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Macroalgae Nursery Methods to Support Marine Forest Restoration

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Macroalgae nursery methods to support marine forests restoration

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Declaração de Honra

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Abstract

In this study, three key species forming marine forests have been investigated: *Himanthalia elongata, Ascophyllum nodosum,* and *Laminaria hyperborea.* These are cold water species, presenting their southern limit of distribution in the North of Portugal. Their life cycle is already known, being like other fucoid and kelp, but their reproduction in the laboratory is still poorly studied. In this work, methods to cultivate germlings in the laboratory have been optimized to be used in reforestation projects. The influence of light intensity (40µ $molm^{-2}s^{-1}$ and 80µ $molm^{-2}s^{-1}$) and three different temperatures (12, 15, and 18°C) on egg and spore germination was assessed. *H. elongata* male gametes were released in lower quantities at 10ºC. All treatments resulted in cultures with a lot of debris. A washing method was tested to try to obtain cultures with fewer residues, but this led to a decrease in gamete release. Considering *A. nodosum*, the receptacles were collected, and the gametes released in the laboratory were placed at different temperatures and light. A higher percentage of germination was obtained at 15ºC. Germlings growth was followed by measuring their length and area. Higher length was obtained at lower light (40µ $molm^{-2}s^{-1}$) and lower temperatures (12ºC). Areas were initially higher at lower temperature and lower light intensity but there was a shift to lower temperature and higher light intensity. These differences indicate different necessities at different developing stages. With *Laminaria hyperborea,* two seeding densities, high (1.42 mg/L) and low (0.71 mg/L), were tested on spools with rope. The lower seeding density showed a higher length of juveniles despite the area of coverage being lower.

Keywords: seaweeds; marine forests; germination

Resumo

Neste estudo, três espécies-chave que formam florestas marinhas foram investigadas: *Himanthalia elongata*, *Ascophyllum nodosum* e *Laminaria hyperborea.* São espécies de águas frias, apresentando o seu limite sul de distribuição no Norte de Portugal. Os ciclos de vida já são conhecidos, sendo como outros fucóides e algas, mas sua reprodução em laboratório ainda é pouco estudada. Neste trabalho, métodos de cultivo de germinação em laboratório foram otimizados para serem utilizados em projetos de reflorestamento. A influência da intensidade luminosa (40µ $molm^{-2}s^{-1}$ e 80µ $molm^{-2}s^{-1}$) e três diferentes temperaturas (12, 15 e 18°C) na germinação de ovos e esporos foram avaliadas. Os gametas de *H. elongata* foram libertados com diferenças significativas a 10ºC. Todos os tratamentos resultaram em culturas com muita contaminação. Um método de lavagem foi testado para tentar obter culturas com menos resíduos, mas isso levou à diminuição da libertação de gametas. Considerando *A. nodosum*, os receptáculos foram recolhidos e os gametas libertados em laboratório foram colocados em diferentes temperaturas e luminosidade. Maior percentagem de germinação foi obtida a 15ºC. O crescimento dos zigotos foi acompanhado medindo o comprimento e área. Maior comprimento foi obtido em menor luminosidade (40µ $molm^{-2}s^{-1}$) e temperaturas mais baixas (12ºC). As áreas eram inicialmente mais altas em temperatura mais baixa e intensidade de luz mais baixa, mas houve uma alteração para maior crescimento a temperatura mais baixa e intensidade de luz mais alta. Esta diferença indica preferências diferentes em estados de desenvolvimento diferentes. Com *Laminaria hyperborea*, foram testadas duas densidades de sementeira, alta (1,42 mg/L) e baixa (0,71 mg/L), em tubos com corda. A menor densidade de sementeira mostrou maior comprimento de juvenis apesar da área de cobertura ser menor.

Palavras-chave: algas; florestas marinhas; germinação

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1. Introduction

1.1 The ecological role of seaweeds and marine forests

Seaweeds are fundamental organisms in marine habitats. Their ecological role is essential and very important for the stability of our ecosystems. They mainly function as habitat and ecological engineers (Harley et al., 2012) since they provide habitat and are primary producers, being a source of food for many organisms (Mann, 1973). Whereas plants require soil for nutrient uptake through their roots, seaweeds just require an attachment site, typically on substrata (Mann, 1973). Water serves as its source of nutrients, and water mobility and wave action help these organisms better absorb light, which is necessary for photosynthesis (Mann, 1973). This ability to photosynthesize is crucial as it allows seaweeds to capture carbon and release oxygen. This means that their role in the food chain is extremely important because they not only contribute to the production of oxygen but also provide a food source for other organisms (Bernardes Batista et al., 2018). These functions are one of the many ecological services provided by seaweeds. They can also range from soil formation, provisioning habitat, regulating services, and even cultural (Heck Jr. et al., 2003; Randall et al., 2020; Cotas et al., 2023) .

The preservation of biodiversity in marine ecosystems depends on these organisms (Graham, 2004). Rocks are transformed into a varied framework of biodiversity by canopy-forming seaweeds like kelps and fucoids (Klinger, 2015). Species richness is also positively impacted by the presence of seaweeds (Christie H et al., 2009).

There are differences in how the habitats are formed depending on the foundation organisms, which influence the biological assemblage composition (Dayton, 1985; Bruno & Bertness, 2001; Bustamante et al., 2017). Canopy-forming seaweeds, which are known for their diversity, productivity, and ecological value, play a crucial role in marine forests (Airoldi et al., 2009, 2014). These underwater ecosystems are primarily comprised of kelps and fucoids and are defined by the abundant presence of large seaweeds. They can achieve great sizes and are often characterized by their high growth rates, biomass production, and ability to provide structure and habitat (Wernberg et al., 2019).

Several studies show that there are differences in the formation of these ecological niches depending on the species (Klinger, 2015; McPherson et al., 2021). This variation can happen due to differences in geography and stress factors (Bulleri et al., 2002). And this structure when altered can change conditions such

as light, source of nutrition, protection from predators, water flow, pH, sedimentation, and physical abrasion (Klinger, 2015).

A drop in biodiversity is linked to canopy loss. Numerous variables, including grazers, ocean acidity, changing temperatures, and the rise in sea level, can contribute to this loss. Additionally, because different organisms are associated with various kinds of seaweeds, the consequences of this biodiversity loss can vary according to the species at stake (Miller et al., 2012). This occurs because of an imbalance in the structure and function, particularly when it comes to productivity, caused by the removal of the canopy (Ellison et al., 2005; Edwards et al., 2020). A study performed in the United Kingdom revealed that patterns of canopy plant biomass, stipe length, and age were all spatially varied, with significant 'between-site' variability seen in each case (Smale DA et al., 2016). Research shows that an increase in habitat volume can lead to more macrofauna associated. For example, the disappearance of sugar kelp in Norway has led to the formation of turf algae. While these turfs showed some presence of organisms, density was lower when compared with the presence of sugar kelp (Christie H et al., 2009). In the Baltic, tests showed that species diversity was higher in plots with a *Fucus* spp. canopy than in those without one, even though net biomass production was lower (Eriksson et al., 2006). Additionally, epiphytes that grow on the stems of kelps offer diversity in the communities and opportunity for colonization (Smale et al., 2013). The abundance of kelp and macroalgal beds, which are used by commercial fish species as nurseries and feeding grounds, increases survival rates, aids in the persistence of fish populations, and boosts fisheries output (Smale et al., 2013).

These underwater forests are highly productive and are a source of carbon (Wernberg & Filbee-Dexter, 2019). They also play an important role in nutrient regulation alongside biogeochemical cycling (Mann, 1973). In the case of kelp forests, biomass increases with the growth of stipes and blades. Through photosynthesis, these organisms grow, and the blade elongates, keeping the younger tissue close to the stipe while older tissue grows towards the end of the blade(Mann, 1972; Tala & Edding, 2007; Wernberg et al., 2019). Often this older tissue can end up deteriorating (Wernberg et al., 2019), resulting in the release of dissolved organic materials, which may provide 35% of the annual energy output (Smale et al., 2013). These particles can migrate to different areas due to water flow and can potentially be used as food (Smale et al., 2013). According to this author, kelps like *Macrocystis* and *Laminaria* have productivity rates of about 3,000

gC m^{-2} per $year^{-1}$ and can photosynthesize at very high rates (Smale et al., 2013). Among phaeophytes, representatives of the genera *Ascophyllum* and *Fucus* have the highest rates of photosynthesis (Chung et al., 2011). Herbivores consume kelp directly, yet more than 80% of the biomass produced enters the carbon cycle (Smale et al., 2013). Kelp forests are still underrepresented in scientific research despite being the biggest and most productive macroalgae biomes, which results in knowledge gaps in this area (Wright et al., 2022).

Their influence in biogeochemical cycling involves mainly their capacity to absorb nutrients and then release them as they decompose. They are very important to the nitrogen and phosphorus cycle, as they are one of the main components of nutrient storage and assimilation in marine habitats. This indicates that interfering with these species can cause significant modifications to the aquatic metabolism (Eger et al., 2020; Layton et al., 2020; Morris et al., 2020; Cotas et al., 2023). Furthermore, their role in the production of oxygen alongside their ability to reduce CO_2 is very important to humans (NOOA, 2022;Cotas et al., 2023).

While the abundant macroalgae growth rates in these coastal ecosystems play a vital role in their importance as natural carbon sinks, a key factor lies in their ability to trap sediment and capture carbon beyond the confines of their habitat. It's important to close knowledge gaps in this area and identify the most affected sites that need conservation or restoration measures (Duarte et al., 2005; Lo Iacono et al., 2008; Mcleod et al., 2011).

Due to the importance of these habitats, it's essential to understand how different types of threats and climate change will affect them.

1.1.1 Threats and Consequences of Climate Change

Anthropogenic and environmental stressors have been the cause of the decrease in macroalgae abundance. These habitats that once were complex and diverse are now shifting to more simple assemblages that are often less diverse and less productive (Johnson et al., 2011; Steneck & Johnson, 2013; Krumhansl et al., 2016; Layton et al., 2019).

Human-induced stressors have resulted in various alterations, with diverse consequences. The temperature rise attributed to global warming has influenced more than 70% of coastlines worldwide (Lima et al., 2007; Straub et al., 2019). European marine ecosystems are highly susceptible to human impacts, with multiple driving forces contributing to their vulnerability. These include habitat destruction, overexploitation of resources, pollution, the introduction of invasive species, and the significant influence of climate change. Climate change disrupts

weather patterns, leading to more frequent and intense storm events that have adverse consequences for these ecosystems (McKinney & Lockwood, 1999; Mineur et al., 2015). All of these impacts have a cumulative effect on biological communities.

While long-term temperature rises have repercussions on ecosystems, the frequency and intensity of Marine Heat Waves, characterized by abnormally high temperatures, are on the rise (Hobday et al., 2016; Straub et al., 2019). The impacts can encompass a variety of changes, including shifts in the distribution, composition, and functions of habitats. When foundation species experience high mortality rates, it often results in the dominance of less complex turf communities, which can pose challenges when attempting to restore the original species (Umanzor et al., 2017, 2019). The Atlantic coast of Europe is experiencing a significant increase in temperature, with sea surface temperatures rising by approximately 0.3–0.8 ˚C per decade since the mid-20th century (Essink et al., 2005; Lima & Wethey, 2012; Smale & Wernberg, 2013; Casado-Amezúa et al., 2019). This warming trend is particularly pronounced in the southernmost region of Europe, namely the Iberian Peninsula, making it one of the areas most significantly impacted by climate change (Belkin, 2009; Casado-Amezúa et al., 2019).

1.2 Portuguese populations and marine forest status

As previously mentioned, canopy-forming seaweeds play a vital role in supporting essential ecosystem services within coastal environments (Casado-Amezúa et al., 2019). Due to the effects of climate change, their distribution and presence have been greatly affected.

Despite their importance, a comprehensive database of the status of kelp forests and species is still lacking. Most data is outdated and monitoring efforts are mainly focused on local, regional, and national levels (Araújo et al., 2016). For example, regarding *Laminaria hyperborea*, some reports suggest expansion, stability, or decline in central Europe, while there are isolated reports of population decrease or extinctions in the Iberian Peninsula (Araújo et al., 2016). *L.hyperborea* has its southernmost distribution limit in the Iberian Peninsula (Maggs et al., 2008; Hoarau et al., 2007; Assis et al., 2017; Casado-Amezúa et al., 2019). The impacts of climate change in this area have been affecting the populations and showing declining trends (Casado-Amezúa et al., 2019). They’re the dominant species along

the European Coast with distribution from the intertidal and subtidal, the last one depending on the clarity of the water (Araújo et al., 2016).

Studies on fucoids have indicated the disappearance of *Himanthalia elongata* from multiple sites, confining its distribution to the northwest corner of the Iberian Peninsula. Furthermore, there have been reports of an ongoing decline in these areas as well (Casado-Amezúa et al., 2019). *Ascophyllum nodosum* is another fucoid with an important presence on the Portuguese coast. In northern Portugal, *A. nodosum* exists as a small, isolated population in contrast to its central distributional range. This southern population exhibits distinctive phenotypic traits, including variations in reproductive output and population growth rate (Viana et al., 2014; Borges et al., 2020).

To better understand what can be done to improve the status of these species, it's important to study different aspects of their biology and ecology, and how they can be applied to restoration efforts.

1.2.1 *Himathalia elongata*

Himanthalia elongata (Linnaeus) S.F. Gray 1821, also known as "Esparguete-do-mar", "Cintas", "Cordas", "Corriolas" (Portugal), and "Sea thong" (England), is a common brown seaweed that presents a two-stage morphology. This seaweed forms a button-like thalli in the first stage, followed by the growth of a strand-like shape frond which holds the receptacles containing the reproductive cells when mature. The reproductive stage is identified in the summer, and at the end of the season, the fronds detach from the button-like structure. New receptacles grow during the winter season. *Himanthalia elongata* typically live for 2 to 5 years and reach maturity, capable of reproduction, by the age of 2 (White, N. 2008).

This species is commonly found in habitats with gently sloping rocky shores, where it attaches to solid surfaces like bedrock or large boulders. It inhabits the lower shore and thrives at the boundary of low tide, facing moderate wave exposure. Its thallus remains consistently submerged in this habitat, categorizing it as an infralittoral species. This species possesses an exceptional life cycle and growth approach, allocating as much as 98% of its overall biomass to reproductive tissue rather than vegetative components (STENGEL et al., 1999). In contrast to fellow Fucales members, *H. elongata* follows a non-perennial growth pattern. Studies conducted by Gibb (1937) and Russell (1990), focusing on population size and age distribution in the Irish Sea, indicated a biennial life cycle for the species. However, certain plants underwent a vegetative growth phase spanning two years before entering the reproductive stage in their third year (Russell, 1990) (STENGEL

et al., 1999). The period of reproduction spans from June to December and is notably influenced by the specific site, likely attributable to variations in water temperature (Stagnol et al., 2016).

During reproduction, the gametes are released and fused to form zygotes. The settlement is very important and happens with the development of rhizoids. The female zygotes are often referred to as oospheres. Upon the release of the sizable oospheres, each is encased within a dense mucilaginous covering. The liberation of the egg from this mucilaginous enclosure is a crucial step for fertilization to occur (MOSS, 1969). In the recruitment stage, the zygotes settle themselves on the substrate (Stagnol et al., 2016).

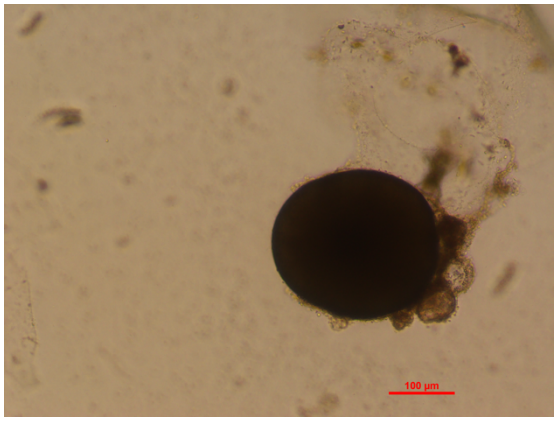


Figure 1. Female gametes of *Himanthalia elongata*

1.2.2 *Ascophyllum nododsum*

Ascophyllum nodosum (Linnaeus) Le Jolis 1863, a brown seaweed closely related to *Fucus*, is a prevalent and substantial species on sheltered rocky shores. This seaweed is also known as "Knotted wrack"," Egg wrack", "Asco" and "Bladderwrack" (England). These names are often used due to the appearance of this species. It falls within the family Fucaceae of the order Fucales, which is a group of brown algae (Phylum Ochrophyta, Class Phaeophyceae). The genus *Ascophyllum* stands out in the Fucaceae family due to its unique feature where groups of fertile branches emerge from specific distinct points along the edge of the main shoot. The species displays elongated strap-like fronds with large egg-shaped air bladders arranged at consistent intervals. They can display a yellow or green color (Pereira et al. 2014).

This species exhibits a single sexual life cycle phase and has a potential lifespan of up to 15 years before fragmenting. It is dioecious, signifying the presence of distinct male and female fronds. Male receptacles may display a greenish-yellow hue, while females can appear dark green. As they mature, males may exhibit orange dots, while females display small green dots. Reproduction takes place during the winter, although variations might arise due to varying seawater temperatures (Pereira et al., 2020).



Figure 2. *Ascophyllum nodosum* fronds. Photo by the author

Male gametes are also known as antherozoids, and female gametes are also known as oospheres. The oogonium is the structure that holds the female gametes. It has a diploid nucleus that divides into eight haploid nuclei by mitoses accompanied by meiosis. From these eight nuclei, only four are formed into one functional oosphere. (Pereira et al., 2020).

However, for fertilization, the eggs must be released from the oosphere (Figure 3). Then the antherozoids can fertilize the eggs. The formation of rhizoids (Figure 4) usually occurs 10 days after fertilization and is essential for the settlement of the zygotes on the substrate. After the settlement, the growth is very slow. The germlings (the zygotes fertilized) often only grow about 0.1 to 0.2 cm in a year, and it takes 2 years to grow a 1.5 cm (Pereira et al., 2020).



Figure 3. *A. nodosum* oosphere with four eggs.

The low growth rate can result in unfavorable consequences, as it renders the germlings more vulnerable to diatoms and grazers. Given that *Ascophyllum nodosum* is a sheltered species, the germlings develop beneath the canopy and are influenced by the presence of other species, with the ability to exist for extended periods. It forms dense, single-species groupings that lead to significant competition within the species for both space and light resources (Pereira et al., 2020).



Figure 4. *A. nodosum* germling with rhizoids.

This species is prevalent in the Northeast Atlantic Ocean. In areas subject to tidal fluctuations, *Ascophyllum* serves as an intertidal species, creating a well-defined zone at slightly variable heights. It inhabits the same vertical level as *Fucus vesiculosus*, often substituting it in tranquil locations where advantageous ecological circumstances are met (Pereira et al., 2020). *Ascophyllum nodosum* attaches itself to rocks and boulders primarily in the middle shore area, inhabiting a diverse range of environments ranging from estuaries to moderately exposed coastal zones. These locations may experience varying levels of wave action,

spanning from low to high, across the entire tidal gradient, although subtidal populations have also been documented. The upper boundaries of *Ascophyllum nodosum*'s distribution are determined by its capacity to endure dehydration and elevated temperatures (Hill, J.M. & White, N. 2008).

1.2.3 *Laminaria hyperborea*

Laminaria hyperborea (Gunnerus) Foslie 1885, significant and easily identifiable kelp, has the potential to reach lengths of up to 3.5 meters under optimal circumstances, although this size is rarely achieved. It belongs to the Laminariaceae family and is characterized by its substantial, leathery structure. The blade is broad, tough, flat, and presents a golden brown color. It's thick at the base and narrows towards the frond. The maximum length can change with the season, age of the plant, and geographical location (Tyler-Walters, H., 2007).



Figure 3. *Laminaria hyperborea* at Vila Chã. Picture by the author.

Laminaria hyperborea is a cold-temperate species found in the European North Atlantic region, with its distribution limited to areas unaffected by Arctic waters. This species is specifically confined to the northeast Atlantic, extending from the northern coast of Iceland to the Russian coast near Murmansk in the north and Cape Mondego in mid-Portugal, in the south of its distribution (Tyler-Walters, H., 2007). Notably, it is present in regions like Norway, the Faroes, northern France, and northern Spain but is absent from the Bay of Biscay (Pereira et al. 2014). The establishment stage is critical and different stages require different light requirements (Kain & Jones, 1964). Unlike other Laminaria species found in the

upper sublittoral zone, which usually have a lifespan of around 3 years, *Laminaria hyperborea* stands out as a long-lived perennial, enduring for 10 to 20 years. This extended lifespan can exhibit variations across its distribution, with the northern regions demonstrating the highest longevity. Spores are generated from sori across the majority of the blade surface, excluding the most distal or proximal regions, during a six to seven-week period in winter, spanning from September to April (Pereira et al. 2014). The development of young sporophytes, known as germlings, primarily occurs during spring but can occur throughout the year depending on prevailing conditions.

Laminaria hyperborea exhibits a clear seasonal growth pattern characterized by its peak growth from November to June, with growth coming to a halt during the summer months, although the metabolic rate remains notably elevated. Every November marks the beginning of new blade growth beneath the older one, creating a distinct boundary or collar between the two; this growth phase typically extends until approximately June (Sjøtun, 1996; Tyler-Walters, H., 2007). The allocation of energy toward growth varies depending on the location. In sheltered conditions, the stipe is thinner and has fewer fronds. In more exposed areas where it faces increased wave action and tidal forces, more energy is directed towards strengthening the stipe to withstand these forces. Additionally, the amount of energy allocated can fluctuate with the season and the age of the plant. It can be located on bedrock or other secure substrates within the cold temperate waters of the sublittoral zone (Sjøtun & Fredriksen, 1995; Tyler-Walters, H., 2007). Furthermore, it can thrive on stable boulders, and pebbles amidst large gravel, the deeper regions of pontoons and moorings, and various other man-made structures (Tyler-Walters, H., 2007).

Within the Laminariales, the diploid sporophyte assumes the role of producing reproductive tissue known as a sorus. This sorus houses sporangia, which generate zoospores through meiosis. Zoospores are then discharged, settle, undergo germination, and develop into either male or female gametophytes, each equipped with male antheridia or female oogonia. Following the maturation and fertilization of the eggs, the zygotes subsequently develop into new sporophytes (Olischläger et al., 2012).

This species is commercially harvested in regions including Norway, Brittany, Scotland, Ireland, and Iceland. Given the long life span and significant ecological role of kelp species within the intertidal and subtidal ecosystems, management strategies have been developed to promote sustainable harvesting

practices for *Laminaria hyperborea*. These strategies often include restrictions on repeated harvesting of the same area, allowing such activity to occur only once within a multi-year cycle (Pereira et al. 2014).

1.3 Restoration Methods and Efforts

The idea of restoring marine habitats has started to gain traction in the last few years to help mitigate the consequences of climate change (Borja, 2014; Abelson et al., 2020). Other efforts of passive conservation have been and are currently being made, such as the control of human activities and the creation of Marine protected Areas (MPAs) (Costello, 1999).

A lot of effort in restoring habitats like coral reefs has been made in the last decade, especially through the building of artificial reefs. Seagrass, mangroves, and oyster reefs also represent groups that have been subjected to restoration (Bayraktarov et al., 2016). Despite the research on other organisms, the attention towards marine forests has only been more prevalent in the last few years.

Conservative strategies involve protection against grazers and predators. Other successful techniques involved restrictions regarding fishing and hunting. However, to help ensure greater results and restore the endangered habitats, more active techniques are being studied (Wood et al., 2019; Layton et al., 2020; Fredriksen et al., 2020).

Active restoration encompasses a variety of methods, including transplanting, sowing seeds and seedlings, and creating artificial habitats (Perrow & Davy, 2002; Bayraktarov et al., 2016). In the Mediterranean, efforts to restore *Cystoseira* species have been successful without the need for the transplantation of adults. The work focused on enhancing restoration methods, and the restored populations became self-reliant (Verdura et al., 2018). Researchers collected fertile branches of the species and allowed for the liberation of zygotes in the laboratory. The zygotes grew on stones and were monitored in the laboratory to then do the deployment (Verdura et al., 2018).

A recent technique, known as 'Green gravel,' has shown promising results when compared to more traditional methods. This approach involves using small rocks for seeding in the laboratory and subsequent field deployment. In 2020, researchers employed this technique to restore the kelp *Saccharina latissima*. It offers the advantage of simplifying the process of habitat restoration. Seeding and maintaining conditions in the laboratory are more accessible, and it eliminates the need for scuba diving during deployment (Fredriksen et al., 2020).

To employ restoration techniques effectively, it's crucial to establish germination protocols tailored to each species' specific requirements. Developing nursery methods entails comprehending the morphological needs that can be applied to restoration efforts.

1.4 Objectives

This thesis was developed in the framework of the project "BLUEFORESTING - Climate Resilient Marine Forests for a Sustainable Future". The objective of this project is to put forth environmentally sound alternatives to strengthen the resilience of marine forests and lessen the effects of climate change. To do so it is necessary to develop protocols for the nursery stage of relevant species. For this work, three species that have their southern distribution limit in the North of Portugal were selected: *Himanthalia elongata*, *Ascophyllum nodosum*, and *Laminaria hyperborea.*

This thesis aimed to develop protocols for reproduction and germination of the target species, to be applied in the restoration of seaweed forests. To do so the following objectives were defined:

- Assess the effect of the factor's temperature, sex, light intensity, different washing treatments, and density of gametes on the development of the initial life stages of the target species.
- Describe the best culture conditions as the base to build a protocol for the spore release and/or spore germination of each species.

2. Material and Methods

2.1 Seaweed species selection

As stated above, in this study, three different seaweed species were examined: *Himanthalia elongata*, *Ascophyllum nodosum,* and *Laminaria hyperborea*. These are cold water species that have distribution ranges extending to the northern region of Portugal, making this region their southernmost distribution limit. This distribution makes them potentially more vulnerable to the impacts of climate change.

2.2 Influence of sex and temperature on the release of *Himanthalia elongata* gametes

To develop a protocol for obtaining seedlings of *H. elongata* in the laboratory, 2 trials were conducted to test the release of their gametes. For the first trial, beach cast receptacles of *H. elongata* were collected at Labruge shore (41.2769° N, 8.7292° W), on September 11th, 2022. The tidal height was 0,4m and the moon phase was full. In this species, similarly to others such as *Fucus vesiculosus*, the gamete release is influenced by a lunar rhythm (Andersson et al., 1994; Andersson & Kautsky, 1996).

The receptacles were washed with sterile seawater and stored overnight at 5ºC with paper embedded with filtered seawater. The next day, the receptacles were identified as male or female using an optical microscope. Male receptacles released orange gametes whereas female receptacles released brown gametes. Three fragments from each sex of approximately 1 cm were placed in Petri dishes with 25ml of sterile seawater and left overnight at three different temperatures (10, 12, and 14ºC) (MOSS, 1969). On the next day, the fragments were removed, and the eggs and male gametes were counted using an inverted microscope.

Since the cultures resulting from this procedure presented high contamination, a washing procedure was tested (Creed JC et al., 1996), in a second trial conducted on the 27th of September 2022, using receptacles freshly collected at the same site. The tidal height was 0,5m and the moon phase was the new moon. The receptacles were washed with sterile seawater and stored at 5ºC. In this trial, males and females, after sex identification, were stored separately in the fridge with paper embedded with filtered seawater.

The receptacles were cut into 5 cm fragments on the following day, 5 male and 5 female. Fragments of each sex were placed in 2 beakers with 300 ml of sterile

seawater. The beakers were stored at 12˚C overnight in a climate chamber to allow for the gamete release.

On the following morning, the fragments were removed, and the washing method was tested. One of the gamete suspensions was filtered using a 100 μm sieve, washed with sterile seawater, and then placed in 300 ml of sterile seawater. Using 18 Petri dishes, 30 ml of the filtered and unfiltered suspensions were distributed in each petri dish, which were then incubated at 12ºC,15ºC and 18ºC (3 replicates per treatment) under a light intensity of 60 $\mu molm^{-2}s^{-1}$. Germinated eggs were counted using an inverted microscope.

2.3 Influence of light intensity and temperature on the germination and growth of *Ascophyllum nodosum* germlings

Receptacles of *Ascophyllum nodosum* were collected, 5 to 10 from each individual at Praia Norte Rocky shore in Viana do Castelo (41.6970˚ N, 8.8510˚ W) on the 25th of January of 2023. Receptacles from each individual were cut with a surgical blade, observed under a light microscope to identify the males and females, and then stored separately in paper moistened with seawater overnight, at 5ºC.

On the following day, the receptacles were washed with sterilized seawater and left to dry for 2 hours at room temperature on top of a clean and dry paper towel (Vadas et al., 1990).



Figure 4. *A. nodosum* receptacles left to dry.

They were then placed over a net with 0.5 cm mesh in a beaker with sterilized seawater for about an hour, allowing the gametes to release.

Every 15 minutes, using a microscope, the gamete release was checked. After removing the net with the receptacles, the gamete suspension was placed in 6 well

plates, 10 ml in each well, at three different temperatures (12˚, 15˚, and 18˚C) and two light intensity levels (40 $\mu molm^{-2}s^{-1}$ and 80 $\mu molm^{-2}s^{-1}$). The nutrient medium was changed every week using half PES (Provasoli's Enriched Seawater Medium).

Due to a malfunction of one of the climatic chambers, only data from temperatures 12 and 18ºC was collected and analyzed. Pictures were captured using an inverted microscope to assess fecundation and growth. To determine germination, we counted the germlings in each well, considering those with rhizoids as germinated. The percentage of germination was calculated by taking the ratio of germinated germlings to the total eggs and multiplying by 100.

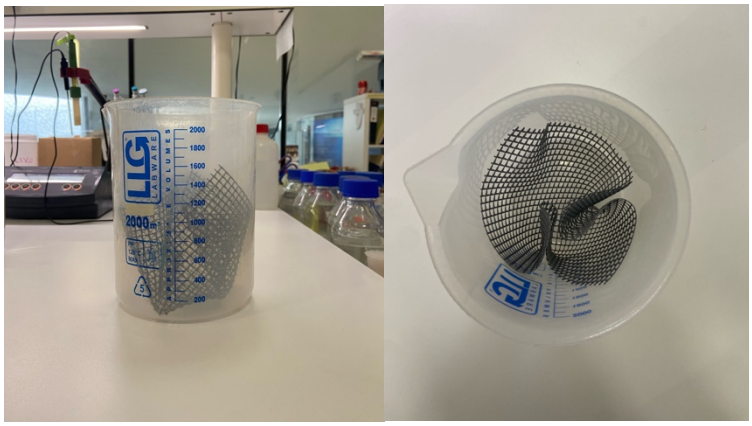


Figure 5. Beaker with the net used.

To measure growth, we recorded the length of rhizoids one week later. The images were captured randomly, with six vision fields for each well, and the length of the rhizoids was measured using the ImageJ program.

Additionally, every month, we used the ImageJ program to calculate the area of the germlings as part of our growth analysis.

2.4 Influence of the seeding density on the growth of *Laminaria hyperborea* seedlings

Specimens were collected at Vila Chã beach and reproductive fronds were brought to the laboratory. The fronds were cleaned with paper and sterilized seawater at the laboratory and then stored at 5ºC for 2 hours. Afterward, they were placed in a beaker with sterilized seawater, to allow for the spore release (Theodorou et al., 2021). After 1 hour, the number of spores released was counted using a Neubauer chamber and an inverted microscope. A density of 492 5000 spores per mL was obtained. The spore suspension was placed in cell culture flasks with 60 ml capacity and kept in red light to allow for spore germination and gametophyte development. The medium was changed every week using half PES (Provasoli's enriched Seawater Medium).

2.5 Optical density measurement to determine the density of gametophyte cultures.

The density of gametophyte cultures may be assessed using optical density, by using a calibration curve that correlates optical density with gametophyte weight (Duarte, 2017). Shortly, the stock gametophyte culture was diluted to different factors. For each dilution, the optical density was measured at 750 nm, and a volume of 10 ml was filtered through the filtration systems, dried in an oven at 60 ºC, and weighed 48 hours later. The following regression equation relating optical density and gametophyte density (in dry weight) was obtained and used afterward for the seeding:

Y (Abs) = 0.0227x (gametophyte density) + 0.0138

2.6 Seeding on spools.

On the 17th of May, 2023, the *L. hyperborea* gametophyte culture was used to seed PVC spools wrapped with polypropylene string. Using the regression equation above, two gametophytes' densities were chosen, high (1.42 mg/L) (40 ml in 200 ml of seawater) and low (0.71 mg/L) (20 ml in 200 ml). They were sprayed evenly on the spools and, after 30 minutes, placed in two separate tanks, three for each tank.

One week later, aeration was provided. Two weeks later the culture medium was changed using half PES with GeO_2 and afterwards, the medium was changed every week (half PES). After three weeks the light intensity was increased from 50 $\mu molm^{-2}s^{-1}$ to 80 $\mu molm^{-2}s^{-1}$. Once the juveniles became visible, their coverage area was assessed weekly. Briefly, spools were taken from the tanks and rotated eight times, and each time a picture was taken of the entire spool with a ruler to provide scale (Figure 6). After the pictures were taken, three vision fields of 21 cm^2 were defined

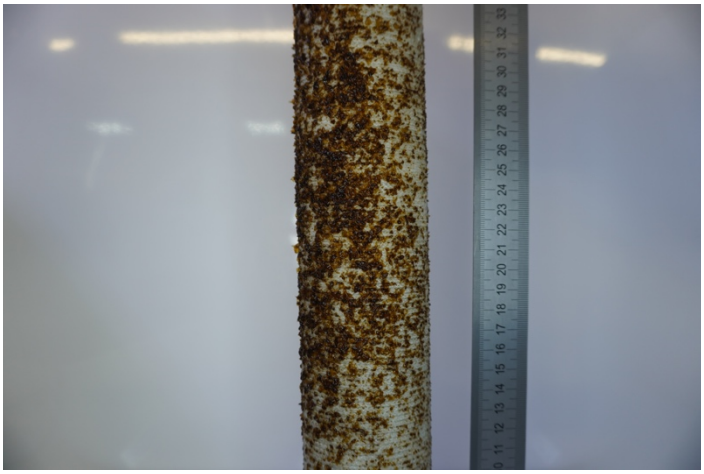


Figure 6. Spool taken off the tank with ruler as scale. Photo by the author.

to calculate the area covered by the juveniles. To measure the length of the blade, 10 juveniles from each vision were selected at random and the blade was measured. Measurements were made using the software ImageJ.

2.7 Statistical analyses

Himanthalia elongata

A two-way analysis of Variance (ANOVA) was performed to assess the effect of sex (2 levels: male, female; fixed) and temperature (3 levels: 10, 12, and 14 °C; fixed) and their interactive effects on the dependent variable density of gametes release. An additional ANOVA was performed to assess the effect of temperature (3 levels: 12, 15, and 18 °C; fixed) and washing procedure (2 levels: washed, no-washed; fixed) and their interactive effects on germination.

When a significant difference between sample means or interaction of factors was revealed by the test, a pairwise comparison among levels of factors was performed and means were considered significantly different at $p<0.05$.

Ascophyllum nodosum

A two-way ANOVA was performed to assess the effect of temperature (3 levels: 12, 15, and 18 °C; fixed) and light intensity (2 levels: low light, high light; fixed) and their interactive effects on the dependent variable percentage of germination. Subsequent ANOVAs were performed to assess the effect of temperature (2 levels: 12 and 18 °C; fixed) and light intensity (2 levels: low light, high light; fixed) and their interactive effects for each dependent variable (length of rhizoids and area of germlings).

Assumptions of normality and homogeneity of variances were tested, the latest by the *Levene* test. Normality was tested using the residuals and building a graph. When homogeneity of the variances was not met data were transformed following recommendations in Underwood (1997). All ANOVAs were conducted using GraphPad Prism and tested at $\alpha = 0.05$. When a significant difference between sample means or interaction of factors was revealed by the test, a pairwise

comparison among levels of factors was performed and means were considered significantly different at $p<0.05$. These pairwise comparison tests applied were Tukey's multiple comparisons.

Laminaria hyperborea

An unpaired t-test was performed to compare the averages and standard variations of the area of coverage and length of juveniles in two groups (high density and low density).

All unpaired t-tests were conducted using GraphPad Prism and tested at $\alpha = 0.05$.

3. Results

3.1 *Himanthalia elongatas'* gametes release and germination.

3.1.1 Influence of the factors sex and temperature on *Himanthalia elongata* gamete release

A higher number of male gametes was released at 10ºC (138.23±39.53) compared to 12 and 14ºC (Figure 9). A significant interaction was obtained between the factors sex and temperature (p= 0.011) (Table 1).

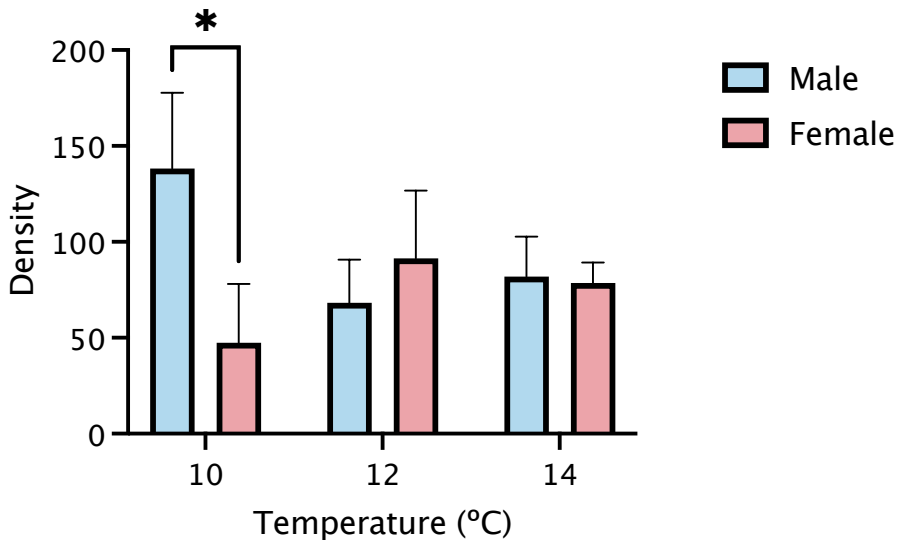


Figure 7. Average number of gametes released per cm^2 of receptacle, at three different temperatures, with standard deviation. The asterisks above bars indicate significant differences in Tukey's pairwise comparisons (*- P ≤ 0.05; **- P ≤ 0.01; ***- P ≤ 0.001; ****- P ≤ 0.0001).

Table 1. ANOVA results to evaluate the effects of sex and temperature on gamete release. Values in bold represent significant differences.

Source	df	SS	MS	F	*P.*
Sex=S	1	2518.13	2518.13	3.13	0.102
Temperature=T	2	655.92	327.96	0.41	0.674
S * T	2	10699.45	5349.72	6.65	**0.011**
Error	12	9653.58	804.47		
Corrected Total	17	23527.09			

Tukey's multiple comparisons indicated that the number of male and female gametes released was significantly different at 10ºC, higher for males (138.23±39.53) than for females (47.33±30.80). No significant differences were obtained between the number of male and female gametes released at 12 or 14ºC.

Table 2. Tukey's multiple comparisons test results. Values in bold indicate significant differences.

Compared groups	*P*- values
10 M vs 10 F	**0.0192**
10 M vs 12 M	0.0869
10 M vs 12 F	0.3839
10 M vs 14 M	0.2184
10 M vs 14 F	0.1769
10 F vs 12 M	0.9393
10 F vs 12 F	0.4456
10 F vs 14 M	0.6766
10 F vs 14 F	0.7545
12 M vs 12 F	0.9087
12 M vs 14 M	0.9898
12 M vs 14 F	0.9971
12 F vs 14 M	0.9981
12 F vs 14 F	0.9924
14 M vs 14 F	>0.9999

3.1.2 Influence of a washing treatment on *Himanthalia elongata* germination

A higher quantity of gametes was germinated per cm^2 of the receptacle when the washing treatment was not applied (61.22±38.20), regardless of the temperature (Figure 10). There was a significant effect of the washing treatment (p=0.006) (Table 3.), significantly reducing the number of gametes germinated per cm^2 of the receptacle (16.56±11.07).

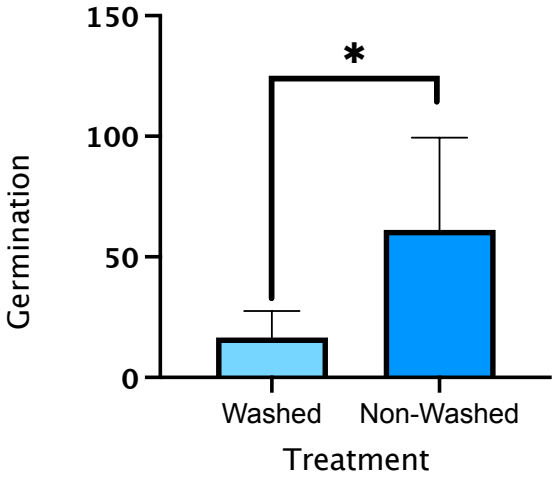


Figure 8. Mean number of gametes germinated per cm^2 of the receptacle, at different treatments, with standard deviation. The asterisk above bars indicates significant differences in ANOVA (*- P ≤ 0.05; **- P ≤ 0.01; ***- P ≤ 0.001; ****- P ≤ 0.0001)

Table 3. ANOVA results to evaluate the effects of a washing treatment and temperature on gamete germination. Values in bold represent significant differences.

Source	df	SS	MS	F	*P*
Temperature= T	2	2177.78	1088.89	1.33	0.3
Treatment= Tr	1	8978.00	8978.00	10.99	**0.006**
T * Tr	2	676.00	338.00	0.41	0.67
Error	12	9802.00	816.83		
Corrected Total	17	21633.78			

3.2 *Ascophyllum nodosum* germination and growth

3.2.1 Influence of light intensity and temperature on gamete germination and germlings growth

The percentage of germinated gametes was quite high in all temperatures and light intensity (Figure 11). The highest percentage was obtained at 15ºC in low light (98.76 % ±0.61). A significant interaction was obtained between light intensity and temperature on the percentage of germinated gametes (p=0.016) (Table 4.). Tukey's multiple comparisons indicated that the percentage of germinated gametes was significantly different at 15ºC and 18ºC at lower light, being higher at 15ºC (98.76 % ±0.61 vs 93.37 % ±4.16). At 18ºC the percentage of germination was higher at high light (96.88 % ±2.13 vs 93.37 % ±4.16). (Table 4).

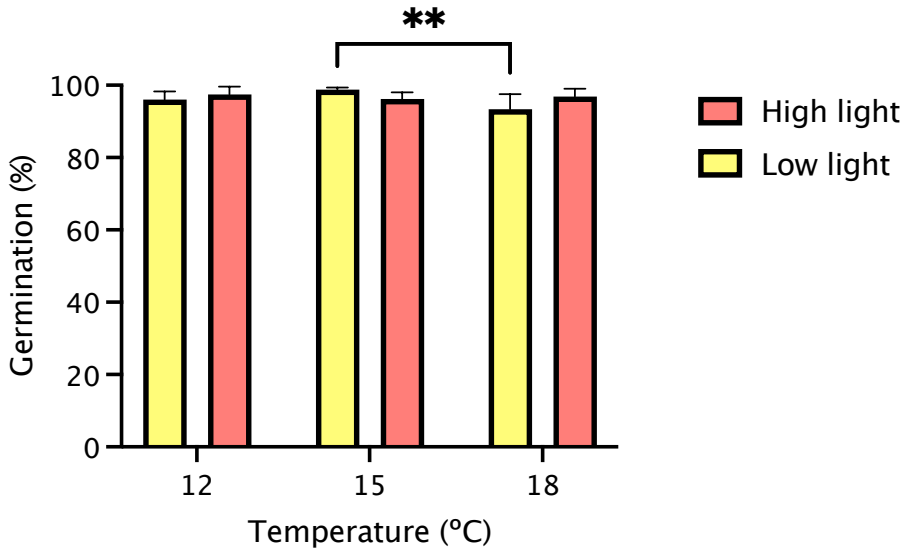


Figure 9. The percentage of germinated gametes (germlings with rhizoids), with standard deviation, at three different temperatures and two light intensities. The asterisks above bars indicate significant differences from Tukey's pairwise comparisons (*- P ≤ 0.05; **- P ≤ 0.01; ***- P ≤ 0.001; ****- P ≤ 0.0001).

Table 5. ANOVA results were performed to evaluate the effects of light intensity and temperature on gametes with rhizoids. Values in bold represent significant differences.

Source	df	SS	MS	F	*P.*
Light_intensity=L	1	5.74	5.74	0.98	0.331
Temperature=T	2	35.34	17.67	3.01	0.064
L* T	2	56.18	28.09	4.79	**0.016**
Error	30	176.10	5.87		
Corrected Total	35	273.35			

Table 4. Tukey's multiple comparisons results. Values in bold represent significant differences.

Compared groups	*P-* values
12 L vs 12 H	0.9148
12 L vs 15 L	0.4011
12 L vs 15 H	>0.9999
12 L vs 18 L	0.4119
12 L vs 18 H	0.9905
12 H vs 15 L	0.9334
12 H vs 15 H	0.9527
12 H vs 18 L	0.0663
12 H vs 18 H	0.9985
15 L vs 15 H	0.4813
15 L vs 18 L	**0.0069**
15 L vs 18 H	0.7606
15 H vs 18 L	0.3374
15 H vs 18 H	0.9972
18 L vs 18 H	0.1524

The length of the rhizoids was measured on the 15th of February after 19 days in culture to assess germlings growth. The average length varied between 59.18 μm and 494.60 μm and was higher at 12ºC and at low light (473.06 μm ±17.01) (Figure 12). Length was lower at 18ºC, but a significant interaction was obtained between light intensity and temperature on the length of rhizoids ($p<0.001$) (Table 6.). Tukey's multiple comparisons indicated that the length of rhizoids was significantly higher at 12ºC at high light when compared to low light (473.06 μm ±17.01 vs 223.44 μm ±40.40) while at 18ºC the differences were not significant (Table 7).

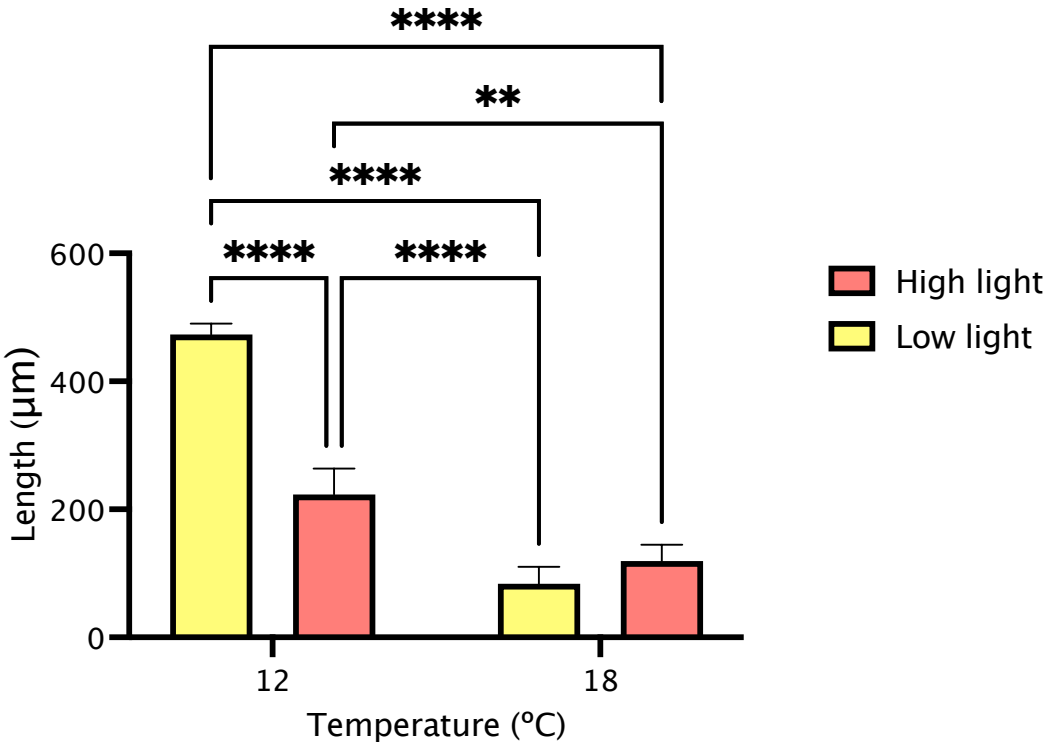


Figure 10. Mean lengths of rhizoids, at two different temperatures and light intensities, with standard deviation. The asterisks above bars indicate significant differences from Tukey's pairwise comparisons (*- P ≤ 0.05; **- P ≤ 0.01; ***- P ≤ 0.001; ****- P ≤ 0.0001) .

Table 6. ANOVA results to evaluate the effects of light intensity and temperature on rhizoid length. Values in bold represent significant differences.

Source	df	SS	MS	F	*P*
Light_intensity=L	1	81065.48	81065.48	99.22	**<.001**
Temperature=T	1	243566.93	243566.93	298.12	**<.001**
L * T	1	46016.69	46016.69	56.32	**<.001**
Error	12	9804.14	817.01		
Corrected Total	15	380453.23			

Table 7. Tukey's multiple comparisons between groups. Values in bold represent significant differences.

Compared groups	*P*- values
12H v 12L	**<0.0001**
12H v 18L	**<0.0001**
12L v 18H	**<0.0001**
18H v 18L	0.3481
12H v 18H	**0.0012**
12L v 18L	**<0.0001**

Since the growth of rhizoids prevented their individual length measurements, germlings' areas started to be measured to assess growth. The initial area was measured on the 17th of February. The mean germlings area was higher at 12ºC at low light (25632.50 $\mu m^2 \pm 8315.68$). However, at 18ºC, the area was higher at high light (21806.44 $\mu m^2 \pm 8679.19$) (Figure 13). A significant interaction was obtained between light intensity and temperature (p= 0.048) (Table 8). Tukey's multiple comparisons indicated that the area of germlings was significantly higher at 12ºC at low light when compared to high light (p=<0.001)(25632.50 $\mu m^2 \pm 8315.68$ vs 10992.52 $\mu m^2 \pm 4034.04$) while at 18ºC the differences between light intensities were not significant (Table 9). It also indicates differences between 12ºC in low light and 18ºC in high light, with 12ºC also being higher (p=0.0009)(25632.50 $\mu m^2 \pm 8315.68$ vs 21806.44 μm^2 ± 8679.19). However, the area under 18ºC low light was significantly higher than the area observed under 12ºC high light (p=0.0027) (10992.52 $\mu m^2 \pm 4034.04$ vs 14582.481μm^2 ± 4229.69).

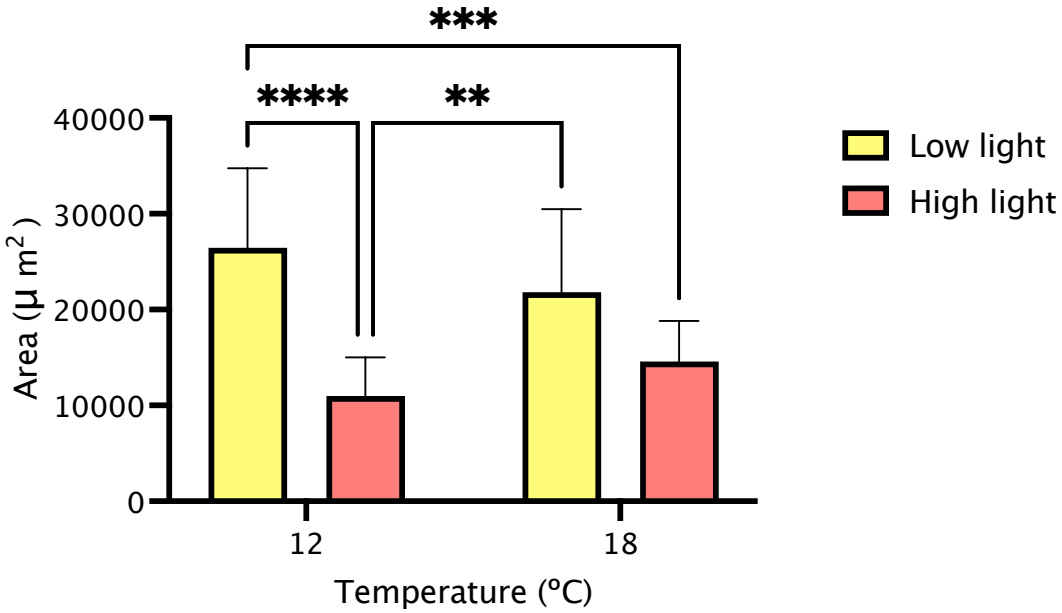


Figure 11. Mean germlings area (17/02/2023), with standard deviation, at two different temperatures and different light intensities. Different letters represent significant differences. The asterisks above bars indicate significant differences from Tukey's pairwise comparisons (*- P ≤ 0.05; **- P ≤ 0.01; ***- P ≤ 0.001; ****- P ≤ 0.0001).

Table 8. ANOVA table was performed to evaluate the effects of light intensity and temperature on the area of germlings. Values in bold represent significant differences.

Source	df	SS	MS	F	*P*
Temperature=T	1	2969277.61	2969277.61	0.07	0.798
Light_intensity=L	1	1412922613.31	1412922613.31	31.61	**<.001**
T*L	1	185769146.15	185769146.15	4.16	**0.048**
Error	40	1788204485.00	44705112.13		
Corrected Total	43	3389865522.07			

Table 9. Tukey's multiple comparisons between groups. Values in bold represent significant differences.

Compared groups	*P*- values
12H v 12L	**<0.0001**
12L v 18H	**0.0009**
12H v 18L	**0.0027**
18H v 18L	0.0698
12H V 18H	0.5937
12L V 18L	0.377

On the 17th of March, the pattern was different. At this time, germlings area was higher at 12ºC in high light (236053.91 $\mu m^2 \pm 60322.94$). At 18ºC, the area was also higher at high light (101075.97 $\mu m^2 \pm 30618.95$) (Figure 14). A significant interaction was obtained between light intensity and temperature ($p<0.001$) (Table.). Tukey's multiple comparisons indicated that the germlings area was significantly higher at 12ºC at high light when compared to low light (473.06 $\mu m^2 \pm 17.01$ vs 223.44 $\mu m^2 \pm 40.40$) while at 18ºC the differences were not significant. Comparing the results in high light, at 12ºC germlings present a higher area when compared to 18ºC (473.06 $\mu m^2 \pm 17.01$ vs 83.94 $\mu m^2 \pm 26.21$).

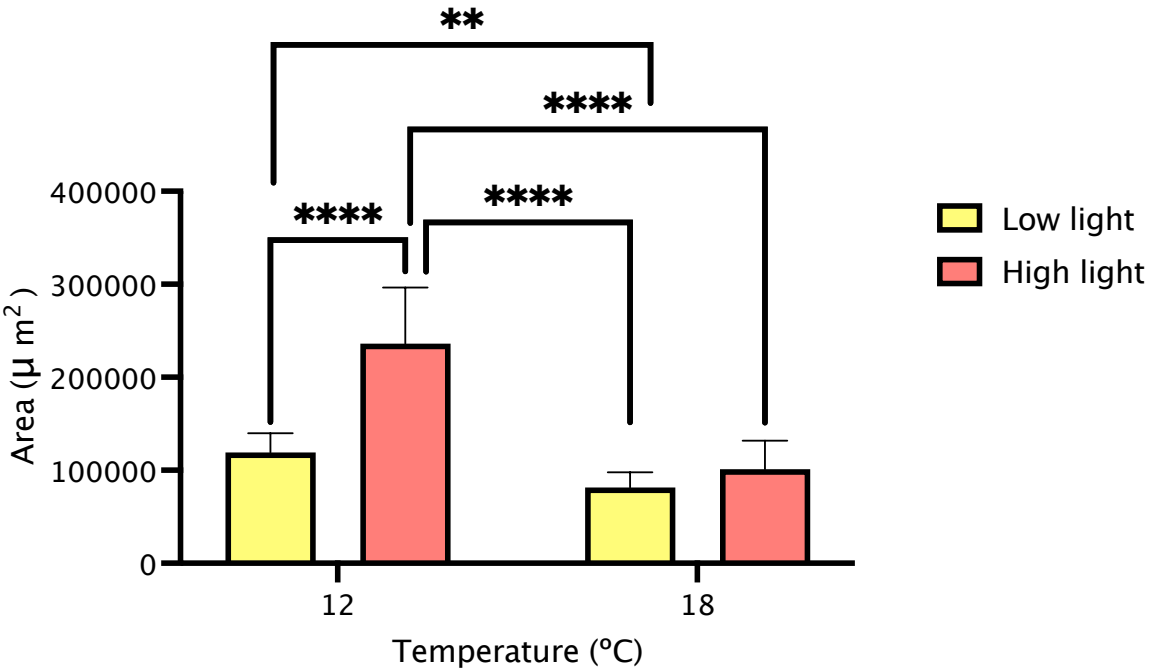


Figure 12. Mean germlings area (17/03/2023), with standard deviation, at two different temperatures and different light intensities. The asterisks above bars indicate significant differences from Tukey's pairwise comparisons (*- P ≤ 0.05; **- P ≤ 0.01; ***- P ≤ 0.001; ****- P ≤ 0.0001).

Table 10. ANOVA table was performed to evaluate the effects of light intensity and temperature on the area of germlings. Values in bold represent significant differences.

Source	df	SS	MS	F	*P*
Temperature=T	1	89625712974.28	89625712974.28	67.94	**<.001**
Light_intensity=L	1	55959135885.38	55959135885.38	42.42	**<.001**
T*L	1	28291829689.33	28291829689.33	21.45	**<.001**
Error	44	58043693184.93	1319174845.11		
Corrected Total	47	231920371733.92			

Table 11. Tukey's multiple comparisons between groups. Values in bold represent significant differences.

Compared groups	*P*- values
12H vs 12L	**<0.0001**
12 L vs 18 H	0.2403
18H vs 18L	0.2482
12H vs 18H	**<0.0001**
12 H vs 18 L	**<0.0001**
12L vs 18L	**0.0025**

The area of the germlings was measured a final time on the 18th of April. A higher mean germlings area was observed at 12ºC (160809.06 μm² ±84184.57) and high light (157697.58 μm² ±85402.33) (Figure 15). A significant effect was obtained for light intensity (p=0.001) and temperature (p<0.001) with no significant interaction between factors (Table 12).

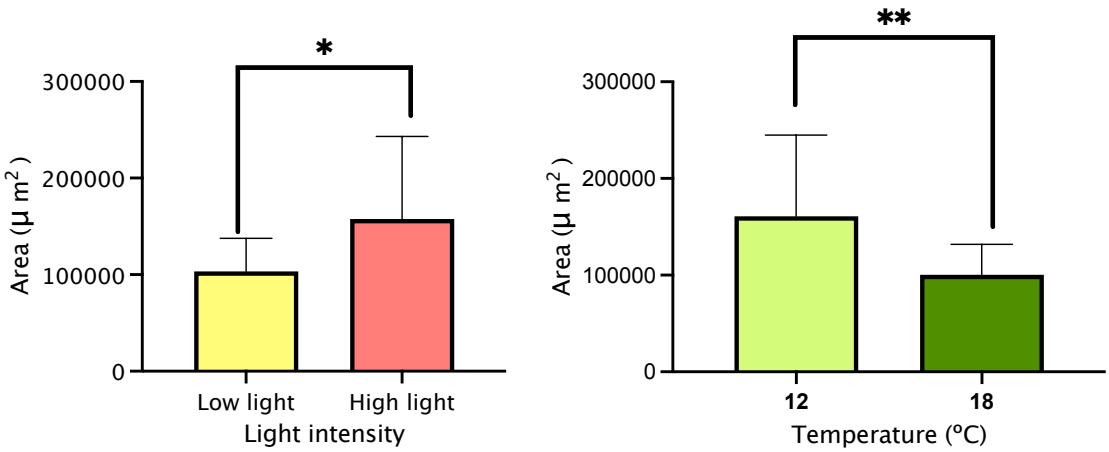


Figure 13. Mean germlings area (18/04/2023), at two different temperatures and different light intensities, with standard deviation. Asterisks above bars indicate significant differences from ANOVA (*- P ≤ 0.05; **- P ≤ 0.01; ***- P ≤ 0.001; ****- P ≤ 0.0001).

Table 12. ANOVA table was performed to evaluate the effects of light intensity and temperature on the area of germlings. Values in bold represent significant differences.

Source	df	SS	MS	F	*P.*
Temperature=T	1	47649748923.31	47649748923.31	14.50	**<.001**
Light_intensity=L	1	38357634749.89	38357634749.89	11.67	**0.001**
T * L	1	6269528224.31	6269528224.31	1.91	0.174
Error	48	157719876895.98	3285830768.67		
Corrected Total	51	249996788793.49			

3.3 Release and germination of *Laminaria hyperborea* spores

3.3.1 Influence of seeding density on area of coverage and length of blade

As expected, a lower coverage was observed in the lower density treatment compared with higher density on all dates. Area coverage increased with time in both treatments, with the highest density registered in the last date (D) (14.42 cm²

±1.66). There was a statistically significant difference between groups on all dates, the first three presenting (p<0.001) and the last date (D) (p=0.027) (Table 13).

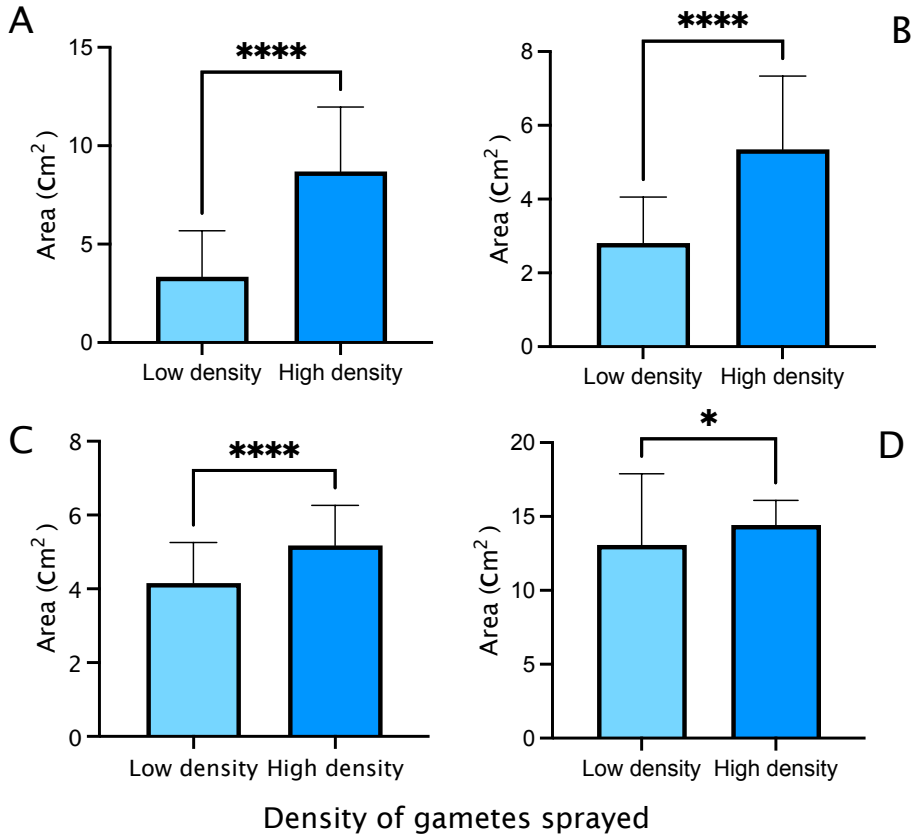


Figure 14. Mean area of coverage on different dates (A-21/06/2023) (B-30/06/2023) (C-07/07/2023) (D-17/07/2023) at two densities, with standard deviation. The asterisks represent significant differences in the area covered (*- P ≤ 0.05; **- P ≤ 0.01; ***- P ≤ 0.001; ****- P ≤ 0.0001).

Table 13. Unpaired t-test were performed to evaluate the difference in the area coverage at two different densities. These were measured first on the 21st of June and assessed weekly, each table representing the four different weeks. Values in bold represent significant differences.

Source	df	t	*P*
Between Groups	2.00	11.28	**<.001**
Within Groups	142		
Total	144		

Source	df	t	*P*
Between Groups	2.00	9.205	**<.001**
Within Groups	142		
Total	144		

Source	df	t	*P*
Between Groups	2.00	2.23	**0.027**
Within Groups	142		
Total	144		

Source	df	t	*P*
Between Groups	2.00	5.6	**<.001**
Within Groups	142		
Total	144		

Initially, a higher average length was observed for the higher density treatment (fig. 17 A) (0.23 cm ±0.07). However, on the last two dates (C and D) the average length was higher at lower densities (0.39 cm ±0.08 and 0.50 cm ±0.10) (Figure 17). There were statistically significant differences between groups at 3 different dates (A, C, D) ($p<0.001$), except for the second (B) which was not significant (Table 14), These results reflect the higher length obtained for the high density treatment initially and the gradual increase in the size of blades in the low density treatment, equaling the high density after 14 days and surpassing it afterward.

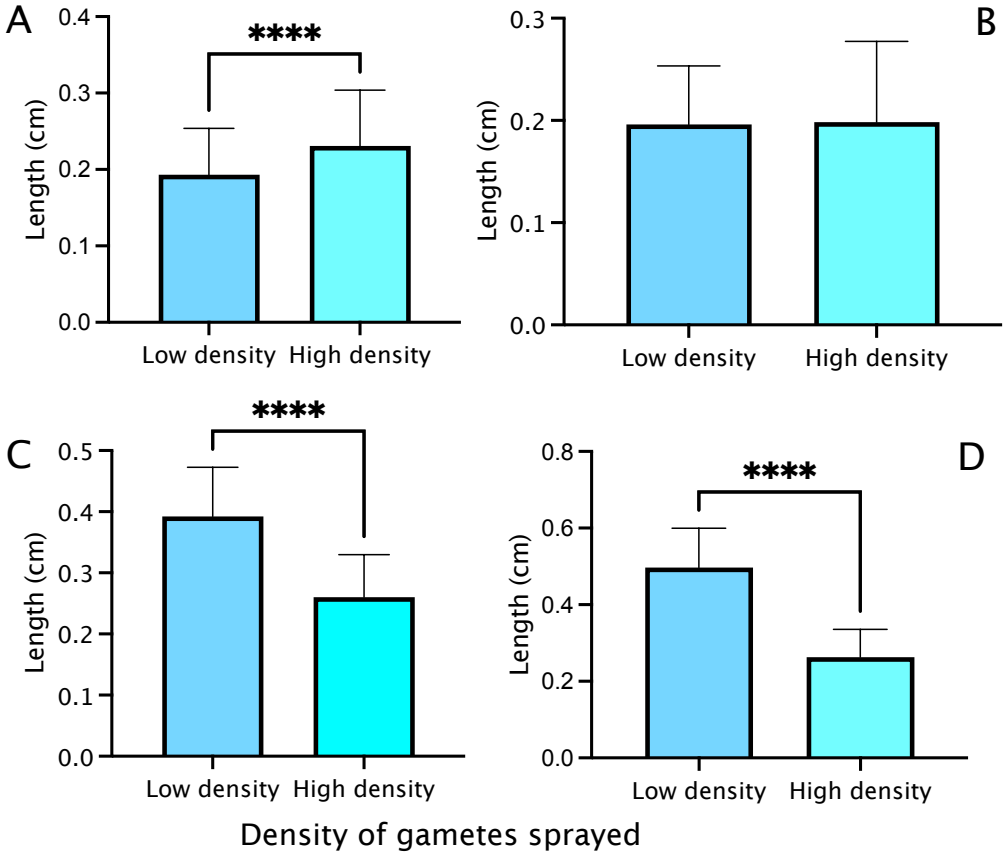


Figure 15. Average length of blades on different dates (A-21/06/2023) (B-30/06/2023) (C-07/07/2023) (D-17/07/2023) at two densities, with standard deviation. Asterisks represent significant differences in the area covered. (*- P ≤ 0.05; **- P ≤ 0.01; ***- P ≤ 0.001; ****- P ≤ 0.0001).

Table 14. Results from the unpaired t-test performed to evaluate the difference in the length of the blade of juveniles at two different densities. These were measured first on the 21st of June and assessed weekly, each table representing the four different weeks. Values in bold represent significant differences.

Source	df	t	*P*
Between Groups	2.00	10.62	**<.001**
Within Groups	1438		
Total	1440		

Source	df	t	*P*
Between Groups	2.00	0.6215	0.5403
Within Groups	1438		
Total	1440		

Source	df	t	*P*
Between Groups	2.00	33.34	**<.001**
Within Groups	1438		
Total	1440		

Source	df	t	*P*
Between Groups	2.00	49.93	**<.001**
Within Groups	1438		
Total	1440		

4. Discussion

4.1 Release of *Himanthalia elongatas'* gametes

4.1.1 Influence of the factors of sex and temperature on the release

The growth and reproduction of this species are thought to be site-dependent (Stagnol et al., 2016) and are thought to be triggered by temperature and photoperiod (Andersson & Kautsky, 1996). Light intensity is also an important factor considered in previous research (Moss et al., 1973), influencing the germination and growth of germlings.

Due to the size of the gametes, the female gametes being larger when compared to males, it is expected to obtain a higher quantity of male gametes released (Gibb, 1937; MOSS, 1969). Such happened at 10ºC, the temperature that presented significant differences between male and female gamete release, while the other two temperatures did not, indicating that this temperature may be more favorable for male gametes release.

Temperature presents as an important factor in the release and germination of gametes. Previous research that focused on the study of this species, maintained cultures at 10ºC (MOSS, 1969; Ramon, 1973). In a study conducted to examine the effects of light intensity and temperature, where the temperatures studied ranged from 3ºC to 25ºC, the highest percentage of germination was obtained at 10ºC (Moss et al., 1973). This matches with the results obtained in our study that showed a higher release of gametes at 10ºC, indicating a preference for this temperature by this species for the release also. This might also be impacted by the release of gametes being in the range of 10-12ºC as explained in (STENGEL et al., 1999). It is crucial to conduct additional research on the impacts of temperature, as it can potentially harm the fertilization process and may even lead to the mortality of gametes at the earliest stages (Moss et al., 1973).

4.1.2 Influence of temperature and washing treatment on the germination

Fertilization only occurs when the oospheres are released from a mucilaginous substance and the gametes fuse to form zygotes (Gibb, 1937; MOSS, 1969). The washing method was unsuccessful since it was harmful to the gamete's germination. This might be because it wasn't successful in removing the mucilaginous substance covering the oosphere. Other works were successful in applying this method (Moss et al., 1973), even with other fucoids such as *Fucus* spp (Creed JC et al., 1996), the last work using a mesh of 100 μm. The reason may

be the mesh size used in this experiment, too small to allow for the removal of the mucilaginous substance. Further trials are necessary to further test washing treatments that also allow us to assess the effect of temperature on germination.

4.2 Release of *Ascophyllum nodosum* gametes

4.2.1 Influence of light intensity and temperature on the germination and growth

Ascophllym nodosum is a species whose reproduction is dependent on water temperature (Pereira et al., 2020). The population in the North of Portugal is expected to react to stressors and temperatures differently than other populations. This population is near the geographic distribution limit of species, so it's expected to be subjected to less optimal conditions (Viana et al., 2014).

The population appears to be influenced by the interaction of light intensity and temperature in the earlier stages of recruitment and growth (Keser et al., 2005). Germination presented high percentages across temperatures and on different light intensities, this could be an indication that when mature this species doesn't present difficulties. The percentage of germination was higher at 15ºC in low light. This temperature is typical of the North of Portugal so this species might be used to it. However, due to complications with the climatic chamber's incubation of the samples at 15ºC further analysis was not possible.

Assessing the length allowed us to accompany the early growth of the germlings. Previous works indicate that coverage with other macroalgae might be important in the earlier stages of reproduction and settlement. Our results showed that longer rhizoids were obtained at lower temperatures (12ºC) and lower light. This can be explained by a better settlement of the species under a canopy and its reproductive season being in winter.

To continue monitoring growth, we analyzed the area of the germlings. The germlings exhibited variations in their growth depending on the measurement date, indicating potential differences in their requirements and responses based on their growth stage.

In February, we found that lower temperatures (12ºC) and reduced light intensity led to a larger area, similar to rhizoid length. These differences in light intensity at 12ºC may indicate the need for a canopy during early growth. Moreover, there were significant differences in growth between lower temperatures (12ºC) with high light and higher temperatures (18ºC) with lower light, with greater area observed in the latter case. This suggests a tendency for better growth under a canopy, even in conditions of higher temperature.

In March, lower temperatures had higher averages, and higher light intensity exceeded February's averages. Lower temperatures significantly differed from higher temperatures under both light conditions. While the interaction of temperature and light in April was not significant, distinct differences persisted. Lower temperatures resulted in higher averages compared to higher temperatures, and higher light intensity also led to increased averages. This shift toward greater growth at higher light intensities may indicate variations in growth requirements during different developmental stages (Viana et al., 2014).

Early research conducted in the United Kingdom by Moss (1975) also found that the percentage of zygote germination was highest at 10ºC, suggesting that growth performance is better at lower temperatures. Furthermore, Moss's work concluded that both light intensity and temperature have an impact on morphological development, which aligns with the outcomes of our study (Moss, 1975). Research conducted by Viana et al. in 2014 emphasizes the significance of other species acting as canopy cover, which in turn influences the amount of light received by zygotes. Additionally, their study concluded that *Ascophyllum nodosum* in Northern Spain faces challenges in terms of poor recruitment and slow recovery, primarily attributed to its geographical location. These findings could hold relevance for future research in Portugal, as similar conditions may impact the species' reproduction and requirements (Viana et al., 2014).

Overall, lower temperatures seem to favor the settlement and growth of this species and the significance of lower light could be important in the earlier stages. Further studies are necessary to better understand how varying light intensities could affect this species and better assess the role of canopy covering in earlier stages. Our work has shown that with the increase in temperature, the growth is predicted to be lower. Studying higher ranges of temperature is also essential to try to understand how shifts in temperature will affect this species further, especially because they are in an area affected by climate change.

4.2 Release and germination of *Laminaria hyperborea* spores

We assessed the most effective seeding density for this species. Our findings in terms of area coverage revealed that spools sprayed with a higher density (1.42 mg/L) of gametophytes exhibited a greater coverage area. This suggests an advantage in using a higher seeding density, which results in greater quantity. Nevertheless, the lower density (0.71 mg/L) approximated the averages of higher density as the weeks progressed.

When analyzing differences in juvenile length, we noticed that earlier dates displayed higher average lengths at higher densities. However, in the last two weeks, spools sprayed with a lower gametophyte density showed significantly greater average lengths compared to those with higher density, in contrast to the area coverage results. It's important to consider the advantages of different densities.

Depending on the goal, lower density appears to be more beneficial. Although higher density resulted in better area coverage, the proximity of averages over time and superior length outcomes favors lower density seeding. This may be due to reduced intraspecies competition for nutrients and light, allowing individuals to grow more effectively compared to higher density (Brawley & Johnson, 1991; Steen & Scrosati, 2004). As it can lead to better results, the usage of a lower density also means less biomaterial is needed. This approach relieves the need to harvest entire individuals, offering a more sustainable method that doesn't harm already endangered populations. It's also economically advantageous to maintain smaller gametophyte cultures.

Previous works involving *Saccharina latissima* tested different seeding treatments, revealing promising results with direct seeding (Forbord et al., 2020). Since this type of seeding has been proven successful before and considering the positive results from our study regarding seeding density, lower seeding density can potentially lead to better recruitment outcomes in *Laminaria hyperborea*.

5. Conclusion

This work has provided valuable insights into the development of germination protocols for the target species.

For *Himanthalia elongata*, it was observed that lower temperatures, such as 10ºC, present the best release results for both male and female gametes. Unfortunately, the washing treatment proved ineffective, suggesting the need for improving this method or test alternative strategies to control contamination.

In the case of *Ascophyllum nodosum*, lower temperatures and lower light intensity are optimal for early development and settlement, while higher light intensity enhances growth during later stages.

As for *Laminaria hyperborea*, lower seeding density appears to promote better growth and more sustainable recruitment.

These findings can aid in the creation of more effective nursery protocols to support marine forest restoration efforts. However, further studies are required to fully understand the effects of these conditions.

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