatments, in which a gradual increase in yield is seen during the heatwave and a significant increase after heatwave administration. The same differences between control and thallus inocula treatments can be observed in the 18 degrees heatwave treatment. However, for the 22 degree heatwave, it can be observed that yield decreases for the control treatment, which is not the case for the thallus inocula treatments. This stipulates

that bacterial inocula might have an effect on gametophyte health during heatwaves. However, no significant evidence has been put forward here yet it still provides promising implementations for future restoration strategies.

Critical reflections on methods during experimentation

As is mentioned by van der Loos and Nijland (2021) in a review on methodology bias, biodiversity is often a proxy for the health of a marine ecosystem and assessing it has to be time- and cost-effective as well as accurate (Aylagas et al., 2018; Goodwin et al., 2017; Porter & Hajibabaei, 2018). DNA metabarcoding simultaneously identifies many taxa within one sample by high-throughput sequencing a pool of genomic DNA (Aylagas et al., 2018). The "bulk DNA" as defined in this review, came from surface swabs obtained from adult *L. ochroleuca* and DNA extraction was done but not the whole genome was sequenced, only the 16S rRNA amplicon of every taxa present in the samples. This was pooled for metabarcoding. The methodology bias as defined in this review yet also presents itself in this research. This is defined as the many choices one makes from sampling up until the metabarcoding for sequencing. While sampling, a certain period, life stage, method, etc is chosen. These choices are also made in the preprocessing steps of the samples: the preservation of the DNA in RNA later, the chosen DNA extraction kit and primers, the PCR and the sequencing method.

For the preserving methods, it has been found that DESS (which are DMSO buffers that contain EDTA and are saturated with salt) is recommended as the better choice by a number of studies because it is said to yield higher quality and quantity of DNA compared to what is preserved in RNAlater and also gives a higher PCR yield (e.g., Fonseca & Fehlauer-Ale, 2012 on nematodes; Gaither et al., 2011 on corals and Ransome et al., 2017) on hard substrate communities). Choosing the right annealing temperature during the PCR step is also crucial. A temperature that is too low can change the outcome by amplifying unwanted products, but setting it too high in contrast will cause a reduction of the PCR yield which means not all the aimed products of the study will be amplified. To evade this problem Korbie & Mattick (2008) have presented a touchdown profile which starts at high annealing temperatures that drop every cycle. This is done in this study. However, a single chosen annealing temperature is still perceived as the better choice in several studies because it has a higher yield still, depending on the study (Aylagas et al., 2018; Clarke et al., 2017)

Sequencing is done using the Oxford Nanopore sequencing technology, as it is critically acclaimed and has already been found an interesting alternative to Illumina sequencing because it can sequence DNA strands of any lengths and thus provide better resolutions, especially for Long ribosomal sequences. The Oxford Nanopore platforms do have a lower accuracy of raw reads but this can be surpassed by the fact that the fragments are longer or by using bioinformatics that can combine multiple raw reads and create a consensus sequence (Nijland et al, in prep). All these considerations on each step of the process makes for difficult choices that truly depend on the study and shows how any of these steps can change the results. Therefore it is important to take this into account when considering the results of this thesis.

Future implications based on previous cultivation experiments

As this thesis aims to provide potential future implications for the restoration success of *Laminaria ochroleuca*, a thorough discussion on previous applications on Laminariales is presented. Kelp cultivation has already been found to be beneficial for restoration and commercial approaches of declining kelp species (Wood et al., 2019). Altering the kelp microbiome has even proven effective in production increase. (Florez et al., 2021; Morris et al., 2016; Wichard, 2023). Cultivation efforts are usually carried out by growing gametophytes in nurseries until they reach the targeted size as a juvenile sporophyte (Redmond et al., 2014) These are then planted onto anchored lines in the open ocean. Microbial manipulations are therefore easily done under controlled conditions in the nurseries as is partly done in this thesis (although not in the same magnitude). This has already been mentioned to have a great impact on the developing host in later life stages (Barret et al., 2015; Damjanovic et al., 2019; Han et al., 2021; Walsh et al., 2021).

The question is however if this developed microbiome from the nursery stages transfers completely to the adult individuals found at the cultivation site. Davis et al (2023) showed a significant difference between microbial composition of seedstring juveniles and outplanted adults of *Alaria marginata* and *Saccharina latissima*. The same has been found by Han et al (2021) on *Saccharina japonica*. Microbiome composition has already been mentioned to differ between life stages in *Mastocarpus spp* by Lemay et al.(2021). As mentioned before, the microbiome is subject to many environmental factors, so the differential microbial composition during life phases is to be expected. If microbial manipulations are to be effective on cultivation sites, it is important to take the “Competitive lottery” hypothesis into consideration. Bacterial recruitment is then dependant on stochastic factors, being the random process at which bacteria are introduced to the seaweed’s surface, and deterministic factors, being the functional requirements of each thallus region determining eventual microbial composition (Burke et al., 2011; Morrissey et al., 2019). If kelp sporophytes are adapted to administered heatwaves due to an increase in microbial diversity or focus on protective and nurturing bacteria, microbial manipulations will probably have a positive effect on overall recruitment success. However, as previously mentioned, the microbiome will most likely change composition when they are out planted at which point previous manipulations might lose their effect. However this has not been extensively researched yet and is one of the future implications for RestoreSeas.

Deterministic factors influencing microbial composition might also be priority effects, defined as microorganisms that influence the structure of surface microbiome by directing further recruitment of other introduced taxa (Fukami, 2015; Nappi et al., 2022; Vass & Langenheder, 2017). Priority effects have been found to have a major impact on the microbiome composition of plant surfaces and the human gut (Carlström et al., 2019; Hiscox et al., 2015; Sprockett et al., 2018)). Early arrival seems to be key for community establishment determined by the bacteria already present (Svoboda et al., 2018). However, the individual effect of many taxa is yet to be determined and especially the effect that one bacteria can have on subsequent assembly of the microbiome. However, Nappi et al (2022) found that manipulative colonization of *Pseudomonas tunicata* D2 and *Pseudovibrio sp*. D323 had significantly different results in subsequent development in comparison to control treatment and between them. The taxonomic changes due to manipulated colonization of these bacteria apparently also changed functional diversity of the microbiome (Nappi et al., 2022).

This implies that if microbial manipulations in the nursery stage could evolve towards a manipulated colonization in the outplanted sites, microbiome composition could in fact also be changed due to priority effects and a subsequent altered functionality. It is plausible to predict that bacteria performing these priority effects could be “chosen” to impact the functionality towards an increased heat stress resistance. However, more studies on this needs to be performed.

Crossbreed experiments between high altitude individuals and their more southern conspecifics might also provide additional possibilities to change the genotype’s response to heatwaves and consequently the functional requirements involved in this response. This might thereby change the host-characteristics, of which microbial composition is so highly dependent as mentioned earlier (Wood et al., 2022), and increase the need for heat-stress mediating bacteria. However this was not the main objective of this study and RestoreSeas.

It is also interesting to note that *L. ochroleuca* has a largely overlapping niche with *L. hyperborea* (Smale et al., 2015) and *L. digitata* (Blight & Thompson, 2008; Wright & Foggo, 2021). It has been found however that the boreal species (*L. digitata and L. hyperborea*) which are normally distributed to the Northeast of the Atlantic have a different pigment content followed by a higher photosynthetic capacity (Wright & Foggo, 2021). Both *L. digitata* and *L. ochroleuca* have an epibiont fauna made up of annelids, bryozoans and molluscs on their holdfast but it has been found that the species richness of these epibiont species is lower in *L. ochroleuca* (Blight & Thompson, 2008). *L. digitata* also has more unique species (Blight & Thompson, 2008). *L. ochroleuca* has some fundamental differences with *L. hyperborea* such as the absence of epiphytes, a longer meristem, more grazers on the blades and a different summer growth rate in the former (Smale et al., 2015). It has been found that *L. ochroleuca* has a higher capacity of withstanding dislodgement caused by waves than *L. hyperborea* (Smale &Vance, 2016; Smale & Moore, 2017) which could provide a large competitive advantage if there would be more storms caused by climate change. Considering the similarities and differences between these *Laminaria* species, insights into the role of the microbiome in stress mediation of kelp and the possibilities of manipulating this toward fitness enhancement, could provide insights in how future climate scenarios will alter interactions between these co-occurring species.

Conclusion

This thesis provides promising results for future restoration strategies of *L. ochroleuca* and other Laminariales. Bacterial inoculation has a significantly positive effect on recruitment success and provides a seemingly positive trend on gametophyte health during heatwave administration. The significantly differential bacterial community composition per thallus region and location, caused by different functional requirements and host-characteristic responses respectively, confirms the close association between the microbiome and its seaweed host. Knowledge of the functions bacteria provide per thallus region and per location is certainly attributable to experimentations in this regard. Future possibilities to further investigate the effect of beneficial bacteria enhancements might however also involve optimalisation of culture conditions, axenization protocols and bacterial isolation. In conclusion, the seaweed

microbiome is dependent on many host-related factors, which makes its bacterial community composition a “snapshot” in time and space. Although this makes long-term manipulations rather difficult, the potential effect of early colonisation by beneficial bacteria on directing further bacterial recruitment by priority effects might provide successful results for later life stages during cultivation experiments.

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Supplementary

Risk assessment

Sampling

During sampling in Normandy, rocky shores were visited at low tide (6 a.m. and 5-7 p.m.) in August. This involved carrying the cooling boxes and goggles towards sampling sites early in the morning and late in the evening. Hazards identified here were falling on the rocks and hurting our feet and head or slipping on exposed seaweeds. During snorkelling for samples, it was necessary to take the tidal cycle into account and beware of being taken away by the current but also the risk of undercooling in the water.

Measures taken to be safe around these hazards were sturdy water shoes and possibly hiking shoes during the walk towards sampling sites; avoiding stepping on seaweeds but rather testing the grip on rocks underneath with every step; taking the tides into account by choosing a reference point to put some equipment on and check the water level every few minutes and avoiding undercooling by wearing wetsuits and taking a towel along.

In case of failure of these safety measures, it was important to make sure no one was ever alone during sampling so help could be acquired since it was not possible to take phones along while in the water. During sample processing at the site, an RNAlater stabilization solution was used, however this solution is marked as non-hazardous (ThermoFisher Scientific, 2021b).

Sample processing

During sample processing in the laboratory, KI (0.5%) was used to clean the tissue samples from the adult *Laminaria* individuals. This solution is considered hazardous for the health and causes organ damage when regularly exposed and swallowed, inhalation must be avoided at all costs. It was therefore handled with gloves and a protective labcoat while the head was kept at a safe distance to avoid inhalation. The tweezers used for handling were continuously sterilized with a STERI360 machine which can get to a temperature of 250 °C, the machine was therefore always placed in a corner of the worktable and covered when not in use while heated tweezers were placed upright on a porcelain dish. In case of burns caused by the STERI360, cool water should be run over the affected area for a minimum of 10 minutes after removing from the heat source and when there is a severe burn, it has to be examined by a professional.

Culture maintenance

For renewal of culture medium of the gametophytes, Provasoli enriched seawater and Germanium dioxide were used. Provasoli enriched seawater aims to mimic the natural seawater that seaweeds are usually exposed to, however measures are necessary to avoid skin irritation and accidental ingestion since it is still a chemical used in the laboratory. Germanium oxide is considered a chemical with serious health hazards and poses an environmental hazard. It damages the organs with long-term exposure or when swallowing or inhaling. Both chemicals were added to the sterilized seawater under a biohazard flow as it was the intension to keep the culture medium sterile. Gloves were also used when handling these chemicals and replaced afterwards to avoid contamination.

Bacterial isolation and maintenance

For bacterial isolation, petri dishes were used with marine agar and saccharine agar. Both are not classified as hazardous according to the GHS classification, however, while pouring the hot agar solution into the petri dishes, a right amount of care was to be taken to avoid getting burned, as this was heated up until it boiled and then immediately used for pouring. Therefore, during this process, gloves were used and extra paper for protection of the hands in case of spillage. The bacteria were isolated using grafting needles, sterilized with the STERI360 under a biohazard flow to avoid contamination since bacterial concentrations were rather high. After handling of the bacteria, gloves were always discarded, and the biohazard flow was carefully disinfected using 70% ethanol. Ethanol is considered highly flammable, so its use was always with careful consideration of avoiding open flames in the proximity. Plastic stencils were used when bacterial isolation was not required in large quantities, these were consistently discarded into a plastic container with an extra plastic zip lock bag to facilitate safer disposal of the needles afterwards. Bacteria were kept in marine broth (Millipore)(Roth GmbH, 2021a) and 30% glycerol (Sigma-aldrich) which are both non-toxic however marine broth has been found to cause serious eye irritation. In case of exposure and contact with the eyes, it had to be rinsed carefully. All materials used for the bacterial isolation were discarded into a specialised bin for contaminated waste. Isolated bacteria petri dishes were sealed off with parafilm and taped together when discarded.

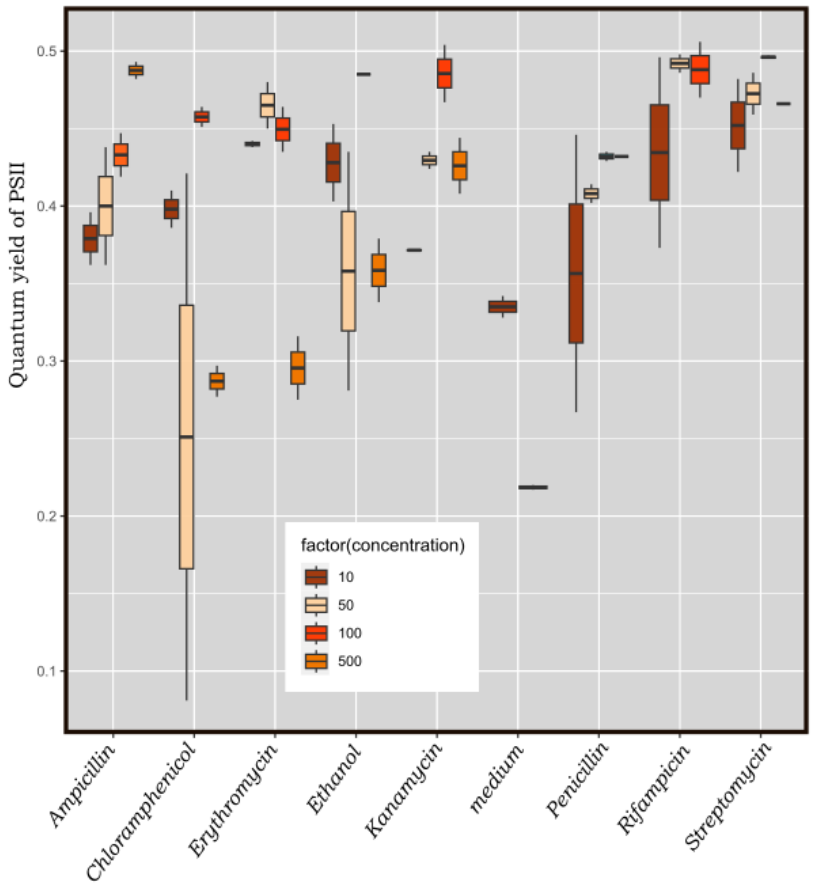
Antibiotics

The antibiotics used (Ampicillin, Chloramphenicol, Kanamycin, Rifampicin, Erythromycin, Penicillin, Streptomycin) were all handled as the most hazardous one. Therefore the serious health risks in close contact with the skin (Streptomycin, Chloramphenicol, Ampicillin, Penicillin, Rifampicin) or the eyes (Rifampicin) and possible respiratory irritation when inhaled (Ampicillin, Penicillin, Rifampicin) were considered as well as the possible reproductive toxicity (Streptomycin, Kanamycin) and carcinogenic effects (Chloramphenicol). Therefore, an FFP2 mask was worn any time these antibiotics were handled at any concentrations. They were acquired from the chemical lab while wearing a protective lab coat and specialised gloves. Equipment was consistently cleaned with ethanol or replaced after every use. While the hands were washed after glove disposal with ample water and soap. In case of contamination of the clothes, they had to be taken of and washed, in case of exposure to the skin or eyes, they had to be washed out, in case of symptoms caused by accidental ingestion (headache, nausea, vomiting, breathing difficulties, irritant effects, allergic reaction) medical advice needed to be acquired. Nystatin was later added to the antibiotic mix; however this has not been found a safety hazard according to GHS regulations. The same goes for the yeast extract used one time. All materials used for the antibiotics were discarded into a specialised bin for contaminated waste. Leftover antibiotic mixes were poured into a “contaminated waste” flask (Pfeizer, 2013; Roth GmbH, 2021b, 2022b; Sigma-Aldrich, 2023a, 2023b; ThermoFisher Scientific, 2021a).

DNA extraction

During DNA extraction, propanol was used in addition to the non-hazardous chemicals in the Wizard® HMW DNA extraction kit (Promega). As propanol is considered corrosive(Roth GmbH, 2022a), highly flammable and irritates the eyes, it's use was kept away from open flames and eye and skin contact was avoided. Gloves and lab coat were worn for this purpose and the bottle was always securely stored back into the freezer as acquired from the protocol of the extraction kit.

Photosynthetic yield of gametophytes under different antibiotic treatments

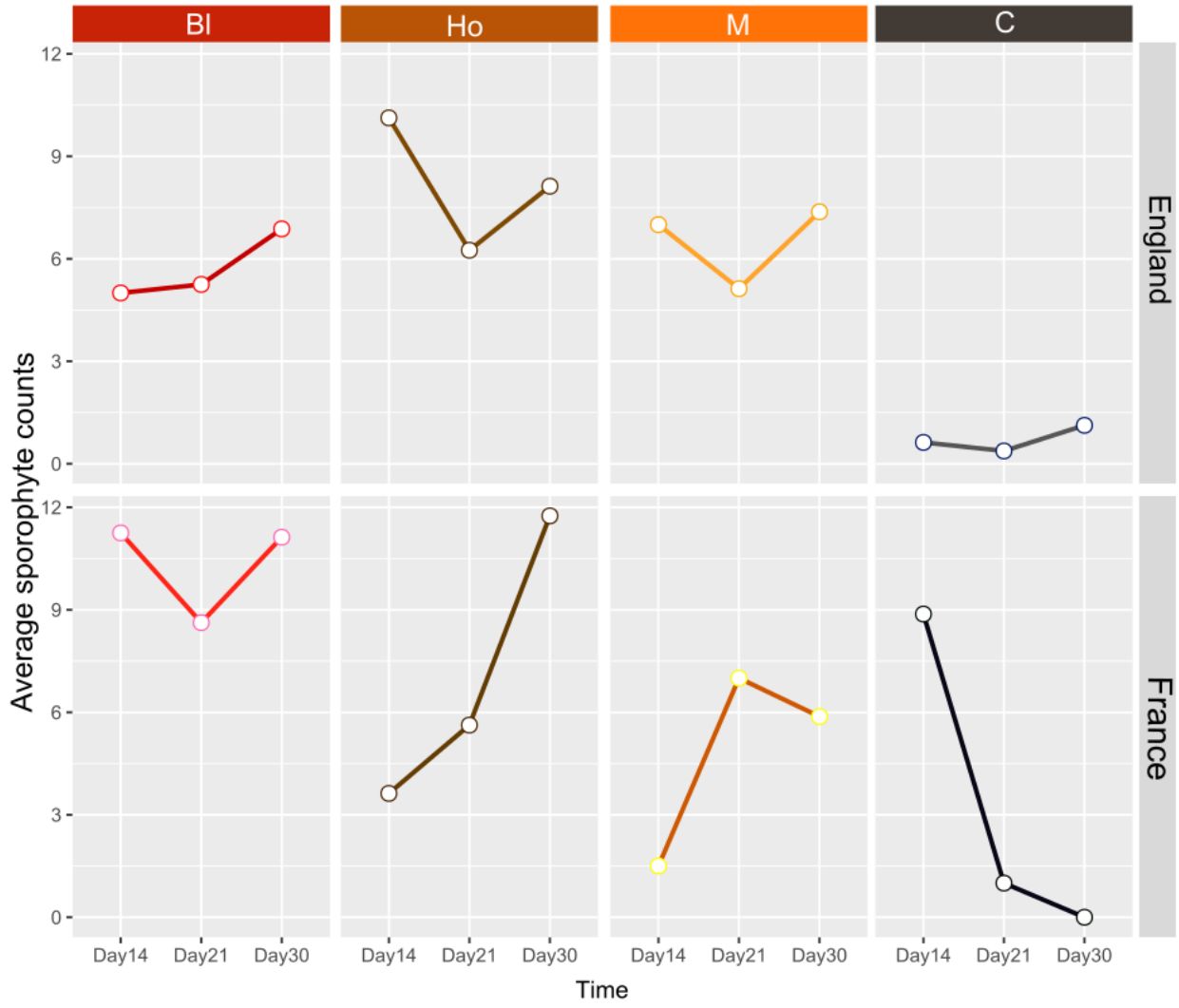


Sporophyte counts induction of gametogenesis after axenization

Individual	Treatment	Origin	Plaat	Day 1	Day 7	Day 14	Day 21	Day 30	TOTAL
NOR G	C	RL	1.1	0	0	0	0	0	0
NOR H	St	Ax	1.1	0	0	0	0	0	5
NOR H	Ho	Ax	1.1	0	2	4	1	2	78
NOR G	Bl	Ax	1.1	1	37	50	28	44	0
NOR G	Bl	RL	1.1	0	0	0	0	0	22
NOR G	Ho	Ax	1.1	1	48	17	5	15	4
NOR G	St	Ax	1.1	1	20	0	4	3	1
NOR D	St	RL	1.1	0	0	0	1	8	0
NOR G	Ho	RL	1.1	0	0	0	0	8	0
NOR H	Bl	Ax	1.1	1	1	0	0	0	0
NOR D	Ho	RL	1.1	0	0	0	0	3	0
NOR G	St	RL	1.1	0	0	0	0	0	1
NOR H	C	Ax	1.1	1	12	0	1	0	20
NOR G	C	Ax	1.1	1	33	14	6	0	1
NOR D	Bl	RL	1.1	0	0	0	1	1	1
NOR D	C	RL	1.1	0	7	0	1	0	1
NOR D	St	RL	1.2	0	0	0	1	3	2
NOR G	Ho	RL	1.2	0	0	0	2	14	33
NOR H	C	Ax	1.2	1	12	33	0	0	0
NOR D	C	RL	1.2	0	0	0	0	0	44
NOR G	Ho	Ax	1.2	1	130	8	36	52	7
NOR H	St	Ax	1.2	1	30	0	7	13	40
NOR D	Bl	RL	1.2	1	28	40	0	3	24
NOR G	C	Ax	1.2	0	0	24	0	0	0
NOR D	Ho	RL	1.2	1	3	0	0	0	54
NOR G	St	St	1.2	1	255	12	42	20	1
NOR H	Ho	Ax	1.2	1	26	0	1	0	1
NOR H	Bl	Ax	1.2	1	5	0	1	3	1
NOR G	St	RL	1.2	0	0	0	1	0	34
NOR G	Bl	Ax	1.2	1	282	0	34	31	5
NOR G	Bl	RL	1.2	0	0	0	5	7	0
NOR G	C	RL	1.2	0	0	0	0	0	0
ENG E	C	RL	2.1	0	3	0	0	0	0
ENG K	C	Ax	2.1	0	0	0	0	0	26
ENG E	Ho	Ax	2.1	1	17	0	26	22	0
ENG E	Ho	RL	2.1	0	0	0	0	2	43
ENG K	St	Ax	2.1	1	1	36	7	8	5
ENG E	C	Ax	2.1	0	0	5	0	0	13
ENG E	St	Ax	2.1	1	75	0	13	19	14
ENG E	Bl	Ax	2.1	1	35	0	14	18	0
ENG L	Bl	RL	2.1	0	0	0	0	0	41
ENG K	Ho	Ax	2.1	1	11	32	9	7	0

ENG L	C	RL	2.1	0	0	0	0	0	0
ENG L	St	RL	2.1	0	0	0	0	0	0
ENG K	Bl	Ax	2.1	0	0	0	0	2	17
ENG L	Ho	RL	2.1	0	0	17	0	2	0
ENG E	St	RL	2.1	0	0	0	0	0	0
ENG E	Bl	RL	2.1	0	0	0	0	1	0
ENG K	St	Ax	2.2	1	20	0	0	2	0
ENG E	Ho	RL	2.2	0	0	0	0	1	42
ENG E	Bl	Ax	2.2	1	90	23	19	27	0
ENG K	Ho	Ax	2.2	1	15	0	0	1	0
ENG E	C	Ax	2.2	0	0	0	0	0	0
ENG E	St	RL	2.2	1	5	0	0	0	26
ENG K	Bl	Ax	2.2	1	20	17	9	7	0
ENG L	Ho	RL	2.2	0	0	0	0	0	0
ENG L	St	RL	2.2	0	0	0	0	0	41
ENG E	St	Ax	2.2	1	70	20	21	30	0
ENG E	Bl	RL	2.2	0	0	0	0	0	47
ENG E	Ho	Ax	2.2	1	112	32	15	30	0
ENG L	Bl	RL	2.2	0	0	0	0	0	0
ENG K	C	Ax	2.2	0	0	0	0	0	3
ENG L	C	RL	2.2	0	0	0	3	0	0
ENG E	C	RL	2.2	1	20	0	0	9	0

Visualisation of sporophyte count over time



Additive treatment and location effect on gametogenesis

	Day 14				Day 21				Day 30			
Effect	E	SE	T	P	E	SE	T	P	E	SE	T	P
Blade	0.0775	0.8135	0.0952	0.9241	1.2289	0.7512	1.6359	0.1019	3.3716	1.1389	2.9605	0.00307**
Holdfast	0.5840	0.7661	0.7623	0.4459	1.0973	0.7451	1.4727	0.1408	3.7509	1.1421	3.2841	0.00102**
Meristem	-0.3018	0.8596	-0.3510	0.7256	1.3762	0.7423	1.8540	0.0637	3.0928	1.1455	2.6999	0.00694**
Location	0.1477	0.5700	0.2592	0.7955	0.6236	0.5076	1.2284	0.2193	0.0624	0.5164	0.1209	0.9037
0|1	1.2100	0.6357	1.9035	0.05697*	1.4878	0.6440	2.3103	0.02087*	2.6953	1.0701	2.5188	0.01178*
1|2	1.3805	0.6421	2.1500	0.03155*	2.5581	0.6910	3.7021	0.00021***	3.8486	1.1058	3.4802	0.00050***

Post-hoc comparison of treatment effects on gametogenesis

Origin = Ax						Origin = RL					
contrast	estimate	SE	df	z.ratio	p.value	contrast	estimate	SE	df	z.ratio	p.value
C - Bl	-3.877	1.195	Inf	-3.244	0.0065**	C - Bl	-3.877	1.195	Inf	-3.244	0.0065**
C - Ho	-4.345	1.213	Inf	-3.582	0.0019**	C - Ho	-4.345	1.213	Inf	-3.582	0.0019**
C - M	-3.495	1.19	Inf	-2.936	0.0175*	C - M	-3.495	1.19	Inf	-2.936	0.0175*
Bl - Ho	-0.468	0.675	Inf	-0.694	0.8995	Bl - Ho	-0.468	0.675	Inf	-0.694	0.8995
Bl - M	0.383	0.683	Inf	0.561	0.9437	Bl - M	0.383	0.683	Inf	0.561	0.9437
Ho - M	0.851	0.692	Inf	1.23	0.6076	Ho - M	0.851	0.692	Inf	1.23	0.6076

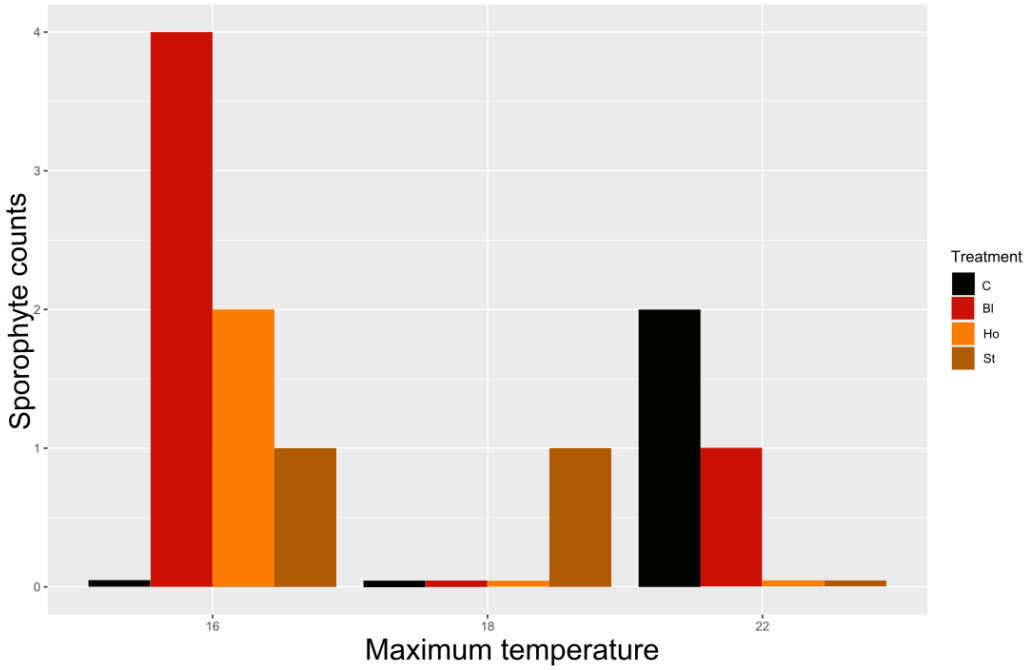
Sporophyte counts culture flasks under aeration

Location	Individual	Temperature	Duplicate	Day 1	Day 7	Day 14	Day 24
ENG	E	18	1	0	0	0	0
ENG	E	18	2	0	0	0	0
ENG	E	18	3	0	0	0	0
NOR	H	18	1	0	0	0	0
NOR	H	18	2	0	0	0	0
NOR	H	18	3	0	0	0	0
ENG	E	16	1	0	0	0	0
ENG	E	16	2	0	0	0	0
ENG	E	16	3	0	0	0	0
NOR	H	16	1	0	0	0	0
NOR	H	16	2	0	0	0	0
NOR	H	16	3	0	0	0	0
ENG	E	12	1	0	0	1	0
ENG	E	12	2	0	0	1	0
ENG	E	12	3	0	0	1	0
NOR	H	12	1	0	0	0	0
NOR	H	12	2	0	0	0	0
NOR	H	12	3	0	0	0	0

Binomial logistic regression

	Estimate	Std.Error	z-value	Pr(>|z|)
(Intercept)	-3.90E+00	1.2097	-3.22	0.00128 **
Blade	7.50E-01	1.2707	0.59	0.55486
Holdfast	0.7503	1.2707	0.59	0.55486
Meristem	1.2154	1.2047	1.009	0.31301
Location (France)	1.2027	0.8481	1.418	0.15618

Visualisation of sporophyte counts after heatwaves



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