Investigating hatchery and cultivation methods for improved cultivation of Palmaria palmata

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Publication date:
2020

Document Version
Publisher's PDF, also known as Version of record

DTU Aqua
National Institute of Aquatic Resources

NTNU
Norwegian University of
Science and Technology

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Peter Søndergaard Schmedes

PhD thesis, April 2020

Danish Shellfish Centre
National Institute of Aquatic Resources

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THE VELUX FOUNDATIONS
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The Research Council of Norway

Data sheet

Title:	Investigating methods for improved hatchery and cultivation of *Palmaria palmata*
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Year of publication:	2020
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Please cite as:	Schmedes PS (2020) Investigating methods for improved hatchery and cultivation of *Palmaria palmata*. PhD thesis. DTU Aqua, Danish Shellfish Centre. 150 pages.
Keywords:	*Palmaria palmata*; dulse; hatchery seeding; spore efficiency; GMA, land-based productivity; cold-water rhodophyte

Funding:	Tandem-PhD program DTU Aqua and NTNU.
	The Tang.NU project (Villum-Velux Foundation) and the two projects MacroSea and MIND-P funded by the Norwegian Research Council additionally provided economic support.
Cover photo:	Peter S. Schmedes
Number of pages:	150

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Preface

This thesis was submitted as part of the requirements for the degree of Doctor of Philosophy (PhD) at the Technical University of Denmark and the Norwegian University of Science and Technology (NTNU). The research presented in this thesis was carried out at the Danish Shellfish Centre (DSC, Nykøbing Mors), National Institute for Aquatic Resources (DTU Aqua) under the supervision of researcher Mette Møller Nielsen (DSC), professor Jens Kjerulf Petersen (DSC) and co-supervised by Kjell Inge Reitan at the Norwegian University of Science and Technology (NTNU). An external research stay was undertaken at NTNU, in association with the seaweed group at SINTEF OCEAN, Trondheim, Norway. Funding for this PhD project was provided by DTU Aqua and NTNU, the Velux Foundation and the Norwegian Research Council through the three projects “MacroSea”, “MIND-P”, and “Tang.NU”. The research stay was partly funded by economic support from the Augustinus Foundation

I have always lived by the coast and been fascinated plant growth. I come from a family of farmers and doctors, so the interest for growing crops and natural sciences is in my blood and at a young age, I enjoyed experimenting with farming vegetables and early on took the lead in my family garden. In 2007, I began my education to become a biologist at Aarhus University, which has given me a solid understanding of ecosystem services - and also a concern for natural resource depletion by human consumption. This coupled with my eagerness for hands-on projects and my ability to think outside the box, lead me to pursue a master’s project in sugar kelp cultivation. This was the beginning of a growing fascination for optimizing seaweed cultivation, boosted by the movement for sustainability and seaweeds as a new superfood being on the raise. My work at the aquaculture company Hjarnø Havbrug during 2013-2015 further drove my interest in cultivating seaweeds. Here I was employed together with Teis Boderskov, my good friend and companion during my master´s education, to start up the first organic large-scale farming of sugar kelp in Denmark. At the same time, my interest in cultivating another seaweed species, *palmaria palmata,* was growing. I read the book *Seaweeds – vegetables of the sea* by professor in gastro physics Ole G. Mouritsen and at a conference, I listened to one of talks of Ole G. Mouritsen, where he expressed his culinary fascination for the use of this red seaweed species in the new Nordic cuisine. I was eager to contribute further to the solution for a future “green deal” regarding food security by promoting a sustainable production of a low trophic marine species, and to take the challenge of farming this red algae species, known for its good taste, but also notorious for the difficulty of cultivating it. It was therefore a quite easy decision to leave my former job as a state-employed biologist, when I in 2016 was fortunate to get the possibility of conducting a PhD project with the aim of developing methods to grow *Palmaria palmata.*

The present PhD project focuses on improving the biological understanding and practical methods in the hatchery and grow out to enable an optimization for its future cultivation. Part 1 of the thesis contains a general background to introduce seaweeds and their aquaculture, as well as a synopsis of the main results of the PhD study and a discussion to elaborate on the status and future trajectory for the aquaculture of *P. palmata*. Part 2 contains five journal manuscripts of which two are published in international peer-reviewed journals, one is under review for publication, and two are in preparation for submission.

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Acknowledgments

First, I would like to thank my supervisor phycologist Mette Møller Nielsen - you gave me the important team play and encouragement for fruitful brainstorming, guidance to experimental designs when I sketched new ideas, and valuable critics for improving our manuscripts. I learn a lot from you in our discussions, although I guess you sometimes feel that I discuss topics too much. You are an enthusiast and expert in listening, and your positivity makes one believe that every day will be a good day – and you had faith in me in times of slow progress that helped me get through this project. Also, big thanks to my main supervisor professor Jens Kjerulf Petersen for hiring me, your guidance throughout the project and taking your time to read and provide valuable feedback on the first manuscript and present thesis. A very big thanks to all my great colleagues at the Danish Shellfish Centre for helping me with fieldtrips, laboratory work and good spirits! I owe Patrick Joyce several lessons in Danish grammar for his help improving my English writing.

I like to give a warm thank you to my Norwegian colleagues, Kjell Inge Reitan (co-supervisor), Aleksander Handå, Jorunn Skjermo, Siv Aniana Etter, and Silje Forbord for taking good care of me during my research stay in Trondheim. You have all been a big inspiration for me during the past years. Whether it was seaweed chitchat over coffee during the period of university courses, stimulating talks and discussions during project meetings, ideas for new projects, fieldwork, or social events I enjoyed your company. I am grateful that we, with my participation in the MacroSea and MIND-P projects, successfully performed the first at sea-based cultivation of *Palmaria palmata* in Norway. Thanks to the laboratory and technicians at NTNU/SINTEF that helped me - you showed me a good access for sampling an intertidal *P. palmata* population near Trondheim, you kindly provided me access to a flow-through seaweed laboratory (SINTEF), and provided me with a beautiful office space and new friendships. Thanks to MSc student Renate Bøe, which I feel privileged to have had as my first co-supervised MSc student - I will like to thank you for finalizing the full year field campaign where we assessed the reproductive phenology in the intertidal *P. palmata* population. Thanks to Jørgen Ulrik Graudal Levinsen, my second co-supervised MSc student, for taking action and for good company during your master´s project testing year-round land-based cultivation using flow-through of water sourced from the Atlantic Sapphire Denmark company, a Recirculated Aquaculture System (RAS) unit producing Atlantic salmon in Hvide Sande, Denmark. Also a big thank you to the great people at Atlantic Sapphire; Thue Holm (CTO and co-founder) for giving us this opportunity, and Tanja and Per for your work ethics and help during this study.

A big thanks also goes out to Lone Thybo Mouritsen (the Kattegat Centre) and Annette Bruhn (Aarhus University) for your engagement in applied phycology, your workshops in the Tang.NU project and hosting the annual Nordic Seaweed Conference in Grenå. Thanks to Dr. Urd Grandorff Bak, talking Palmaria issues with you was fruitful for my study. I will also like to thank my former MSc-supervisor Michael Bo Rasmussen for field collections of biological samples for this PhD project and inviting me on scientific vessel cruises for macroalgae surveys in inner Danish waters. Together with PhD-fellow and friend Teis Boderskov, you introduced us to the fundamental skills and understanding of setting up a reliable seaweed grow-out unit back in the days. At university, I enjoyed our study group with you and Mads Fristrup Schou. Teis, we think differently, but share the same enthusiasm for seaweeds and together we generate great thoughts, at least in our opinion - I don't recall a single day with you where we haven't laughed. Thank you for all the good time we have spent so far.

Lastly, I will like to thanks peer-reviewers for their constructive feedback improving my publications.

I dedicate this PhD project to my parents, sisters and my beloved wife Line and our son Asger.

Peter S. Schmedes Nykøbing Mors, February 2020

Referat

Udnyttelsen af dyrket tang (makroalger) til mange forskellige formål er ved at opnå momentum i Europa. En af fokusarterne er rødalgen *Palmaria palmata*, kendt som dulse, eller søl på dansk. Nuværende teknikker til klækkeri produktion og dyrkning af søl er underudviklede og ineffektive i forhold til stor-skala produktion, og kompleksiteten af livscyklussen for søl har været begrænsende for optimal dyrkning af denne tangart i tidligere forsøg. Især er det nødvendigt at etablere fundamental viden angående 1) produktionen af fertilt væv (sori) til at producere sporer, 2) betingelserne for sporefrigivelse og optimal spredning samt fastgørelse på dyrkningssubstrater, 3) betingelserne for spiring og vækst af robuste kimplanter, og til sidst 4) optimal konfiguration af de spire-bærende substrater ved udplantningen til havs (eller i land-baserede opsætninger) til biomasse vækst. Disse fire arbejdstrin er det primære fokus i dette ph.d. projekt, med det samlede formål at optimere klækkeri og kultiverings-teknikker for søl, for at kunne imødekomme den stigende efterspørgsel på opdrættet søl i Europa.

Denne ph.d. afhandling dokumenterer det relativt korte sæsonvindue for den naturlige fertilitet hos søl i den nære kystzone i både danske og norske populationer med et maksimum på 50-70% fertilitet i perioden december-marts, og fraværet af fertilitet om sommeren. For at optimere klækkeri-produktionen, understreger dette hermed nødvendigheden af at inducere fertilitet i sommermånederne for at forlænge adgangen til sporer, og muliggøre udplantning i af spirer i det tidlige efterår, hvilket ikke har været muligt førhen, men anses for at være optimalt for tangdyrkning. Et højere indhold af udvalgte polyaminer blev fundet i fertile planter, og indikerer deres potentielle involvering i søl tetrasporogenese eller modning af tetrasporangium. Dog, viste forsøg med eksogent administreret spermidin, allantoin samt vandopløst ætylen ingen effekt på induktionen af fertilitet. I kontrast til dette viste forsøg med manipulation af miljøfaktorer at 5° C, kort dagslængde (8 timers lys), tilstedeværelse af meristematisk væv og forsøgstid at kunne frembringe ”sporangiale initialer” i søl om sommeren. En høj koncentration af næring var nødvendig for at modne sporangia, hvorimod en lav temperatur (5° C) og lav lysstyrke blev vist at stimulere udviklingen af fertilitet i både hankønnede gametofytter og tetrasporofytter, når de blev udsat for behandling om sommeren, dog med forskellige responstider (hanner: 14 dage; sporofytter: 3-5 måneder). Ved indsamling af søl i vegetativ tilstand allerede i april viste det sig, at med en for-kultivering på to måneder, med høj næringstilgængelighed og vækstrate før eksponering til vinterlignende tilstande, kunne inducere 6-60% fertilitet indenfor 1-3 måneder om sommeren og i det tidlige efterår. Selvom antallet af replikationer var lavt og resultatet ikke stærkt underbygger at fertilitet kan induceres året rundt, har jeg vist et udgangspunkt til videre udvikling af en metode og strategi for at opnå helårlig induktion af fertilitet hos søl.

Dette ph.d. studie fandt, at sporefrigivelsen blev positivt påvirket ved at påføre relativt høj agitation af vandvolumen og at fertile sporofytter frigiver sporer i mindst 20 dage under klækkeri-forhold, hvilket er en klar forskel fra for hvad opnås hos eksempel brune makroalger, såsom sukkertang, hvor zoosporerne frigives i løbet af få timer. I denne afhandling findes en nyudviklet metode til såning af vertikale net substrater ved at bruge gennemstrømnings-betingelser og flere efterfølgende såninger, hvorved det samme sori-væv bruges til a pode tre net i løbet af ni dage. Dette medførte en god spiredensitet på nettene, uanset mængden af sori som blev brugt (5, 10 eller 15 g). Resultaterne viser også, at man ved at inkludere et fertiliseringstrin under den tidlige klækkeri-vækst kan fordoble spiredensiteten, sandsynligvis ved at aktivere hunkønnede gametofytter, som ellers ville være gået

tabt. Alene med brug af den præsenterede metode, påpeger de præsenterede resultater en øgning af klækkeri-effektiviteten hos søl på faktor 5-17 per km reb eller 65-201 gange per hektar, som dog er afhængig af sori modenhed. Derudover, kan dette studie dokumentere en alternativ metode til at producere substrater med søl spirer. Denne metode baseres på at bruge aggregater af frigivne tetrasporer, som efter en modningsperiode på 29-39 dage (**G**ermination) udvikler sig til et mix af han og hun gametofytter. Efter en adskillelses-forbehandling (**M**aceration) og tilførsel til pode-kar under gennemboblende forhold (**A**gitation) etablerer disse propaguler en gen-fasthæftelse via deres disc organ til dyrkningssubstrater ("GMA-metoden"). Dermed fungerer disse gemte propaguler som et alternativ inokulum frem for en nødvendig adgang til spore-frigivende sori. GMA-metoden blev testet succesfuld op til dag 39 efter påbegyndt modningsperiode og kræver yderligere forskning for at øge dets anvendelse til helårs produktion af tilsåede substrater.

Ved at beholde tilsåede substrater under klækkeri-forhold, som giver langsom vækst, i en lang periode (7-10 måneder), opnåede robuste spirer en længde op til 5 cm, kunne modstå begroning af epifytter og udviste vækst ved lysintensiteter, som er relevant ved udplantning ud i efteråret i de indre danske farvande, end ved brug af unge kimplanter. Ved at beholde tilsåede substrater under forhold som nærmer sig hvile-tilstand i 11 måneder, blev den genoptagede vækstrate af de små spirer stimuleret ved stigende lys intensitet og mættet ved 20 µmol fotoner $m^{-2}s^{-1}$ PAR. Tilsammen viste unge og ældre spirer at vokse bedst ved lavest salinitet testet (15 psu).

Succes og opnået biomasse tilvækst ved kultivering af søl til havs var positivt påvirket ved brug af en lang klækkeri varighed før udplantning, samt af beliggenheden af opdrætsanlægget. Lav tilvækst (27±10 g vådvægt m^{-1}) blev opnået i den centrale del af Limfjorden (Færker Vig) ved brug af den konventionelle metode, hvor en tynd line omvikles en bæreline of dyrket i 1,5 meter under overfladen. I samme vandybde blev der opnået et biomasseudbytte på 289±33 g vv m^{-1} i den vestlige del af Limfjorden (Nissum Bredning) og 1991±49 g vv m^{-1} i Kattegat (Hjarnø Farm). Substrater som giver en høj rebdensitet (net) synes at være fordelagtige i at opnå et ligelig fordelt og højt biomasseudbytte ved dyrkning i de øverste 1.5-2.5 meter af vandsøjlen, afhængig af den optimale lyseksponering i det givne område og sæson. Den dyrkede søl indeholdte 40-190 µg kainsyrer g^{-1} DW, hvilket er 3-12 gange lavere end det reporterede for vild søl i dansk farvand.

Kultivering af spirer, opnået fra kanten af ældre søl, viste sig at være en god som en kilde til vegetative stiklinger til opformering af søl biomasse i en landbaseret opsætning som i en tofaset kultiveringsstrategi viste at kunne øge den areal-specifikke produktivitet og fjernelse af næring. Gennem 6 ugers for-kultivering med udsultende næringsbetingelser voksede disse stiklinger ca. 4 % d^{-1} mens deres røde pigmenter blev nedbrudt. Med et skifte i vækstfase, ved at tilsætte en enkelt høj dosis næringsmedie, udviste stiklingerne en vækstrate på ca. 6.5 % d^{-1} og et forhøjet optag af næringsstoffer igennem to følgende ugers forsøg. Skiftende næringsforhold er tidligere vist at være fordelagtigt for tangarters vækst og kan være en strategi til at optimere produktiviteten og samtidig undgå begroning af epifytter, som tidligere har vist sig at være et problem i landbaseret tangdyrkning. Resultaterne understreger at mætning af vækstraten ved 114-178 µmol photons m^{-2} s^{-1} PAR og afhænger af næringsbetingelserne.

Resultaterne i denne afhandling bidrager med ny, opdateret viden til forbedringer af klækkeri og dyrknings-teknikker, som er anvendelige for videre udvikling af stor-skala produktion af *P. palmata*, som anses nødvendigt for at efterleve den stigende efterspørgsel på søl samt til at sænke produktionsomkostningerne.

Summary

The exploitation of cultivated seaweed (macroalgae) for multiple applications is currently gaining momentum in Europe. One of the species in focus is the red algae *Palmaria palmata*, commonly known as dulse. Current hatchery and cultivation techniques for *P. palmata* are underdeveloped and inefficient for large-scale production, and moreover, the complexity of its life cycle has previously restricted optimal cultivation of this species. In particular, there is a need to establish fundamental knowledge on 1) the production of fertile tissue to provide the spore seedstock for seeding growth substrates, 2) the conditions for spore release and optimal settlement, 3) the conditions for germination and growth into robust seedlings ready for deployment, and finally 4) the out-planting at sea (or in land-based setups) for biomass propagation. These four lines of work were the main focus of this present PhD project with the overall aim to optimize the hatchery and cultivation methods for cultivation of *P. palmata* to meet the growing demand for farmed dulse in Europe.

The present thesis documented a relatively short seasonal window of natural fertility in *P. palmata* from both Danish and Norwegian intertidal populations. Fertility peaked reaching 50-70% occurrence during December to March but was absent during summer months. Hatchery optimization requires fertility induction to occur during summer months to prolong annual spore access. Year-round access to spores will allow grow out to begin during autumn which will optimize harvesting in the following spring. A higher content of selected polyamines in fertile sporophytes indicated their potential involvement in tetrasporogenesis or sporangia maturation. Here however, neither high concentrations of exogenous administrated spermidine, allantoin, nor ethylene triggered fertility. In contrast, fertility was induced by manipulation of environmental factors with significant effects of temperature manipulation (maintenance at 5° C), short daylength (8 h light), presence of meristems and time on the formation of sporangial initials. For maturation of sporangia, a high nutrient concentration was required. Low temperature (5° C) and low irradiance was found to stimulate the development of fertility in both male gametophytes and tetrasporophytes during summer, although response times differed (males: 14 days, sporophytes: 3-5 mth). By collecting vegetative fronds in spring, pre-cultivating at high nutrient and growth conditions for two months induced 6-60% fertility within 1-3 months during summer and early autumn by applying winter-like conditions. Although replication of the results was relatively low here, I provide a strong starting point for manipulation of fertility induction in *P. palmata* with regards to cultivation commercialisation.
During this research, I also found that *P. palmata* spore release was positively affected by applying high levels of agitation, and that fertile sporophytes released spores for at least 20 days under hatchery conditions, which differs from spore release in other species, such as kelps, where zoospores can be released within 1-2 hours.
The present PhD study developed a new method for seeding net substrates in vertical manner using flow-through conditions, three consecutive seeding events and by using the same sori over the course of nine days. This resulted in a good seedling density on the nets regardless of the amount of sori used (5, 10, or 15 g). In addition, the inclusion of a fertilization step during nursery growth led to a doubling of the seedling density, presumably by activating female gametophytes that otherwise would have been lost. Collectively, the presented methods identified techniques to increase to hatchery efficiency of sori use by a factor of 5-17 times per km rope, or 65-201 times per hectare in dulse grow out using seeded growth substrates, depending on the ripeness

of fertile sori. Furthermore, this study demonstrates an alternative seeding method based on using aggregates of released tetraspores. After 29-39 days of **G**ermination, the aggregates of spores developed into a mix of male and female gametophytes. Following a dislodgement treatment (**M**aceration), I showed that macerated propagules were able to establish firm discoid reattachment to substrates by adding a solution to **A**gitated seeding tanks ("GMA-seeding" method). Maintenance of germinated propagules resulted in sufficient seeding quality up to 39 days after germination was initiated, thus this method could be used as an alternative seeding material in contrast to newly released spores. Thus the GMA- method can extend the potential seeding period for *P. palmata* but, to extend this to a year-round method of seeding, further research is required.

Retaining seeded substrates in slow-growth nursery conditions for 7-10 months resulted in robust seedlings up to 5 cm in length, depending on position in nursery tanks. These robust seedlings resisted epi-fouling and grew at irradiance levels that are common during the autumn deployment season in inner Danish waters, whereas these irradiance levels has previously shown to reduce the growth of young seedlings. After keeping spore-seeded twines at dormancy for 11 months, the resumed growth of the small seedings increased by increasing irradiance and saturated at 20 µmol photons $m^{-2}s^{-1}$ PAR. The spore-derived seedlings exhibited increased growth in length at the lowest salinity of water tested (15 psu).

The success in sea-based grow out trials and biomass growth of *P. palmata* increased after longer periods of nursery maintenance but differed between farm locations. Poor harvest yield was obtained (27±10 g FW m^{-1}) in the central part of the Limfjorden (Færker Bay) where a conventional cultivation method of thin twine coiled upon a carrier rope was grown at a depth of 1.5 m bsl. By using same cultivation method, the Western part of the Limfjorden (Nissum farm) and the farm in the Kattegat (Hjarnø Farm) sustained a biomass yield of 289±33 g FW m^{-1} and 199±49 g FW m^{-1} at a depth of 1.5 m below sea level, respectively. Substrates providing a high rope density (nets) seem advantageous for obtaining an even and high biomass yield by cultivating the nets in the upper 1-2 meters of the water column. The optimal cultivation depth depends on the optimal light exposure in the given location and season. A content of Kainic acid of 40-190 µg g^{-1} DW was found in the cultivated dulse, which is 3-12 times lower than levels reported for wild harvested dulse in Danish waters.

In land-based grow out, the use of marginal proliferations were suited as a vegetative seedstock in tumble cultures by which a two-phased cultivation strategy increased growth and area-specific nutrient removal of *P. palmata*. During 6 weeks pre-cultivation, the tumbled seedstock grew ≈4 % d^{-1} and were able to withstand an irradiance of 280 µmol photons m^{-2} s^{-1} PAR using single low-dose nutrient pulses (nutrient starved growth), which resulted in depigmentation. By shifting growth phase, using a single addition of high nutrient concentration, the seedstock grew ≈6.5 % d^{-1} and exhibited a high nutrient take up during the following 2 weeks. Alternating nutrient availability has previously been shown to benefit productivity and a means of avoiding epiphytes. Results highlight a saturation of growth rate at 114-178 µmol photons m^{-2} s^{-1} PAR, depending on the nutrient conditions.

In conclusion, the present thesis provides new, updated knowledge for further development of the hatchery and cultivation techniques applicable to large-scale *P. palmata* production, which is required to lower the production costs and meet the increasing demand.

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Part 1: Introduction, synopsis and discussion

1. General introduction

1.1 Brief introduction to seaweeds

Taxonomically, seaweeds belong to three kingdoms: Plantae, Chromista and Bacteria (Guiry 2020). Seaweeds are phylogenetically diverse reflecting their evolutionary origin, different life cycles, as well as their ubiquitous distribution and adaptation to different environments from the warm, sunlit tropics to the ice-cold polar regions with long periods of darkness. Worldwide, at least 10,000 different seaweed species have been recorded, with an estimated 221-400 species being commercially important, depending on region (Guiry 2015, FAO 2018). Seaweed, as a crop, can provide biomass yields higher than that of genetically modified and conventional fertilized land-based crops (Feng et al. 2004; Duarte et al. 2009). Seaweed divisions are grouped according to their distinct pigment groups: green (Clorophyceae), brown (Phaeophyceae), and red seaweeds (Rhodophyceae). The rhodophytes are the most valuable phylum of today's seaweed aquaculture with approximately 20 species in cultivation, yet only very few European companies farm rhodophytes. The life cycle of rhodophytes are more complex than, for instance, kelp species that are widely cultivated in aquaculture, with either two or three alternating iso- or heteromorphic sexual generations (gametophyte, carposporophyte, and tetrasporophyte) (Cole and Sheath 1990). The different generations produce specific types of sexual or asexual reproductive spores (neutral spores, archeospores, carpospores, tetraspores) (Drew 1949, Santelices 1990, Clayton 1992, Fletcher and Callow 1992, Brodie and Irvine 2003, Mizuta et al. 2003, Saito et al. 2008), which provides the basis for aquaculture hatchery production (see section 1.5). In temperate regions, the growth of wild and cultured seaweeds follows a seasonal pattern with a rapid growth rate during spring, and reduced or negative growth during autumn and winter (Chapman and Craigie 1977, Fortes and Lüning, 1980, Sanderson 2006). Correspondingly, the biochemical composition of seaweeds in temperate regions also follows a seasonal pattern, which determines the nutritious value seaweeds (Box 1). Environmental factors, such as daylength and temperature, control life history stages in autotrophic organisms, which are adapted genetically to the seasonality through their evolution (Lüning and Dieck 1989, Bartsch et al. 2008, Liu et al. 2017). The life history stages of seaweeds are reproduction and growth, with crucial stage gates such as spore release, settlement, survival and early growth (Scott and Dixon 1973, Hoffmann 1988, Santelices 1990), which are especially affected by water motion (Charters et al. 1973, Hurd 2000). A substantial amount of previous research provides a consensus that life history stages of algae can be manipulated by controlling exposure levels of environmental factors (Lüning 1988, review by Bartsch et al. 2008). For perennial seaweeds in the North Atlantic, this includes the use of distinct spectral light qualities and short daylength, high nutrient conditions, winter-like temperatures and removal of meristematic tissue zones (Buchholz and Lüning 1999, Pang and Lüning 2004b, Bogaert et al. 2016). Annual seaweed species display rapid growth during a peak season to produce and liberate reproductive spores for hibernation before the thallus disintegrates. Perennial species produce and liberate sexual/asexual spores, which settle onto substrate before germination. After germination, the spore-derived seedling may enter dormancy during the winter, while the adults reduce in biomass by breakage of thallus in storms before regrowth starts in early spring (Flores-Moya et al. 1996, Faes and Viejo 2003).

1.2 *Palmaria palmata* (dulse)

Palmaria palmata, commonly known as dulse, is an example of a rhodophyte that is in focus for aquaculture. There is a growing demand for *P. palmata* proteins, peptides and biomass to use it in food, feed and cosmetics (Walsh and Watson 2011, Harnedy and FitzGerald 2013). *P. palmata* grows in the subtidal and lower intertidal zones (1-20 m depth) of temperate regions in the northern hemisphere, from arctic Russia and Canada to Portugal and New Jersey (Guiry 1975). From a single spherical holdfast disc, which enlarges ~ 2mm y^{-1} (fig. 1F-G), small stipes proliferate into a thallus (frond). The frond grows in a marginal meristematic zone along the frond edge (Fritsch 1961). Fronds usually grow to 10-40 cm in length but can reach 100 cm in length, with a seasonal peak in growth during the first half of the year (Kain 1986, Faes and Viejo 2003). *P. palmata* is characterized as a pseudo-perennial seaweed as new tissue grows from the edges after breakage of older fronds during storms (fig. 1B). The presence of these marginal proliferations depends, however, on the environment (Faes and Viejo, 2003). *P. palmata* exhibits high levels of diversity in morphology and color depending on the growth conditions and age (Fig. 1A-I), ranging from a single frond to a highly dichotomously or palmately divided frond.

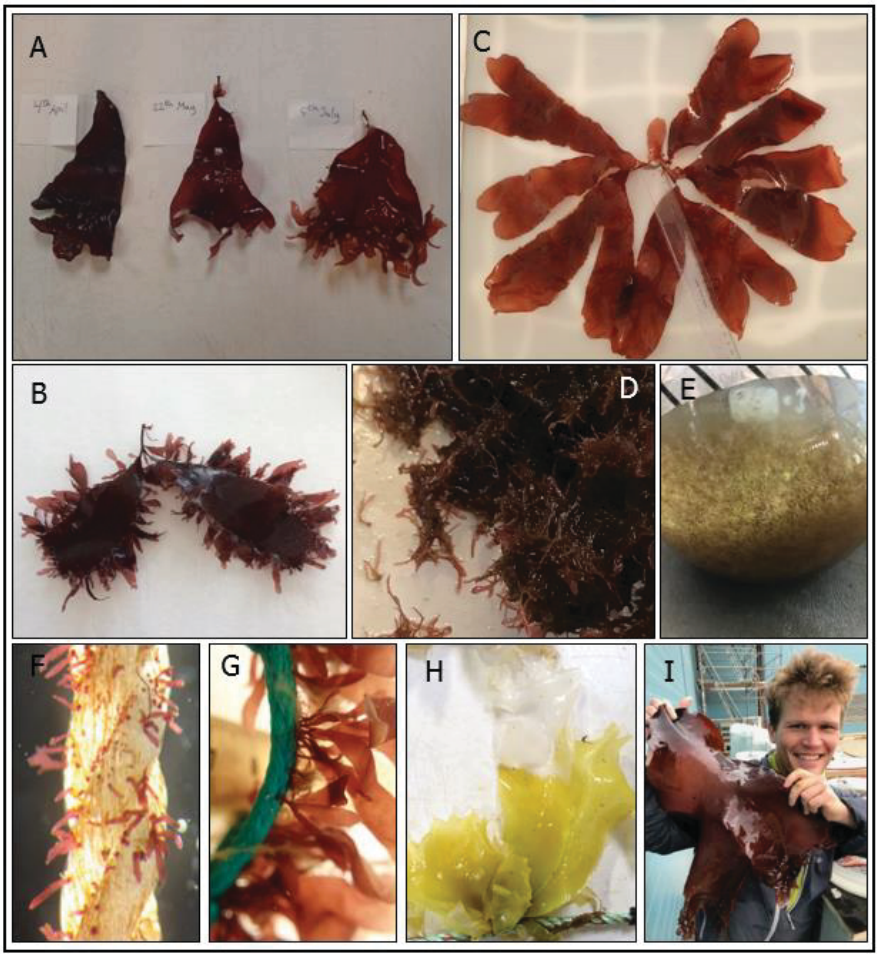


Figure 1. Presentation of the diversity of *P. palmata* grown under different conditions. A-C: Morphology, number of fronds, and the growth of marginal proliferations varies individually. D-E: Sporelings of *P. palmata* grown in bubble cultures appear red or green regarding high or low nutrient status. F-G: spore-seeded kuralon twine (Ø=2 mm) with seedlings. H: Bleached fronds with apical parts deteriorating after open water cultivation in June 2017. I: Large fronds obtained in May 2019 by cultivating vegetative shoots in flow-through bubble culture sourcing nutrient rich water from land-based salmon production.

Two research studies (van der Meer and Chen 1979, van der Meer and Todd 1980) documented the diplohaplontic life cycle of *P. palmata* (fig. 2, centre) in which the diploid tetrasporophyte and haploid male gametophyte fronds are isomorphic and only distinguishable at ripe fertility. The tetrasporophyte disperses haploid tetraspores from the cortex embedded tetrasporangia (fig. 2A, D) while the male gametophyte releases spermatangial gametes at ripe fertility (fig. 2D). Fertility is not restricted to the basal section and can occur in distal sections except the very marginal frond edges (fig. 2, centre). Tetraspores settle and attach to stones or on stipes of kelps, fucoids and some other rhodophytes (Irvine and Guiry 1983). Spores are covered in mucilage made of proteins and carbohydrates, which remains as a surrounding layer after spore release and, due to its sticky nature, is involved in the initial substrate attachment (Chamberlain and Evans 1973; Scott and Dixon 1973; Vesk and Borowitzka 1984), but is speculated also to cause bacterial growth on seeded substrates in hatchery. Spore settlement promotes a thigmotactic response by which the adhesive vesicles on the outside of spores release their content to enable firm attachment within 1-3 days (Pueschel 1979, Le Gall et al. 2004, Edwards 2007, Edwards and Dring 2011). The female gametophytes (fig. 2B) grow into microscopic (~200µm), crustose-like individuals, develop a carpogonium carrying an egg, and proliferate trichogynes, i.e., cellular tubes, into the environment to receive male gametes. Presumably, females are susceptible to fertilization within the first couple of months, but have been found to live up to 8 months without fertilization (Mine and Tatewaki 1994, Le Gall et al. 2004). After fertilization of the egg, a new sporophyte develops and overgrows the female gametophyte. Male gametophytes develop into erect fronds (fig. 2C, seedlings) immediately after spore settlement and become fertile within 9-12 months. Thus, male gametes (spermatia) for fertilization must come from a previous generation of males. Unlike many other rhodophytes, which commonly have tri-phasic life cycles with two diploid stages (carposporophyte and tetrasporophyte), the bi-phasic life cycle of *P. palmata* shows only a single diploid generation (tetrasporophyte) alternating with the haploid generation (gametophyte), which is ordinal to Palmariales (Guiry and Irvine 1981).

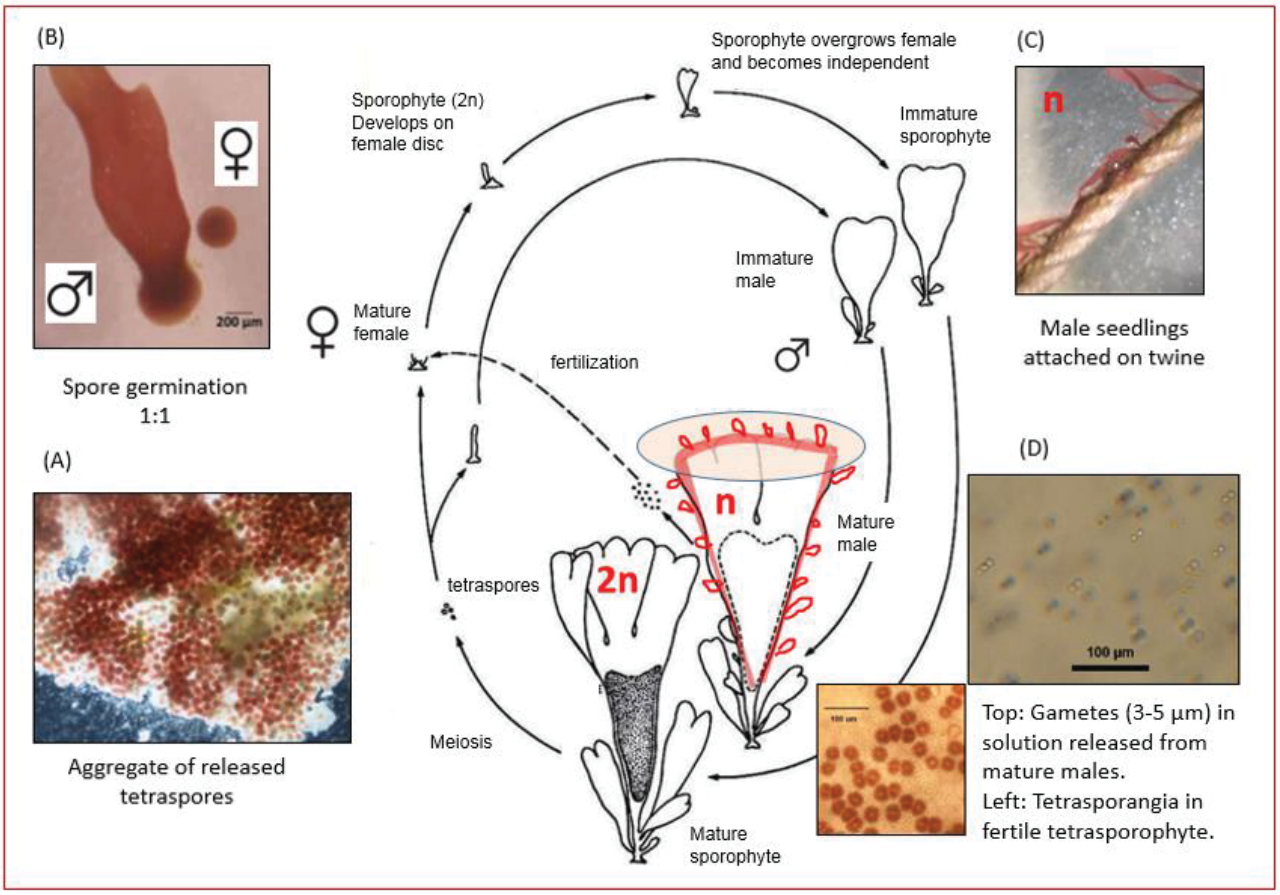


Figure 2. Centre: Diagrammatic presentation of the diplohaplont life cycle of *Palmaria palmata* with alternating isomorphic generations of the diploid tetrasporophyte (2n) and the haploid male gametophyte (n) (modified after van der Meer and Todd 1980). The dark-shaded tetrasporangial basal sorus (2n) and the dashed line (n) indicate fertile tissue. Peripheral photos: A) aggregate of tetraspores after release. B) After attachment, spores germinate into male and female gametophytes (*photo credit: C. Constanza*). C) Male seedlings attached to a thin twine. D) Top: solution containing male gametes (spermatia) Left: cortex embedded tetrasporangia in a fertile tetrasporophyte.

1.3 The use of seaweeds

Utilization of seaweeds by humans, especially *P. palmata*, dates back millennia. *P. palmata*, commonly known as dulse was used as a source of essential minerals, vitamins and fibers for poor people and monks in Ireland, while other seaweeds were a part of fine dishes served to the Asian Emperors some 1500 years ago (Tseng and Wu 1962, Kraan and Guiry 2006). In the 1900s, extraction of fiber carbohydrates from the matrix phase of cell walls (phycocolloids), such as agars (in Japan), alginates (FMC Corporation in USA) and carrageenans (GENU/Copenhagen Pectin Factory in Denmark, now CP Kelco), have been taken to full-scale production. These compounds are refined and used as thickeners, gelling agents and food binders in the science, chemical, medicine and food industries. For example, canned meat uses sodium alginate to bind liquid for consistency, while agars and carrageenans act as gelling agents to control the viscosity in toothpaste, ice cream, cosmetics etc. (FAO 1983, 2018). The use of seaweeds is now also receiving growing interest in

Western countries due to the recognition of seaweed as a sustainable source of renewable biomass and because of their reputation as health-promoting superfoods (Box 1). The growth in seaweed aquaculture lags behind the increase in demand (societal need) and has stimulated a growing interest in farming this sustainable biomass in Europe (Callaway 2015), undergoing a technical and economic development to find feasible solutions (fig. 3). The European blue bio-economy program stimulates a prosperous development of seaweed-based products, such as functional food ingredients, nutraceuticals and cosmetics (Hasler 2000, McHugh 2003, Smit 2004, Holdt and Kraan 2011, Hafting et al. 2015). Today, seaweed is also a valuable supplement in feed for livestock and fed aquaculture (Evans and Critchley 2014, Makkar et al. 2016, Wan et al. 2019). Further research in using seaweed in feed may help to reduce the climate footprint in beef production (Wan et al. 2019), as a recent Australian study showed promising effects of 5% supplementary inclusion of the red algae *Asparagopsis sp.* in cow feed reducing their methane emissions by 99%, though a long-term effect study is lacking (Roque et al. 2019). Sheep that consumed feed containing red seaweed also exhibited methane reduction (Li et al. 2016). However, using seaweed in feed has to follow a cautionary approach as bromoform, produced from ingested seaweed, is likely the chemical reducing the number of methanogen gut microbes, but is also a human carcinogen (Smith 2019, *Scripps Institution*). Red seaweeds, like *P. palmata* and *Pyropia sp.*, show crude protein contents of 185-277 g/kg DW, which can be estimated by use of total N conversion factor 4.52-4.65 (Gaillard et al. 2018) and is comparable to rapeseed meals (Woods et al. 2003). Improving cultivation methods is crucial to lower the cost of producing seaweed in Europe (Kraan 2013, Kim et al. 2017), as well as optimizing bio-refinery methods to extract different high-valued molecules (Torres et al. 2019 and references within). *P. palmata* has a long tradition of use in human diet, e.g., as ingredient or as a tasty snack, and has a high nutritious value (Morgan et al. 1980, Mouritsen et al. 2013).

Today, the demand for *P. palmata* by European retailers and producers is outstripping the supply (Edward 2007; Walsh and Watson 2011*, gourmettang.dk 2019 pers. comm.).* Wild harvest of seaweed to supply existing industrial sectors is widely performed in Europe and Asia (FAO 2009, 2018). However, wild harvesting can lead to a depletion of wild populations of seaweeds (Chopin et al. 1992) and the use of dredges and other mechanical harvesting gear has further raised concern about the sustainability of this method. Beach casted seaweeds are also being used commercially, however, in several countries this method has to comply with the restrictions of the Rio convention (1992), since beach-casted seaweed can be an important foraging ground for protected bird species. Because of this, there is an emerging demand for seaweed cultivation that is environmentally sustainable and links to societal needs, social acceptance and economic prosperity (fig. 3) (Chopin et al. 2001, FAO 2009, SAPEA 2017). At present, there is no stable methods or protocols for *P. palmata* cultivation as there is for other species, e.g., brown seaweed species like the sugar kelp, and only a few companies successfully cultivate other red seaweed species. Consequently, the supply of *P. palmata* and other red seaweeds is limited in Europe, thus the markets for seaweed-based products are still underexploited and immature in many member states (Chapman et al. 2015).

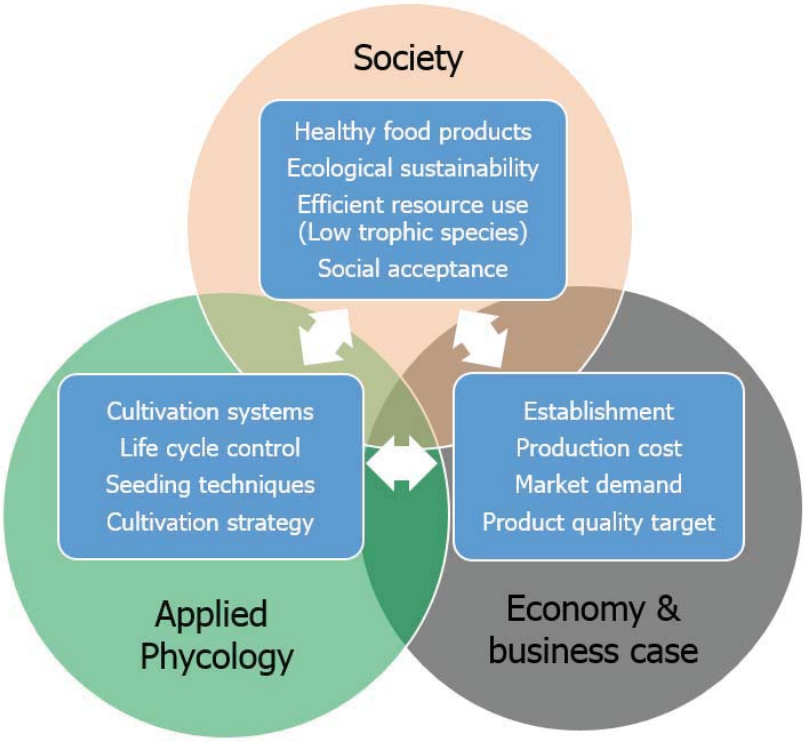


Figure 3. Farming seaweed is a discipline in applied phycology (green) based on controlling the life cycle and growth of desired seaweed crops by using cultivation systems. The economic prospects (gray) of farmed seaweed require a successful business case based on a demand from society (orange). Society, economy, and applied phycology are linked by overlapping relevance.

Box 1: The nutritional value of seaweed and health claims of seaweed-based products

Seaweeds were recently denoted one of the “foods of the future” and an important vegan protein food due to their sustainable production. Seaweeds like *P. palmata* and nori (*Pyropia yezoensis*) are valued in a variety of food products and have high concentrations of some of the essential nutrients for a balanced diet, i.e., minerals, vitamins, and some amino acids (Morgan et al. 1980, Mabeau and Fleurence 1993, Morrisey et al. 2001). The bioavailability of nutritional and bioactive compounds is seasonally dependent, as the concentrations of readily absorbed amino acids, proteins and polysaccharides, along with ash, varies throughout the year (Black 1950, Galland-Irmouli et al. 1999, Rødde et al. 2004, Harnedy et al. 2014). *P. palmata* and nori contain up to 35-47% (of dry weight; DW) crude protein, with 26-90% of the amino acids (eAAs) being essential to humans (Morgan at al. 1980, Galland-Irmouli et al. 1999, Wong and Cheung 2000, Murata and Nakazoe 2001).

Red seaweeds are a source of dietary fibers, such as xylans and galactans. By eating flakes or powdered red seaweeds, these fibers have a prebiotic positive effect on human gut health by stimulating gut exercise, modulating microbe compositions and their fermentation with potential prospects of seaweed products as health-promoting superfoods (Simpson and Campbell 2015, Liu et al. 2015, Cherry et al. 2016). However, human digestion is poor in degrading algal cell wall polymers, impairing the assimilation of algal proteins, as most humans do not have enzymes to degrade cell wall polysaccharides (Harnedy and FitzGerald 2011, 2013a). The crude dulse protein digestibility of 1.5 % without pretreatment can be greatly improved by washing and powdering the biomass to 29% of the crude protein (Marrion et al. 2003), while >90% of the proteins can be extracted from dulse using N-acetylcysteine assisted enzyme hydrolysis (Naseri et al. 2020). Further, the production of bioactive peptides and protein hydrolysates from seaweeds is gathering interest for application in food and pharmaceutical industries (Harnedy et al. 2014, Admassu et al. 2018). Seaweeds contain iodine, which is important for human thyroid function, and can be a good source of iodine to substitute iodized kitchen salt. By precautionary reasons, the European Food Safety Authority (EFSA) recommends a daily intake of 600 µg iodine for adults. In dulse, the iodine content ranges between 10 and 100 µg g^{-1} DW^{-1} depending on the location and season from which it is collected (Mouritsen et al. 2013). Thus, a daily intake of 15-60 grams of dried *P. palmata* provides an adequate daily iodine intake for adults by these regulations. The Danish food authority recommends a maximal consumption of 20 g DW of dulse d^{-1} for adults due to concern about Kainic acid, which potentially causes neurotoxicity at a relative high dose (Hanne Boskov Hansen, Danish Food Authority, pers. comm. 2018). In addition, due to a relatively high content of potassium in *P. palmata* (McGrath et al. 2010), children, pregnant women and people with renal impairment should be careful eating *P. palmata*. A daily consumption of 16-25 g dried *P. palmata* would be sufficient to obtain 250 mg of the n-3 fatty acid eicosapentaenoic acid (EPA) (Morgan et al. 1980), which is found to reduce the risk of coronary death by 35% (van Ginneken et al. 2011). While the content of fatty acids is relatively low in red algae like dulse (0.3-3.8 % DW), the relative content of poly-unsaturated fatty acids (PUFA) omega-6 and omega-3 (n-6 and n-3) is 20-40 % of the total fats (Morgan et al. 1980). In general, the ratio of n-6/n-3 PUFAs in seaweeds is low (0.05-2.75), which is beneficial for human health regarding reduced risk of inflammatory disorders (Sánchez-Machado et al. 2004; Mouritsen et al. 2013).

Pure seaweed extracts have shown health-related properties e.g., polyphenols (anti-oxidant), pigments (anti-inflammatory), sulfated polysaccharides (anti-viral), anti-proliferating peptides and acids (Yuan et al. 2005, O`Sullivan et al. 2010, Harnedy and FitzGerald 2013b; Lee et al. 2017, Suwal et al. 2019). A study found elevated acute inflammatory serum proteins (CRP) in humans consuming bread with dulse, and the authors suggested an increased innate immune readiness within clinical normal range (Allsopp et al. 2016). Applying pure extracts or whole unprocessed seaweed into products, companies may claim it as functional food or nutraceuticals, which require some standard testing of the efficacy of claimed function, besides nutrition (Hasler 2000, Hafting et al. 2015, Millian-Linares et al. 2019). Recently, the Chinese authorities conditionally approved a seaweed-based medicine called Oligomannate to treat mild to moderate Alzheimer´s disease by oligosaccharide-mediated suppression of the gut bacteria causing the disease (Wang et al. 2019).

1.4 Farming systems

Sea-based cultivation is the main supply of biomass for the seaweed industry (Rebours et al. 2014, FAO 2018), taking place in many developing countries where farmers use off-bottom lines, floating rafts, or longline suspended ropes, nets and tubular nets (Góes and Reis 2011, Reis et al. 2015, Hayashi et al. 2017).

Since the first European grow out trials in 2007, ropes deployed as vertical droppers or continuous loops have been used (fig. 4a, b) (Bostock et al. 2016, Stévant et al. 2017). Since then, research in substrates that provide more surface area, such as thick ropes, nets, textile ribbons and textile sheets (Werner and Dring 2011, AtSeaNova, formerly At~SEA Technologies, Belgium), has been conducted to provide an optimal cultivation strategy in the pursuit of lowering production cost (fig. 4c-e). Rope cultivation is still used in Chinese kelp farming by deploying vertical droppers on horizontal longlines and controlling the seedling density by thinning the seedlings prior to deployment, or by inserting small pieces of seeded twine with substantial spacing (fig. 4A-B).

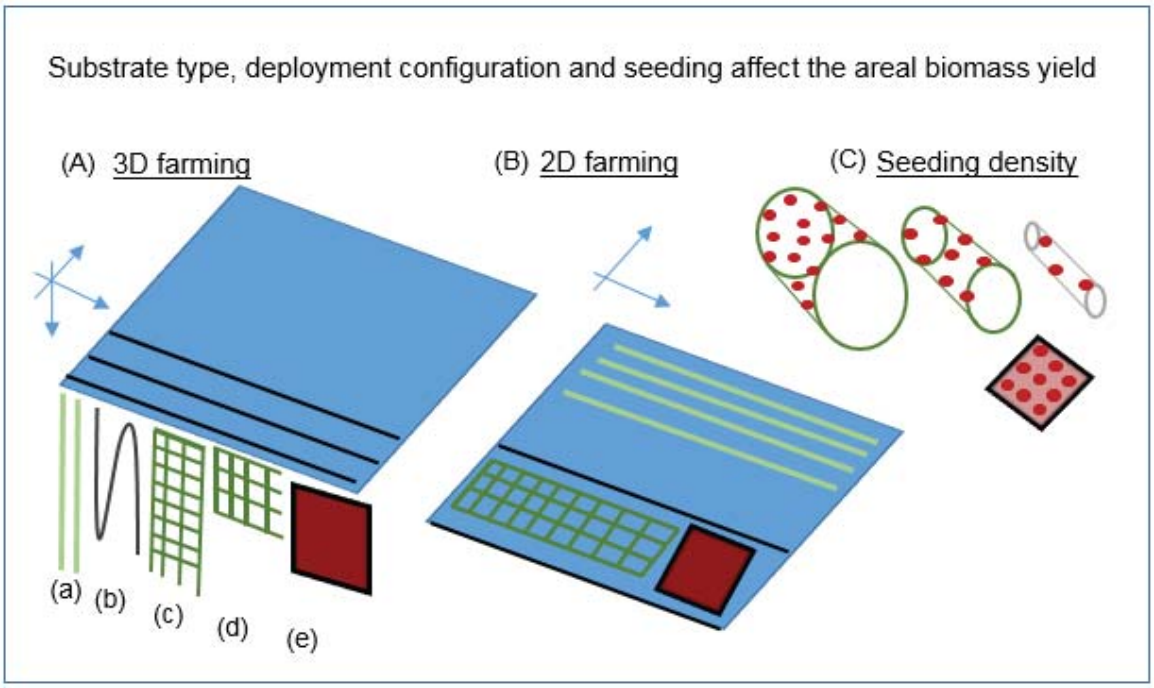


Figure 4. Diagrammatic illustration of the areal usage of a farm site with longlines (black lines) either with a 3-dimensional (A) or 2-dimensional (B) growth substrate configuration providing different degrees of utilization of the marine surface. (C) Illustration showing that pieces of cultivation growth substrate of equal length will differ in spore settlement density (red dots) proportional to the amount of surface area. (a) single vertical dropper lines, (b) rope hanging as continuous loops, (c) vertical deployment configuration of net, (d) horizontal deployment configuration of net and (e) vertical deployed textile sheet.

Roesijadi et al. (2008) emphasized the negative effects of sea-base grow out at large scale, which impose water flow stagnation at a certain substrate density. Slower water motion leads to impairment of bicarbonate diffusion rate to thalli, and furthermore, at high biomass density, a high portion of the incoming light is absorbed

in the upper part of the water column, which results in lower light penetration through the water and likely result in reduced seaweed growth with increasing water depth (Hurd 2000, Sanderson 2006). Hence, the optimal deployment configuration to maximize yield depends on species and site characteristics.

Aquaculture of the relatively small-sized rhodophyte *Pyropia sp*. in temperate and sub-tropic regions appears to be optimized regarding the choice of method for seedling production, grow out location and deployment configuration. Grow out is conducted by using horizontal floating nets in coastal waters or by using a pole system in tidal zones (Mumford and Miura 1988, Hayashi et al. 2017). In combination, the areas provide moderate exposure or high currents and the horizontal configuration using nori nets optimizes the amount of deployed growth substrates per area (2D) (fig. 4B) to sustain the highest growth rate. The method for *Pyropia* grow out in tidal zones allows desiccation during ebb tides to kill settled epiphytes and thereby maintain a clean crop. Conversely, vertical deployment configurations (fig. 4A) are used in European grow out trials of kelps and *P. palmata*, as well as in Chinese kelp farming industry, to exploit several meters of the water column to maximize the area-specific yield (Werner and Dring 2011). In a farmed area, large spacing of growth substrates, e.g. mono-lines or floating rafts, can positively affect the growth rate of individual seaweeds, because of more available resources per unit biomass. However, the biomass yield is potentially reduce if spacing are too large, due to less growth substrate per unit space. The seedling density on growth substrates affects the growth rate of cultivated seaweeds (Kerrison et al. 2017). The seedling density depends on the spore concentration in the spore-seeding phase and the type of growth substrate (fig. 4C). The spore concentration is relatively easy to control with kelps as their motile, photo-tactic zoospores remain in suspension, while it is a challenge in seeding with non-motile tetraspores of *P. palmata.* In addition, Palmarian tetraspores have a sticky surface due to mucilage, which makes them clump together. The dimension of the growth substrate influences the spore settlement density, as the amount of surface available for spore settlement on the growth substrate (fig. 4C, red dots) increases per length unit, proportional to the rope diameter and by use of sheets.

The use of seaweed as a biological filter to reduce nutrient concentrations in effluent water, for example from finfish aquaculture (Ryther et al. 1975, Kim et al. 2013, Corey et al. 2014), may facilitate land-based seaweed cultivation because it can reduce the operational costs of seaweed production (Blouin et al. 2007, Watson et al. 2012). A potential co-cultivation of an existing land-based fish farm e.g., a Recirculated Aquaculture System (RAS) unit, with a seaweed hatchery and production unit producing commercial red seaweed, has previously been mentioned (Blouin et al. 2007). A RAS salmon farm releases a substantial amount of filtered seawater (wastewater) with elevated nutrient concentration. This valuable resource flow can source a land-based seaweed hatchery to save expenses on pumping, cleaning and cooling of seawater as well as buying nutrients (fig. 5). In addition, seaweed biomass grow out in land-based tanks act as a biological filter to reduce the nutrient concentration before the water is released into the environment. Connecting different production units will allow utilization of the flow of resources and is a potential way to optimize the establishment and operational costs of a seaweed cultivation company. Land-based cultivation allows for full control of environmental parameters such as light exposure, salinity, temperature and nutrient availability (Neish et al 1977, Morgan and Simpson 1981ab, Hafting et al. 2015). The control of environmental factors is a way to elevate the

concentration of specific molecules in *P. palmata*, for example pigments and mycosporine-like amino acids (MAAs), and potentially vitamin B12 (Kanazawa et al. 1966, Yuan et al. 2009). In addition, the use of vertical mixing ensures high area/volume specific photosynthetic rates (Grobbelaar 2009), which can result in high productivities of up to 20-100 g DW m^{-2} day^{-1} of *Ulva sp.* and *P. palmata* (Matos et al. 2006, Mata et al. 2010, Nielsen et al. 2012). The stocking density and age of seaweed thalli also affects productivity. By use of multiple cropping as harvest method with time intervals during the growth, the amount of biomass returns to optimal stocking density (Morgan and Simpson 1981ab, Kim et al. 2013, Corey et al. 2013). The few studies that have investigated whether land-based cultured *P. palmata* can maintain productivity over a full year production show that the growth rates decline during summer. Thus, further research is required to develop an optimal growth strategy (Corey et al. 2013, Tremblay-Gratton et al. 2018).

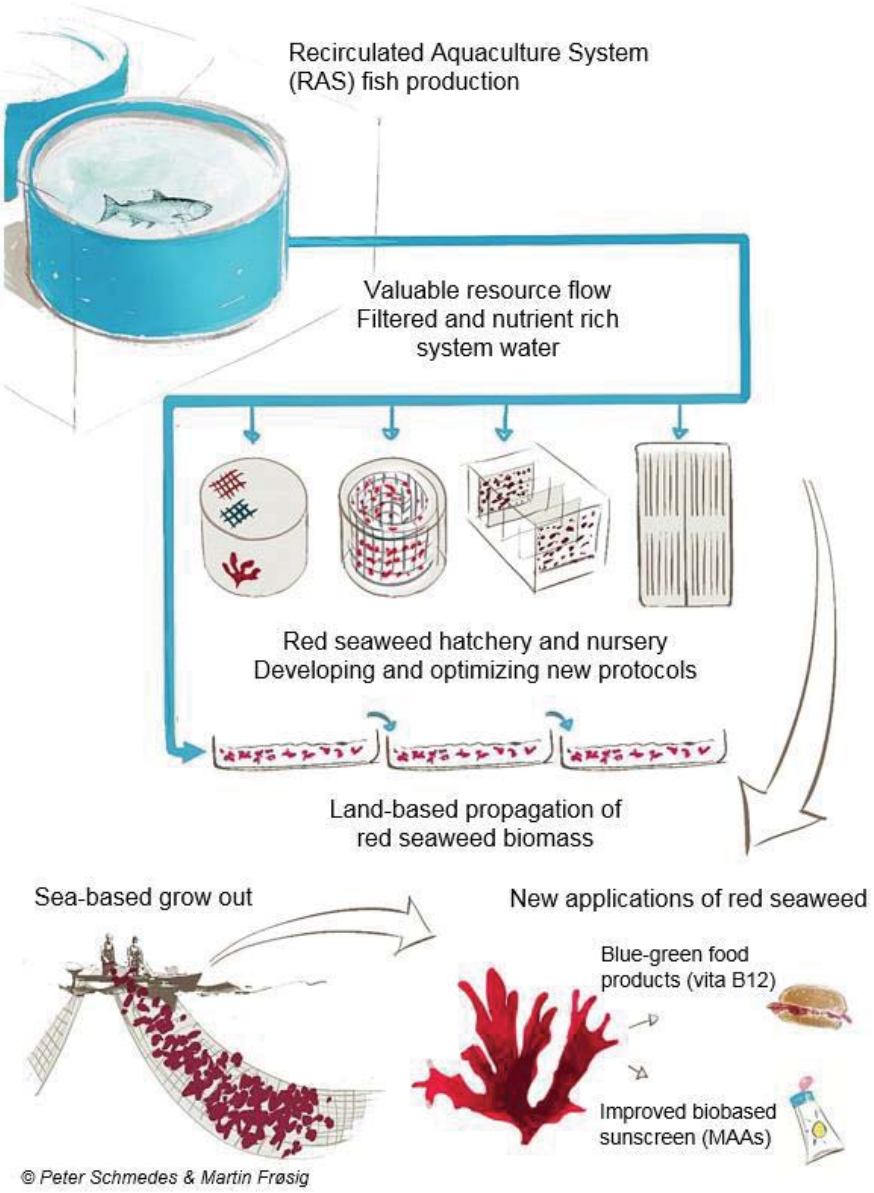


Figure 5. Schematic of the potential integration of a land-based salmon farm using RAS with seaweed cultivation processes including hatchery, biomass propagation and grow out.

1.5 Cultivation and productivity of seaweeds

Cultivation of seaweed presumably started in Korea in the 15th century with *laver* (*Pyropia sp.*), known as nori or sushi seaweed. Today, seaweed cultivation takes place in many developing countries and is an important income for many companies and family enterprises, which have adopted cultivation techniques to increase the supply of seaweeds (Alveal et al. 1997, Avila et al. 1999, FAO 2009). The current global seaweed cultivation produces some 30 million tonne of fresh weight, of which America and Europe only cultivate 54,000 tonne (FAO 2018). While momentum is gathering for large-scale seaweed production and cultivation is seen as a core activity in the development of the European blue bio-economy, the present cultivation methods are still cost-ineffective and suboptimal. Overall, two methods are in use for cultivating seaweed biomass at sea (fig. 6). The spore-based cultivation methods used for kelps, *Pyropia sp.* and *P. palmata* (fig. 6-1), normally require a land-based hatchery phase before the actual cultivation, whereas vegetative propagation at sea using tissue fragments of species, such as *Euchema/Kappaphycus sp.*, does not require a hatchery (fig. 6-2).

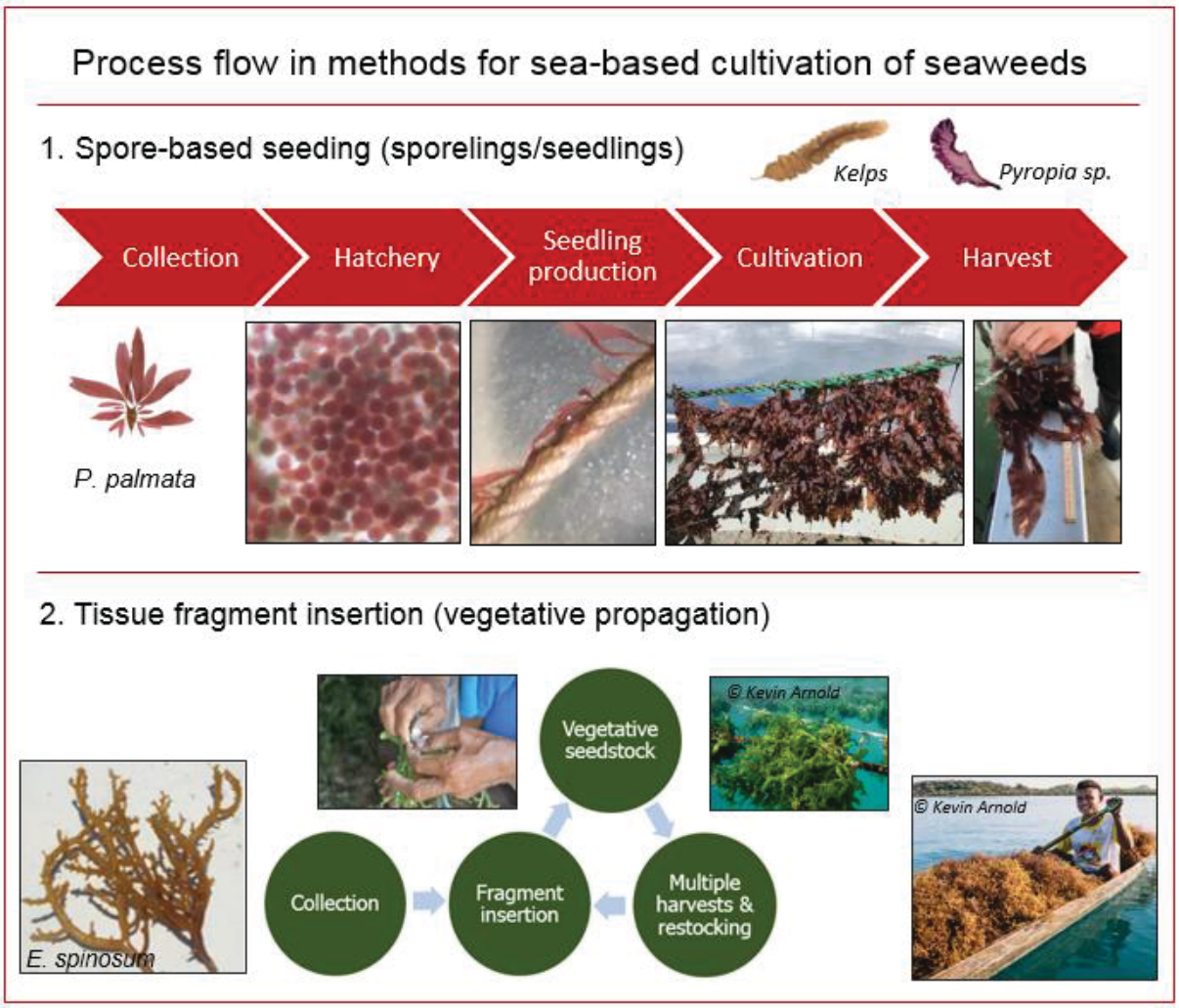


Figure 6. General process flow using two different methods for sea-based cultivation of seaweeds. 1: The spore-based method relies on collection of sporophytic tissue that is either fertile upon collection or that require maturing in nursery before spores are released, and can then be used in hatchery seeding of growth substrates. Spores germinate into seedlings during a nursery phase. Sea-based cultivation for grow out of

seeded substrates results in new seaweed biomass for harvest. 2: In vegetative propagation, tissue fragments are prepared from collected material to produce a batch of vegetative seedstock and inserted in polyfilament monolines. In the tropics, this method results in fast biomass acquirement, thus multiple harvests, where a part of the harvested biomass is used to produce new fragments for restocking the lines, are possible.

The area-specific productivity of farmed seaweed depends on multiple factors including geographical location, environmental conditions, farm size and depth, species, cultivar strain, seedling density, cultivation system and harvest method (Harrison and Hurd 2001, Kerrison et al. 2015, Bruhn et al. 2016, Kim et al. 2017).

Seaweed farming in Europe is still a relatively new industry and the initial focus on kelp species has resulted in reliable cultivation manuals (Forbord et al. 2018) leading to several European countries creating seaweed cultivation companies that farm kelp species. However, the relatively low value of the raw biomass of kelp species and the immature local markets means only a few companies are economically sustainable. Grow out trials using wild sugar kelp (*Saccharina latissima*) have reported relatively low area-specific yields, while the biomass yield per meter (m^{-1}) of seeded rope can be high (1-25 kg m^{-1}), thus showing a potential for large-scale farming (Sanderson 2006, Taelmann et al. 2015, Bruhn et al. in prep., Petersen et al. 2016). With very few actual reports on large-scale cultivation trails in North Atlantic (NA) waters, the extrapolations from different studies generate highly variable yields of farmed seaweed in general, which also reflects a difference in farm environmental parameters. Further, there are ongoing developments to ensure cost-effective sea-based grow out and suitable farming sites in Europe (Marinho et al. 2015, Bruhn et al. 2016, Boderskov et al. in prep). A production of 25.2 tonne fresh weight per hectare per year (FW ha^{-1} y^{-1}) was extrapolated from reported yields in Denmark by assuming a deployment of 5000 meters of seeded lines ha^{-1} (Bruhn et al. in prep). In comparison, higher yields of 58.5 tonne FW ha^{-1} y^{-1} has been reported in Spain (Peteiro and Feire 2009) and 125 tonne FW ha^{-1} y^{-1} in Ireland have been reported, with differences in biomass yields largely attributed to the differences in water motion (Taelman et al. 2015). In China, area specific yields of 24.3 tonne dry weight (DW) ha^{-1} y^{-1} (corresponding to 202.5 tonne FW ha^{-1} y^{-1} FW; DW/FW=0.12) of *Saccharina japonica* hybrids (Dongfang no.6) have also been reported (Li et al. 2007, 2016).

Farming of red seaweeds has been conducted in many continents for several decades, however, cultivation in Europe has only developed relatively recently. The main species have been *Gracillaria sp*. and *Eucheuma/Kappaphycus* for phycolloid extraction and *Pyropia sp.* for food, such as nori snacks and sushi rolls (FAO 2018). Recently, red seaweeds have gained more attention in European seaweed aquaculture (Pang and Lüning 2004a, 2006, Sanderson 2006 Edwards 2007, Sanderson 2015) due to their high content of pigments, poly-unsaturated fats, vitamins, proteins and amino acids making them high valuable in developing new products (Morgan et al. 1980, Fleurence 1999, Mouritsen et al. 2013, Moroney et al. 2015). Although *Palmaria palmata* production has been in focus for the last two decades, the hatchery and cultivation methods for large-scale are still suboptimal.

1.6 Previous trials of cultivating *P. palmata* in Europe – scoping the challenges

Seminal work on developing protocols for open water dulse cultivation have been developed in Ireland and Scotland (Browne 2001, Sanderson 2006, Edwards 2007, Werner and Dring 2011, 2013). The relatively few studies have documented the use of hatchery techniques or wild seeding (Bak 2019) to seed growth substrates with spores. These previous studies have underlined the need for optimized hatchery methods, high-yield cultivation systems and cultivation strategies to lower the production cost of dulse. A low efficiency of using the spore-releasing sori is the main challenge in *P. palmata* hatcheries, especially regarding the timing of spore release, dispersal, settlement, germination and initial growth (Edward and Dring 2011, Werner and Dring 2011, Wood 2018). Thus, an optimization of its cultivation requires research on conditions affecting each cultivation step, with emphasis on developing new hatchery methods as well as investigations of new cultivation strategies to improve growth and enhance biomass yields. The previous hatchery trials relied on collecting and using a large amount of spore-donors (sori), where the period from January to March was found to be the optimal window for collecting fertile spore-donors (Werner and Dring 2011). However, the authors found high spore mortality (60-95%) in the early nursery phase of the seeded growth substrates, which resulted in highly variable hatchery quality of the seeded growth substrate, with patchy seedling density (Sanderson 2006, Werner and Dring 2011). Together with the high sori requirement, the basis for upscaling was not feasible.

Methods to improve the access of fertile spore-donor have been based on exposing tissue to low temperature and short daylength to induce fertility in *P. palmata* tetrasporophytes and to enhance spore release (Mine and Takewaki 1994, Le Gall et al. 2004, Titlyanov et al. 2006, Pang and Lüning 2006). However, another study, treating vegetative fronds but within the natural season of fertility occurrence, did not find sufficient evidence for an inductive effect of using that such treatments as ambient conditions resulting in similar fertility (Edwards 2007), which emphasizes the need for further investigation and improvement of hatchery techniques to increase the efficiency in seedling production. A previous study used male gametes to investigate the zygote formation and early development of the new tetrasporophyte in lab scale (Mine and Takewaki 1994). The application of male gametes in hatchery production have been mentioned as a way to improve the seedling output for *P. palmata* seedling production (Le Gall et al. 2004) as fertilization will activate the female gametophytes and enable a higher density of seedlings on the growth substrates. However, this has not yet been trialed at relevant scale.

Previous trials of sea-based cultivation of dulse have reported yields in the range of 0.94-1.85 kg FW m^{-1} of seaweed line in Ireland and Scotland (Sanderson 2006, Edwards and Dring 2011, Werner and Dring 2011, Sanderson et al. 2012). An earlier study reported an extrapolated yield of 86.3 kg m^{-1} longline (Browne 2001) by assuming equal growth rate down to 7 meters depth on dropper lines with little spacing. By assuming 2000 meters of longline in a farmed hectare (20 longlines of 100 meter), these studies provide estimated yield of 1.8-172.6 tonne FW ha^{-1} y^{-1} of *P. palmata* biomass (=0.21-20.7 t DW, dry/wet= 0.12). Werner and Dring (2011) investigated sea-based grow out from which a biomass yield of 36 tonne FW ha^{-1} is estimated with this density of longlines. The validity of the high estimates is speculative and requires validation through large scale testing. Based on previous developments, there is currently a need to improve the hatchery and cultivation methods to enable an optimization and upscaling in grow out of dulse biomass to meet the growing demand in Europe.

2. Aims and work flow of the thesis

The overall aim of this PhD study was to improve the cultivation of *P. palmata* based on investigating the hatchery techniques and the methods for sea- and land-based grow out. To achieve this, I intended to advance current methods of dulse cultivation by providing data driven insights into the core chronological steps of dulse cultivation:

Fertile spore donors → spore release and settlement → growth of seedlings → propagation of dulse biomass.

Manipulation of environmental factors has been the basis for controlling algal life history in cultures and is crucial for domestication and a successful cultivation of seaweed. I have thus used manipulation of environmental conditions as a basis for the working hypotheses of my studies to investigate the control of life history stages and to optimize grow out of *P. palmata*.

As I intended to examine the production of tetraspores, which is fundamental for sea-based grow out, I planned and executed field collections of wild *P. palmata* to assess the natural reproductive phenology. I combined these results with laboratory experiments using manipulative exposure treatments aiming to induce fertility year-round in vegetative tissue to see if differences occurred between wild and manipulated individuals (Paper 1). Different means were tested as catalysts to induce fertility in *P. palmaria* spanning from exposures to different metabolites, to manipulation of environmental factors, such as light, day length, nutrients and temperature.

In the following cultivation step, my aim was to improve the spore release, spore settlement on substrates and the initial growth of spore-derived seedlings by manipulating the physical conditions in the seeding tanks (Paper 2). I hypothesized that the spore release yield would increase by use of water agitation assuming water agitation promotes liberation from the sticky cortex surface. Furthermore, I performed a seeding experiment based on the “tumble spore release method” (Sanderson 2006), where I used agitation level as a treatment to assess the effect on even spore settlement on rope substrates. Doing this, I tested whether an alginate gel coating of growth substrate surface, mimicking the surface of kelp stipes where *P. palmata* attaches in nature, enhanced spore settlement. Following this, I examined whether the light saturation point for spore-seedling growth improved with increasing nursery duration. In paper 3, my main aim was to develop new methods to improve the sori use efficiency (amount of sori per unit substrate) in hatchery seeding based on modifying the physical configurations for spore-seeding and the conditions for augmented control of spore germination and growth. For this, I used air bubble agitated conical tanks in flow-through with a “sori-package” placed in a central position where the air hit the surface. I used the sori package in three consecutive seeding periods to enable an increase in the number of nets seeded per gram sori. In addition, I retained effluent tetraspores and used them as an alternative seeding material.

In paper 4, I aimed to improve the biomass production and nutrient removal rate by using small vegetative seedlings as a seedstock for biomass propagation in a two-phased grow out method with manipulated light intensity and nutrient availability. In addition, I aimed to estimate the light saturation point of this seedstock in nutrient deprived and replete conditions. This aimed to add knowledge on how to adjust the timing and depth of deployment of seeded growth substrates regarding the optimal irradiance levels.

Finally, in paper 5, I aimed to investigate the cultivation success and biomass growth in different parts of inner Danish waters by deploying different types of seeded growth substrates based on using a short or long nursery duration. I assessed the growth rate and biomass yield on the substrates, which can be used to estimate the potential biomass yield on a larger scale. The aim was to provide knowledge for an optimized strategy for cultivating *P. palmata* at sea in Danish waters.

The following synopsis summarizes the objectives and main results of the investigations. In part 2 of this thesis, five research articles (Paper 1-5) provide a more comprehensive description of each study.

3. Synopsis

3.1 Access to *P. palmata* tetraspores – by field collection and induction of fertility

Sea-based grow out of *P. palmata* relies on tetraspores for seeding. As spore accessibility in nature is sparse, the main aim is to induce fertility to secure access to spores in the hatchery independently of the natural fertility cycle. Access to spores during summer would enable a production of seeded substrates well in advance of the optimal season for deployment for biomass grow out, which is autumn to spring (Sanderson et al. 2012).

In nature, sexual reproduction in North Atlantic (NA) perennial seaweeds show synchronization with the winter season and is believed to be triggered by changes in few environmental factors, such as day length, temperature and nutrient content of tissue (Kain 1982, Martínez and Rico 2002, Le Gall et al. 2004, Bartsch et al. 2008). In addition, it is speculated that metabolites, such as polyamines and ethylene, induce tetrasporogenesis in rhodophytes (Guzmán-Urióstegui et al. 2002, García-Jiménez and Robaina 2012, Takagi et al. 2016). Manipulated exposure treatments, e.g., winter-like conditions, have been shown to result in the production of a high number of motile zoospores for hatching sugar kelp (Forbord et al. 2012), whereas the effect of exposing *P. palmata* to similar treatments is questionable (Edwards 2007, Wood 2018). Further, previous efficient methods for inducing fertility, such as isolating small submeristematic tissue fragments, have not yet been tested during summer (Titlyanov et al. 2006). In addition, the isomorphic appearance of the sporophyte and male gametophyte is a challenge in managing seedstock for a large-scale hatchery production. The present study provides a description and photo-guide for identification of maturing sporophytes at an early stage by detection of sporangial initials (fig. 7). This guide is based on results from Paper I.

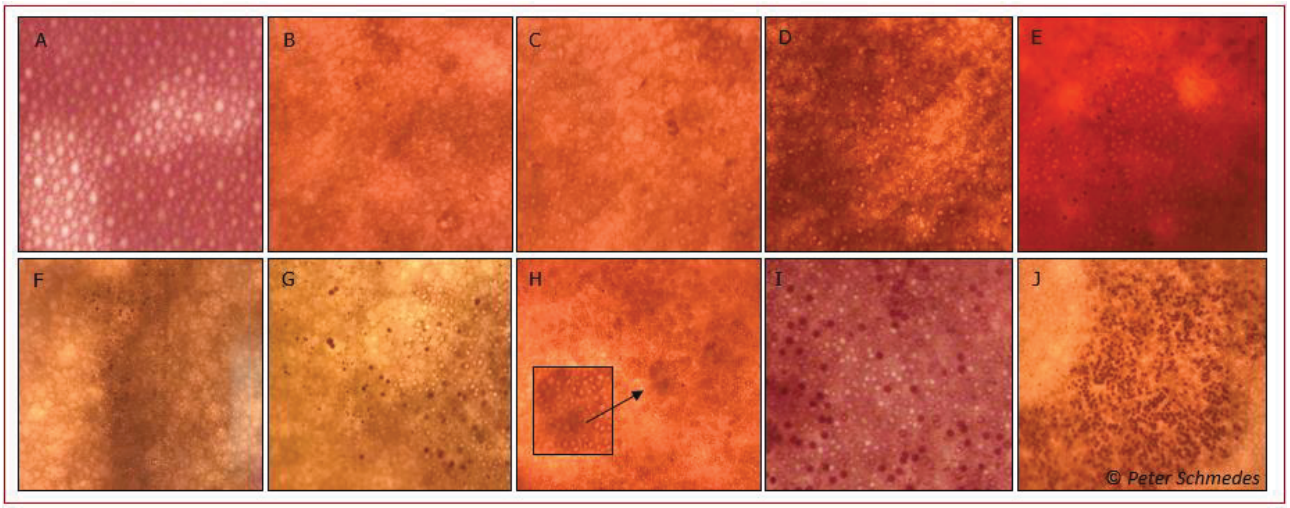


Figure 7. A photo-guide for early sporophyte identification. Stereomicroscopy views of the morphological transition from vegetative (A) to ripe fertile tetrasporophytes (I-J). B-E: Development of tiny, distinct hyaline cells embedded in the inner cortex of maturing sporophytes. F-G: progressed sporangial development. The sporocytes enlarge and mature to form sorus containing ripe fertile tetrasporangia visible as various marbling patterns. H: The box magnifies a cortex field (arrow) showing a brown indistinct pattern.

From a full year survey[1] in a Norwegian seaweed population and during the winter months in three Danish populations, a peak season of natural fertility occurrence and spore release yields during December-March with a fertility occurrence of 50-70% was shown, whereas no fertility was seen during summer (May-September). Thus, results point to the importance of inducing fertility in *P. palmata* to secure the proper spore availability for hatchery production.

The manipulative exposure experiments found short daylength, presence of meristem, low temperature and experimental time to induce sporangial initials after 2-3 months following collection in late August. High nutrient concentration was crucial for the maturation of ripe sporangia. Paper I suggests that the induction of sporangia and spermatangia requires 3-5 months duration by collecting material in the field during summer and late summer, which is in accordance to a previous study (Pang and Lüning 2006). Furthermore, Paper I shows that by collecting fronds in April with subsequently 2 months of pre-cultivation, 6-8 % of the sporophytes were fertile after a month (mid-August) and 20-60% after 2-3 months (late September and early October), , which is a higher fertility in comparison to the occurrence in natural environments, and suggest a beneficial effect of the chosen induction strategy. Exogenous administrated polyamines did not exert any effect on reproduction, however, a screening revealed a relative high content of tyramine and putrescine in fertile fronds from the wild, which suggests a potential involvement of such compounds in tetrasporogenesis of *P. palmata*. Future studies investigating induction of sporogenesis in *P. palmata* during summer should aim to quantify the polyamine modulations coupled to a RNA transcriptome analysis, as used by Garcia-Jimenez et al. 2018, on a weekly basis to reveal the involvement of metabolite-photo-receptor signaling in tetrasporogenesis.

The potential for inducing tetraspore production during summer (off-season) seems to require collection and pre-cultivation of vegetative thalli in early April to build up nutrient reserves before the exposure to winter-like conditions promotes fertility. However, despite thorough investigations within this topic, all year spore induction is still not been convincingly achieved, and more research is still needed. Before this achievement, the narrow time window of spore access from collecting material from wild populations imply a demand for efficient seeding methods to sustain a large-scale production of seedlings (paper 2) or a shifted season, i.e., to perform the hatchery activity the year before the grow out (paper 5).

[1] Major contributions to the phenological study is attributed to MSc graduate Renate Bøe from NTNU, who conducted 8 of the 11 sample collections in the Trondheimsfjorden (Bøe 2019).

3.2 Spore release and settlement - testing new hatchery methods to improve *P. palmata* cultivation

Spore release in *P. palmata* is hard to predict due to variation in sori ripeness causing a high variation in the timing and yield of spore release (Werner and Dring 2011, Wood 2018). Further, with high spore mortality during the first three days after seeding, the previous method is associated with a low efficiency (Werner and Dring 2011). As seeding is performed in horizontal tanks with calm water, the method also suffers from poor spore dispersal due to the large size and non-motility of the spores. This leads to patchy spore settlement, and therefore requires a high amount of sori to reach desired spore density (Sanderson 2006, Edwards 2007, Werner and Dring 2011). Alternatively, use of water agitation has been found to provide a good spore yield (Pang and Lüning 2006), even within one hour (Le Gall et al. 2004) and by tumbling the fertile sporophytes in tanks (Sanderson 2006, the "tumble method"). However, the attempts so far to develop a large-scale seeding protocol (fig. 8, top), all rely on high use of sori (Browne 2001, Sanderson 2006, Werner and Dring 2011) because a substantial amount of the tetraspores sink to the bottom of the non-agitated tanks and form dense aggregates, thus being unable to inoculate substrates.

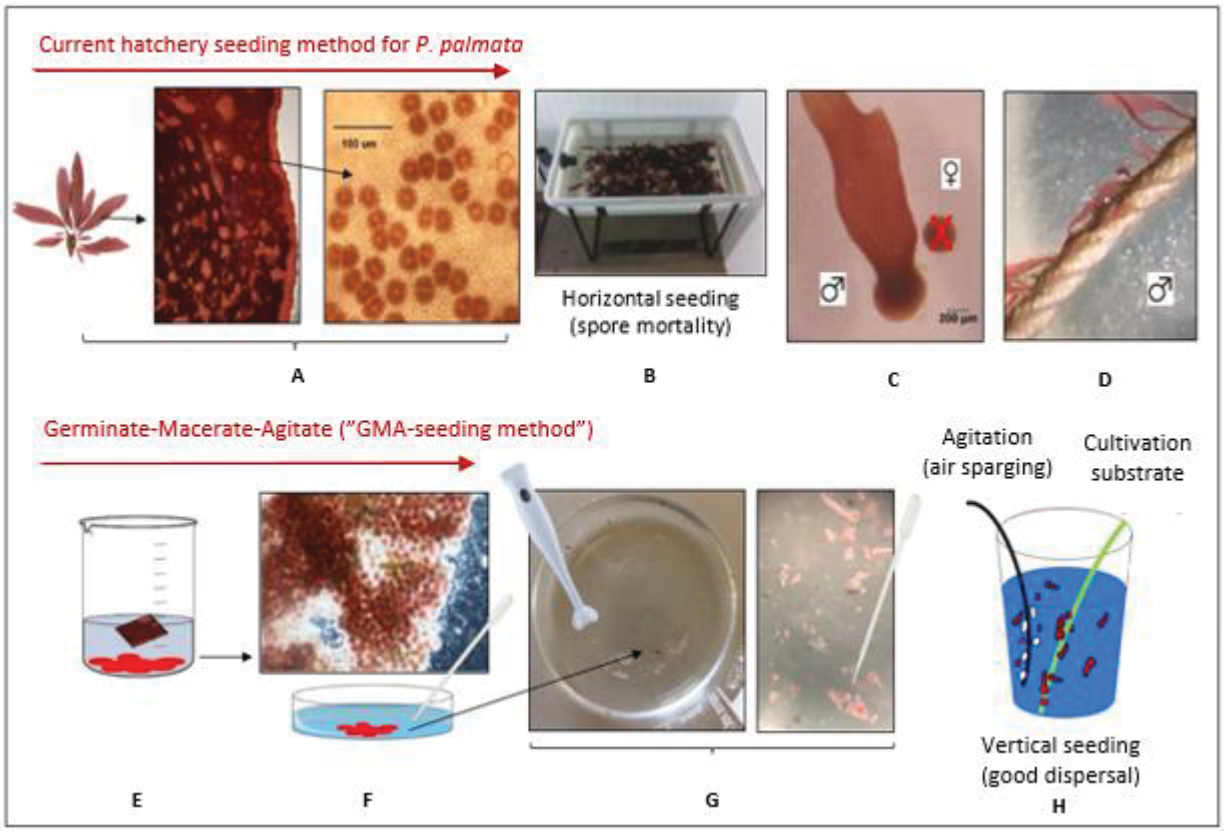


Figure 8. Illustrations showing the steps in previous hatchery method for cultivating dulse (top) and the "GMA-seeding method" proposed in paper II (bottom). A: Tetrasporophyte with sori containing tetrasporangia. B: 1:1 areal cover of net substrates in horizontal tank during spore release *(photo-credit Werner and Dring 2011)*. C: Gametophytes germinate after settlement. Males develop into an erect thallus; females are microscopic and die unless being fertilization *(photo credit Constanza C. Cerda)*. D: Male seedlings after 2-3 months of nursery. E: Released tetraspores settle in dense aggregates. F: Spore aggregates washed and transferred for

germination in petri dish. G: Dislodging and macerating aggregates to obtain a solution of propagules (spores and tiny seedlings). H: Adding propagule to agitated inoculation tank for settlement.

The present PhD study investigated means to improve the seeding hatchery protocol for *P. palmata* by improving spore release and developing new methods for efficient seeding. The results highlight a significant positive effect of agitation on spore release, providing a better spore dispersal on cultivation substrates (paper 2, 3). From a single piece of sori, spore release showed to last from one hour and up to 20 days, providing an opportunity of using the same sori in consecutive seeding events, thereby enhancing the efficiency of available sori. In addition, I developed a new seeding method based on the use of aggregated tetraspores. The three steps in this method, **G**ermination, **M**aceration and **A**gitation provide the acronym "GMA-seeding method" (fig. 8, bottom). After spore release, bottom-aggregated tetraspores (fig. 8E-F) showed to be a suitable inoculum (seeding material) for later seeding activity as the germinated propagules (mix of microscopic females and sprouting male seedlings after maceration, fig. 8G) showed the ability to reattach to the growth substrates and disperse homogeneously by the use of agitation (fig. 8F).

To upscale both the spore-seeding method, where a new tank configuration was tested, and the propagule-seeding method (GMA), I used high agitation and vertical oriented growth substrates, which is a space-efficient setup, besides benefitting the spore dispersal. Doing this, I aimed to increase the sori use efficiency, by taken advantage of the long-term spore release seen in fertile plants. Thus, I applied three consecutive seeding events of three days each, which enabled the seeding of three nets with the same sori. I assumed that sori packages of 5, 10, or 15 g placed in the surface water of agitated tanks (fig. 9A-C) would promote spore dispersal with the attachment ensured, due the sticky mucilage covering the spores (Boney 1981). Paper 3 verified a suitable spore density on the nets using this updated method. This approach combines the use of high-agitated flow-through tanks (fig. 9B-C) and a good water flow in the tanks, as proposed in earlier studies (Le Gall et al. 2004, Browne and Edwards 2008, unpublished, in Wood 2018). By this, the present method build-in the positive effects of washing spores after release (Pang & Lüning 2006) and the use of multiple water renewals (Le Gall et al. 2004) on spore survival without imposing extra handling. Besides, flow-through condition stimulates biomass growth and frond health (Morgan and Simpson 1980a). The setup allowed the effluent tetraspores to aggregate in detaining tanks (fig. 9D), which I used to test the GMA-method at a larger scale and the temporal extend of the method (fig. 9F-J). The results highlight that both germinated females and males successfully reattach to the growth substrates up to 39 days after spore release, but not by testing after 240 days (over-summering), which suggests a temporal limitation of the GMA-method affected by germination time. The propagule-seeded ropes were cultivated at sea, as described in paper 5 (fig. 9J-L).

Inspired by a previous fertilization study (Mine and Tatewaki 1994), I aimed to quantify the effect of including a fertilization step during the nursery phase, by adding male spermatia to recently spore-seeded substrates. The obvious expectation of a successful fertilization is a higher seedling density on substrates because of a zygote formation and the development of new sporophytes. Here, I found that seedling density was up to 2 times higher than without adding spermatia after 20-32 days of nursery, and that germination success

increased with up to 80 % of the settled spores. This emphasizes a relatively easy way to increase the spore use efficiency in *P. palmata* hatchery by activating the settled females.

The evaluation of sori use efficiency using the proposed spore-seeding method compared to a previous seeding method (Werner and Dring 2011) reveals a substantial improvement with a factor 5-17 (per km rope) or 65-201 times (per hectare). This is based on the amount of sori used for seeding (104-312 g vs. 1785 g fresh weight sori to seed one km linear rope). The latter number reflects the need to collect 22 kg of fronds to obtain 1.784 kg sori. This optimization mainly attributes to the use of consecutive spore release events in the present method, i.e., the same sori release spores for nine days. Furthermore, the use of highly agitated flow-through conditions enhance spore dispersal, survival and growth of seedlings.

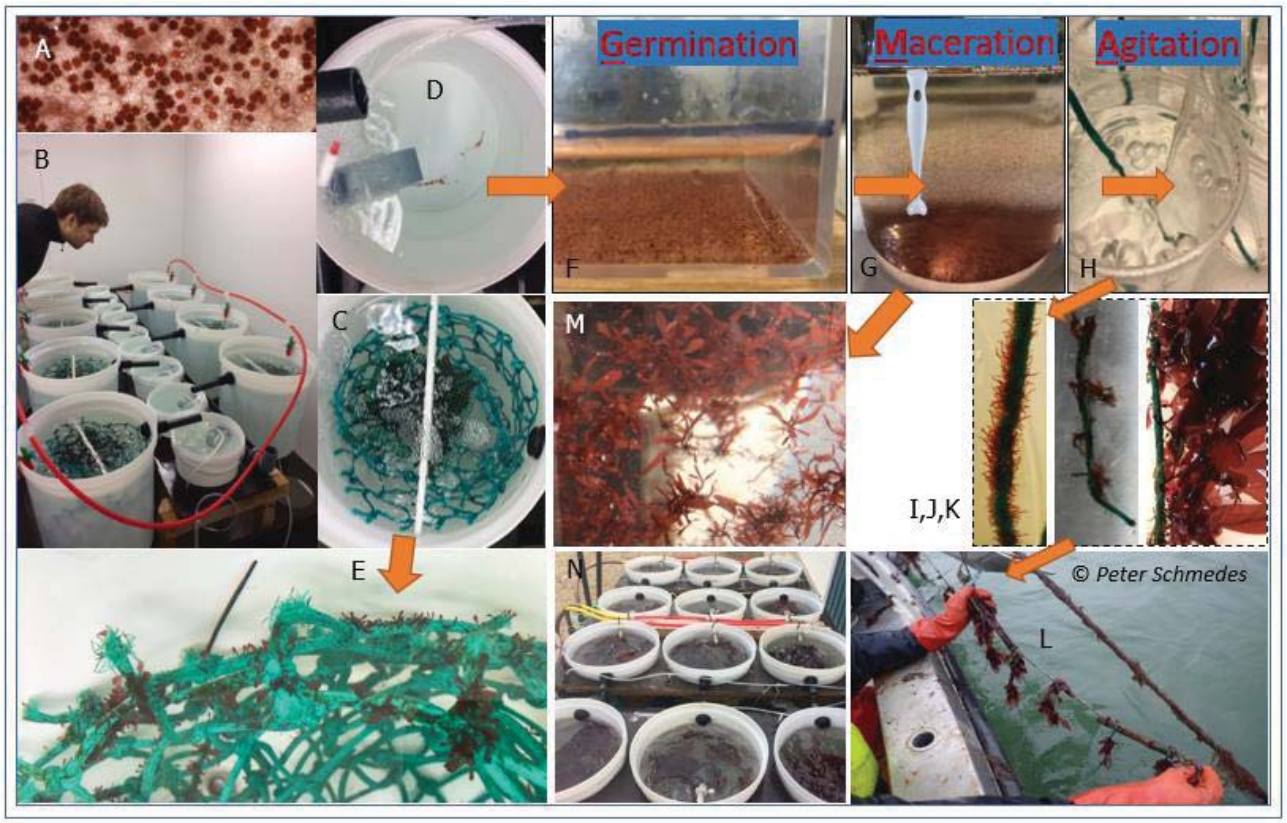


Figure 9. Photo documentation of the seeding method proposed in the current PhD study. A: Sori used for seeding. B: Parallel setup of tanks sourced by flow-through water (red tube) recirculating after UV treatment and agitated with air from the bottom. C: Net-spirals in tanks with a sori package fixed above. The outlet (black fitting) was diagonal to the inlet (gray). D: the effluent water led into spore detaining tanks (SDTs). E: seedlings on nets after 3 months in nursery. F: Transferring tetraspores from SDTs to germination tanks. G: Maceration of germinated propagules for use as seeding inoculum. H: Adding mix of propagules to bubble cultures for reattachment on rope. I: Substrate propagule density after 10 days agitation and J: after 8 months of nursery. K-L: Growth of propagule-seeded ropes cultivated from October to February at sea (~12 cm). M: Macerated propagules as inoculum in propagating biomass as free-floating cultures. N: Tank setup for testing cultivation of vegetative tissue fragments sourcing nutrient rich seawater from a land-based salmon farm.

In future developments of large-scale seeding methods of *P. palmata*, studies should include determination of optimal water flow-through rate, the effect of water mixing within the seeding tank for spore release, dispersal

and settlement, as well as optimal conditions for keeping seedstock in nursery for long periods in the pursuit of all year seeding ability. Further, due to poor temporal resolution in paper 3, the ability of propagule discoid reattachment between day 39 and day 240 after spore release needs further investigation to enable an all year application of this seedstock.

3.2.1 Early growth of seedlings

After spore settlement, securing spore survival and germination is paramount, where low irradiance seems crucial in the first 12 weeks during development of spore-derived seedlings (Edwards and Dring 2011). In a study by Edwards and Dring (2011) they found that a relative low irradiance (above 25 µmol photons m^{-2} s^{-1} PAR) impairs growth and above 50 µmol photons m^{-2} s^{-1} PAR spore-derived seedlings may suffer necrosis, even when nutrient availability is optimal. Thus, at lower light intensities, seedlings may survive but this implies a longer nursery duration (2-3 months) before seedlings reach the minimum length of 6-8 mm recommended for deployment (Werner and Dring 2013). In contrast, paper 2 found the growth rate of seedlings after 11 months nursery to saturate at 20 µmol photons m^{-2} s^{-1} PAR and growth showed no signs of impairment at irradiances up to 78 µmol photons m^{-2} s^{-1} PAR (fig. 10). Hence, long nursery duration were shown to provide robust seedlings that can cope with higher irradiance. By autumn deployment, which is the optimal season for achieving highest biomass yield (Browne 2001, Sanderson 2006), the irradiance levels was previously found to exceed 25-50 PAR in the upper water column (Schmedes and Boderskov 2013). Thus, considering the seedling age (robustness) at the deployment time and the water depth of substrates, to prevent harmful irradiances, seems important. Furthermore, by laboratory assessment, I found high agitation to stimulate the growth rate of seedling, obtained by GMA-seeding. This result supports that water motion mediates better resource availability and stimulates growth (Harrison and Hurd 2001, Ryder et al. 2004), which emphasize the importance of farm site selection (Werner and Dring 2011). Conversely to the common recording of P. palmata along Atlantic coasts (Morrisey et al. 2001), I found the highest seedling growth at low salinity (15 psu), which seems to suggest a broad salinity tolerance of intertidal *P. palmata* or some degree of adaptation of *P. palmata* in inner Danish waters (Paper 1 and 3).

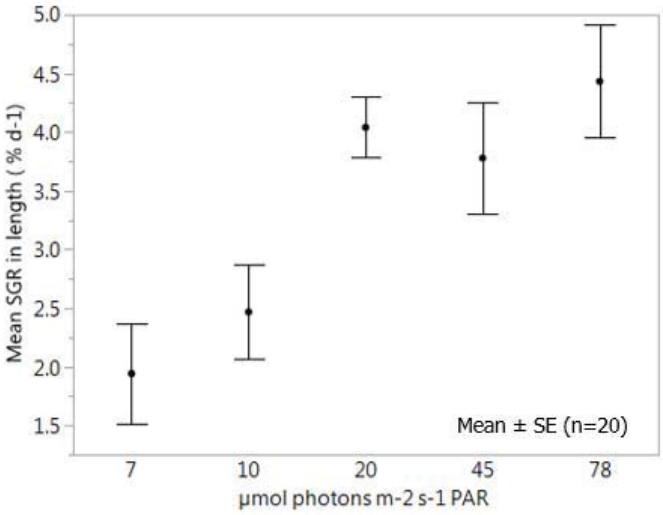


Figure 10. Spore-derived seedlings on kuralon twine cultivated in high nutrient concentration (50 % F/2+) at five different irradiances after 11 months of nursery. The data points show the specific growth rate (SGR % d^{-1}) (n=5) based on measured the length of five longest fronds during 5 weeks (mean±standard error (SE).

An additional objective of my investigations of the early growth was to examine whether marginal proliferations are a suitable seedstock for land-based grow out (paper 4). Palmarians show numerous proliferating shoots along a wide marginal meristem (Fritsch 1961) known to grow into new fronds (Faes and Viejo 2003) and suggested suitable as propagation units for tank-based grow out (Titlyanov et al. 2006). Land-based cultivation using a vegetative seedstock is a promising method for propagating biomass and for nutrient reduction in land-based fish production (Kim et al. 2013, Corey et al. 2013, 2014), yet a scalable source of seedstock for such grow out is still incomplete. Isolating meristematic cell aggregates or tiny fragments is also promising for increasing biomass yields (Titlyanov et al. 2006) but requires good hygiene (Reddy et al. 2003) and a long-term cultivation.

By six weeks of starved growth in nutrient deprived conditions (paper 4), using weekly nutrient pulse additions of low concentration, marginal proliferations grew into small seedlings and suffered depigmentation at an irradiance of 150-280 µmol photons m^{-2} s^{-1} PAR at 10 °C (16:8 h light). After this pre-cultivation, the growth, biomass and nutrient removal rates significantly increased after adding a single high-concentrated nutrient addition by using this starved seedstock. Growth rate (up to 6.9 % d^{-1}) and nutrient removal rate peaked at 200-280 µmol photons m^{-2} s^{-1} PAR and saturated (I_{SAT}) at 114-178 PAR, depending on nutrient status, which is in the high end of previous reported I_{SAT} (Sagert & Schubert 1995, 2000, Manríquez-Hernández et al. 2016). Tissue nutrient status affects the capability of algae to tolerate light-induced stress (Zhao et al. 2017), but the use of constant high nutrient availability has shown to promote growth of epiphytes (Neish et al. 1977, Sanderson 2006). Based on this, the present thesis suggests testing a continuously alternation of such a two-phased cultivation strategy, or consecutive restocking by using starved seedstock, as a potential way to optimize land-based cultivation of dulse while avoiding epi-fouling.

3.3 Grow out of *P. palmata*

3.3.1 Sea-based grow out from seeded substrates

Based on the hatchery seeding experiments, cultivation trials at different farms in the inner Danish waters were performed (paper 5). The aim was to investigate the effect of using short versus long nursery duration and investigate the use of different deployment configurations on the growth of *P. palmata*.

Water motion and nutrient availability promote nutrient uptake and growth rate in seaweeds (Hurd 2000, Harrison and Hurd 2001, Sanderson 2006, Barr et al. 2008). Nutrient depletion of the water during late spring and summer results in depigmentation of dulse (Neish et al. 1977, Martínez et al. 2006, Martínez and Rico 2008), which can be reinforced by increasing temperatures and light intensity, resulting in bleaching or necrosis of fronds (Werner and Dring 2011). Bleached fronds compromise the commercial value of the biomass. Thus, site selection in relation to environmental parameters, such as nutrients, exposures, light, and salinity, and additionally, incidence of epi-fouling will affect the success of seaweed cultivation in Danish waters (Bruhn et al. 2016).

From paper 2 and 4, we know that the optimal mean irradiance for survival and growth of seedlings after a long nursery period and older thalli is in the range of 78-280 µmol photons m^{-2} s^{-1} PAR at 10 °C (12-16 h light d^{-1}). How is this span in light tolerance consistent with experienced irradiance levels in inner Danish waters? By using a 20 year mean of down-welling light and secchi depth measurements in different areas of the inner Danish waters, the level of irradiance at different meters below sea level (bsl) has previous been estimated (Schmedes and Boderskov 2013). This study found irradiance levels (µmol photons m^{-2} s^{-1} PAR) at two locations; one location close to the seaweed farm in the eutrophic Limfjorden (Færker Bay) and one location close to the mesotrophic Kattegat (Hjarnø farm) in mid-September; 200 and 280 PAR at 1 m bsl and 70-200 µmol photons m^{-2} s^{-1} PAR at 3 m bsl. Moreover, I recorded irradiance in Færker Bay (January 2017) at 1.5 m bsl revealing potentially lethal light intensities for young spore-derived seedlings (>50 µmol photons m^{-2} s^{-1} PAR), according to findings by Edwards and Dring (2011). This suggests that the period for deployment and the part of the water column providing suitable light intensities for deploying seedlings <12 weeks old is relative short and narrow, which emphasize the benefit of nursing seedlings for a longer time.

Paper 5 showed that seedlings nursed for a long period resulted in the most successful cultivation trials. Deployments of seeded substrates, after a short nursery duration, in the central Limfjorden was poor, with 27±10 g FW m^{-1} seaweed line due to severe fouling during the first months of grow out. With similar deployment, the Western part of the Limfjorden sustained the longest frond growth and provided a biomass yield of 287±33 g FW m^{-1}. Paper 5 showed that deployment in autumn benefit frond growth to a depth of 1.5 m bsl in the Limfjorden. Conversely, growth in fronds length was better on the vertical dropper lines down to 2.5 m bsl on the east coast of Jutland (Hjarnø Farm), which suggests better light conditions at this location. However, in the Hjarnø farm, tissue bleached severely in the upper 0.5 m bsl, and to some extent at 2.5 m bsl, where fronds were reddish-pale green and resulted in a biomass yield of 199±49 g FW m^{-1}. This suggests that a cultivation depth of 1.5 m bsl is optimal during the early growth season, while 2.5 m bsl or deeper seems

optimal for extending grow out into the early summer season, which is in accordance with earlier findings from Ireland and Scotland (Browne 2001, Sanderson 2006). In contrast to the deployments of the very small seedlings on kuralon twine obtained from long nursery durations, the deployments of larger seedlings of about 3 cm in length, obtained from a nursery period of 9-10 months, provided the best grow out success in the present thesis. However, a Norwegian pilot study, showed that deploying spore-seeded nets after 3 months of nursery, where seedlings were < 6 mm in length, resulted in decent frond growth up to 18±3 cm in length (mean±std, n=3) after 4 months grow out (Bøe 2019). This indicates that a combination of sufficient nursery time and optimal site selection results in a successful grow out. Grow out on net substrates seems to result in much higher biomass yield than single ropes, based on the observed growth over 4 month duration. The advantage of using nets reflects a higher amount of linear rope compared to single dropper lines or continuous twine coiled upon a carrier rope. The two deployment configurations that I tested extrapolates to 1-6.3 km linear rope per 100-meter longline. The preliminary results suggest that farming in Kattegat provide a longer growth season, as fouling occurs 1-2 months later here than in the Limfjorden. A linear regression mixed-effects model ought to reveal the importance of cultivation depth, farm site, seedling density and frond length on the dulse biomass yield cultivated in inner Danish waters.

Regarding the application of sea-based cultivated *P. palmata*, previous research has documented a seasonal variation in the content of nutritious and potential health-promoting compounds in wild *P. palmata* (Galland-Irmouli et al. 1999, Martinez and Rico 2002, Harnedy et al. 2014, Schiener et al. 2017). The reported levels of heavy metals are generally low in *P. palmata* and can be reduced by post-harvest treatment (Schiener et al. 2017, Bruhn et al. 2019). The potentially toxic compounds in Palmariales, i.e., Kainic acids, show considerable variation in tissue concentrations and may exceed acceptable limits depending on the origin of the biomass (Ramsey et al. 1994, Mouritsen et al. 2013). This thesis reports a content of Kainic acid in cultivated dulse between 40-190 µg g^{-1} DW, which is 3-12 times lower than previously reported in wild collected dulse in Denmark (Jørgensen and Olesen 2018). Augmented control for enhancing or reducing the content of a specific seaweed compound, like pigments or iodine, is promising in land-based farming, which will increase the value and dulse biomass (Parjikolaei et al. 2013, Hafting et al. 2012, 2015, Lüning and Mortensen 2015).

3.3.2 Land-based grow out using vegetative propagation of biomass

Maintaining a high growth rate, harvesting of a clean biomass, and targeting a specific biochemical composition is a challenge in sea-based grow out due to poor control of ambient environmental factors (Hafting et al. 2015). Land-based grow out of *P. palmata* may thus be a promising alternative (Watson et al. 2012, Kim et al. 2013). In order to test land-based grow out we used outdoor tanks (fig. 11) supplied with thermal stable, filtered and nutrient enriched seawater before the denitrification step from a Recirculated Aquaculture System (RAS) unit producing *Salmo salar*.

The aim was to examine whether the biomass yield and tissue quality of *P. palmata* will remain high during the summer period, by cultivating vegetative tissue pieces in constant water flow and using high air agitation. We aimed to perform multiple harvests to return initial biomass density (cropping and reducing the number of plants) (Levinsen in prep.). The concentrations of ammonium (1 mg L^{-1}) and nitrate (200 mg L^{-1}) in the water were stable during the period. These conditions assumed to alleviate light-induced stress (Zhao et al. 2017), however, we also applied a light treatment (shaded; 90 % reduction of ambient light vs. ambient irradiance) from early May to assess the phenotypic response of photosynthesis parameters and algal stress in the two groups. We assessed this by measuring the chlorophyll fluorescence quenching based on pulse amplitude modulated fluorometer (PAM) (see Appendix 1).

Figure 11. Experimental setup before shading (green nylon nets) half of the tanks. Each tank had a volume of 30 liters and an initial biomass of 90-100 g FW.

Until May 28 2019, where PAM was measured, tank water temperature was stable (14-16 °C), Overall, the mean summer temperature was stable at 15-16 °C, although measurements at six occasional summer days revealed instable water resulting in temperatures of 17-23 °C in both shaded and ambient tanks.. During May, most days were sunny with irradiance surpassing 1000 μmol photons m^{-2} s^{-1} in the ambient group. The specific growth rate (SGR, % d^{-1}) was highest during April and May in the ambient light group, but crashed during June. The SGR in the shaded group was half of the ambient group during the peak growth phase, but remained positive during summer, though some fronds died. By performing only two harvests by cropping, we were unable to answers whether fronds would sustain a high growth rate during summer by this harvest method at ambient irradiance, as fronds deteriorated. Necrosis was likely caused by a combination of too high temperatures and light-induced stress due to instable flow of water. In late May, where all fronds appeared healthy and at rapid growth, the PAM investigations revealed a higher level of non-regulated heat dissipation, i.e., photosynthetic stress Y(NO), in the ambient group, while the shaded fronds showed higher regulated heat dissipation, i.e., stress parameter Y(NPQ), to dissipate the excess energy received (Appendix 1). I found that the distal part of fronds show highest light tolerance (Appendix 1), and has previously been found to be the part of thallus with the highest growth rate due to the meristematic tissue (Morgan and Simpson 1981b). Eventually, this suggest distal parts as optimal propagation units by using multiple cropping.

By using optimal tank dimensions to ensure only short-term exposures of stressful light intensities (1600 μmol photons m^{-2} s^{-1} PAR) and by tumbling of thalli (Pang and Lüning 2004a), *P. palmata* might sustain growth in ambient summer conditions. In Paper 4 I showed a light tolerance of marginal proliferations up to 280 μmol photons m^{-2} s^{-1} PAR (16:8 h) during six weeks in nutrient deprived conditions at 10 °C.

Besides, the control of light exposures to secure biomass growth and quality, pulse addition of nutrients instead of constant availability is an effective way to minimize growth of epiphytes (Neish et al. 1977, Morgan and Simpson 1981b). At the same time, pulse additions enhance the nutrient mitigation service (reduction in effluent concentration) of using a seaweed as a biological nutrient filter (Demetropoulos and Langdon 2004, Kim et al. 2013, Corey et al. 2013, 2014). By using constant high nutrient availability, we observed severe epi-fouling in the ambient light group, while moderate levels in the shaded group (Levinsen in prep.). In accordance, by means of chemical cleaning (Kerrison et al. 2016, Bøe 2019), the cropped biomass might sustain growth and remain clean of epiphytes during summer grow out using present co-cultivation setup.

Performing two harvests during April and May by reducing the number of fronds in tanks led to a slower SGR (% d^{-1}) during summer months, where the basal part of fronds were overgrown by filamentous brown algae (Levinsen in prep.). The PAM recordings indicate that the distal parts of the fronds showed higher light stress tolerance. In addition, the distal part of the fronds show highest meristematic activity (Morgan and Simpson 1981b, Pang and Lüning 2004a, Sanderson 2015), which collectively provide the argument for propagating distal frond parts and harvesting the rest, when performing multiple harvest using cropping. Based on these results, I suggest that land-based grow out can be optimized by adjusting the light exposure, i.e., shifting between high and low irradiance on a fine temporal scale using an automated shading system or securing an irradiance at I_{SAT} ≈178 PAR, besides maintaining temperature within optimal range 6-14 °C (Morgan and Simpson 1981a). Further, the optimal harvest method to maximize the harvest of clean biomass yield require more research, as reducing the incoming irradiance by 90% was found to be insufficient. This would include the multiple cropping method tested against the use of restocking with new crops (all-in-all-out).

4. General discussion

This thesis has demonstrated successful induction of fertility for spore production in *P. palmata* during autumn and a method with potential to induce fertility during summer (Paper 1). In Paper 1, I have tested the effects of multiple environmental parameters and metabolites on fertility induction in *P. palmata* during seasons of low natural spore occurrence. I have developed and tested a new hatchery method with high sori use efficiency and elevated germination success, compared to earlier trials, due to optimal seeding conditions and by including a fertilization step. The developed seeding setup allowed retention of excess spores for further use. I have tested the potential of "re-using" the retained spores in a seeding protocol relying on germination, maceration and agitation (GMA-seeding) of the spores (Paper 2 and 3). To provide more knowledge surrounding cultivation methods as well as method optimization, Papers 4 and 5 tested a new method for land-based grow out and examined sea-based grow out of *P. palmata*, respectively. The following sections discuss the main findings of this thesis and the potential implications of these findings from a farming perspective, as well as out of general academic interest.

4.1 Is year-round spore induction reliable for future hatchery production of *P. palmata*?

Absence or low occurrence of fertility in *P. palmata* during late summer is a commonly accepted phenomenon (Kain 1982, Le Gall et al. 2004, Edwards 2007, Werner and Dring 2011, Wood 2018), which has further been confirmed in this thesis. Absence of fertility appear from end March in the assessed Danish population, while a Norwegian population first showed absence in fertility from May (Paper 1). Despite using several means of fertility induction, I found no strong evidence for year-round production and release of tetraspores in *P. palmata*, which differs from previous studies (Pang and Lüning 2006, Titlyanov et al. 2006). These previous investigations infer that disconnecting thallus from meristem regions promotes sporangia formation in dulse. This disconnection prevents these sporulation inhibitor compounds, in the meristems, from occurring in thallus parts where sexual structures then start to develop. While this mechanism triggers sporogenesis in some kelp and Ulva species (Buchholz and Lüning 1999, Wichard and Oertel 2010), results found here showed that the same process does not occur under all conditions in *P. palmata*. In accordance with earlier work, I found that by collection of *P. palmata* in the periphery of the natural peak season and subsequent exposure to winter-like conditions induced fertility (Pang and Lüning 2006, Edwards 2007), which supports the finding that manipulation of environmental conditions can accelerate the maturation of sporangial tissue also in other rhodophytes (Guzmán-Urióstegui and Robledo 1999). However, no previous studies have reported successful induction of tetraspores in *P. palmata* during summer. In Paper 1, I showed that a combination of short day length, presence of meristems and cold temperature triggered sporangial initiation during summer and autumn. Conversely, long day length exposure in winter, which is the peak season of tetrasporogenesis, did not prevent sporangial maturation, during which high nutrient availability was crucial, as also seen under natural conditions. This supports the persistent "short-day fertility response" in several seaweeds tested in "night-break" studies, i.e., interrupting long dark periods with short-term irradiance (Terry and Moss 1980, Dring and Lüning 1983,

Dring and West 1983, Breeman and Hoopen 1987), and indicates that sexual reproduction is not solely under environmental control (Liu et al. 2017). Thus, some seaweeds continue to develop sexual structures even at summer daylength, which suggests that photoperiod is only important to initiate the fertility induction, while the amount of light and nutrient availability are important for the ongoing development (Dring 1974, 1984, Guiry and Dawes 1992, Nimura et al. 2002). Induction of fertility outside of the natural fertility season will enable hatchery production prior to cultivation deployments in autumn but seems to require a certain level of accumulated energetic reserves in fronds as a prerequisite. In wild populations, energy reserves are at maximum after summer (Morgan et al. 1980), while N and P accumulate from winter to spring (Martínez and Rico 2002). Pre-cultivation during spring is thus required.

The induction of sexual reproduction in *P. palmata* is under strong endogenous control, which is reflected in the adapted synchronization with season. Moreover, ripening of tetrasporangia and spermatangia is required before spore release is possible, which appears to occur 1-3 months after sexual structures are visible (Pang and Lüning 2006, personal observation). By exogenous application, metabolites, such as polyamines and gaseous ethylene, can accelerate sporangia maturation and ripening in other rhodophytes (Guzmán-Urióstegui et al. 2002, García-Jiménez and Robaina 2012). Furthermore, ethylene, and ethylene precursor (ACC) and allantoin exposures induced the formation of gametes and archeospores in *Pyropia yezoensis* (Mizuta et al. 2003, Uji et al. 2016). Polyamines and ethylene are naturally occurring metabolites in plants and some algae known to influence on cell membrane stability, cell division, ripening and stress tolerance (Cohen et al. 1984, Galston and Kaur-Sawhney 1990, Lee 1998, Marián et al. 2000). Paper 1 documented a higher content of endogenous polyamine putrescine and tyramine in fertile *P. palmata* fronds compared to vegetative fronds. Conversely, externally administered metabolites previously shown to be involved in rhodophyte reproduction (spermidine, allantoin and ethylene) were unable to trigger fertility in the present study. Although collection of ripe, fertile material during the peak fertility season may be able to sustain *P. palmata* hatchery production, year-round fertility induction is required to increase commercialization. However, I show that year-round fertility induction is not yet a reliable strategy for securing a propagation seedstock and remains a bottleneck for establishing commercial *P. palmata* hatcheries relying on spore production to seed substrates (Sanderson 2006, Edwards 2007, Werner and Dring 2011, Wood 2018), thus further investigation is required.

Augmented control of fertility induction and spore release for successful commercial scale aquaculture of *P. palmata* require more research. Future work includes developing methods to manipulate the seasonality of the metabolic adjustment accumulating reserve carbohydrate floridoside (floridian starch) in *P. palmata* (Morgan et al. 1980). Selection of sun-acclimated fronds might serve as a basis for such work, showing higher rates of carbon fixation (Martínez and Rico 2008). The present thesis showed the potential of optimizing growth of fronds in early spring to build up energy reserves, following a shift to winter-like conditions during early summer to induce fertility. Further research should also be conducted to understand how changes in proximate factors (e.g. light intensity, spectral composition, nutrient availability and temperature) during pre-cultivation can lead to manipulation of the timing of energy reserve accumulation and reproduction. Methods to manipulate the non-photosynthetic receptor signaling should also be conducted as this also shows promise in controlling the timing of fertility induction, and thus could be another method of potential year-round fertility control. Model

species for studying rhodophyte reproduction, such as *Pyropia sp.* (Nakamura et al. 2013) and *Chondrus crispus* (Collén et al. 2013), ought to provide new insights in the genetic and metabolomics contribution on signaling pathways involved in triggering fertility. Further, by investigating dose-response curves in the candidate species *Pterocladiella capillacea*, that is, applying various concentrations of metabolite compounds to trigger fertility or to mature sporangia, could provide knowledge that can be transferred to *P. palmata*. As commercial scale production requires large numbers of tetrasporophytes to produce spores, future research in fertility induction should include high levels of replication to ensure results are valid. These studies should also be followed up using qualitative evaluation of spore release and germination, which would provide further knowledge for improved aquaculture.

4.2 How to secure seedstock in future *P. palmata* cultivation?

Paper 2 and 3 demonstrated an alternative method to seed substrates, which did not directly rely on available ripe, fertile sori during the peak season. Instead, this alternative method relies on collection, culturing and maintaining released spores for germination in a mix of propagules during the whole season of natural fertility, thus is a way to prolong the spore availability in the hatchery. After 29-39 days of germination, the aggregated spores develop into a mix of male and female gametophytes. Following a dislodgement treatment, these macerated propagules were able to establish firm discoid reattachment to substrates by adding a solution to agitated seeding tanks ("GMA-seeding" method). This method prolonged hatchery production up to 39 days after spore release, while the ability of reattachment was unsuccessful when tested at day 240. Controlling the factors, such as light intensity and temperature involved in dormancy processes (Faes and Viejo 2003), is a potential way to store macroalgae seedlings and prolong the phase during which seedlings are able to reattach to substrates. Additionally, by culturing propagules in tumble cultures they developed into sporelings, thereby being a suitable seedstock for propagation units in land-based biomass production. Until now, in order to store seedstock for more than 39 days, it is better to inoculate substrates and maintain them in a nursery, instead of storing the seedstock in bottles at high density.

Securing seedstock for sea-based grow out is limited by access to tetraspores, while an increase in the seeding efficiency is crucial, which entails optimal dispersal and even settlement of spores, as well as "re-use" of retained spores with the GMA-seeding method to prolong the hatchery season. The GMA-method relies on germinated propagule reattachment. By this, the method avoids the reduction in the initial seed density, as seen immediately after spore-seeding of substrates. Furthermore, it shows the potential to obtain an optimal seedling density in a controlled way to avoid self-thinning of the density and self-shading of fronds (Flores-Moya et al. 1996, Scrosati 2005), which impose a low hatchery and growth efficiency. Although this thesis provides a starting point for improving *P. palmata* production, further research is required to secure year-round seedstock, extend storage of seedstock beyond 39 days post spore release and maintain germinating seedlings close to dormancy. Manipulating light and temperature exposure to secure minimum conditions required to compensate respiration (Bidwell and McLachan 1985) might be a potential method for keeping isolated propagules or seeded material as seedstock ready for year-round deployment. Further research in

optimizing the biotic and abiotic control of propagule reattachment is requested (Santelices and Varela 1994). This includes optimizing the maceration treatment, substrate surface. Enhancing the adhesion strength of propagule disc organs by stimulation of disc growth or excretion of adhesive compounds at dish surface would potentially increase the temporal extent of using the GMA-method. Lastly, it would be intriguing to test whether the "freeze technology", i.e., storing nori nets with *Pyropia* seedlings at ~ -20° C (Kurakake and Hori 1966) is a suitable method to also store *P. palmata* seedstock.

4.3 What are the optimal methods for *P. palmata* spore release and seeding?

Desiccation and rehydration of sori can trigger the release of zoospores in various kelp species and provide a high spore concentration in just 1-3 hours (Forbord et al. 2018). Though a similar osmotic mechanism has been found in red algae spore release (Scott and Dixon 1973), the use of desiccation to boost *P. palmata* tetraspore release is not effective, unless sori is at its maximum ripeness (Werner and Dring 2011, Wood 2018). To account for differences in ripeness of sori, the results in Paper 2 and 3 suggest that a seeding period of 3-20 days can optimize the release of the non-motile tetraspores. A long spore release period may be beneficial especially when seeding of growth substrates (rope, net, textile sheet etc.) is based on fronds collected during the early stages of natural fertility, where sori covers only 8-10% of the frond surface (Werner & Dring, 2011), and because spore release varies with sori ripeness (Wood 2018, Paper 1).

A long spore-seeding period is inefficient for large-scale application, as it prevents the use of the same seeding tanks and spore donors to seed new substrates for a long period. In contrast, a short seeding period allows for high turnover of seeding tanks and sori, which can facilitate the production of more seeded substrates within a limited timeframe. Thus, the use of high agitation during sporulation seems a useful alternative method to decrease spore release time (Le Gall et al. 2004, Paper 2). A combination of increased agitation and water flow-through of the seeding tanks and low sori requirement (5-15 g) was shown to promote survival and germination, as well as provide an even spore distribution on the settlement substrates (Paper 2 and 3). Potentially, the seeding of substrates could be shortened to a few hours, but it will depend on multiple factors including sori ripeness (Werner and Dring 2011), the actual need for spores as determined by the ratio of sori-to-substrate and the desired settlement density. Seeding efficiency depends on spore distribution and substrate settlement. An even spore settlement is a prerequisite to obtain an even biomass distribution on growth substrates and can greatly affect the final harvest yield per meter of longline (Sanderson 2006, Sanderson et al. 2012). A density above 10-20 seedlings cm^{-1} substrate was stressed to be the minimum quality for deployment (Werner and Dring 2011, 2013), which requires an initial settling of about 100 spores cm^{-1} to account for mortality of up to 90% of the initial spore settlement. Paper 3 demonstrates a new method to improve the spore-seeding efficiency in *P. palmata* hatchery, by reducing the amount of sori per seeding tank and by applying three consecutive seeding periods using the same sori. The ratio of settled spores that germinate (=survival) was up to 80% when using flow-through conditions during seeding, and by securing fertilization of females by adding male gametes during the nursery phase. The addition of male gametes during nursery days 20-32 elevated the final seedling density by up to 2 times compared to the controls, which did

not have male gametes added. By using the seeding methods presented in this thesis, a higher germination success can be obtained compared to previous methods, which required a high amount of sori and used calm water conditions during the nursery period (Werner and Dring 2011). The proposed new spore-seeding method required just 0.1-0.3 kg of fertile sporophytes per km rope and an additional 0.3 kg of fertile males to secure fertilization during the nursery phase. In contrast, previous seeding protocols required 22 kg of fertile sporophytes to seed a net substrate equivalent to one km rope (Werner and Dring 2011). This suggests that the new method results in a 5-17 times higher efficiency in sori usage per km substrate and a 65-201 times higher efficiency of sori use per hectare.

Future studies should investigate seeding efficiency using large-scale tanks, including the ratio of substrate-to-spore donor density to improve sori use efficiency. Moreover, the importance of shape, volume and flowrate of seeding tanks, which affect water mixing and thus spore dispersal and settlement, have not yet been investigated, although they are of particular importance when up-scaling production. Smaller scale conical tanks (25 L) promoted a hemispherical water mixing when applied with central agitation using air bubbles and provided sufficient spore dispersal of spores and settlement to growth substrates. However, air agitation of large volume in upscaling is yet to be tested with regards to sufficient spore dispersal. This links to the request of identifying optimal tank shape, as constant air agitation of large volumes requires big and costly pumps (Caines et al. 2014). Inspired by nori hatcheries (Tseng 1981), rolling nets on big tubes into a low volume of water with suspended tetraspores could also be a suitable solution for seeding dulse. Having secured the seedstock supply, the next important task in dulse production is to identify the best-suited seeding and grow out strategy with regards to the choice of production volume, harvest technology and operational costs. Net substrates appear to be advantageous compared to single ropes and future research should investigate optimal nets dimensions, i.e. mesh size, rope diameter, rope type, surface corrugation (Kerrison et al. 2017). This will have implications for the maintenance and handling of seeded substrates in the nursery when kept for long periods prior to deployment. At a small scale, keeping seeded substrates under nursery conditions for several months benefit seedling quality, such as seedling size. Large-scaled, temperature-controlled hatcheries, however, would increase maintenance costs and thus may prove to be cost-ineffective. An additional consideration is that seedlings might be vulnerable to detachment during handling of large substrates, which requires more research.

4.4 How to improve grow out of *P. palmata*?

While improving the biological quality of seedstock is important, optimizing grow out of *P. palmata* is another vital step that could enhance production. For the grow out phase, farm site characteristics, such as water motion, water renewal rate, nutrients, light penetration, salinity and fouling, can also affect seaweed biomass yield (Holt 1984, Hurd 2000, Harrison and Hurd 2001, Kraan and Guiry 2001, Sanderson 2006, Marinho et al. 2015, Bruhn et al. 2016, Boderskov et al. in prep). Further, the effect of substrate type (Kerrison et al. 2017, 2019) and optimal substrate configuration in sea-based outgrow (Sanderson 2006, Bak 2019, Boderskov et al. in prep) is also of key importance to lower the production cost of seaweed grow out.

Paper 5 showed that the success in field trials and the growth of *P. palmata* using seeded ropes in different areas of inner Danish waters varied considerably. Cultivation depth interacted with the time of year and affected frond growth, which is in accordance with previous growth trials showing light penetration greater than that of inner Danish waters (Browne 2001, Sanderson 2006), while high wave force resulted in frond breakage (Martínez et al. 2006). Biomass yields of *P. palmata* in Denmark of ≈300 g FW m^{-1} seaweed line compared to 937-1875 g FW m^{-1} seaweed line obtained in Ireland and Scotland (Browne 2001, Sanderson 2006), suggest that optimal site selection is crucial to maximize biomass yield. Besides the effect of farm location and optimizing the individual seedling size for deployment, grow out of *P. palmata* is likely to be improved by more even settlement, as the presented yield obtained from kuralon twines showed patchy coverage of fronds.

Deployment of grow out units containing light tolerant seedlings near the surface in autumn seems to be optimal for utilization of the scarce incoming light during winter (Paper 5). Later in the growth season, light intensity may still be limited in the upper water column of eutrophic and shallow water bodies, where phytoplankton and dissolved material result in low light penetration. In contrast, in less turbid waters, light intensity becomes excessive and can result in bleaching or deterioration of the biomass during late spring. Hence, the actual cultivation depth after deployment and later in the season is important to investigate in future trials for optimizing biomass yield.

Keeping seeded substrates in the nursery for an extended period (8-11 months) improves *P. palmata* growth in Danish waters, in accordance with the “over-summering nursery” used by Werner and Dring (2011). A long nursery phase provides several benefits compared to using young seedlings: it provides seedlings of desired size and age, boosts growth and resistance to epi-fouling (Paper 5) and improves light tolerance of seedlings at deployment (fig. 10). Deploying large-sized seedlings is a strategy also used in Asian kelp and *Pyropia* aquaculture to mitigate epi-fouling and improve biomass yield (Tseng 1981, Su and Pang 2017). However, Dion and Delépine (1981) and Bøe (2019) demonstrated successful grow out trials by deploying small seedlings attached to substrates after just one and three months of nursery growth, respectively. These studies emphasize the importance of farm site choice and local growth conditions.

Substrates other than rope can also sustain high biomass yields. Deployments of net substrates resulted in the best growth (Paper 5) and is expected to result in the highest biomass yield, however, this method also required a long period of nursery growth to succeed in the Limfjorden. The advantage of using nets was demonstrated by Sanderson (2006) and Werner and Dring (2011) and is attributed to the high amount of substrate per meter of longline. These studies deployed nets of 3-4 m in a vertical configuration, which resulted in the biomass being significantly reduced at the deepest point due to light attenuation and self-shading.

Biomass evenness may also be a factor affecting final biomass yield of *P. palmata*. A potential method of improving biomass evenness on the deployed substrates is to reduce plant density or to narrow the deployment depth of substrates. The simplest deployment configuration is to deploy horizontal lines (two-dimensions). However, in the pursuit of maximizing the yield per farm area of *P. palmata*, previous studies have investigated the deployments of 7 m long vertical single dropper lines with 15 cm spacing (Browne 2001) and nets of 3 meters height (Werner and Dring 2011). These methods resulted in lower biomass yield with increasing depth

due to light limitation. In paper 5, I examined the effect of water depth on growth and potential biomass yield per unit longline by deploying nets at 0.25 and 1.4 m depth in October, the start of the growing season. Preliminary results suggest that growth is limited at the bottom of the 1.4 m configuration until end of February. In this coming summer (2020), frond length on both nets is expected to be highest in the top part, as the growth advantage throughout deployment will persist. The deployment using the high net, which provide up to 6 times more growth substrate per meter longline, is expected to provide the highest biomass yield, assuming that frond growth will catch up in the lower part of this net in the later part of the growth season.

The approaches investigated in this thesis have addressed the fundamental trade-off of intensive seaweed farming, which is to balance biomass per unit of substrate with the amount of substrate units within the cultivation area. This ultimately determines the yield per area (fig. 4). To maximize biomass growth per unit of substrate, seedling density of *Saccharina japonica* is often reduced to 1-2 plants cm^{-1} to optimize resource availability (Su and Pang 2017). At high seedling density, competition for resources can limit growth potential (Harrison and Hurd 2001), while too low a density can reduce potential biomass yield. Maximizing biomass yield per hectare depends on the growth on all units. Hence, at too high a seedling density per unit of substrate, combined with a high density of substrates per hectare, the harvest yield per hectare may be impaired (Roesijadi et al. 2008). For sea-based grow out of *P. palmata*, a density of 10-20 individuals cm^{-1} is suggested to be optimal, but the combination of density per unit of substrate area and substrate density per area requires more research (Sanderson 2006, Werner and Dring 2011, 2013).

Farming of valuable rhodophyte species, in genera of *Pyropia*, *Gracillaria* and *Eucheuma*, has facilitated developments of hatchery techniques and cultivation systems for yield optimization (Drew 1949, Alvial 1994, Sahoo and Yarish 2005). Sea-based grow out of these fast growing and relatively small-sized rhodophytes is typically conducted just below the sea surface in a two-dimensional structure (nets suspended between vertical poles, i.e., the “pole-system” and off-bottom monolines). Due to their fast growth, the use of multiple cropping or restocking cultivation systems with new crops enables up to 6-15 harvests per year (Tseng 1981). The aquaculture and multiple harvests of these smaller rhodophyte species can therefore yield similar yearly biomass production as larger kelp species (125-249 tonne FW ha^{-1} y^{-1}) (Chueh and Chen 1982, Moss and Doty 1987, Li et al. 2016). In comparison, the previous extrapolated biomass yields in dulse aquaculture using nets in North Atlantic waters range between 34-172 tonne FW ha^{-1} y^{-1} (Browne 2001, Werner and Dring 2011).

Based on the present findings, future grow out trials of *P. palmata* should test different farming densities and cropping intensities using multiple substrate types. There appears to be a focus on using solid textile sheet as growth substrates in spore-seeding methods. Solid textile sheets seeded with *S. latissima* meiospores have previously provided a high seedling density and resulted in double the biomass yield compared to traditional twine methods (Kerrison et al. 2018). This grow out unit is currently used in the emerging European kelp cultivation (e.g. companies Hortimare and AtSeaNova) and is promising to fulfill the requested needs for an optimal substrate, which could potentially be transferrable to *P. palmata* cultivation. Thus, further research should include sheets and nets with a shallow deployment depth and should be tested against likewise substrates and lines in a horizontally deployment. Testing these substrates should include different seedling

densities to assess the effect of self-shading in the pursuit of maximizing the biomass harvest per farm area per season. Adjustment of the cultivation depth, according to the light saturation point, may also optimize growth rates and potentially increase harvest yield. Furthermore, investigations of depth adjustment should include an evaluation of the feasibility of adjusting the cultivation depth during the season compared to multiple cropping of horizontal lines. This would reveal the optimal seedling density to minimize self-shading, bleaching of biomass in late spring, as well as the frequency of multiple cropping that is optimal for *P. palmata* aquaculture in Danish waters. Optimal deployment configurations are likely to differ among farm sites, as light availability varies considerably in the last part of the growth season in Danish waters. A key outcome of this research will suggest suitable substrates with potential for automated handling and shed light upon whether heavy or lightweight harvest technology is required to reach the most cost-efficient cultivation. Automated harvesting technology (e.g. developed by the company AtSeaNova), is promising to increase the production efficiency of seaweeds, which ought to lower the production cost for large-scale seaweed aquaculture.

4.5 Land-based grow out

Today, land-based grow out provides high quality seaweed for human food with or without co-cultivation of finfish, where the level of heavy metals is within limits of food safety (Ratcliff et al. 2016), though only few companies practice it. By tank cultivation, seaweed works as a biological filter by taking up nutrients. Paper 4 demonstrated how a nutrient deprived seedstock can boost the nutrient removal rate up to 20.6 mg N m^{-2} d^{-1} in *P. palmata* within range of the growth saturating light level at 10 ° C. This removal rate was in the high range of otherwise reported removal rates for *P. palmata* (Corey et al. 2013, Tremblay-Gratton et al 2018).

The specific growth rate (SGR) and accumulated biomass yield was shown to stagnate and decline from early summer and the rest of the year, by culturing small whole fronds in agitated flow-through tanks, using thermally stable, nutrient rich water in flow-through from April (Levinsen in prep.). The stagnation in SGR suggests that returning initial stocking density by reducing the number of fronds or partial cutting/cropping is not in itself optimal for culturing *P. palmata*. Further, deterioration of tissue and fouling of the basal part of fronds during summer resulted in unpalatable biomass, which is in accordance with previous studies (Corey et al. 2013, Tremblay-Gratton et al. 2018). This emphasizes the need for a new cultivation strategy for land-based *P. palmata* grow out to ensure high growth rate and palatable biomass and to increase the concentration of desired molecules (Abreu et al. 2001, Neori et al. 2004, Parjikolaei et al. 2013). Combining a better control of light exposure within growth saturation (Paper 4), use of intermittent water renewals or nutrient pulse additions during night times (Nagler et al. 2003, Demetropoulos and Langdon 2004), and performing multiple cropping could result in alternating periods of nutrient replete and depleted conditions. In turn, this could potentially minimize growth of ephemeral epiphytes, which is a general problem in seaweed aquaculture, and secure a good biomass growth year-round (Neish et al. 1977, Fletcher 1995, Pedersen and Borum 1996, Sanderson 2006). By undergoing growth at starving conditions (nutrient deprivation), seaweeds perform a luxury uptake, surpassing what is needed to sustain instantaneous growth rate, to replenish nutrient pools (Hanisak 1990, McGlathery et al. 1996). Hence, pulse fertilization or intermittent water renewals would allow a high nutrient

removal rate, as the requirement for nutrients build up until ambient nutrient concentration is replete (Morgan and Simpson 1981ab). Performing multiple cropping implies a decision on propagating distal or basal thallus parts, of which the distal part is the youngest and most light tolerant tissue (Appendix 1). As young seaweed tissues, like marginal proliferations and distal tips, exhibit higher growth rates than older tissues (Morgan and Simpson 1981b, Martínez et al. 2006), I recommend future studies to test a cultivation strategy using multiple crops ("all-in-all-out" cultivation strategy) by restocking with young, nutrient deprived tissue. This strategy requires a steady supply of seedstock, which could be marginal proliferations (Paper 4), or spore-derived seedlings. Lastly, testing of other methods to prevent or minimize the amount of epi-fouling might be required during summer, if the cultivation strategy relies on cropping instead of new crops (personal observation). As shown in previous studies, cleaning frond surfaces by short-term suspension in a mild dose of oxidant agent can prevent growth of fouling species (Kerrison et al. 2016, Bøe 2019, Levinsen in prep.).

4.6 Selection of ecotypes and breeding

A last topic to be looked into regarding future research of *P. palmata* aquaculture is selective breeding. Currently, only a single company (Big Island Abalone Corporation, Hawaii) performs commercial grow out of *P. palmata,* while several companies worldwide are performing pilot tests using both vegetative propagation for land-based grow out and spore-seeded substrates for sea-based trials. All of the current cultivation activities rely on collecting spore donors from wild populations. In wild seaweed populations, a degree of intraspecific phenotypic plasticity in traits, such as morphology, frond size, growth rate, timing of fertility and stress tolerance exists (Gerard et al. 1987, Guiry and Dawes 1992, Matos et al. 2006, Bulboa et al. 2008, FAO 2014, Manríquez-Hernández et al. 2016, Liu et al. 2017). Paper 2 and 4 indicate that *P. palmata* in inner Danish waters grows high in the intertidal zone with potential adaptation to grow in low salinity water. Levinsen (*in prep.*) show frond growth up to 50 cm in these population by using tumble cultivation. It can also be postulated that plasticity in tetrasporophyte energy reserve accumulation (Martínez and Rico 2008), as well as timing of sporogenesis could exist and thus, potentially increase spore production in the *P. palmata* hatchery. However, this requires further research. Samples from a population showing early fertility or high growth could be the basis for strain optimization with high light and low salinity tolerances by continuously and selectively breeding the highest quality individuals to increase frond size, increase stress tolerance and manipulate reproduction period. Hybridization, through protoplast fusion, could also potentially enable cultivar strain optimization (Polne-Fuller et al. 1986, Polne-Fuller 1988, Baweja et al. 2009). The work from Chinese kelp hatcheries has shown that, by performing specific crossings for >6 generations of in-line breeding, new cultivar strains can be produced that express the desired phenotypic traits (Zhang et al. 2007, Li et al. 2016, Su et al. 2017).

4.7 Overall conclusion

For aquaculture of *P. palmata* to improve and develop, a full control of the production of spore-donors, as well as the triggering of spore release still remain a bottleneck for establishing large, commercial hatcheries. Although some progress has been made in this thesis, the key to unlocking sporogenesis induction of *P. palmata* is still to be found. Given the scarce availability of spore-donors, a new spore seeding method with high sori use efficiency (5-15 g sori per 48 m substrate) using flow-through conditions is an important step forward. This method resulted in even spore settlement and high germination rate (up to 80%) of seedlings on vertical nets. Additionally, the inclusion of a fertilization step, to activate female gametophytes that otherwise stay microscopic, resulted in up to twice the number of seedlings. After long nursery duration, the seedling density was 9 individuals cm^{-1} prior to deployment. This seeding efficiency was obtained using only 16% of the spore release, while 84% of the spores were retained from the effluent water. In order to utilize the retained spores, an alternative seeding method using germinated propagules to prolong the hatchery seeding production and to enhance spore use efficiency was provided. The method relies on three steps to seed substrates based on discoid reattachment of the propagules: Germination-Maceration-Agitation (GMA-method). Thus, the use of germinated propagules as seedstock and propagation units for land-based grow out might act as an alternative to inducing fertility to secure year-round production. However, the method verified propagule reattachment up to 39 days after initial spore release and thus, further research is required to verify its potential application year-round.

In conclusion, optimization of grow out of *P. palmata* relies on: 1) securing year-round seedstock supply; 2) development of optimal methods to achieve evenly seeded substrates for large-scale grow out; 3) optimal biological quality of seedlings; 4) optimal deployment configuration (e.g. substrate density per hectare); and; 5) optimal site selection (sea-based cultivation) and synergies with land-based production units providing a valuable resource flow (land-based cultivation). Importantly, a harvest strategy to maximize the yield and biomass quality within a season and to lower production costs, demands cost-effective, automated methods for optimal handling of seeded substrates.

This thesis provides new and important insights for further development of an optimal commercial cultivation method for farming *P. palmata*, which is still at an immature level compared to the methods for cultivating kelp species in Europe. Although this thesis provides a good starting point regarding dulse production, more research is needed to strengthen the scientific knowledge and practical methods for dulse production.

Appendix 1

Measuring chlorophyll fluorescence quenching using pulse amplitude modulated fluorometer (PAM) to estimate photosynthetic parameters and stress in *P. palmata* grown in a land-based setup.

Background. Light stimulates algae photosynthesis rate with a linear relationship at low irradiance, where the slope (alfa) defines the light utilization efficiency. The maximal electron transfer rate (ETR_{max}) between the thylakoid membrane embedded photosystems II and I, is a relative measure of photosynthesis, and defines the light intensity where the actual photosynthesis rate is expected to be highest. At a higher light level, photosynthesis is negative affected and the tissue experience photosynthetic stress. The intercept of the initial linear slope and the y-value of maximal photosynthesis defines the saturation point (I_K). The light utilization efficiency reduces with increasing light exposure and by receiving high light exposures, seaweed and plants dissipate excessive energy by either heat or re-emit light as fluorescence (F). The relative fluorescence measure of PS II quantum yield ($Y(II) = F_{max} – F_0 / F_{max}$) refers to the ratio of absorbed light that is photochemical converted by the photosynthesis apparatus. The Y(II) identifies the photochemical efficiency at different light levels and can be used to estimate the relative electron transmission rate (ETR) between photosystems using the equation ETR = Y(II) * PAR *0.5 * AF, where PAR is µmol photons m^{-2} s^{-1}, and AF is tissue absorption factor (=0.84) (Genty et al. 1989). The absorbed energy in excess is dissipated as heat in two pathways and can be estimated by measuring chlorophyll fluorescence quenching based on pulse amplitude modulated fluorometry (PAM). The PAM fluorometry measures the relative electron flow from photosystem (PS) II to I and is used to estimate photosynthetic stress by energy dissipation. In short, a red-light diode in the PAM apparatus emits beams of actinic light (<0.15 µmol photons m^{-2} s^{-1}), which promotes an excitatory state of the PSI + PSII main pigments. To return to normal state, the PSII + PSI distribute the excessive energy in several pathways for dissipation, where fluorescence is one. The non-photochemical fluorescence quenching (Y(NPQ)) in algae is the regulated heat dissipation pathway. The non-regulated heat dissipation pathway (Y(NO)) indicates a photosynthetic stress which the algae cannot control.

Materials and methods. A sample of fronds collected in early March in Little Belt, Denmark, were cultivated in ambient light conditions by sourcing flow-through nutrient rich seawater from the landbased Recirculated Aquaculture System unit (RAS) producing salmon (Atlantic Sapphire, Denmark) from April 4 to April 30 (2019). Then, fronds (n=6) were subjected to a light treatment by applying shading (90% reduction of ambient light) and tested against an ambient light condition until May 28. Subsequently, thallus discs from the basal, middle, and distal part of the frond were cut and dark-acclimated (10-15 min) in seawater before PAM measurements (fig. 12).

Fig 12. Preparing basal, middle and distal thallus discs of *P. palmata.*

Results. Regarding the photosynthetic parameters, the sun-acclimated (ambient) and the shaded fronds showed similar ETRmax, in accordance with Martínez and Rico (2008), while a slight tendency in higher light utilization efficiency (alfa) in shaded fronds, which was expected (**fig. 13)**. Correspondingly, the sun-acclimated fronds showed saturation (I_K) of photosynthesis at a higher light intensity than shade-acclimated fronds, supportive to earlier work (Young and Smith 1980, Sagert and Schubert 2000) and attributes the phenotypic acclimation of the enzymatic reactions in the Calvin-Benson cycle. The effective quantum yield of PSII (Y(II)) and the regulated stress energy dissipation (Y(NPQ)) was higher in shaded fronds, while the non-regulated energy dissipation (Y(NO)) was higher in fronds cultured in ambient light, regardless of thallus section (**fig. 14**).

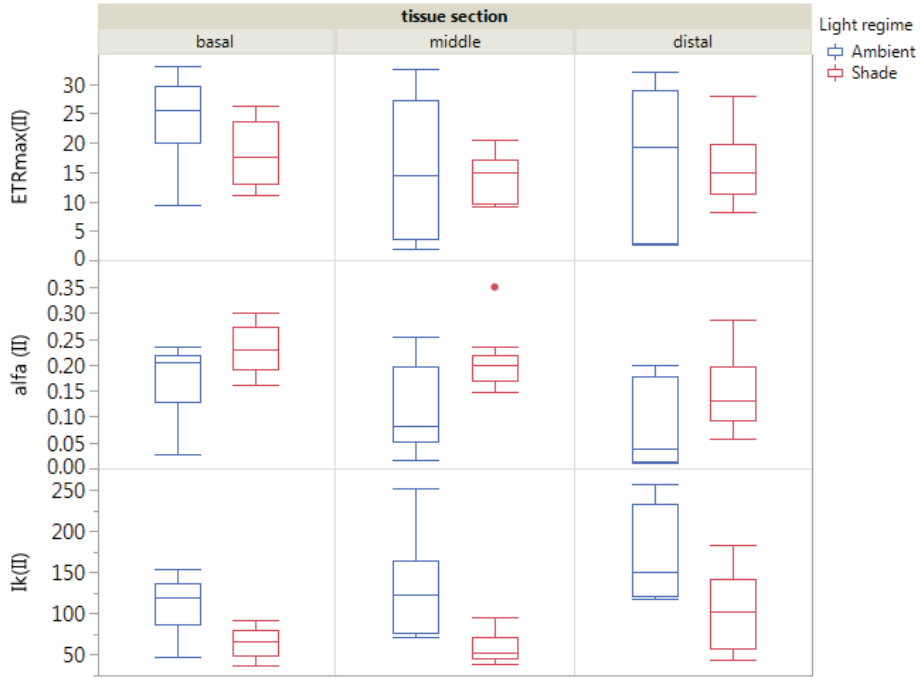


Fig. 13. ETRmax, alfa and I_K for basal, middle and distal parts of *P. palmata* cultivated in ambient or shaded light conditions shown as outlier box plots (n=3). The box represents the inter quartile range (IQR) framed by the the 1st and 3rd quartile around the median. The the upper and lower horizontal lines (whiskers) represent the quartile value*1.5 IQR.

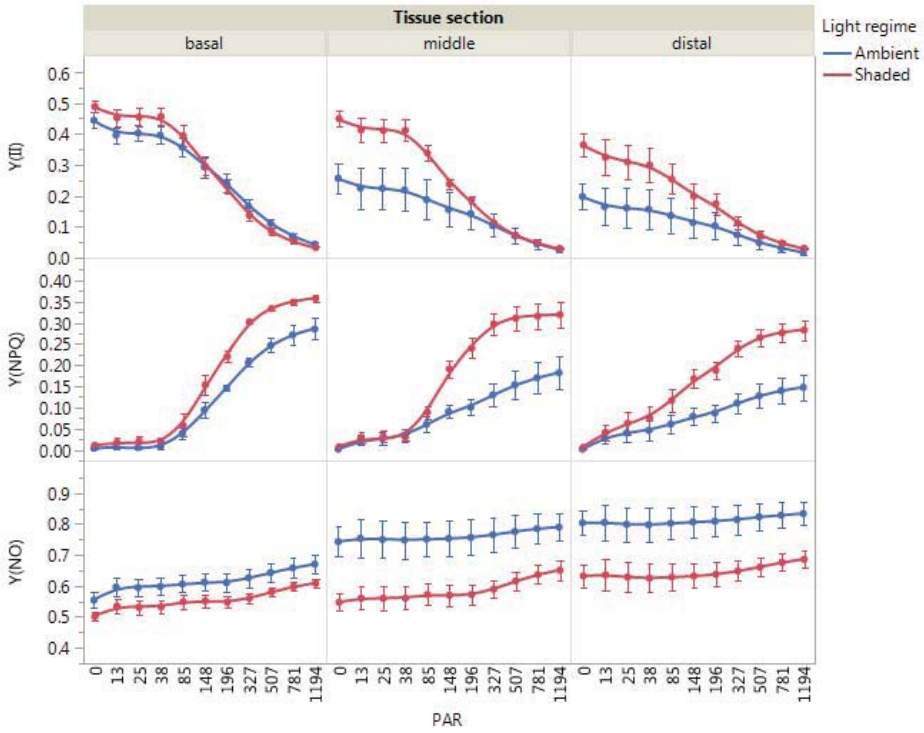


Fig. 14. Measures of Y(II), Y(NPQ) and Y(NO) for basal, middle and distal parts of *P. palmata* cultivated in ambient (blue) or shaded (red) light conditions. Data represent mean ± standard error (n=3).

5. References

Admassu H, Gasmalla MAA, Yang R, Zhao W (2018) Bioactive peptides derived from seaweed protein and their health benefits: antihypertensive, antioxidant, and antidiabetic properties. J Food Sci 83: 6–16.

Allsopp P, Crowe W, Bahar B, Harnedy PA, Brown ES, Taylor SS, Smyth TJ, et al. (2016) The effect of consuming Palmaria palmata-enriched bread on inflammatory markers, antioxidant status, lipid profile and thyroid function in a randomised placebo-controlled intervention trial in healthy adults. Eur J Nutr 55: 1951–1962.

Alveal K, Romo H, Werlinger C, Oliveira EC (1997) Mass cultivation of the agar-producing alga Gracilaria chilensis (Rhodophyta) from spores. Aquaculture 148: 77–83.

Avila M, Ask E, Rudolph B, Nunez M, Norambuena R (1999) Economic feasibility of Sarcothalia (Gigartinales, Rhodophyta) cultivation. Hydrobiologia 398/399: 435–442.

Bak UG (2019) Seaweed cultivation in the Faroe Islands: An investigation of the biochemical composition of selected macroalgal species, optimised seeding technics, and open-ocean cultivation methods from a commercial perspective. Kgs. Lyngby, Denmark: Technical University of Denmark.

Barr NG, Kloeppel A, Rees TAV, Scherer C,Taylor RB, Wenzel A (2008) Wave surge increases rates of growth and nutrient uptake in the green seaweed Ulva pertusa maintained at low bulk flow velocities. Aqautic Biol 3: 179-186.

Bidwell RGS, McLachlan J (1985) Carbon nutrition of seaweeds: photosynthesis, photorespiration and respiration. J Exp Mar Biol Ecol 86: 15–46.

Black WAP (1950) The seasonal variation in wheight and chemical composition of the common british Laminariaceae. Scottish Seaweed Research Association UK 29: 45–72.

Bartsch I, Wiencke C, Bischof K, Buchholz CM, Buck BH, Eggert A, Feuerpfeil P et al. (2008) The genus Laminaria sensu lato: recent insight and development. Eur J Phycol 43: 1–86.

Bogaert K, Beeckman T, De Clerck O (2016) Abiotic regulation of growth and fertility in the sporophyte of Dictyota dichotoma (Hudson) JV Lamouroux (Dictyotales, Phaeophyceae). J Appl Phycol 28: 2915–2924.

Boney AD (1981) Mucilage: the ubiquitous algal attribute. Br Phycol J 16: 115-132.

Bostock J, Lane A, Hough C, Yamamoto K (2016) An assessment of the economic contribution of EU aquaculture production and the influence of policies for its sustainable development. Aquac Int 24: 699–733.

Blouin N, Xiugeng F, Peng J, Yarish C, Brawley SH (2007) Seeding nets with neutral spores of the red alga Porphyra umbilicalis (L.) Kützing for use in integrated multi-trophic aquaculture (IMTA). Aquaculture 270: 77–91.

Breeman AM, Hoopen AT (1987) The mechanism of daylength perception in the red alga *Acrosymphyton purpuriferum*. J Phycol 23: 36-42.

Brodie JA, Irvine LM (2003) Seaweeds of the British Isles, Vol. I: Bangiophycidae. Part 3b. British Museum, Natural History, London. 167 pages.

Browne KL (2001) Mariculture of the edible red alga, *Palmaria palmata*. PhD Thesis, Queen's University, Belfast.

Browne and Edwards (2008, unpublished) Palmaria aquaculture : Programme of experiments on spore viability. Unpublished data, in Wood E 2018.

Bruhn A, Dahl J, Nielsen HB, Nikolaisen L, Rasmussen MB, Markager S, Olesen B, Arias C, Jensen PD (2011) Bioenergy potential of Ulva lactuca: biomass yield, methane production and combustion. Bioresour Technol 102: 2595–2604.

Bruhn A, Tørring D, Thomsen M, Canal-Vergés P, Nielsen M, Rasmussen M, Eybye K, Larsen M, Balsby T, Petersen J (2016) Impact of environmental conditions on biomass yield, quality, and bio-mitigation capacity of Saccharina latissima. Aquac Environ Interact 8: 619–636.

Bruhn et al. (in prep.) Chapter "Synopsis for marine mitigation tools – Cultivation of sugar kelp". In: Marine virkemidler. DCE 2021.

Bruhn A, Brynning G, Johansen Aa, Lindegaard MS, Sveigaard HH, Aarup B, Fonager L, Andersen LL, Rasmussen MB, Larsen MM, Elsser-Gravesen D, Børsting ME (2019) Fermentation of sugar kelp (Saccharina latissima)—effects on sensory properties, and content of minerals and metals. J Appl Phycol 31: 3175-3187.

Buchholz C, Lüning K (1999) Isolated, distal blade discs of the brown alga *Laminaria digitata* form sorus, but not discs, near to the meristematic transition zone. J Appl Phycol 11: 579–584.

Bulboa C, Macchiavello J, Oliveira E, Véliz K (2008) Growth rate differences between four Chilean populations of edible seaweed Chondracanthus chamissoi (Rhodophyta, Gigartinales). Aquacult Res(39): 1550-1555.

Bøe RR (2019) Investigation of important steps in *Palmaria palmata* cultivation. Master´s thesis (NTNU), Norwegian University of Science and Technology Faculty of Natural Sciences, Department of Biology.

Caines S, Manríquez-Hernández JA, Duston J, Corey P, Garbary DJ (2014) Intermittent aeration affects the bioremediation potential of two red algae cultured in finfish effluent. J Appl Phycol 26: 2173-2181.

Callaway E (2015) Lab staple agar hit by seaweed shortage. Nature 528: 171–172.

Chapman ARO, Craigie JS (1977) Seasonal growth in *Laminaria longicruris* : Relations with dissolved inorganic nutrients and internal reserves of nitrogen. Mar Biol 40: 197–205.

Chapman AS, Stévant P, Larssen WE (2015) Food or fad? Challenges and opportunities for including seaweeds in a Nordic diet. Bot Mar 58(6): 423-433.

Chamberlain AHL, Evans LV (1973) Aspects of spore production in the red algae Ceramium. Protoplasma 76: 139-159.

Charters AC, Neushel M, Coon D (1973) The effect of water motion on algal spore adhesion. Limnology and Oceanography 18(6): 884-896.

Cherry P, Yadav S, O'Callaghan C, Popper ZA, Ross PR, McSorley EM, Allsopp PJ, Stanton C (2016) In-vitro fermentation of whole seaweed and a polysaccharide-rich extract derived from the edible red seaweed Palmaria palmate. Proc Nut Soc 75(OCE2). Doi: 10.1017/S0029665116000471

Chueh CT, Chen C (1982). Seaweed economics in Taiwan: pp. 9-16 in Tsuda RT, Chiang YM, eds. Proceedings of Republic of China—United States cooperative science seminar on cultivation and utilization of economic algae. University of Guam Marine Laboratory, Mangilao, Guam.

Clayton MN (1992) Propagules of marine macroalgae: structure and development. Br Phycol J 27: 219–232.

Chopin T, Pringle JD, Semple RE (1992) Impact of harvesting on frond density and biomass of Irish moss (*Chondrus crispus* Stackhouse) beds in the southern Gulf of St. Lawrence. Can J Fish Aquat Sci 49: 349–57.

Chopin T, Buschmann, A, Halling C, Troell M, Kautsky N, Neori A, Kraemer G, Zertuche-Gonzalez J, Yarish C, Neefus C (2001) Integrating seaweeds into marine aquaculture systems: a key toward sustainability. J Phycol 37: 975–986.

Cohen E, Shoshana A, Heimer YH, Mizrahi Y (1984) Polyamine biosynthetic enzymes in the cell cycle of Chlorella. Plant Physiol 74: 385–8.

Cole KM, Sheath RG (1990) *Biology of the red algae*. Book. Cambridge University Press. ISBN: 978-0-521-20246-6.

Collén J, Porcel B, Carré W, Ball SG, Chaparro C, Tonon T, Barbeyron T, Michel G, Noel B, Valentin K (2013) Genome structure and metabolic features in the red seaweed Chondrus crispus shed light on evolution of the Archaeplastida. Proc Natl Acad Sci USA 110: 5247–5252.

Corey P, Kim JK, Duston J, Garbary DJ, Prithiviraj B (2013) Bioremediation potential of Palmaria palmata and Chondrus crispus (Basin Head): effect of nitrate and ammonium ratio as nitrogen source on nutrient removal. J Appl Phycol 25: 1349–1358.

Corey P, Kim JK, Duston J, Garbary DJ (2014) Growth and nutrient uptake by Palmaria palmata (Palmariales, Rhodophyta) integrated with Atlantic halibut recirculating aquaculture. Algae 29: 35–45.

Demetropoulos CL, Langdon CJ (2004) Enhanced production of Pacific dulse (Palmaria mollis) for co-culture with abalone in a land-based system: nitrogen, phosphorus, and trace metal nutrition. Aquaculture 235: 433–455.

Dion P, Delépine R (1981). Studies on the development of Palmaria palmata (Rhodophyceae) using in situ controlled cultures. Xth International Seaweed Symposium. Proceedings of the International Seaweeds Symposium. Walter de Gruyter, Berlin. pp. 265–270.

Drew KM (1949) Conchocelis-phase in the life-history of Porphyra umbilicalis (L.) Kútz. Nature 164: 748–749.

Dring MJ (1974) Reproduction. In, Algal physiology and biochemistry, edited by Stewart WDP, Blackwell Scientific Publications, Oxford, pp. 814-837.

Dring MJ, Lüning K (1983) Photomorphogenesis of marine macroalgae. In: Encyclopedia of plant physiology, N.S. vol. 16: Photomorphogenesis, pp. 545 568, Shropshire, W.,Mohr, H., eds. Springer, Berlin Heidelberg New York.

Dring MJ, West JA (1983) Photoperiodic control of tetrasporangium formation in the red alga Rhodochorton purpureum. Planta 159: 143–150.

Dring MJ (1984). Photoperiodism and phycology. Prog Phycol Res 3: 159–192.

Duarte CM, Holmer M, Olsen Y, Soto D, Marba N, Guiu J, Black K, Karakassis I (2009) Will the oceans help feed humanity? BioScience 59: 967–976.

Edwards MD (2007) The cultivation of the edible red alga, *Palmaria palmata* for aquaculture. *PhD Thesis*, Queen's University, Belfast.

Edwards MD, Dring MJ (2011) Open sea cultivation trails of the red alga, Palmaria palmata from seeded tetraspores in Strangford Laugh, Northern Ireland. Aquaculture 317: 203–209.

Evans FD, Critchley AT (2014) Seaweeds for animal production use. J Appl Phycol 26: 891-899.

Faes VA, Viejo RM (2003) Structure and dynamics of a population of Palmaria palmata (Rhodophyta) in northern Spain. J Phycol 39: 1038–1049.

FAO (1983) Food and Agriculture Organization. ADB/FAO Second Fish Market Study. Vol. 6. The world seaweed industry and trade: Developing Asian producers and prospects for greater participation. Asian

Development Bank and the Food and Agriculture Organization of the United Nations, South China Sea Fisheries Development and Coordinating Programme (SCSP), Marketing Information and Advisory Service for Fish Products in the Asian/Pacific Region (INFOFISH).

FAO (2009) Integrated mariculture - a global review (Edited by Soto D). Technical Paper no. 529. Rome,

FAO (2014) The state of world fisheries and aquaculture 2014, pp. 223. Food and Agriculture Organization of the United Nations, Fisheries and Aquaculture Department, Rome.

FAO (2018) Food and Agriculture Organization of the United Nations) The State of World Fisheries and Aquaculture 2018 - Meeting the Sustainable Development Goals, Rome.

Feng YY, Hou LC, Ping NX, Ling TD, Kyo CI (2004) Development of mariculture and its impacts in Chinese coastal waters. Reviews in Fish Biology and Fisheries 14: 1–10.

Fletcher RL, Callow ME (1992) The settlement, attachment and establishment of marine algal spores. Br Phycol J 27(3): 303-329.

Fletcher RL (1995) Epiphytism and Fouling in Gracilaria Cultivation - an Overview. J Appl Phycol 7(3): 325-333.

Fleurence J (1999) Seaweed proteins: biochemical, nutritional aspects and potential uses. Trends in Food Science & Technology 10: 25–28.

Flores-Moya A, Fernández JA, Niell FX (1996) Growth pattern, reproduction, and self-thinning in seaweeds. J Phycol 32: 767–9.

Forbord S, Skjermo S, Arff J, Handå A, Reitan KI, Bjerregaard R, Lüning K (2012) Development of Saccharina latissima (Phaeophyceae) kelp hatcheries with year-round production of zoospores and juvenile sporophytes on culture ropes for kelp aquaculture. J Appl Phycol 24(3): 393-399.

Forbord S, Steinhovden K, Rød KK, Handå A, Skjermo J (2018) Cultivation protocol for Saccharina latissima. In: Protocols for Macroalgae Research, edited by Charrier B, Wichard T & Reddy CRK. CRC Press, Taylor & Francis Group. Pp: 37-59.

Fortes MD, Lüning K (1980) Growth rates of North Sea macroalgae in relation to temperature, irradiance and photoperiod. Helgoländer Meeresuntersuchungen 34: 15–29.

Fritsch FE (1961). The Structure and Reproduction of the Algae. University Press, Cambridge. 791 pages.

Galland-Irmouli AV, Fleurence J, Lamghari R, Luçon M, Rouxel C, Barbaroux O, Bronowicki JP, Villaume CH, Guéant JL (1999) Nutritional value of proteins from edible seaweed Palmaria palmata (dulse) J. Nutr Biochem 10: 353-359.

Galston, AW, Kaur-Sawhney R (1990) Polyamines in plant physiology. Plant Physiol. 94:406–10.

García-Jiménez P, Robaina RR (2012) Effects of ethylene on tetrasporogenesis in *Pterocladiella capillacea* (rhodophyta). J Phycol 48: 710-715.

Garcia-Jimenez P, Llorens C, Roig FJ, Robaina RR (2018) Analysis of the Transcriptome of the Red Seaweed *Grateloupia imbricata* with Emphasis on Reproductive Potential. Mar Drugs 16(490) doi.org/10.1007/s10811-019-01998-0.

Gaillard C, Bhattia HS, Novoa-Garridob M, Lind V, Roleda MY, Weisbjerg MR (2018) Amino acid profiles of nine seaweed species and their in situ degradability in dairy cows. Animal Feed and Technology 241: 210-222.

Genty B, Briantais JM, Baker NR (1989) The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. Biochem Biophys Acta 990: 87–92.

Gerard VA, DuBois K, Greene R (1987) Growth responses of two Laminaria saccharina populations to environmental variation. Hydrobiologia 151/152: 229-232.

Góes HG, Reis RP (2011) An initial comparison of tubular netting versus tie-tie methods of cultivation for Kappaphycus alvarezii (Rhodophyta, Solieriaceae) on the south coast of Rio de Janeiro state, Brazil. J Appl Phycol 23: 607-613.

Grobbelaar JU (2009) Upper limits of photosynthetic productivity and problems of scaling. J Appl Phycol 21: 519–522.

Guiry (1975) An assessment of *Palmaria palmata* forma mollis (S. et G.) comb. nov. (= Rhodymenia palmata forma mollis S. et G.) in the eastern North Pacific. Syesis 8: 245-261

Guiry, M.D. & Irvine, D.E.G. (1981). A critical reassessment of infraordinal classification in the Rhodymeniales. Proc. Int. Seaweed Symp. 8: 106-111

Guiry MD, Dawes CJ (1992) Daylength, temperature and nutrient control of tetrasporogenesis in Asparagopsis armata (Rhodophyta). J Exo Mar Biol Ecol 158:197-217

Guzmán-Urióstegui A, Robledo D (1999) Factors affecting sporulation of *Gracilaria cornea* (Gracilariales, Rhodophyta) carposporophytes from Yucatan, Mexico. Hydrobiologia 399: 285–290.

Guiry MD (2015). The seaweed site: information on marine algae. Retrieved from: http://www.seaweed.ie/.

Guzmán-Urióstegui A, García-Jiménez P, Marián F, Robledo D, Robaina R (2002) Polyamines influence maturation in reproductive structures of *Gracillaria cornea* (Gracialariales, Rhodophyta). J Phycol 38: 1169-1175.

Guiry MD, Guiry GM (2020). AlgaeBase. Worldwide electronic publication, National University of Ireland, Galway. http://www.algaebase.org

Hafting JT, Critchley AT, Scott MLC, Hubley A, Archibald AF (2012) On-land cultivation of functional seaweed products for human usage. J Appl Phycol 24: 385–392.

Hafting JT, Craigie JS, Stengel Dagmar B, Loureiro RR, Buschmann AH, Yarish C, Critchley AT (2015) Prospects and challenges for industrial production of seaweed bioactives1. J Phycol 51: 821-837.

Hanisak, MD (1990) The use of *Gracilaria tikvahiae* (Gracilariales, Rhodophyta) as a model system to understand the nitrogen nutrition of cultured seaweeds. Dev Hydrobiol 58: 79–87.

Harnedy PA, FitzGerald RJ (2011) Bioactive proteins, peptides, and amino acids from macroalgae. J Phycol 47: 218–232.

Harnedy PA, FitzGerald RJ (2013a) Extraction of protein from the macroalga Palmaria palmata. LWT - Food Science and Technology 51: 375-382

Harnedy PA, FitzGerald RJ (2013b) In vitro assessment of the cardioprotective, anti-diabetic and antioxidant potential of *Palmaria palmata* protein hydrolysates. J Appl Phycol 25: 1793-1803.

Harnedy PA, Soler-Vila A, Edwards M, Fitzgerald RJ (2014) The effect of time and origin of harvest on the in vitro biological activity of *Palmaria palmata* protein hydrolysates. Food Research International 62: 746–752.

Harrison PJ, Hurd CL (2001) Nutrient physiology of seaweeds: application of concepts to aquaculture. Cahiers de Biologie Marine 42: 71–82.

Hasler CM (2000) The changing face of functional foods. J Amer Coll Nutr 19: 499-506.

Hayashi L, Reis RP, dos Santos AA, Castelar B, Robledo D, de Vega GB, Msuya FE, Eswaran K, Yasir SM, Ali MKM, Hurtado AQ (2017) The cultivation of Kappaphycus and Eucheuma in tropical and sub-tropical waters. In Hurtado AQ, Critchley AT, Neish IC (Eds.), Tropical seaweed farming trends, Problems and opportunities: Vol. 9. Developments in applied phycology. Cham: Springer: 55-90.

Hoffmann AJ (1988) Day length and light responses in growth and fertility of Glossophora kunthii (Phaeophyta, Dictyotales) from Pacific South-America. J Phycol 24: 203–208.

Holt TJ (1984). The development of technique for the cultivation of Laminariales in the Irish Sea. Port Erin Marine Laboratory, University of Liverpool. Doctor of Philosophy.

Holdt SL, Kraan S (2011) Bioactive compounds in seaweed; functional food applications and legislation. J Appl Phycol 23: 543–597.

Hurd CL (2000) Water motion, marine macroalgal physiology, and production. J Phycol 36: 453–472.

Jørgensen K, Olesen PT (2018) Kainic acid in the seaweed Palmaria palmata (dulse). Food Additives & Contaminants: Part B, DOI: 10.1080/19393210.2018.1462258

Kain JM (1982) The reproductive phenology of nine species of Rhodophyta in the subtidal region of the Isle of Man. Br Phycol J 17: 321–331.

Kain JM (1986) Frond size and reproduction phenology of six species of Rhodophyta in subtidal Isle of Man. Br Phycol J 21: 129–138.

Kanawaza A, Saito A, Idler DR (1966) J Fish Res Bd Canada 23(6). 2 pp.

Kerrison PD, Stanley MS, Edwards, MD, Black KD and Hughes AD (2015) The cultivation of European kelp for bioenergy: Site and species selection. Biomass Bioenerg 80: 229-242.

Kerrison PD, Le HN, Twigg GC, Smallman DR, MacPhee R, Houston FAB, Hughes AD (2016) Decontamination treatments to eliminate problem biota from tank cultures of Osmundea pinnatifida (Rhodophyta), Palmaria palmata (Rhodophyta) and Ulva lactuca (Chlorophyta). J App Phycol 28: 3423-3434.

Kerrison PD, Stanley MS, Black KD, Hughes AD (2017) Assessing the suitability of twelve polymer substrates for the cultivation of macroalgae *Laminaria digitata* and *Saccharina latissima* (Laminariales). Algal Res 22: 127-134.

Kerrison PD, Stanley MS, Hughes AD (2018). Textile substrate seeding of Saccharina latissima sporophytes using a binder: An effective method for the aquaculture of kelp. Algal Res 33: 352-357.

Kerrison PD, Stanley MS De Smet D, Buyle G, Hughes AD (2019). Holding (not so) fast: Surface chemistry controls kelp bioadhesion. Eur J Phycol 54(3). https://doi.org/10.1080/09670262.2018.1547924

Kraan S, Guiry MD (2001) Phase II: strain hybridisation field experiments and genetic fingerprinting of the edible brown seaweed *Alaria esculenta*. Marine Resource Series. Martin Ryan Institute, Galway: 1–33.

Kraan S (2013) Mass-cultivation of carbohydrate rich macroalgae, a possible solution for sustainable biofuel production. Mitigation and Adaptation Strategies for Global Change 18 (1): 27–46.

Kraan S, Guiry MD (2006). A genetic investigation of two morphotypes of *Palmaria palmata* (Palmariales, Rhodophyta) using Rubisco spacer and ITS 1 and ITS 2 sequences. Cryptogamie Algologie 27: 17-30

Kim JK, Duston J, Corey P, Garary DJ (2013) Marine finfish effluent bioremediation: effects on stocking density and tem- perature on nitrogen removal capacity of Chondrus crispus and Palmaria palmata (Rhodophyta). Aquaculture 414–415: 210–216.

Kim JK, Yarish C, Hwang EK, Park M, Kim Y (2017) Seaweed aquaculture: cultivation technologies, challenges and its ecosystem services. Algae 32(1) 1.13

Kurakake T, Hori A. (1966). A method of keeping laver (*Porphyra*) culturing net in low temperature. Refrigeration (Japanese Association of Refrigeration, Tokyo) 41: 15–29.

Lee TM (1998) Investigations of some intertidal green macroalgae to hyposaline stress: Detrimental role of putrescine under extreme hyposaline conditions. Plant Sci. 138:1–8

Lee D, Nishizawa M, Shimizu Y, Saeki H (2017) Anti-inflammatory effects of dulse (Palmaria palmata) resulting from the simultaneous water-extraction of phycobiliproteins and chlorophyll a. Food Res Int 100: 514-521.

Le Gall L, Pien S, Rusig A-M (2004) Cultivation of Palmaria palmata Palmariales, Rhodophyta from isolated spores in semi-controlled conditions. Aquaculture 229: 181–191.

Levinsen JUG (in prep.) Cultivation of Palmaria palmata in land based Integrated multitrophic aquaculture systems – From spore to harvestable resource. MSc thesis. DTU Aqua - National Institute of Aquatic Resources. Kgs. Lyngby

Li XJ, Cong YZ, Yang GP, Shi YY, Qu SC, Li ZL, Wang GW, et al. (2007) Trait evaluation and trial cultivation of Dongfang no. 2, the hybrid of a male gametophyte clone of *Laminaria longissima* (Laminariales, Phaeophyta) and a female one of *L. japonica*. J Appl Phycol 19: 139–151.

Li X, Norman HC, Kinley R, Laurance M, Wilmot M, Bender H, de Nys R, Tomkins N (2016) *Asparagopsis taxiformis* decreases enteric methane production from sheep. Animal Production Science 58: 681–688.

Liu J, Kandasamy S, Zhang Z, Kirby CW, Karakach T, Hafting J, Critchley AT, Evans F, Prithiviraj B (2015) Prebiotic effects of diet supplemented with the cultivated red seaweed Chondrus crispus or with fructo-oligo-saccharide on host immunity, colonic microbiota and gut microbial metabolites. BMC Complementary and Alternative Medicine 15(279): 2-12.

Liu X, Bogaert K, Engelen AH, Leliaert F, Roleda MY, De Clerck O (2017) Seaweed reproductive biology: environmental and genetic controls. Bot Mar 2(60): 89-108.

Lüning K (1988) Photoperiodic control of sorus formation in the brown alga Laminaria saccharina. Marine Ecology Progress Series 45: 137–144.

Lüning K, Dieck IT (1989) Environmental Triggers in Algal Seasonality. Bot Mar 32: 389–397.

Lüning K, Mortensen L (2015) European aquaculture of sugar kelp (Saccharina latissima) for food industries: Iodine content and epiphytic animals as major problems. Bot Mar 58(6): 449-455.

Mabeau S & Fleurence J (1993) Seaweed in food-products - biochemical and nutritional aspects. Trends Food Sci Tech 4: 103-107.

Makkar HPS, Tran G, Heuzé V, Giger-Reverdin S, Lessire M, Lebas F, Ankers P (2016) Seaweeds for livestock diets: a review. Anim Feed Sci Technol 212: 1–17.

Manríquez-Hernández JA, Duston J, Garbary DJ (2016) Effect of irradiance on bioremediation capacity of the red algae Chondrus crispus and Palmaria palmata. Aquaculture International 24: 39–55.

Marián FD, García-Jimenez P, Robaina RR (2000) Polyamines in marine macroalgae: Levels of putrescine, spermidine and spermine in the thalli and changes in their concentration during glycerol-induced cell growth in vitro. Physiologia Plantarum 110(4): 530-534.

Marinho GS, Holdt SL, Birkeland MJ, Angelidaki I (2015) Commercial cultivation and bioremediation potential of sugar kelp, *Saccharina latissima*, in Danish waters. J Appl Phycol 27: 1963–1973.

Marrion O, Schwertz A, Fleurence J, Gueant JL, Villaume C (2003) Improvement of the digestibility of the proteins of the red alga Palmaria palmata by physical processes and fermentation. Food/ Nahrung 47: 339-344.

Martínez B, Rico JM (2002) Seasonal variation of P content and major N pools in *Palmaria palmata* (Rhodophyta). J Phycol 38(6): 1082–1089.

Martínez B, Viejo R, Rico JM, Rødde R, Faes V, Oliveros J, Alvarez D (2006) Open sea cultivation of *Palmaria palmata* (Rhodophyta) on the northern Spanish coast. Aquaculture 254(1–4): 376–387.

Martínez B, Rico JM (2008) Changes in nutrient content of Palmaria palmata in response to variable light and upwelling in Northern Spain. J Phycol 44: 50-59.

Mata L, Schuenhoff A, Santos R (2010) A direct comparison of the performance of the seaweed biofilters, *Asparagopsis armata* and *Ulva rigida*. J Appl Phycol 22: 639–644.

Matos J, Costa S, Rodrigues A, Pereira R, Sousa-Pinto I (2006) Experimental integrated aquaculture of fish and red seaweeds in Northern Portugal. Aquaculture 252: 31–42.

McGlathery KJ, Pedersen MF (1999) The effect of growth irradiance on the coupling of carbon and nitrogen metabolism in Chaetomorpha linum (Chlorophyta). J Phycol 35:721–731.

McGrath BM, Harmon JP, Bishop G (2010). *Palmaria palmata* (Dulse) as an unusual maritime aetiology of hyperkalemia in a patient with chronic renal failure: a case report. J Med Case Rep 4**:** no. 301.

McHugh DJ (2003) A guide to the seaweed industry. FAO Fisheries Technical Paper No. 441. pp. 105. FAO, Rome.

Millan-Linares MC, Martin ME, Rodriguez NM, Toscano R, Claro C, Bermudez B, Pedroche J, Millan F, Montserrat-de la Paz S (2019) Nutraceutical Extract from Dulse (Palmaria palmata L.) Inhibits Primary Human Neutrophil Activation. Mar Drugs 17(610). Doi:10.3390/md17110610.

Mine I, Tatewaki M (1994) Attachment and fusion of gametes during fertilization of Palmaria sp. (Rhodophyta). J Phycol 30: 55–66

Mizuta H, Yasui H, Saga N (2003) A simple method to mass produce monospores in the thallus of *Porphyra yezoensis* Ueda. J Appl Phycol 15**:** 345-349.

Morgan KC, Wright JLC, Simpson FJ (1980) Review of chemical constituents of the red alga *Palmaria palmata* (dulse). Economic Botany 34(1): 27–50.

Morgan KC, Simpson FJ (1981a) The cultivation of Palmaria palmata. Effect of light intensity and temperature on growth and chemical composition. Botanica Marina 24: 547–552.

Morgan KC, Simpson FJ (1981b) Cultivation of *Palmaria palmata* (Rhodymenia): Effect of high concentrations of nitrate and ammonium on growth and nitrogen uptake. Aquatic Botany 11: 167–171.

Morrisey J, Kraan S, Guiry MD (2001) A guide to commercially important seaweeds on the Irish coast. Bord lascaigh Mhara (BIM), Dublin.

Moroney NC, Wan AH, Soler-Vila A, Fitzgerald RD, Johnson MP, Kerry JP (2015) Inclusion of *Palmaria palmata* (red seaweed) in Atlantic salmon diets: effects on the quality, shelf-life parameters and sensory properties of fresh and cooked salmon fillets, J. Sci. Food Agric. 95: 897–905.

Moss JR, Doty MS (1987) Establishing a seaweed industry in Hawaii: and initial assessment. Aquaculture Development Program of the Hawaii State Department of Land and Natural Resources, Honolulu, Hawaii. Pp. 73.

Mouritsen OG, Dawczynski C, Duelund L, Jahreis G, Vetter W, Schröder M (2013) On the human consumption of the red seaweed dulse (Palmaria palmata (L.) Weber & Mohr. J Appl Phycol 25: 1777–1791.

Mumford TF, Miura A (1988) *Porphyra* as food: cultivation and economics. In Lembi C. A. & Waaland, J. R. (Eds.) *Algae and Human Affairs*. Cambridge University Press, London, pp. 87-117.

Murata M, Nakazoe J (2001) Production and use of marine algae in Japan. Japan Ag Res Quarterly 35: 281–290.

Nagler PL, Glenn EP, Nelson SG, Napolean S (2003) Effects of fertilization treatment and stocking density on the growth and production of the economic seaweed Gracilaria parvispora (Rhodophyta) in cage culture at Molokai, Hawaii. Aquaculture 219: 379-391.

Nakamura Y, Sasaki N, Kobayashi M, Ojima N, Yasuike M, Shigenobu Y, Satomi M, et al. (2013) The first symbiont-free genome sequence of marine red alga, Susabi-nori (*Pyropia yezoensis*). PLoS One 8: e57122. doi.org/10.1371/journal.pone.0057122.

Naseri A, Marinho GS, Holdt SL, Bartela JM, Jakobsen C (2020) Enzyme-assisted extraction and characterization of protein from red seaweed *Palmaria palmata*. Algal Res 47: https://doi.org/10.1016/j.algal.2020.101849

Neish AC, Shacklock PF, Fox CH, Simpson FJ (1977) The cultivation of Chondrus crispus. Factors affecting growth under greenhouse conditions. Can J Bot 55: 2263-2271.

Nielsen MM, Bruhn A, Rasmussen MB, Olesen B, Larsen MM, Møller, HB (2012) Cultivation of *Ulva lactuca* with manure for simultaneous bioremediation and biomass production. J Appl Phycol 24: 449–458.

Nimura K, Mizuta H, Yamamoto H (2002) Critical contents of nitrogen and phosphorus for sorus formation in four Laminaria species. Bot Mar 45: 184–188.

O'Sullivan L, Murphy B, McLoughlin P, Duggan P, Lawlor PG, Hughes H, Gardiner GE (2010) Prebiotics from marine macroalgae for human and animal health applications. Marine Drugs 8: 2038–2064.

Pang S, Lüning K (2004a) Tank cultivation of the red alga Palmaria palmata: effects of intermittent light on growth rate, yield and growth kinetics. J Appl Phycol 16: 93–99.

Pang S, Lüning K (2004b) Breaking seasonal limitation: year-round sporogenesis in the brown alga Laminaria saccharina by blocking the transport of putative sporulation inhibitors. Aquaculture 240: 531–541.

Pang S, Lüning K (2006) Tank cultivation of the red alga *Palmaria palmata*: year-round induction of tetrasporangia, tetraspore release in darkness and mass cultivation of vegetative thalli. Aquaculture 252: 20–30.

Parjikolaei BR, Kloster L, Bruhn A, Rasmussen MB, Fretté XC, Christensen KV (2013) Effect of Light Quality and Nitrogen Availability on the Biomass Production and Pigment Content of Palmaria palmata (Rhodophyta). Chemical Engineering Transactions 32: 967-972.

Pedersen MF, Borum J (1996) Nutrient control of algal growth in estuarine waters. Nutrient limitation and the importance of nitrogen requirements and nitrogen storage among phytoplankton and species of macroalgae. Mar Ecol Prog Ser 142: 261-272.

Peteiro C, Freire Ó (2009) Effect of outplanting time on commercial cultivation of kelp *Laminaria saccharina* at the southern limit in the Atlantic coast, N.W. Spain. Chinese J Oceanol Limnol 27: 54–60.

Petersen JK, Bjerre AB, Hasler B, Thomsen M, Nielsen MM, Nielsen P (2016) Blå biomasse – potentialer og udfordringer for opdræt af muslinger og tang. DTU Aqua-rapport nr. 312-2016 (2. ed). Institut for Akvatiske Ressourcer, Danmarks Tekniske Universitet. 37 pp.

Polne-Fuller M, Saga N, Gibor A (1986) Algal cell, callus, and tissue cultures and selection of algal strains. Nova Hedwigia 83: 30–6.

Polne-Fuller M (1988) The past, present and future of tissue culture and biotechnology of seaweeds. In Stadler, T., Mollion, J., Verdus, M. C., Karamanos, Y., Morvan, H. and Christan, D. (Eds) Algal Biotechnology. Elsevier Applied Science, London, pp. 111–118.

Pueschel CM (1979) Ultrastructure of tetrasporogenesis in *Palmaria palmata* (Rhodophyta). J Phycol 15: 409-424.

Ramsey UP, Bird CJ, Shacklock PF, Laycock MV, Wright JLC (1994) Kainic acid and 1'-hydroxykainic acid from Palmariales. Nat Toxins 2: 286–292.

Ratcliff JJ, Wan AHL, Edwards MD, Soler-Vila A, Johnson MP, Abreu MH, Morrison L (2016) Metal content of kelp (Laminaria digitata) co-cultivated with Atlantic salmon in an Integrated Multi-Trophic Aquaculture system. Aquaculture(450)-1: 234-243.

Rebours C, Marinho-Soriano E, Zertuche-González JA, Hayashi L, Vásquez JA, Kradolfer P, Soriano G, Ugarte R, Abreu MH, Bay-Larsen I, Hovelsrud G, Rødven R, Robledo D (2014) Seaweeds: an opportunity for wealth and sustainable livelihood for coastal com- munities. J Appl Phycol 26: 1939–1951.

Reddy CKR, Kumar, RK, Siddhanta AK, Tewari A, Eswaran K (2003) In vitro somatic embryogenesis and regeneration of somatic embryos from pigmented callus of Kappaphycus alvarezii (Doty) Doty (Rhodophytaa, Gigartinales)1. J Phycol 39: 610-616

Reis RP, Pereira RRC, Góes, HG (2015) The efficiency of tubular netting method of cultivation for Kappaphycus alvarezii (Rhodophyta, Gigartinales) on the southeastern Brazilian coast. J Appl Phycol 27: 421-426.

Roesijadi G, Copping AE, Huesemann MH, Forster J, Benemann JR (2008) Techno-economic feasibility analysis of offshore seaweed farming for bioenergy and biobased products. Independent Research and development report. IR Number: PNWD-3931. Battelle Pacific Northwest Division. Jane Thomas, IAN, UMCES. 105 pp.

Roque BM, Brooke CG, Ladau J, Polley T, Marsh LJ, Najafi N, Pandey P, Singh L, Kinley R, Salwen JK, Eloe-Fadrosh E, Kebreab E Hess M (2019) Effect of the macroalgae *Asparagopsis taxiformis* on methane production and rumen microbiome assemblage. Animal Microbiome 3 https://doi.org/10.1186/s42523-019-0005-3.

Ryder E, Nelson SG, McKeon C, Glenn EP, Fitzsimmons K, Napolean S (2004) Effect of water motion on the cultivation ofthe economic seaweed Gracilaria parvispora (Rhodophyta) on Molokai, Hawaii. Aquaculture 238, 207–219.

Ryther JH, Goldman JC, Gifford CE, Huguenin JE, Wing AS, Clarner JP, William LD, Lapointe BE (1975) Physical models of integrated waste recycling marine polyculture systems. Aquaculture 5: 163-177.

Rødde RSH, Vårum KM, Larsen BA, Myklestad SM (2004) Seasonal and geographical variation in the chemical composition of the red alga Palmaria palmata (L.) Kuntze. Bot Mar 47: 125–133.

Sahoo D, Yarish C (2005) Mariculture of seaweeds. In: Andersen R (ed) Phycological Methods: algal Culturing Techniques. Academic Press, New York, pp. 219–237.

Sagert S, Schubert H (1995) Acclimation of the photosynthetic apparatus of Palmaria palmata (Rhodophyta) to light quali- ties that preferentially excite photosystem I or photosystem II. J Phycol 31: 547–54.

Sagert S, Schubert H (2000). Acclimation of *Palmaria palmata* (Rhodophyta) to light intensity: Comparison between artificial and natural light fields. J Phycol 36(6): 1119–1128.

Saito A, Mizuta H, Yasui H, Saga N (2008) Artificial production of regenerable free cells in the gametophyte of *Porphyra pseudolinearis* (Bangiales, Rhodophyceae). Aquaculture 281**:** 138-144.

Sanchez-Machado DI, Lopez-Cervantes J, Lopez-Hernandez J, Paseiro-Losada P (2004) Fatty acids, total lipid, protein and ash contents of processed edible seaweeds. Food Chem 85: 439–444.

Sanderson JC (2006). Reducing the environmental impact of sea-cage fish farming through cultivation of seaweed. *PhD Thesis Submission*. Open University, 339 pages.

Sanderson JC, Dring M, Davidson K, Kelly M (2012) Culture, yield and bioremediation potential of *Palmaria palmata* (Linnaeus) Weber & Mohr and *Saccharina latissima* (Linnaeus) C.E. Lane, C. Mayes, Druehl & G.W. Saunders adjacent to fish farm cages in northwest Scotland. Aquaculture 354–355: 128–135.

Sanderson JC (2015) Maximising production of *Palmaria palmata* (Linnaeus) Weber & Mohr, 1805. MASTS Report.

Santelices, B., 1990. Patterns of reproduction, dispersal and recruitment in seaweeds. Oceanography and Marine Biology Annual Review 28: 177-276.

Santelices B, Varela D (1994) Abiotic control of reattachment in Gelidium chilense (Mongtagne) Santelices & Montalva (Gelidiales; Rhodophyta). J Exp Mar Biol Ecol(177): 145-155.

SAPEA (2017) Evidence Review Report: Food from the Oceans - How can more food and biomass be obtained from the oceans in a way that does not deprive future generations of their benefits?

Schiener P, Zhao S, Theodoridou K, Carey M, Mooney-McAuley K, Greenwell C (2017) The nutritional aspects of biorefined Saccharina latissima, *Ascophyllum nodosum* and *Palmaria palmata*. Biomass Conv Bioref 7: 221-235.

Schmedes PS, Boderskov T (2013) Cultivation of two kelp species, *Laminaria digitata* and *Saccharina latissima*, in Danish waters – geographic variation in growth and biochemical composition. MSc thesis. Faculty of Bioscience, Aarhus University.

Scott JL, Dixon PS (1973) Ultrastructure of tetrasporogenesis in the marine red alga *Ptilota hypnoides*. J Phycol 9: 29–46.

Scrosati R (2005) Review of studies on biomass-density relationships (including self-thinning lines) in seaweeds: Main contributions and persisting misconceptions. Phycol Res 53: 224-233.

Simpson HL, Campbell BJ (2015) Review article: dietary fibre-microbiota interactions. Aliment Pharmacol Ther 42:158–179.

Smit AJ (2004) Medicinal and pharmaceutical uses of seaweed natural products: a review. J Appl Phycol 16: 245–262.

Smith JE (2019) - https://scripps.ucsd.edu/labs/coralreefecology/research/seaweed-as-a-tool-for-methane-reduction-in-livestock/

Stévant P, Rebours C, Chapman A (2017) Seaweed aquaculture in Norway: recent industrial developments and future perspectives. Aquaculture International 25: 1373–1390.

Takagi H, Ishiga Y, Watanabe S, Konishi T, Egusa M, Akiyoshi N, Matsuura T, et al. (2016) Allantoin, a stress-related purine metabolite, can activate jasmonate signaling in a MYC2-regulated and abscisic acid-dependent manner. J Exp Bot 67:2519–2532.

Terry LA, Moss BL (1980) The effect of photoperiod on receptacle initiation in *Ascophyllum nodosum* (L.) Le Jol. Br Phycol J 15: 291-301.

Su L, Pang SJ, Shan TF, Li X (2017) Large-scale hatchery of the kelp Saccharina japonica: a case study experience at Lvshun in northern China. J Appl Phycol 29: 3003-3013.

Suwal S, Perreaulta V, Marciniaka A, Tamigneauxc E, Deslandesd E, Bazineta L, Jacquesb H, Beaulieua L, Doyen A (2019) Effects of high hydrostatic pressure and polysaccharidases on the extraction of antioxidant compounds from red macroalgae, Palmaria palmata and Solieria chordalis. J Food Engineering 252: 53-59.

Taelman SE, Champenois J, Edwards MD, De Meester S, Dewulf J (2015) Comparative environmental life cycle assessment of two seaweed cultivation systems in NorthWest Europe with a focus on quantifying sea surface occupation. Algal Res 11: 173-183.

Titlyanov EA, Titlyanova TV, Kadel P, Lüning K (2006) New methods of obtaining planlets and tetraspores of fragments and cell aggregates of meristematic and submeristematic tis- sue of the red alga Palmaria palmata. J Exp Mar Biol Ecol 339: 55–64.

Tseng CK, Wu CY (1962) Mariculture of *Saccharina japonica*. Science Press, Beijing.

Tseng CK (1981) Commercial cultivation. In: Lobban CS, Wynne MJ (Eds.) The Biology of Seaweed. Blackwell Scientific Publication, Oxford, pp. 680–725.

Torres MD, Kraan S, Domínguez H (2019) Seaweed biorefinery. Reviews in Environmental Science and Bio/Technology 18: 335–388.

Uji T, Matsuda R, Takechi K, Takano H, Mizuta H, Takio S (2016) Ethylene regulation of sexual reproduction in the marine red alga *Pyropia yezoensis* (Rhodophyta). J Appl Phycol 28: 3501–3509.

van Ginneken VJT, Helsper J, de Visser W, van Keulen H, Brandenburg WA (2011) Polyunsaturated fatty acids in various macroalgal species from north Atlantic and tropical seas. Lipids in Health and Disease 10: 104. https://doi.org/10.1186/ 1476-511X-10-104.

van der Meer JP, Chen LCM (1979) Evidence for sexual reproduction in the red algae Palmaria palmata and Halosacction ramentaceum. Can J Bot 57: 2452–2459.

van der Meer JP, Todd ER (1980) The life history of Palmaria palmata in culture. A new type for the Rhodophyta. Can J Bot 58: 1250–1256.

Vesk M, Borowitzka MA (1984) Ultrastructure of tetrasporogenesis in the coralline alga *Haliptilon cuvieri* (Rhodophyta). J Phycol 20: 501-515.

Wan AHL, Davies SJ, Soler-Vila A, Fitzgerald R, Johnson MP (2019) Macroalgae as a sustainable aquafeed ingredient. Reviews in Aquaculture 11: 458-492.

Wang X, Sun G, Feng T, Zhang J, Huang X, Wang T, Xie Z et al. (2019) Sodium oligomannate therapeutically remodels gut microbiota and suppresses gut bacterial amino acids-shaped neuroinflammation to inhibit Alzheimer's disease progression. Cell Res 29: 787-803.

Walsh M, Watson L (2011) Part 1A: Market analysis towards the further development of seaweed aquaculture in Ireland. Bord Iascaigh Mhara (BIM), Dublin, unpublished report.

Watson L, Mahona FO, Edwards M, Dring MJ, Werner A (2012) The economics of seaweed aquaculture in Ireland Laminaria digitata and Palmaria palmata. World aquaculture society meeting. Meeting abstract.

Werner A, Dring MJ (2011) Cultivating Palmaria palmata. 'Aquaculture Explained' Series No. 27 Bord Iascaigh Mhara (BIM), Dublin.

Werner A, Dring MJ (2013) Recommendations for optimal ongrowing and harvesting techniques for *Palmaria palmata* in different Irish sites with indications of yield. Irish Sea Fisheries Board. Report - PBA/SW/07/001.

Wichard T, Oertel W (2010) Gametogenesis and gamete release of *Ulva mutabilis* and *Ulva lactuca* (Chlorophyta): regulatory effects and chemical characterization of the "swarming inhibitor". J Phycol 46: 248–259.

Wood E (2018) Cultivation of *Palmaria palmata*: spore release, vegetative propagation and decontamination techniques. Scottish Association for Marine Science (SAMS) and University of the Highlands and Islands.

Woods VB, Moloney AP, Calsamiglia S, O'Mara FP (2003). The nutritive value of concentrate feedstuffs for ruminant animals. Anim Feed Sci Technol 110: 145–157.

Wong KH, Cheung PCK (2000) Nutritional evaluation of some subtropical red and green seaweeds: Part I — proximate composition, amino acid profiles and some physico-chemical properties. Food Chem 71(4): 475-482.

Zhao LS, Li K, Wang QM, Song XY, Su HN, Xie BB, Zhang XY, Huang F, Chen XL, Zhou BC, Zhang YZ (2017) Nitrogen Starvation Impacts the Photosynthetic Performance of Porphyridium cruentum as Revealed by Chlorophyll a Fluorescence. Sci Rep 7: 1-11. Doi: 10.1038/s41598-017-08428-6.

Zhang QS, Tang XX, Cong YZ, Qu SC, Luo SJ, Yang GP (2007) Breeding of an elite Laminaria variety 90-1 through inter-specific gametophyte crossing. J Appl Phycol 19: 303–311.

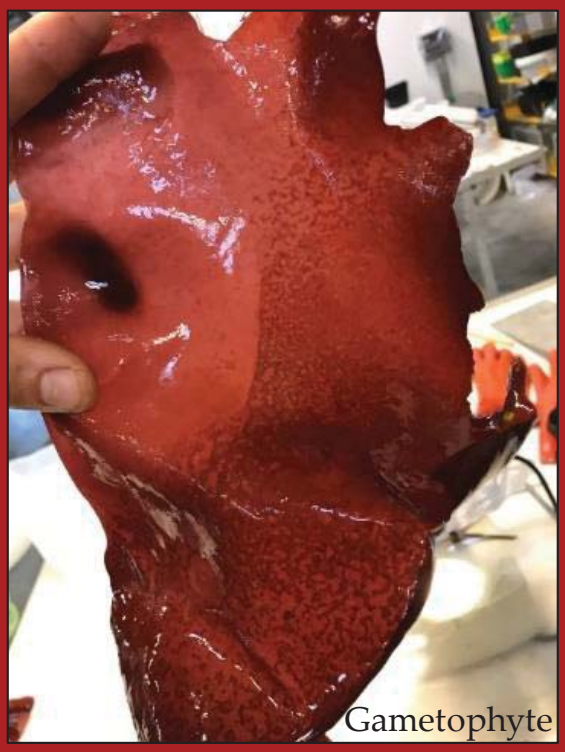
Young DR, Smith WK (1980) Influence of sunlight on photosynthesis, water relations, and leaf structure in the understory species Arnica cordifolia. Ecology 61: 1380–90.

Yuan YV, Bone DE, Carrington MF (2005) Antioxidant activity of dulse Palmaria palmata extract evaluated in vitro. Food Chemistry 91: 485–494.

Yuan YV, Westcott ND, Hu C, Kitts DD (2009) Mycosporine-like amino acid composition of the edible red alga, Palmaria palmata (dulse) harvested from the west and east coasts of Grand Manan Island, New Brunswick. Food Chem 112:321–328.

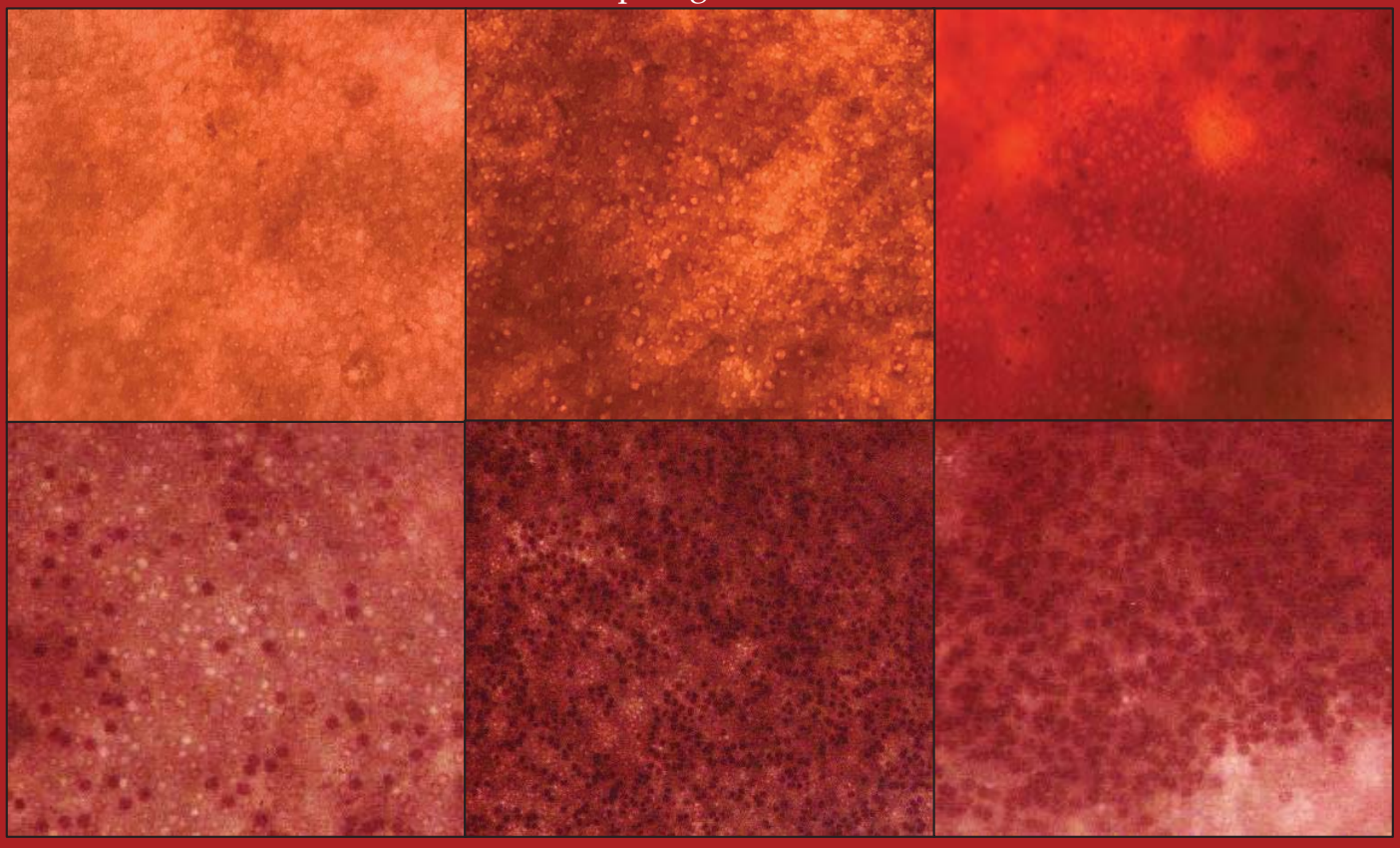
Paper 1: Reproductive phenology and the induction of fertility in *P. palmata*

Sporophytes



Gametophyte

Sporogenesis



Reproductive phenology and investigation on the induction of fertility in *Palmaria palmata* (Rhodophyta)

(Manuscript draft for submission in *Botanica Marina*)

Peter S. Schmedes & Mette Møller Nielsen

Author statement: Peter Schmedes conceptualized the experimental idea constituting fig. 5, who also did the data collection during the first three months October, November, and December, which was finalized by NTNU MSc student Renate Rimstad Bøe until September.

Abstract

Induction of fertility (sorus) in *P. palmata* tetrasporophytes is paramount for the timing of spore seeding in the hatchery to propagate biomass in sea-based cultivation. During summer and early autumn, absence of fertility overlap the desired season initiating the hatchery. Manipulating proximate factors induce fertility; yet, few studies have investigated the success during the season of low natural occurrence. The present study investigates the reproductive phenology of a Norwegian intertidal population by monthly collections and screening of biological polyamines of interest. Furthermore, present study investigate fertility induction in *P. palmata* collected at different seasons in Danish waters using manipulative exposure experiments. The phenology study showed fertility occurrence from early October to June in the Norwegian population with increasing spore yield approaching the peak season (Jan-March) with an overlap in fertile sporophytes and gametophytes. In December, fertile fronds significantly upregulated the content of the polyamine tyramine. The induction experiments document that short day length, presence of meristems and experimental days significantly affect the formation of sporangial initials. High nutrient concentration is crucial in the formation and maturation of fertile sporangia. Low temperature (5 °C) promotes sorus and spermatangial tissue. Red light exposure seems to promote sporangia formation, while blue light showed inhibitory effect. Antibiotics seems to prevent induction and development of tetrasporogenesis suggesting the involvement of surface-associated microbes. By only 6-20 % sporophyte induction obtained during summer, management of year-round spore seeding for large-scale cultivation of *P. palmata* seems challenging. This study discuss other hatchery strategies to circumvent spore supply for extended seedling production.

Key words: Sporogenesis; reproduction; rhodophyte cultivation; polyamines; tetraspores

1. Introduction

The control of algal life history stage gates to access tetraspores is crucial in sea-based cultivation of seaweeds like the cold-water rhodophyte *P. palmata* (Linnaeus) F. Weber & D. Mohr as seeding in hatchery commonly use spore-releasing fertile plants (sorus tissue) to inoculate substrates (fig.1A-C). Since van der Meer and Chen (1979) and van der Meer and Todd (1980) documented the diplohaplont life cycle of *P. palmata* (fig. 1, center) a seminal hatchery protocol to produce seedlings for *P. palmata* cultivation in North Atlantic waters prescribes best access to spore donor material by collection during winter where peak in tetrasporophyte fertility occurs (Werner and Dring 2011). The diploid tetrasporophyte and haploid male gametophyte frond are isomorphic until fertility, see dark dashed area on 2n and dashed area in the male “n”. The tetrasporophyte disperse haploid tetraspores (fig. 1A-B) while male the releases spermatangial gametes at ripe fertility (fig. 1D). Fertility does not restrict to the basal section, like shown in the diagrammatic drawing as sporangia is also found in distal sections, except the marginal frond edges, which are meristematic. The hatchery protocol rely

on inter-dependent steps, thus terminating the seedling production by the lack of success in any step, e.g., by incidence of lacking sporulation (Wood 2018) or high spore mortality (Edward 2007, Werner and Dring 2011). Therefore, a continuous access to spores will provide basis to iterate the spore-seeding step by incidence of failure, and optionally extend the hatchery production. While studies have shown methods to improve the spore release rate, the success in spore release and spore yield ultimately depends on the maturity status of sporangia (Le Gall et al. 2004, Werner and Dring 2011, Wood 2019, Paper 2). Full control of the spore availability by year-round induction of spore-containing tissue have been suggested but is still incomplete (Pang and Lüning 2006, Titlyanov et al. 2006, Sanderson 2006, Edward 2007, Wood 2018). For cultivation of *P. palmata* in NA waters, the hatchery production is desirably conducted during late summer, as seedlings require a couple of months in nursery to grow into a suitable size for deployment in autumn, thereby increasing the success of the cultivation effort and maximizing the biomass yield (Edwards and Dring 2011, Paper 5). Despite recent improvements in spore-seeding technique and extensional use of propagule seedstock for *P. palmata* (Paper 2, Paper 3), the access to ripe sorus in well advance of the growth season is a bottleneck for large-scale, standardized and reliable cultivation (Werner and Dring 2011).

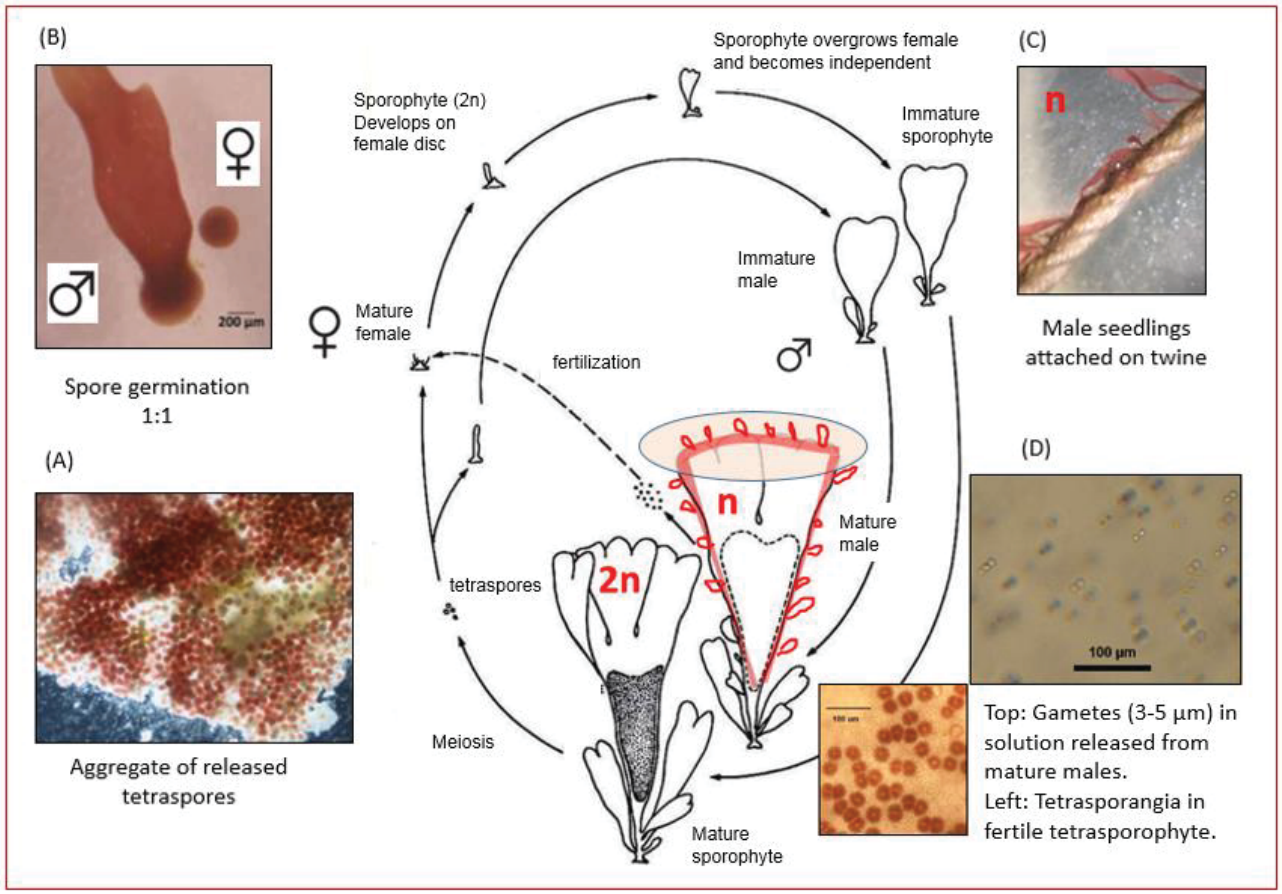


Figure 1. Centre: Diagrammatic presentation of the diplohaplont life cycle of *Palmaria palmata* with alternating isomorphic generations of the diploid tetrasporophyte (2n) and the haploid male gametophyte only distinguishable at ripe fertility (modified after van der Meer and Todd, 1980). The dark-shaded tetrasporangial basal sorus (2n) and the dashed line (n) indicate fertile tissue. Fronds grow by meristematic activity in apical

tips (exemplified on the male; orange oval), however, growth also displays as numerous primary and secondary proliferations along frond edges in both generations (red sprouting shoots along light red shaded frond edge). Peripheral photos: A) aggregate of tetraspores after sporulation in the seeding tanks. B) After attachment, spores germinate equally into male and female gametophytes (photo credit C. Constanza). Female gametophytes grow into microscopic crustose-like individuals and seem to live up to 8 months without fertilization. By fertilization, a new sporophytic thallus overgrow the tiny female gametophyte. Thus, inclusion of fertilization in hatchery production theoretically enhance spore use efficiency by factor 2. C) Male sporelings attached on twine develop frond thallus immediately after spore settlement and become fertile within 9-12 months, thus male gametes (spermatia) to ensure fertilization must come from a previous generation male plant. D) Upper: solution containing male gametes (spermatia) lower: cortex embedded tetrasporangia in a fertile tetrasporophyte.

The growth and reproduction of macroalgae is seasonal dependent and controlled by complex interactions between sensing changes in the environment, especially day length which they perceive using their photoreceptors, and stored nutrient reserves (Cortel-Breeman and Hoopen 1978, Dring 1984, Dring 1988, Agrawal 2012). In NA waters, *P. palmata* elicit highest growth rate (length) from spring and well into summer where solar light fuel autotrophic photosynthesis and promotes the build-up of nutrient reserves (Faes and Viejo 2003, Martinez et al. 2006, Sanderson 2006). In the following stationary growth phase photosynthates are put into accumulating energy reserves (floridian starch, floridosides) while the relative content of nitrogen are low (Martinez and Rico 2002, Simon-Colin 2003, Martinez et al. 2006). In autumn, the decreasing day length is of its highest rate along with decreasing water temperatures and replenishment of ambient nutrients, which in combination constitute the seasonal effect involved in algae reproduction (Bartsch et al. 2008, de Bettignies et al. 2018).

The basis for perceiving the seasonal change in light is photoreceptors which conduit a modulation of the cellular transcriptome, collectively in control of the signaling pathways for the circannual rhythm of seaweed growth and reproduction. This signaling mobilizes the stored energy reserves to fuel the formation of reproductive structures and spores (Hegemann et al. 2001, Liu et al. 2017). The formation of cortical sporangial tissue (sorus) in *P. palmata* is a gradual `reproductive morphogenesis´, i.e. the transition of the vegetative tissue into the reproductive state, (van der Meer and Todd 1980, Kain 1986, Henry 1988, Guiry and Dawes 1992) (fig. 2). In the pursuit of year-round induction of tetrasporangia in *P. palmata*, it is desirable to distinguish sporophytic fronds from males at an early point, which currently is recognized at mature fertility. Thus, a description and photo-guide to make an early identification of tetrasporophytes would benefit cultivators management of spore donor stock needed for a planned seedling production, but has yet not been formulated.

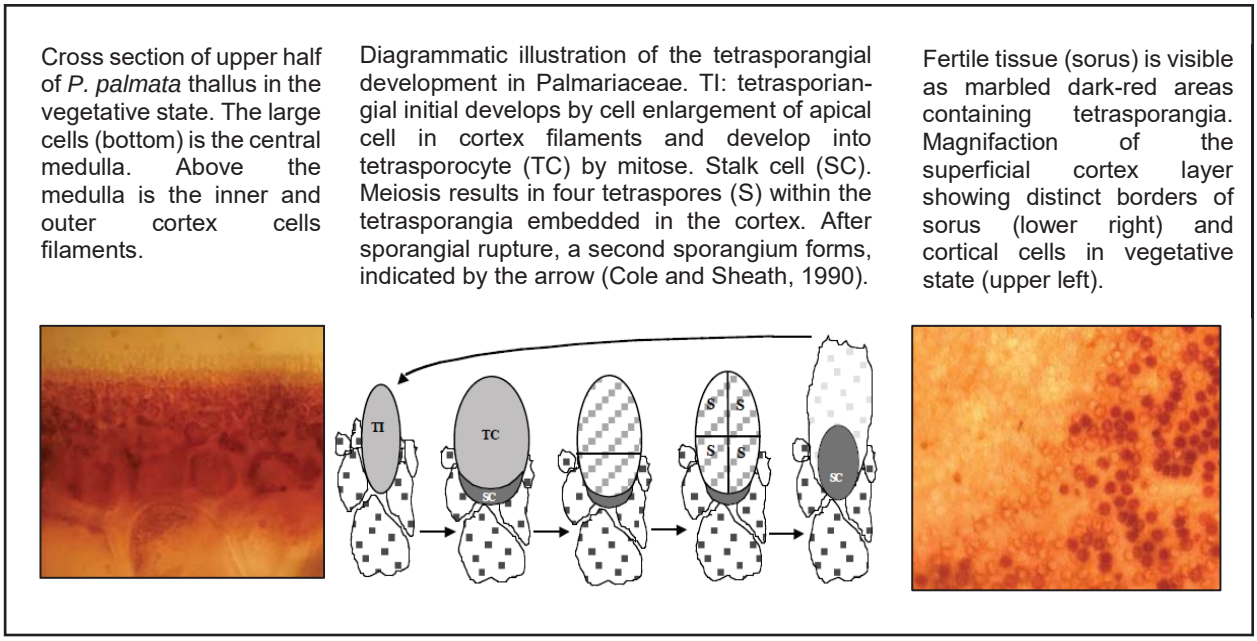


Figure 2. Diagrammatic explanation of the tetrasporangial development in Palmariaceae (Rhodophyta).

Recently, revisit of the coupling of algal environmental sensing and their cellular signaling pathways control of the onset of sexual reproduction, focus on the involvement of metabolites (García-Jiménez et al. 2018). The biosynthesis of biogenetic polyamines, like putrescine, allantoin, spermidine, spermine, as well as exogenously administrated gaseous ethylene and other phytohormones is researched (Bradley 1991; Marián et al. 2000, Gúzman-Urióstegui et al 2002, García-Jiménez and Robaina 2012, Kumar et al. 2015, Takagi et al. 2016, Uji et al. 2016). Using transcriptome analysis, research shed light on genes presumably governing the pathways regulating these compounds in red alga reproduction, which probe relevant for seaweed aquaculture by administrating metabolites for algal uptake. Potentially, exogenous administrated metabolites taken up by the alga induce fertility, yet their coupling to photoreceptor control and ethylene receptor signaling in algae is not fully documented (Kumar at al. 2015, Uji et al. 2016, García-Jiménez and Robiana 2015). Spectral quality of light shows to affect sporogenesis, where blue light induced sporangial tissue earlier than red and white light in *Laminaria japonica* (Mizuta et al. 2007). In contrast, red light showed to increase sporogenesis in another brown macroalgae *Dictyota dichotoma* (Hudson JV Lamouroux) (Bogaert et al. 2016). The in-depth understanding of the light sensing-hormone interaction in rhodophytes and the involvement of blue light perceiving cryptochromes and potentially the co-action with ultraviolet-photoreceptors are being researched (Cashmore et al. 1999, Collén et al 2013, Liu et al. 2017). Moreover, seaweed surface-associated microbes seems to produce phytohormone-like substances, which potentially is involved in the regulation of reproduction of some seaweeds (Prasad et al. 2010, Agrawal 2012).

Several studies have documented the reproductive phenology of *P. palmata* in NA waters (Ireland, France, Portugal), showing that fertility depends on the geographic location and season (Kain 1982, Le Gall et al 2004). In general, December to April is the peak season for tetrasporangia-, or spermatia-containing *P. palmata* in intertidal populations, whereas absence of sorus seems to occur from summer until autumn (Kain 1986, Faes and Viejo, 2003, Werner and Dring, 2011). The absence of fertile plants during summer and autumn pose a

mismatch in timing of hatchery production and outgrow season, which potentially compromise the harvest yield in the following spring by postponing the deployment, assuming a one-year production cycles. Optionally, spore-seeded substrates nursed for an extended period to develop seedlings in controlled `over-summering´ conditions is applicable, however, this practice implies an additional hatchery cost (Werner and Dring 2011, Paper 3).

Recent studies suggest year-round induction of tetrasporangial tissue in *P. palmata* by culturing submeristematic tissue, to avoid sporulation inhibitor substances produced in the meristems, at manipulated winter-like conditions, using short daylength and 10 ° C (Titlyanov et al. 2006, Pang and Lüning 2006). By this, the manipulated culture conditions ought to trigger cellular signaling pathways to modulate any circannual reproductive rhythm or accelerate the ongoing morphological process of sporangial formation (García-Jimenez and Robaina 2012).

Though promising for the induction of fertility, it remains unrevealed whether manipulation of proximate factors and exogenous administrated polyamines induce tetrasporogenesis during summer, where fertility is absent in *P. palmata*. With the increasing attention on cultivating *P. palmata* in European, a documentation of the annual reproductive status and the effect of manipulating environmental factors on sporogenesis is of interest.

Present study aims to document the reproductive status in a population in the Trondheimsfjorden (Norway), assess the spore yield in autumn as well as screening the content of common red-algal polyamines in vegetative and reproductive fronds. Additionally, the study investigates a stepwise identification of the reproductive morphogenesis in *P. palmata* to provide a practical guide for early identification of maturing sporophytes. Taking into account different pre-acclimation (season, culture condition) present study evaluate the effect of environmental factors on the onset of tetrasporogenesis by use of comprehensive stereomicroscopy of the cortex cell layer undergoing a transitional morphological change.

2. Material and methods

2.1.1 Reproductive status, spore yield and screening of selected polyamines in P. palmata in a Norwegian intertidal population

P. palmata was handpicked monthly (n=146-266 individual fronds) to assess the status (%) of reproductive, vegetative and unhealthy fronds in the intertidal population at Storsteinan (Trondheimsfjorden, NO) during October 2017 to September 2018 (Bøe 2019). Visual inspection of fronds revealed the apparent fertility status before verified as either fertile sporophyte or maturing/mature male gametophyte by stereoscope magnification. The ratio of fertile, healthy/unhealthy vegetative plants was evaluated by each monthly collection, except July.

2.1.2 Spore yield assessment. From fertile fronds collected in October, November, and December pieces of sori (0.5-1 cm^2) were immediately cut after rinsing and fresh weight noted (n=6) before submerged in individual wells (16-well plate) containing clean seawater (5 mL). The daily spore yield g^{-1} FW was estimated by leaving the sori in stagnant conditions at 10 °C and 12:12 h day length (15 µmol photons m^{-2} s^{-1} PAR) and by

transferring the sori to new wells on a daily basis, for 7-13 days. After scratching the bottom, well-mixed subsamples (125 µL) was poured into a raft-counting well on an objective glass and red tetraspores was by use of magnification manually counted. The counts was used a basis to estimate the spore release yield g FW^{-1} for each month.

2.1.3 Screening biogenic polyamines. For the plants collected on December 4 2017, the content of selected biogenic polyamines was analyzed in vegetative or reproductive whole fronds from different plants (n=3) after verifying their status as either vegetative or reproductive sporophyte by 400 times magnified inspection. The material was stored immediately (-20 °C) in separate zip lock bags until analysis. By solvent extraction and formation of dansylchlorid derivatives, samples run through a HPLC Waters Spherisorb ODS2 column to separate the derivatives. By using an internal standard, the concentration of selected polyamines was estimated using a PDA detector (254 nm) providing chromatographic peaks. The concentration of selected polyamines was calculated based on the integrated area of peaks for tryptamine, phenylethylamine, putrescine, histamine, serotonin, tyramine, spermine, and spermidine by limit of quantification of 13 ppm (National Food Agency, DANAK accredited).

2.1.4 In culture induction and blocking. Vegetative fronds from November (n=200) and December (n=41) batches were cultured (SINTEF OCEAN Sealab, Trondheim) in flow-through tanks (25 L) to assess the time required for fertility development. The November fronds were cultured using local filtered seawater from 80 meters depth (5-8° C) and exposed to a short day (SD) length of 8:16 h in four separate tanks. Three tanks were setup up for the December batch exposing fronds to either SD 8:16 h (white or blue light) of 50 µmol photons m^{-2} s^{-1} PAR or long day (LD) length of 16:8 white light, receiving the same daily dose of photons (25 µmol photons m^{-2} s^{-1} PAR). Placing two layers of blue silk paper above the container provided the blue light treatment. The development in fertility was monitored in all batches by weekly inspections.

2.1.5 Observations on reproductive status in Danish populations. In different seasons, *P. palmata* (n=10-100) was collected from subtidal populations in inner Danish waters (2016-2019) as a basis to evaluate the gradual reproductive morphogenesis (black crosses, fig. 3). The collected vegetative plants showed no signs of fertility (tissue color pattern or lumpiness).

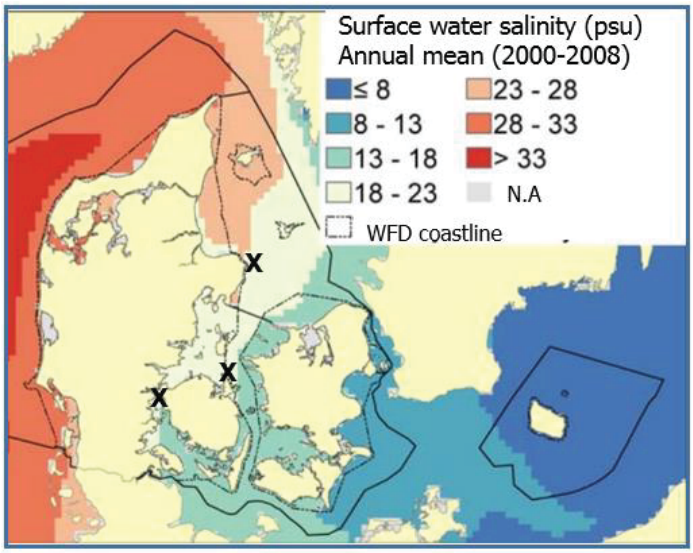


Figure 3. Map of Denmark showing (crosses) coastal sites for collection of *P. palmata* samples.

2.2 Induction experiments

Three experiments were conducted to induce fertility in *P. palmata* by manipulating different environmental parameters. In aquaculture of *P. palmata* the demand for fertility induction is highly relevant for improving the timing of hatchery seeding. Hence, the *P. palmata* used in following experiments was collected in different seasons (early summer, late summer, and spring) to cover the season of absence of fertility.

2.2.1 Induction exp.1.

A batch of vegetative plants was sampled at 2.5 meters depth near Fornæs lighthouse, Denmark, (56.443534 N, 10.958985E) August 31, 2016. Plants were temperature acclimated for one week by gradually lowering the temperature from 13 ° C to 10 ° C and cultured in 1 µm filtered and UV treated seawater with a salinity of 26-28 psu (400 L). Nutrients (20% F/2+ media, Guillard and Ryther 1962) was added twice per month to provide a concentration of ~180 µM-NO_3 and warm white fluorescent tubes provided surface irradiance of 80 µmol photons m^{-2} s^{-1} PAR in a day length of 16:8 h (L:D). On October 25, 80 fronds (length ranging 3-20 cm) were detached from the base of ten individual plants. Fronds were individually divided into 80 beakers (400 mL), using weekly water renewals, to constitute a factorial cultivation design of 16 different exposure treatments (n=5) by combining four factors with each two levels (4 lower rows in table 1).

Table 1. Description of dependent variables in induction exp. 1.

Class of variable	Level	Values
Dates	5	04.01.2017; 21.12.2016; 14.12.2016; 07.12.2016; 30.11.2016
Photoperiod (day length)	2	LD (10:16 h), SD (5:19h) of 35 µmol photons m^{-2} s^{-1} PAR
Meristem (+M/-M)	2	Presence/Absence
Temperature	2	Autumn-like (10° C) Winter-like (5 ° C)
Nutrient concentration	2	High (100% 882 µM-N) Low (10 % 88.2 µM-N)

Presence or absence of meristem (+M/-M) was prepared by removing the margin tissue edge (0.5 cm) of fronds. LD and SD denotes long and short day length. All beakers were cultured at 10 ° C for 24 days before

half of the containers were transferred to 5 ° C. The treatment effects were assessed by weekly stereoscope inspection of five random fields per frond during November 30 to January 4 (2017) and evaluated by morphological changes in the cortex layers at 5 dates (table 2). The cortex status was asserted one of two categories using binomial scores (0,1), where “1” denotes a positive identification of a morphological feature underlining the sporogenesis and “0” denotes no signs of fertility. Further, the category “1” was denoted either “c” (initial onset of sporangia) or “C” (progressed sporangial development) and refers to the reduced or full size of the hyaline distinct cells embedded in the cortex layer, interpreted as the sporangial initials or progressed young sporocytes, according to Pueschel (1979), see fig. 2. Thus, “c” is a prerequisite of “C”. At the “C” state and by further cell enlargement the cortex surface reveal a “lumpy texture” (Werner and Dring 2011). When lumpy cortical tissue areas showed dark-red cells a frond scored “F” in the fertility (“1”) category, regardless presence or absence of the distinct tetrad cruciate pattern in tetrasporangia, thus this category does not strictly infer ripe fertility.

2.2.2 Induction exp.2 (a+b)

a) Vegetative plants were collected by divers July 9 (2017) at 4-6 meter depth at Northern Funen (Fyns Hoved, 55.610895°N 10.594308°E) and regarded an “early summer collection”. Fronds were thin-leafed and grew in rosette-like appearance from a basal blade. The largest fronds appeared pale green while smaller fronds (3-15 cm) were light red and detached from the basal blade and pre-cultured in indoor tank facilities at 10 ° C. These smaller fronds (n=15) were stocked in ten experimental tanks of 6 liters and exposed to different treatments (table 2). During the experimental duration, the number of fronds reduced in some tanks (n=5-15). Allantoin is found to enhance to archeospore formation in foliose *Pyropia yezoensis* (Mizuta et al. 2003), why the effect of this polyamine is relevant to investigate on the induction of tetrasporogenesis in *P. palmata*. In general, physiological stress is known to trigger sexual reproduction in plants and algae, thus the application of short spells of desiccation and alternating temperatures is supposed to stress *P. palmata*. During four months, the occurrence (%) of fertile fronds was estimated on a weekly basis by inspection. A final assessment was done after additional two months (January 11, 2018).

Table 2. Description of treatment parameter levels used in induction exp.2. Ten tanks containing *P. palmata* fronds (n=5-15) were exposed to each of ten treatments. Daylength regimes (light:dark h) provided an irradiance of 30 µmol photons m^{-2} s^{-1} PAR. Covering tank surfaces (red light filter) provided red light treatment. Daily, fronds in tank 5 were drained and left in air for 2 h, while fronds in tank 6 were placed in an empty tank covered by a black plastic bag at room temperature for 2 h (darkness). –M=meristematic tissue cut off as in exp. 1.

Container/ Treatment	Frond status	Light color and intensity	Day length (L:D)	Nutrient (% F/2+)	°C	Add-on
1	(-M)	White 30 PAR	8:16	10	5	
2	Whole	White 30 PAR	8:16	10	5	
3	Whole	Red 30 PAR	8:16	10	5	
4	Whole	White 30 PAR	8:16	10	5	10 mM Allantoin
5	Whole	White 30 PAR	8:16	10	5	Desiccation (2h/d)
6	Whole	White 30 PAR	6:18	10	5-15	2 h darkness + alternating °C
7	Whole	White 30 PAR	16:8	10	5	
8	Whole	White 30 PAR	16:8	10	10	
9	Whole	White 30 PAR	8:16	0	5	
10	Whole	White 30 PAR	8:16	100	5	

b) An two separate experiments the effect of exogenous administrated spermidine (n=20) and ethylene (n=5) was tested using vegetative fronds (n=20) collected in field August 23, 2017, constituting a “late summer” sample and were cultured with similar conditions container no. 10), but only 50 % F/2+ nutrient addition. The polyamine spermidine was added (Sigma Aldrich) to provide a concentration of 239 µM during culture. Spermidine has been associated in maturing red algae reproductive structures by exogenous administration (Guzmán-Urióstegui et al. 2002). However, yet to be investigated for the effect in *P. palmata* tetrasporogenesis. Ethylene was administrated via gaseous infusing using a setup of 1 L DURAN blue cap bottles. Ethylene (C2H2) was bubbled (2 L min^{-1}) through a wooden air stone (microbubbles) into the seawater for 0, 10, 30, 60, and 120 seconds before ceiling the bottles containing a vegetative frond of *P. palmata*. Ethylene is a ubiquitous plant growth regulator and potentially involved in regulation reproduction in some algae. Plants were inspected every week for two months.

2.2.3 Induction exp.3

On April 4, 2019, small fronds of *P. palmata* (5-10 cm) was sampled at 2-3 meters depth in the Little Belt (Middelfart, Denmark) and cultivated in flow-through (0.5 flushing h^{-1}) tumble cultures (25 L) sourced with nutrient rich (>1000µM-NO_3) filtered seawater (14 °C) from the land-based salmon RAS facility (Atlantic Sapphire, Denmark) receiving ambient irradiance. After two months (May 28), the largest fronds (15-40 cm) were brought to indoor tank facilities and cultured in filtered seawater with nutrient addition twice a week (20 % F/2+). At May 28, (tissue %C, %N is currently being analyzed, n=3) fronds (n=5-29) were exposed to a combination of four treatments including light intensity (15 or 70 µmol photons m^{-2} s^{-1} PAR) and temperature (5 ° C or 10 ° C). The daylength was 16:8 h (L:D) for all treatments during the first 47 days (until July 15), then adjusted to 8:16 h (L:D) to simulate an abrupt shift.

By weekly inspection of fronds, signs of reproductive morphogenesis was noted like in exp1. Additionally, maturing and matured male gametophytes were noted, according to fig.4. The presence of maturing or mature males showed no presence of distinct hyaline cells (sporangial initials) and were asserted by the spermatangial tissue pattern and intensity of color (see fig.3).

After 92 days (September 1), some of the thalli in the group denoted “10°C-70 PAR” was transferred to constitute three additional transplant treatment groups cultured at 5 °C and denoted “T-70 PAR-5°C”, “T-15 PAR-5°C”, and “T-15 PAR-5°C +blocking” (n=10). In the latter, a solution of antibiotic Penicillin G-sodium and Streptomycin sulfate salt at a concentration of 50 mg/L of each was added twice during the first 20 days. This was assumed to remove (block) gram-positive surface-associated microbes potentially involved in sporogenesis.

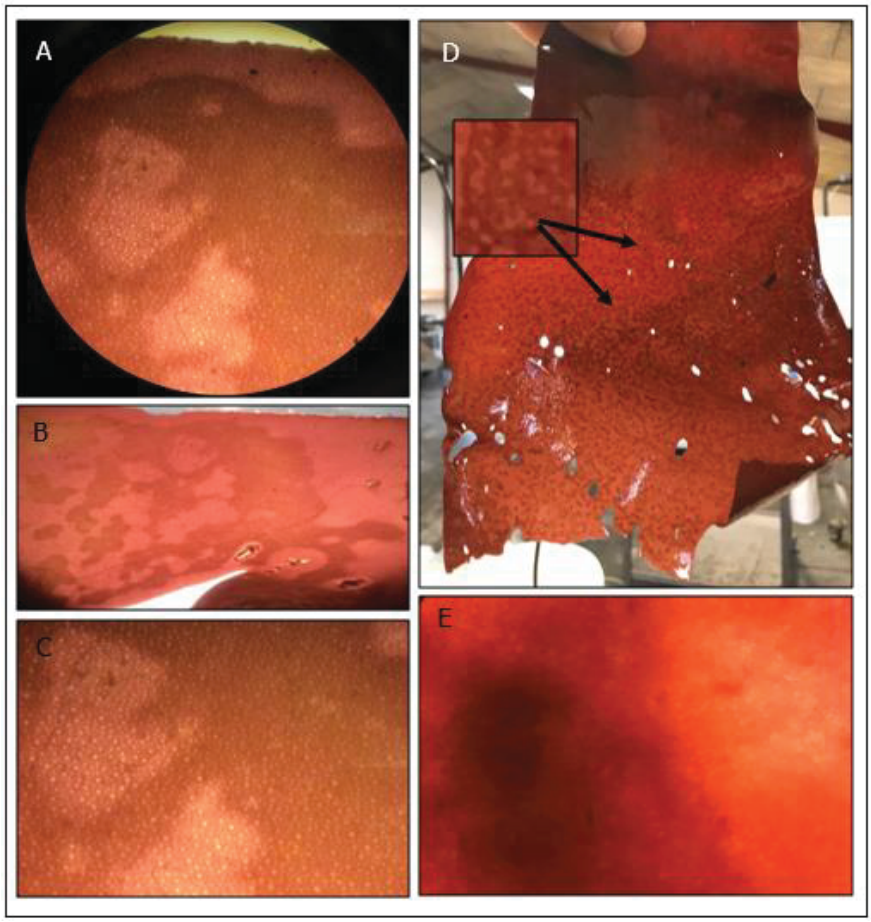


Figure 4. A-C: Inspection views asserting tissue apperance as maturing male, while D-E asserting mature males.

3. Statistics

Induction exp.1. To account for the use of the same containers during the experiment “dates” was included as nested within replicate as a random factor, using Proc Glimmix in GLM-mode to run a generalized linear mixed model (SAS 9.3) assuming Gaussian random effects. The response variables (c, C, F) all followed a

binomial distribution. The model included photoperiod, meristem, temperature, nutrient concentration and dates, which were treated as categorical variables (table 2). Differences in the mean total content of polyamines as well as between the mean of spore yield (log-transformed) were analyzed by use of the Welch´s anova test. Alfa was 0.05.

4. Results

4.1 Frond status, spore yield and screening of selected polyamines in the intertidal population at Storsteinan (Trondheimsfjorden)

The collection of *P. palmata* in Trondheimsfjorden revealed an overlapping peak in fertile sporophytes (32-50%) and male gametophytes (23-33%) during January to March (fig. 5). In November and April about 10-20 % of fronds revealed as fertile sporophytes. The spore yield (g^{-1} FW d^{-1}) in December was significant higher (welch´s $_{(1, 105)}$, F=25: p<0.0001) compared to November, while no sporulation occurred until day 7 in the October batch (fig. 6). The polyamine analysis revealed that content of tyramine in fertile tissue was above limit of quantification, while spermidine and spermine provided no peaks. The sum of the three detected polyamines (total concentration of tyramine, putrescine, and serotonin) was significantly higher in fertile compared to vegetative fronds in December (welch´s $_{(1,2.8)}$, F=52:p=0.0067), mainly due to the higher content of tyramine in fertile tissue (fig. 7).

In November, 14 % of the vegetative fronds cultured *in vitro* at 8:16 h (L:D) in flow-through had developed sorus within 24 days. By continued cultivation for 18 days, only additional 4 % of the vegetative from this batch turned fertile. This indicates a potential suboptimal tank effect on developing sporangia from November to December as 32% was fertile in the field in early December. By culturing the vegetative fronds from the December batch *in vitro* 71-73% developed fertility within 14 days regardless of day length (8:16 h vs. 16:8 h), while only 51 % in the SD blue light treatment was fertile sporophytes (n=68). This indicates a high maturation in tank cultures regardless day length and potentially a negative effect of blue light.

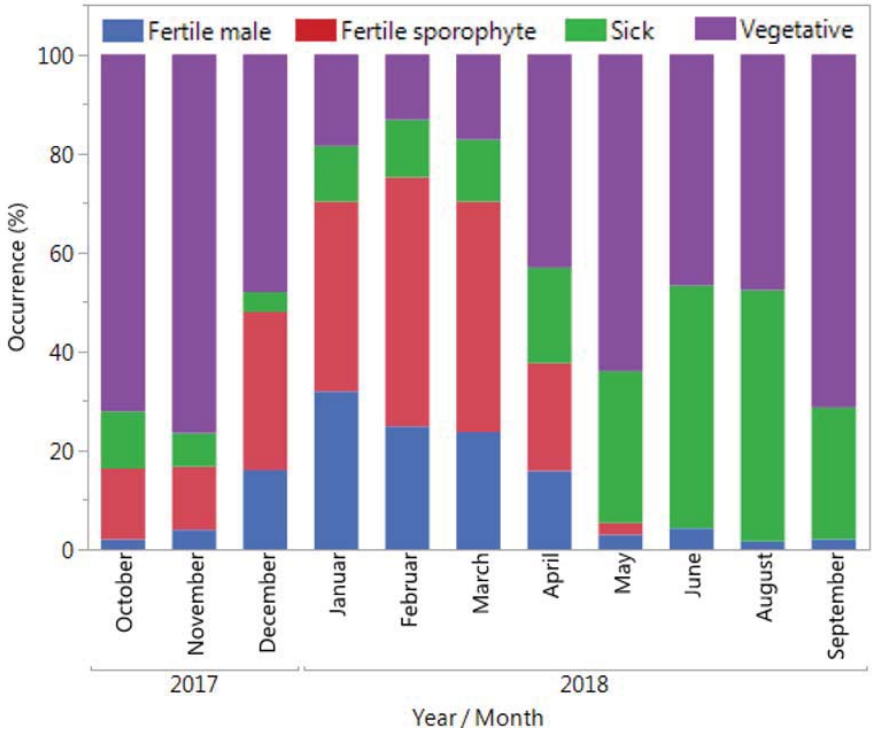


Figure 5. Seasonal change in the percentage of *P. palmata* plants with the appearance of fertile male (n), fertile sporophyte (2n), sick, or in vegetative state from the Storsteinan population (Trondheimsfjorden). The number of collected plants ranged from 141-266 with no collection in July. (Modified after Bøe 2019).

4.2 Spore yield in October, November, and December

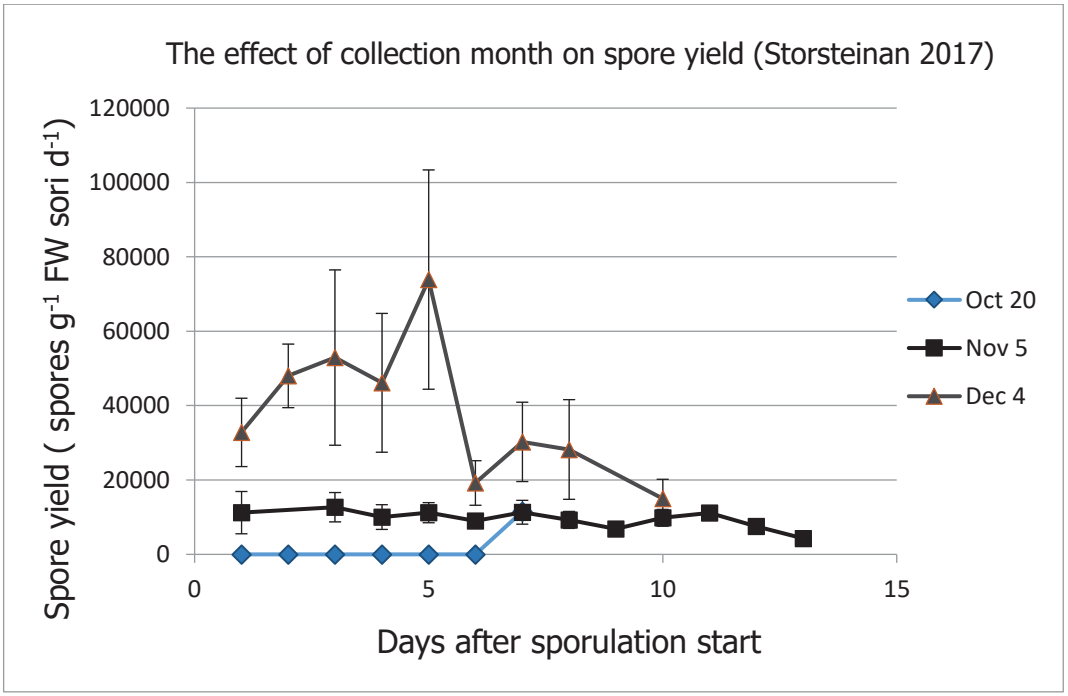


Figure 6. The release of tetraspores (spores g^{-1} FW sori d^{-1}) as a function of sporulation days in pieces of fertile sori (0.5-1 cm^2) from fertile sporophytes collected from the intertidal population at Storsteinan (Trondheimsfjorden, Norway) in Oct, Nov, and Dec 2017. The single pieces of sori sporulated in wells (5mL) and daily transferred to a new well. Data points are mean±SE (n=6).

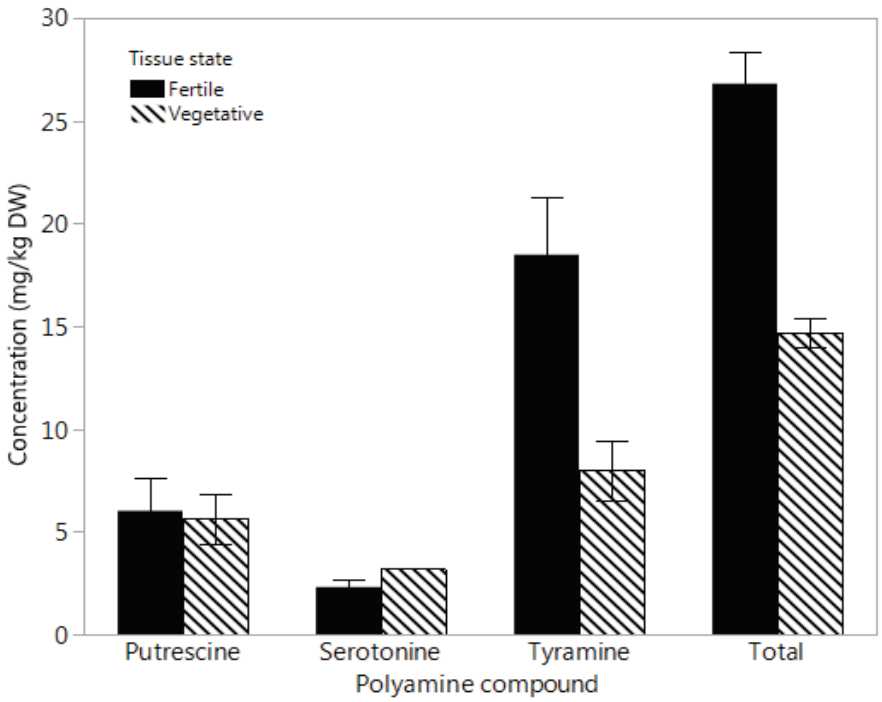


Figure 7. Concentrations (mg/kg; ppm) of three biogenetic polyamines detected in (whole frond) fertile and vegetative *P. palmata* collected from the intertidal population at Storsteinan (December 4, 2017) and their total concentration (n=3) at a limit of quantification of 13 mg/kg (ppm). Spermidine and spermine was not detected. Data points represent the mean±SE (n=3).

4.3 Observations on reproductive status in some Danish populations

By sporadically collections of *Palmaria palmata* in different intertidal and upper subtidal populations in inner Danish waters (Fornæs Light house, Fyns Hoved, and Little belt) fertility was not present from May to early October (n=20-100), see fig. 3. In January, fronds (n=100) collected in the splash zone at the Fornæs lighthouse site revealed 70 % maturing males and 30 % fertile sporophytes, with sporophytic fertility in fronds bigger than 3 cm in length. On March 23, fronds showed no signs of dark-red marbling patterns (n=100, 2-20 cm), indicating that the peak in fertility occurs between late October and early March. However, only 60-70 km south of the Fornæs population (Kalundborg, near Fyns Hoved), plants collected August 28 from 8-16 meters depth all showed fertile or maturing tissue patches on older blades (n=10). This indicate a positive interaction of effect of water depth and season for early formation of sporangia in *P. palmata* (Norall et al 1981).

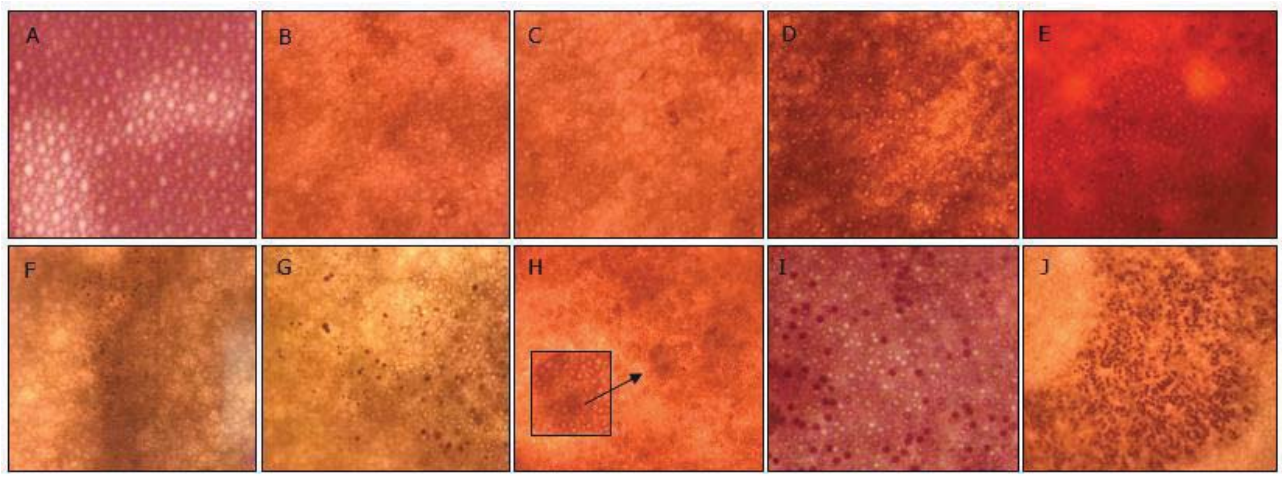


Figure 8. A guide for early sporophyte identification. Stereomicroscopy views of the cortical morphological transition from vegetative (A) to ripe fertile tetrasporophytes (I-J) which show pronounced variation within fronds – a practical guide for early identification (B-D) of sporophytic fronds prior to mature fertility. B-E: The tiny, distinct hyaline cells (tetrasporangial initial) embedded in the inner cortex of maturing sporophytes can be found before and concomitantly with bigger cells forming the tetrasporocyte (E-I; progressed sporangial development). The sporocytes enlarge and mature to form sorus containing ripe fertile tetrasporangia visible as various marbling patterns. Sorus may cover the whole frond surface in peak season.

4.4 Induction exp.1.

The sporangial initials (“c”) were significantly induced by short day length, the presence of meristems, 5 ° C and time (days) (table 3). As the presence of “c” and “C” (progressed development of sporangia) was overlapping, the exposure of short day length also affected the “C” significantly. While the meristem treatment and time did not affect “C”, only high nutrients concentration induced this state and these parts of the tissue appeared with lumpy surfaces. The following fertility stage “F” was also significantly affected by high nutrient concentration (table 3).

Table 3. Statistical results of the Proc Glimmix GLMM analysis in induction exp.1.

Type III Tests of Fixed Effects				
Effect ("c")	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Photo period	1	347	12.56	**0.0004**
Meristem	1	347	7.23	**0.0075**
Temp	1	347	27.99	**<.0001**
Nut	1	347	1.29	0.2572
Day	4	44	9.35	**<.0001**
Effect ("C")	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Photo period	1	347	4.76	**0.0298**
Meristem	1	347	0.15	0.6966
Temp	1	347	0.02	0.8965
Nut	1	347	7.16	**0.0078**
Day	4	44	1.75	0.1556
Effect ("F")	**Num DF**	**Den DF**	**F Value**	**Pr > F**
Photo period	1	347	0.16	0.6912
Meristem	1	347	2.10	0.1482
Temp	1	347	0.86	0.3553
Nut	1	347	11.88	**0.0006**
Day	4	44	0.71	0.5890

4.5 Induction exp.2 (a+b).

In the field-collected material (early summer; July 9 2017), no signs of sporogenesis induction was observed in any of the treatment groups during the first four months from July to early November. At the final assessment (January 11, 2018), after additional two months, some of the fronds had developed sporangia (fig. 9). There was a tendency that sporangia was associated in fronds which had grown the fewest number of marginal proliferations (data not shown), which suggest a localized trade-off between energy allocation for either growth or reproduction. The exogenous administrated polyamines allantoin promoted a thick layer of slime to cover the fronds, which deteriorated the fronds. Spermidine and ethylene exposures did not exert any effect – all fronds remained non-fertile, even after 3 months.

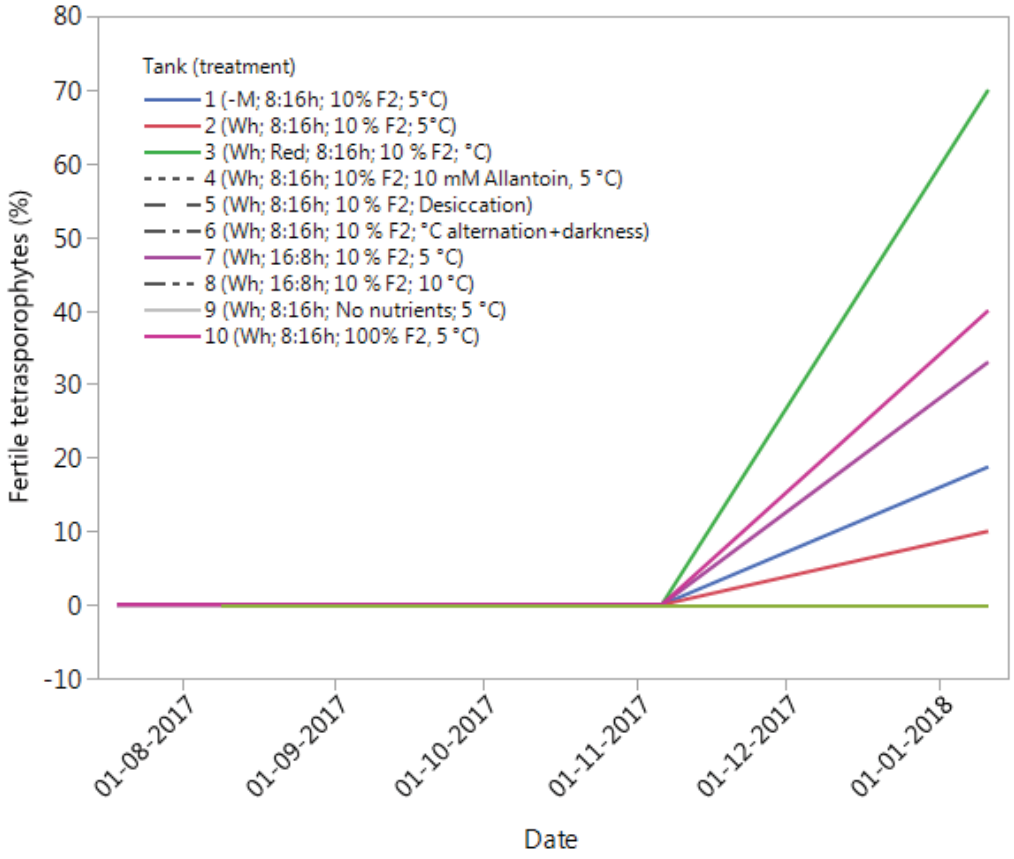


Figure 9. The percentage of fertile sporophytes induced in vegetative tetrasporophytes collected early summer July 9 (2017) after treatment exposure cultivation in ten tanks (6L). Ten different treatments groups were cultivated (n=10-12). No sign of fertility revealed during the first four months where some treatment groups were abandoned (gray colored). After additional two month (Nov-Jan), some fronds had developed fertile sporangia.

4.6 Induction exp.3.

The spring field-collected material, growing rapidly in nutrient rich flow-through bubble before sampled and exposed to *in vitro* induction exposures from May 28 to October 7, 2019 (fig. 9). Figure 9 shows the results of the different induction exposures after 75 days until 130 days after initiating the experiment.

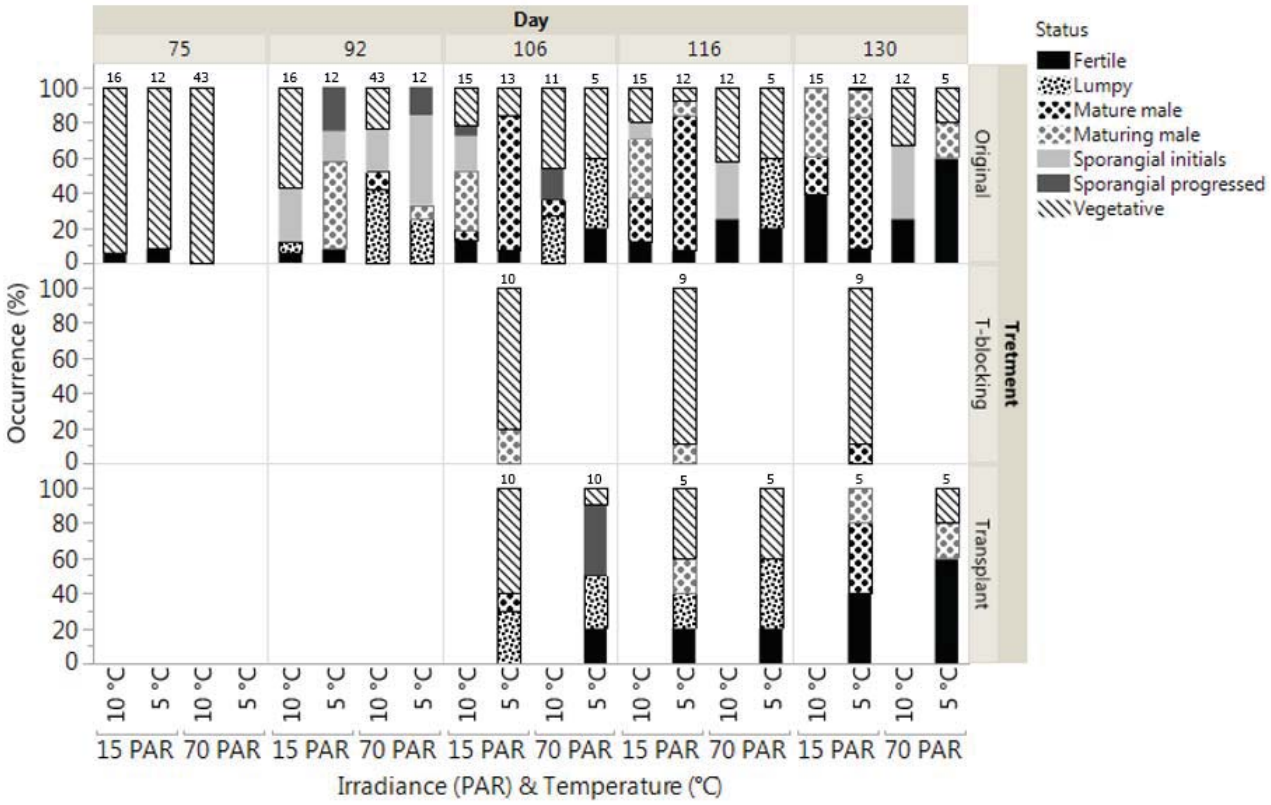


Figure 9. The temporal development in tissue status (percentage occurrence, %) of P. palmata fronds cultured for 130 days since May 28, 2019. The plants were sampled in field April 4 and grown in nutrient rich flow-through seawater sourced from a landbased salmon RAS farm before exposed to different combinations of induction exposures. After 92 days, the "original" treatment group "10°C 70 PAR" was split into three transplant groups at 5 °C (15 vs. 70 PAR and a group denoted T-blocking which was added antibiotics. The number of fronds in each treatment varied during the experiment, indicated by the number above the columns.

After two and a half months of exposure (day 75, August 12) 6 % (1 out of 16) and 8 % (1 out of 12) of fronds at 15 PAR-10°C and 15 PAR-5°C, respectively, was fertile sporophytes. Beside this, none of the other fronds (70 PAR, n=43) showed signs of reproductive morphogenesis, indicating some interspecific differences in maturation. The majority of the fronds at 5 °C appeared soft in texture and with a slimy surface, in contrast to the rigid texture in fronds at cultured at 10 °C, regardless of light intensity. From the original treatment groups (fig. 9 upper), the results showed overall a trend for mature males (n=13) was present after 106 days (15 PAR-5° C) and after 130 days at higher irradiance promoted 60% occurrence of fertile sporophytes (n=5). After 37 days, the transplant group added antibiotic solution revealed only 11 % maturing males (n=9). The transplants group T-5°C-70 PAR revealed 60 % fertile sporophytes and 20 % maturing males (n=5) while the T-5°C-15 PAR revealed 40 % fertile sporophytes, 20 % maturing and 40 % mature males (n=5).

5. Discussion

The present study demonstrated the induction of tetrasporophytic fertility in *P. palmata* during summer and early autumn when the natural occurrence of spore-containing fronds are absent. The fertility response in male gametophyte was faster than tetrasporophytes using manipulation treatments of winter conditions. The short seasonal window of fertile *P. palmata* sporophytes in inner Danish waters emphasizes the temporal mismatch between desired season for spore-seeding in hatchery and the period for out-grow deployment, which highlight the need for successful induction methods during summer. The absence of fertile sporophytes from May - likely already from late March - until September in the intertidal Norwegian and Danish populations is in line with previous studies from Northern Portugal (Faes and Viejo 2003), the coasts of Brittany (Le Gall et al. 2004), but differs to recordings from and Isle of Man (Kain 1982, 1986). Results of the induction experiments suggests that the formation of sporangial initials are significantly induced during summer, in the Danish samples, by short day exposure at 5-10° C and with presence of meristems. By winter collection, vegetative fronds (Norwegian *in vitro* cultures) became fertile at all conditions. These results are in line with previous studies on several temperate short-day macroalgae (van der Meer and Todd 1980, Guiry and Dawes 1992, Buchholz and Lüning 1999, Pang and Lüning 2006, Agrawal 2012) and support the present findings on the reproductive phenology in the Storsteinan population (fig. 5), where fertility is likely induced during early or mid-September. The positive effect of meristem presence in *P. palmata* supports Pang and Lüning (2006) who induced sporangia within 3-5 months in apical tissue pieces. In contrast, the present result is potentially contrasting to Titlyanov et al. (2006). The mentioned studies induced sporangia formation during January and May in isolated tissue submeristematic tissue fragments and in freeze-thawed cell aggregates and stressed that that release of sporulation inhibitor compounds might be triggering sporogenesis in *P. palmata*.

High nutrient concentration was not involved in the early fertility induction (table 3), however was crucial for the ongoing sporocyte development resulting in the presence of fertile sorus (induction exp. 1), and in support to previous literature (Dring 1974, 1984). At insufficient nutrient availability sporangia maturation turned into black cells, indicating a termination of its development (personal observation). During the early season of natural peak in fertility (November-December), 14-73% of the vegetative fronds collected at Storsteinan exposed to either SD or LD white light treatments developed fertility within 24-14 days respectively. This suggests that long daylength do not inhibit fertility induction in this season, which is supportive to Edwards (2007). The use of blue light filters showed to reduce sporophytic fertility in December (Norwegian cultures) while red light – in the summer induction experiment - seemed to promote sporogenesis (exp. 2, fig. 8). This suggests that the reproductive morphogenesis in *P. palmata* is under photoreceptor control with potential effects of light spectral quality. Hence, by collection in autumn the fronds have perceived the change in daylength and spectral quality which triggers a cellular mechanism to onset of sporangial initials, of which their maturation is not reversible by abrupt chance to long daylength exposures. This is supportive to previous studies (Dring and West 1983, Breeman and Hoopen 1987). In the December batch, spores started releasing within the first hour and continued for at least 10 days providing a daily mean spore yield of 38,487±13,854 spores g^{-1} FW (mean±SE, n=6). In contrast, fertile tissue collected in October provided a spore yield of 11,500±3,138 spores g^{-1} FW (mean±SE,n=6) by sporulation on day 7. This result suggests substantial variation in sporangia ripeness and spore release readiness in the months relevant to hatchery activity (Paper 2).

Relevant for hatchery planning, present study found a required duration of more than 5 months from mid-summer to induce the formation of sporangia by using winter-like conditions (high nutrient concentration and short daylength), which is in accordance to Pang and Lüning (2006) testing the other end of the reproductive window (January-June). In contrast, using high growth and nutritious pre-cultivation of vegetative fronds in ambient light during April-June showed to develop sporophytic and male fertility in late August early September after 1-4 months of winter-like exposure (exp. 3). By this pre-cultivation, fronds obtained a relative high tissue nitrogen content (being analyzed) prior to the induction exposures emphasizing the importance of sufficient nutrient reserves as a pre-requisite for induction. The fact that fertile sporophytes is some habitats can be found almost year-round, for instance at temperature stabile intertidal population in Ireland, and during late summer in deeper water populations suggests that the interaction with nutrient status and the individual perception of the environment controls when a frond becomes fertile (Edwards 2007). In support, no clear temporal trend in the occurrence of tetrasporangia was found in a Portuguese population due to high fluctuations in abiotic factors on small scales (Martinez and Rico, 2002).

The elevated content of the polyamine tyramine in fertile fronds, suggests some involvement in the reproductive initiation or maturation. Tyramine is a well-known polyamine with vasoactive effects found in many eukaryote organisms. The content of tyramine was not analyzed in the August material when fronds showed soft and slimy presence (exp. 2). However, it is likely that tyramine could be involved in this `softening of thallus´, required for the enlargement of reproductive cortical cells in *P. palmata*, which is believed to be an early event in the red algae reproduction (García-Jiménez and Robiana, *Insight into...*). The content of putrescine, a precursor in the biosynthesis of spermidine and spermine (Guzmán-Urióstegui et al. 2002), showed similar contents in fertile and vegetative fronds while spermidine and spermine was not detected. Likewise, the exogenous administrated polyamines and ethylene exerted no effect on frond reproductive status, which either indicate a narrow time frame for their metabolism (anabolism-catabolism) of specific polyamines, or, that these substances have no biological relevance in *P. palmata*. Whether *P. palmata* possess biochemical pathways for synthesis of polyamines and ethylene is yet unknown.

Adding a solution of antibiotics seems to restrict the maturation of males, and potentially prevent sporophytic fertility. However, with only nine biological replicates, it remains speculative whether antibiotics mediates a reduction in fertility by preventing the effect of surface-associated microbes.

Conclusions and perspectives

The required duration of exposure treatment to obtain fertility and access *P. palmata* tetraspores is greatly depended on season, pre-acclimation and nutrient status. During summer, the natural occurrence of fertile plants is very low or absent in Danish and Norwegian intertidal populations. The cultivation of *P. palmata* in large-scale by annual cultivation cycles at sea using spore-seeded substrates pose a challenge for optimizing hatchery production. In present study, the best manipulative exposure treatments, promoted only 6-60% sporophytic fertility induction within 1-4 months during summer, though with no incidence of spore release, which seems insufficient for a reliable hatchery production at commercial scale. Hence, the achievement of high success in fertility induction (e.g. 60-90% fertility) during low natural season (July-Sept) demands more research for this rhodophyte. As of yet, year-round spore production within a reasonable timeframe seems

unlikely by collection of wild material, where fertility is only inducible in the peripheral season (autumn and spring) by use of winter-like environmental conditions. Potentially, by applying high nutrient and growth conditions during spring and early summer, fertility in *P. palmata* achieves by an abrupt shift to autumn or winter-like conditions. Present study indicates that consecutive exposures of good growth and nutrient conditions followed by autumn conditions (5-10 °C and elevated ratio of red light) during summer is potentially a useful method for inducing fertility during low natural season. Alternatively, by recent modifications in the hatchery spore-seeding methods, the sori usage efficiency in *P. palmata* cultivation based on the natural occurrence of tetraspores was greatly improved (Paper 3). In addition, a secondary seedling production, using the GMA-seeding method greatly improves the use of overall efficiency of tetraspores by utilizing the adhesive germinated propagules as seedstock. This method enables an extension of the hatchery production of seeded substrates by at least 39 days based on the ability of germinated propagules to form discoid reattachment, which result in good quality seedlings. Still, year-round access to tetraspores would benefit a stable hatchery production. In perspective, utilizing the emitted resources (nutrients, clean cooled water) from a landbased fish farm to sustain the required nutritional pre-acclimation of tissue that natural habitats does not deliver in late spring and augments a resource synergy which benefit the environmental sustainability and economics of running a seaweed hatchery.

Overall, induction of sporophytic fertility is demanding for optimized biomass yield within one-year production cycle. Using efficient spore-seeding methods in the hatchery may sustain a feasible cultivation relying on fertile plants from wild stocks and using a shifted production strategy, whereas the induction of male fertility might become increasingly important to improve the utilization of tetraspores and screening for robust diploid sporelings in selective breeding. Half of the spores develop into females only by fertilization, which potentially is more robust than haploid males seedlings. Revealing the application of female gametophytes entails a substantial improvement of the hatchery production by extending the seeding activity using females as seedstock. This demands further investigation in their cryptic life and reproductive susceptibility.

Acknowledgements

Parts of this work is done in collaboration with Norwegian University of Science and Technology (NTNU) and SINTEF OCEAN, and was partly funded by the Joint Doctoral Degree
Agreement between the National Institute of Aquatic Resources (DTU Aqua) at Technical University of Denmark and the Norwegian University of Science and Technology (NTNU), Norway, as well as the Tang.nu project (under Grant Agreement No. 13744, Velux Foundation) and the MacroSea project (grant no. 254883) and MIND-P (grant no. 268338/E50) funded by the Research Council of Norway. We thank Jorunn Skjermo providing laboratory and field equipment at SINTEF OCEAN and Renate Rimstad Bøe for finalizing the collection of samples and major contribution to the fertility assessment in the Trondheimsfjorden population. We thank Kasper Lenda Andersen for his help maintaining the cultures during the induction experiments and Thorsten Johannes Skovbjerg Balsby for statistical analysis of induction exp.1.

References

Agrawal SC (2012) Factors controlling induction of reproduction in algae-review: the text. Folia Microbiol 57: 387-407. DOI 10.1007/s12223-012-0147-0

Bartsch I, Wiencke C, Bischof K, Buchholz CM, Buck BH, Eggert A, Feuerpfeil P, et al (2008) The genus *Laminaria* sensu lato: recent insight and development. *Eur. J. Phycol. 43*: 1–86.

Bogaert K, Beeckman T, De Clerck O (2016) Abiotic regulation of growth and fertility in the sporophyte of *Dictyota dichotoma* (Hudson) JV Lamouroux (Dictyotales, Phaeophyceae). J Appl Phycol 28: 2915–2924.

Bradley PM (1991) Plant hormones do have a role in controlling growth and development of algae. J Phycol 27: 317–321.

Breeman AM, Hoopen AT (1987) The mechanism of daylength perception in the red alga *Acrosymphyton purpuriferum*. J Phycol 23 36-42.

Buchholz C, K Lüning (1999) Isolated, distal blade discs of the brown alga *Laminaria digitata* form sorus, but not discs, near to the meristematic transition zone. J Appl Phycol 11: 579–584.

Bøe RR (2019) Investigation of important steps in *Palmaria palmata* cultivation. Master´s thesis (NTNU), Norwegian University of Science and Technology Faculty of Natural Sciences, Department of Biology.

Cashmore, A.R., Jarillo, J.A., Wu, Y., Liu, D., 1999. Cryptochromes: blue light receptors for plants and animals. Science 284, 760–765.

Cole KM, Sheath RG (1990) *Biology of the red algae*. Book. Cambridge University Press. ISBN: 978-0-521-20246-6.

Collén J, Porcel B, Carré W, Ball SG, Chaparro C, Tonon T, Barbeyron T, et al. (2013) Genome structure and metabolic features in the red seaweed Chondrus crispus shed light on evolution of the Archaeplastida. Proc. Natl. Acad. Sci. USA 110: 5247–5252.

Cortel-Breeman AM, Hoopen A ten (1978) The short-day response in *Acrosymphyton purpuriferurn* (J. Ag.) Sjost. (Rhodophyceae, Cryptonemiales). Phycologia 17: 125-132

de Bettignies T, Wernberg T, Gurgel CFD (2018) Exploring the influence of temperature on aspects of the reproductive phenology of temperate seaweeds. Front Mar Sci 5: 218. doi: 10.3389/fmars.2018.00218

Dring, MJ (1974) Reproduction. In: (W.D.P. Stewart, ed) Algal physiology and biochemistry. Blackwell Scientific Publication, Oxford. pp. 814–837.

Dring MJ, West JA (1983) Photoperiodic control of tetrasporangium formation in the red alga *Rhodochorton purpureum*. Planta 159: 143–150.

Dring MJ (1984) Photoperiodism and phycology. Prog Phycol Res 3: 159–192.

Dring MJ (1988) Photocontrol of development in algae. Annu Rev Plant Physiol Plant Mol Biol 39: 157–174.

Edwards MD (2007) The cultivation of the edible red alga, *Palmaria palmata* for aquaculture. PhD Thesis, Queen's University, Belfast.

Edwards MD, Dring MJ (2011) Open-Sea cultivation trial of the red alga, *Palmaria palmata* from seeded tetraspores in Strangford Lough, Northern Ireland. Aquaculture 317:203–209.

Faes VA, Viejo RM (2003) Structure and Dynamics of a Population of *Palmaria Palmata*. J Phycol39: 1038-1049.

García-Jiménez P, Robaina RR (2012) Effects of ethylene on tetrasporogenesis in *Pterocladiella capillacea* (rhodophyta). J Phycol 48: 710-715.

García-Jiménez P, Robaina RR (2015) On reproduction in red algae: further research needed at molecular level. Frontiers in Plant Science. doi.org/10.3389/fpls.2015.00093.

García-Jiménez P, Robaina RR *Insight into the mechanism of red alga reproduction. What else is beyond cystocarps development?* Chapter in *System Biology*. Doi: 10.5772/intechopen.83353.

García-Jiménez P, Llorens C, Roig FJ, Robaina RR (2018) Analysis of the Transcriptome of the Red Seaweed *Grateloupia imbricata* with Emphasis on Reproductive Potential. Mar Drugs 16(490).

Guiry MD, Dawes CJ (1992) Daylength, temperature and nutrient control of tetrasporogenesis in *Asparagopsis armata* (Rhodophyta). J Exp Mar Biol Ecol 158:197-219.

Guillard RRL, Ryther JH (1962) Studies on marine planktonic diatoms I. Cyclotella nana Hustedt and *Detonula confervacea* (Cleve) Gran. Can J Microbiol 8:229–239.

Guzmán-Urióstegui A, García-Jiménez P, Marián F, Robledo D, Robaina R (2002) Polyamines influence maturation in reproductive structures of *Gracillaria cornea* (Gracialariales, Rhodophyta). J Phycol 38: 1169-1175.

Hegemann P, Fuhrmann M, Kateriya S (2001) Algal sensory photoreceptors. J Phycol 37: 668-676.

Henry EC (1988) Regulation of reproduction in brown algae by light and temperature. Bot Mar 31: 353-357.

Kain JM (1982) The reproductive phenology of nine species of Rhodophyta in the subtidal region of the Isle of Man. British Phycological Journal 17: 321–331.
Kain JM (1986) Plant size and reproductive phenology of six species of rhodophyta in subtidal isle of man, British Phycological Journal 21(2): 129-138.
Kumar M, Reddy CRK, Ralph PJ (2015) Polyamines in morphogenesis and development: a promising research area in seaweeds. Frontiers in Plant Science 6: 27.
Le Gall L, Pien S, Rusig AM (2004) Cultivation of *Palmaria palmata* (Palmariales, Rhodophyta) from isolated spores in semi-controlled conditions. Aquaculture 229: 181–191
Liu X, Bogaert K, Engelen AH, Leliaert F, Roleda MY, De Clerck O (2017) Seaweed reproductive biology: environmental and genetic controls. Bot Mar 60(2): 89-108.
Lüning K (1988) Photoperiodic control of sorus formation in the brown alga *Laminaria saccharina* . Mar Ecol Prog Ser 45(1): 137–144.
Lüning K, Wagner A, Buchholz C (2000) Evidence for inhibitors of sporangium formation in *Laminaria digitata* (Phaeophyceae) during the season of rapid growth. J Phycol 36(6): 1129–1134.
Marián FD, García-Jimenez P, Robaina RR (2000) Polyamines in marine macroalgae: Levels of putrescine, spermidine and spermine in the thalli and changes in their concentration during glycerol-induced cell growth in vitro. Physiologia Plantarum 110(4): 530-534.
Martínez B, Viejo R, Rico JM, Rødde, RH, Faes V, Oliveros J, Alvarez D (2006) Open sea cultivation of *Palmaria palmata* (Rhodophyta) on the northern Spanish coast. Aquaculture 254: 376–387.
Mizuta H, Yasui H, Saga N (2003) A simple method to mass produce monospores in the thallus of *Porphyra yezoensis* Ueda. J Appl Phycol 15: 345-349.
Mizuta H, Kai T, Tabuchi K, Yasui H (2007) Effects of light quality on the reproduction and morphology of sporophytes of Laminaria japonica (Phaeophyceae). Aquac. Res. 38: 1323–1329.
Norall TL, Mathieson AC, Kilar JA (1981) Reproductive ecology of four subtidal red algae. J Exp Mar Biol Ecol 54: 119-136.
Pang S, Lüning K (2006) Tank cultivation of the red alga *Palmaria palmata*: Year-round induction of tetrasporangia, tetraspore release in darkness and mass cultivation of vegetative thalli. *Aquaculture*, 252(1): 20–30.
Prasad K, Das AK, Oza MD, Brahmbhatt H, Siddhanta AK, Meena R, Eswaran K, et al., (2010) Detection and quantification of some plant growth regulators in a seaweed-based foliar spray employing a mass spectrometric technique sans chromatographic separation. J Agric Food Chem 58: 4594–4601.
Pueschel CM (1979) Ultrastructure of tetrasporogenesis in Palmaria-palmata (Rhodophyte). J Phycol 15: 409–429.
Sanderson JC (2006) Reducing the environmental impact of sea-cage fish farming through cultivation of seaweed. PhD thesis, UHI Millenium Institute & Scottish Association of Marine Science (SAMS).
Simon-Colin C (2003) Complete ^{1}H and ^{13}C spectral assignment of floridoside. Carbohydr Res 337(3): 279–80.
Takagi H, Ishiga Y, Watanabe S, Konishi T, Egusa M, Akiyoshi N, Matsuura T, et al. (2016) Allantoin, a stress-related purine metabolite, can activate jasmonate signaling in a MYC2-regulated and abscisic acid-dependent manner. J Exp Bot 67: 2519–2532.
Titlyanov EA, Titlyanova TV, Kadel P, Lüning K (2006) New methods of obtaining plantlets and tetraspores from fragments and cell aggregates of meristematic and submeristematic tissue of the red alga *Palmaria palmata*. J Exp Mar Biol Ecol 339(1): 55–64.
Uji T, Matsuda R, Takechi K,Takano H, Mizuta H, Takio S (2016) Ethylene regulation of sexual reproduction in the marine red alga *Pyropia yezoensis* (Rhodophyta). J Appl Phycol 28: 3501–3509.
van der Meer JP, Todd ER (1980) The life history of *Palmaria palmata* in culture. A new type for the Rhodophyta. Can J Bot 58:1250–1256.
Werner A, Dring MJ (2011) Aquaculture explained: cultivating *Palmaria palmata*. Irish Sea Fisheries Board, report no. 27 - project PBA/SW/07/001. Dublin.
Wood E (2018) Cultivation of *Palmaria palmata*: spore release, vegetative propagation and decontamination techniques. Scottish Association for Marine Science (SAMS) and University of the Highlands and Islands.

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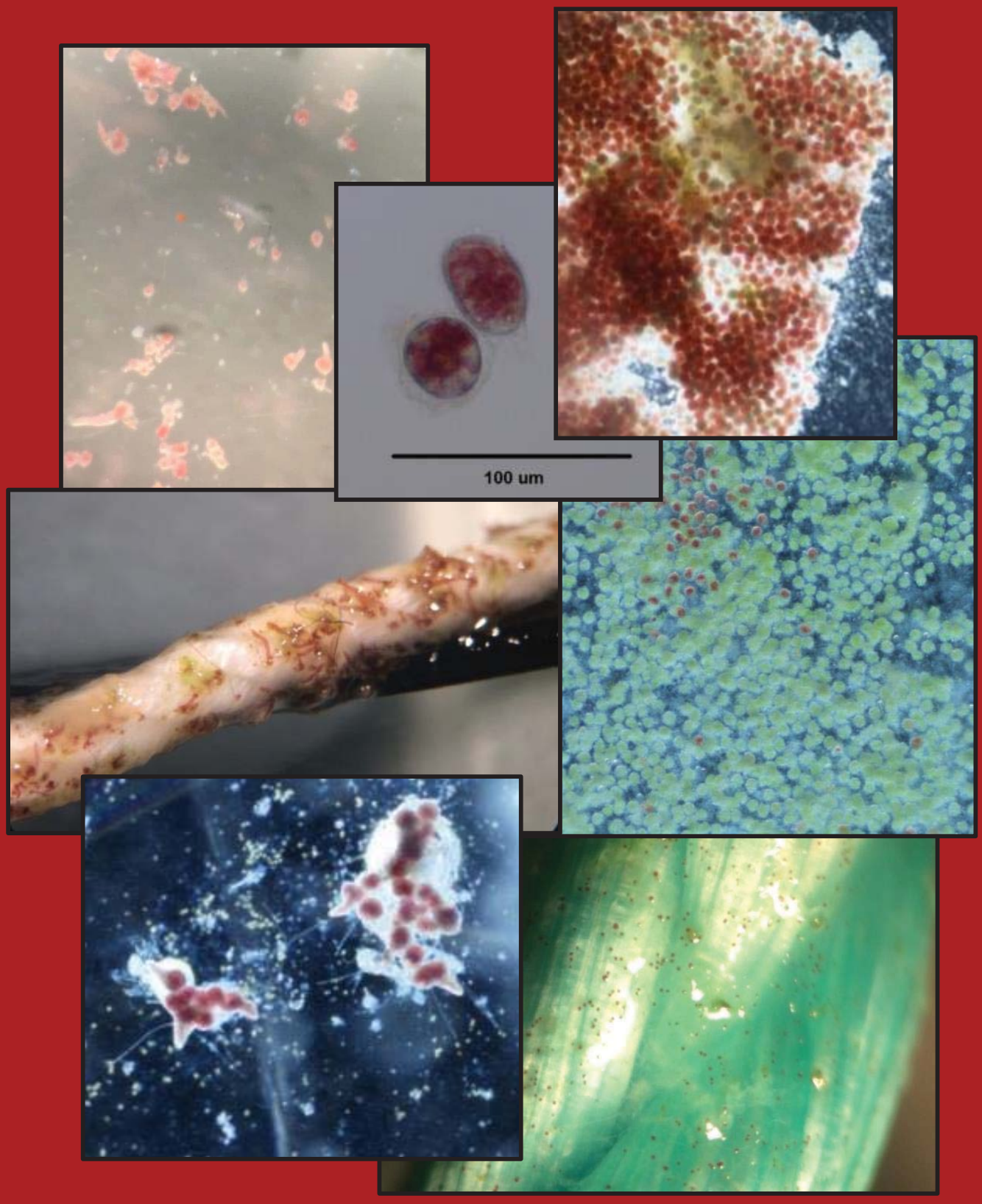
Paper 2: Release and settlement of tetraspores – and the concept of propagule seeding (GMA-seeding method)



Algal Research 40 (2019) 101494

Contents lists available at ScienceDirect

Algal Research

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Improved *Palmaria palmata* hatchery methods for tetraspore release, even settlement and high seedling survival using strong water agitation and macerated propagules

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ARTICLE INFO

Keywords:
Palmaria palmata
Spore release
Spore settlement
Seeding
Seedling survival
Re-attachment

ABSTRACT

Cultivated *Palmaria palmata* is highly valued as a nutritious source of biomass. Yet, current hatchery techniques using tetraspores show low spore-to-seedling efficiency, normally imposing a high requirement of sori for seeding in large-scale cultivation, and pointing to a need for developing current hatchery techniques. This study shows new hatchery strategies to improve tetraspore release, efficiency of spore use as well as seedling distribution on seeded substrates for *P. palmata* cultivation, based on germination, maceration and agitation (GMA-method).

We showed increased spore yield by using high-agitated sporulation tanks (67,906 ± 11,303 spores g FW^{-1}) compared to calm water (17,889 ± 3652 spores g FW^{-1}). In addition, twine substrates cultured in high water agitation (2.5 L air min^{-1}) resulted in highest settlement and dispersal of spores and seedlings compared to non-agitated cultures. Using alginate coated twine showed no effect after 70 days nursery growth, despite higher initial spore density after a 22 days spore release phase in some treatments.

Spore release time did not affect spore yield when comparing 1 and 3 h, whereas the yield increased during long-term sporulation (22 days) in some treatments. Released tetraspores settled in dense aggregates that germinated into a mixture of spores and seedlings (propagules) during 30 days and showed an ability of discoid re-attachment and growth after a maceration pretreatment. Here, the level of water agitation affected the re-attachment success and 39% of the added seedlings reattached after 14 days of nursery. The cultivation strategies presented here provide a way to increase the overall spore-to-seedling survival and might serve as a new seeding strategy for *P. palmata*. Present findings are important knowledge in the quest of optimizing large-scale hatchery production of *P. palmata*.

1. Introduction

Interest in cultivating of *Palmaria palmata* Linnaeus O. Kuntze (dulse seaweed) in European countries for human consumption and feed ingredient, has increased as knowledge about its nutritious and potential health benefits is increasingly valued [1–5]. Globally, seaweed cultivation is the fastest growing aquaculture sector and provides a low trophic and nutrient extractive source of biomass [5–7]. Several red algae species have been cultivated at small to larger scales in open water, such as *Sarcothalia atropurpurea, Gracilaria lemaneiformis and Pyropia umbilicalis (*former *Porphyra umbilicalis)* to secure a source of raw material for agar and carrageenan extraction as well as food, respectively [8–10]. Those studies used spore inoculation methods and a period of calm water to promote settlement of tetra-, and carpospores on bottom-laid structures, such as mollusks valves or nets. Yet, the exploitation of seaweed in Europe is 99% based on harvest from wild stocks [5]. In Europe, Irish seaweed companies contributed with 99% (< 100 t FW) of the annual harvest of *P. palmata* in 2011 [11]. *P. palmata* is dried and retailed for high-valued niche products at prices reaching ~245 € per kg dry weight (DW). Besides a high content of essential minerals and rich flavor [1,12,13] and relative high content of crude protein of up to 19.3–35% [4,14], *P. palmata* has been characterized as promising diet supplement for humans and salmon feed due to the content of bioactive peptides, protein hydrolysates and antioxidants [2,15,16]. As demands for *P. palmata* increase, focus on cultivation rather than wild harvest of this species is important to avoid diminishment of wild populations and to secure a sustainable crop production [1,17]. Cultivated *P. palmata* requires a relative high

Abbreviations: psu, practical salinity unit; DW, dry weight; FW, fresh weight

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https://doi.org/10.1016/j.algal.2019.101494
Received 2 October 2018; Received in revised form 28 March 2019; Accepted 3 April 2019
Available online 12 April 2019

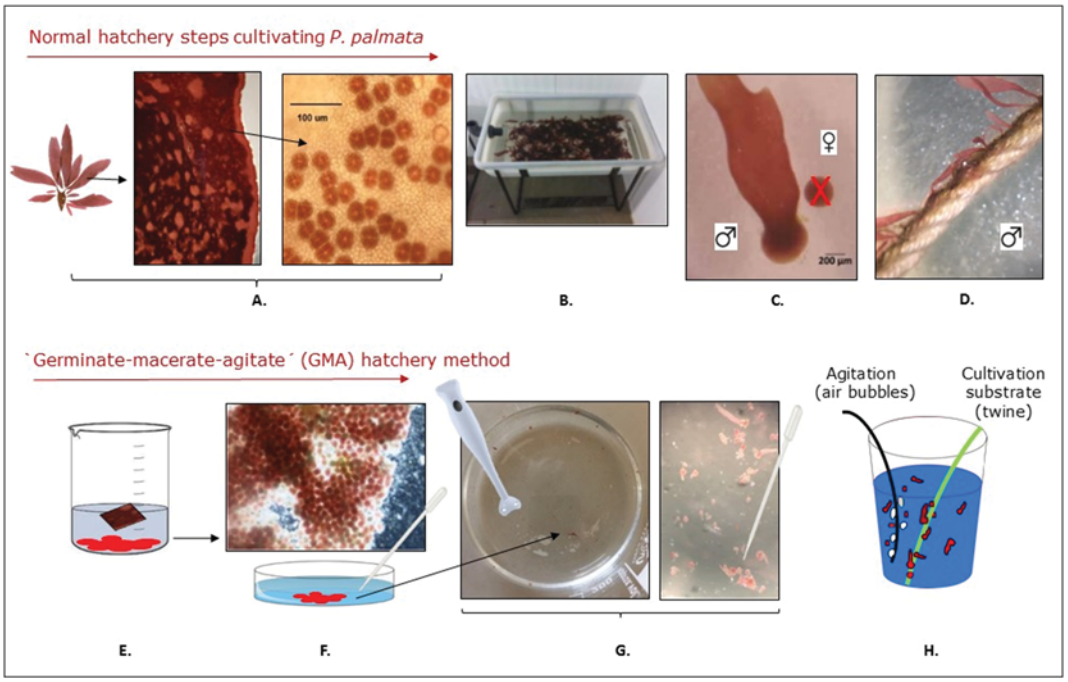

Fig. 1. Schematic illustration of two hatchery methods for *P. palmaria*. Top: Schematic illustration of normal hatchery steps cultivating *P. palmata* (A–D). A: Zoom-in on fertile tetrasporophytic frond (sori) containing tetrasporangia, each with four tetraspores. B: To inoculate substrates, a high amount of sori is normally used to cover the substrates during sporulation. C: Male gametophyte (seedlings) germination after few days while females remain microscopic or die off (red cross). D: After 1–3 months of nursery, male seedlings are visible on twine substrate. Bottom part: `Germinate-macerate-agitate´ (GMA) method (E–H). E: Released tetraspores settle in dense aggregates due to mucilage. F: Spore aggregates washed and transferred for germination in petri dish. G: To dislodge spores and tiny seedings after germination, aggregates were subjected to maceration to obtain a solution of propagules (spores and tiny seedlings). H: Adding a certain volume to the agitated inoculation tank provide dispersal of propagule settlement. Substrates (green) orientated in near-vertical position to increase space efficiency. Photo-credit: Werner & Dring, 2011 (B.), Constanza Chianala Cerda (C.). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

nitrogen availability during growth to maintain the dark-red color due to phycobilliproteins [18], which is an important trademark for the species as commercial product. This have led to the idea of farming *P. palmata* in nutrient riched areas [7,19,20]. However, in near-coastal waters, like Danish inner waters where nutrient levels may be elevated due to run-off and fish farms, water bodies show great spatial and temporal variation in salinity [21], even down to 15 psu (National monitoring survey data, NOVANA). Despite the potential of cultivating in nutrient-rich semi brackish waters [22], the growth of *P. palmata* seedlings in low saline waters, as inner Danish waters, is yet to be investigated.

Open-sea cultivation of *P. palmata* requires a land-based hatchery phase to produce seeded substrate, like twine or textile, using released meiotic tetraspores as seeding material [17,23]. However, as of yet, existing seeding and hatchery methodologies for large-scale cultivation are suboptimal and need further development [24]. The normal hatchery process can be divided in three important steps: spore release, spore attachment and seedling growth (Fig. 1A–D).

Tetrasporophytes release tetraspores during fall and winter from mature fertile tissue (sori) [17,25] (A). Current seeding methodology for *P. palmata* is based on a gravity principle due to the relative large size (Ø~30 µm) and non-mobility of the tetraspores [26,27]. By this method, fertile sori submerged in inoculations tanks release tetraspores, which settle during few days [17]. The spore release is highly unpredictable as ripeness and rupture of the tetrasporangia varies even within tissue sections [25,28,29]. Furthermore, the sticky nature of *P. palmata* tetraspores often result in settlement of nested spore aggregates [29] of high density on the substrate or on bottom of the tank [24]. As a result, the spore density on substrates can be highly variable and cause low quality seedling lines [24,30]. To compensate for the low spore use efficiency inherent in this method, a 1:1 areal coverage of sori-to-substrate has been used in the seeding phase (B). However, this impose a high demand of sori [26]. Thus, the ratio between the amount of sori needed to seed substrates and the harvest yield is high, which indicate a low seeding efficiency. Using this method, Werner & Dring (2011) estimated a requirement of 135 kg of fertile plants to seed 70 (1.2 ∗ 3 m) to obtain an extrapolated harvest yield of 1.8 ton FW. After settlement, half of the tetraspores germinate into male gametophytes (seedlings), whereas the other half (female gametophytes) remain microscopic or die off (C) as no fertilization step to initiate the development of new tetrasporophytes is implemented in the normal cultivation practice. Young male seedlings are typically nursed for 1–5 months before deployed at sea (D). After 5–8 months of growth at sea, biomass is ready for harvest in late spring when sea temperature is still within the optimal range for growth (6–17 °C) [14,24].

Agitation mediates a physical disturbance at the water-sporangia interface, which likely enhance sporangia wall rupture [28] and augment the spore release rate. In addition, the risk of contamination increases as fertile tissue remains in the sporulation tank for several days [31]. Thus, agitation of water might be important for a faster spore release rate and seedling survival in batch cultures. Likewise, water agitation might increase evenness of settled spores as it simulates the conditions with moderate exposure and strong currents found in the lower intertidal and sublittoral habitat where *P. palmata* live. In this habitat, *P. palmata* is often observed in high densities on stipes of *Laminariales* [25], suggesting that the stipe surface provides a higher chance for spore settlement, survival and growth. After release, spores settle and attach to kelp stipes mainly as result from higher encounter rate enforced by small-scale water eddies [32,33], though other factors, such as stipes surface roughness and herbivory might also be important [34,35]. The stipes of kelps contain an extracellular alginate matrix to which sticky tetraspores might show preference of settlement attributed

to the outer layers of mucilage sheath and the adhesive mucilage vesicles [28,36]. Mimicking an alginate surface on cultivation substrates might enhance the settlement and survival of *P. palmata* seedlings.

Based on the previous findings and challenges as summarized above, we aimed to investigate a modified strategy (Germinate-macerate-agitate; GMA) to optimize release and handling of *P. palmata* tetraspores to produce seedlings on cultivation substrate, as shown in Fig. 1(E–H). First step was to test the effect of agitation and spore release duration on spore yield (E). Aggregates of released tetraspores were transferred and germinated in petri dish (F) obtaining a mix of spores and seedlings (propagules). After germination, the aggregates were subjected to maceration as pretreatment to dislodge propagules (G) and used as seeding inoculum in agitated tanks to test the ability of propagules to re-attach on substrates. Three levels of agitation and two types of twine were tested. After settling propagules were denoted 'seedlings' (H). Finally, we tested the effect of alginate-coated substrate and the level of water agitation on tetraspore settlement density and salinity effect on seedling growth.

2. Material & methods

Sterile *P. palmata* (n = 50) were collected by divers the 23rd of August 2016 near Fyns Hoved, Denmark (55.610895°N 10.594308°E) at 4–5 m depth and water temperature of 15 °C. The salinity of the upper water column at the collection site varies seasonally between 14 and 27 psu (National monitoring survey data, NOVANA) but was not measured at sampling time. The plants were transported to indoor tank facilities and cultured in 400 L aerated filtered seawater (1 μm) at 10 °C and exposed to 12:12-h light:dark regime with a surface irradiance of 80 PAR (E_{PAR}; μmol photons $m^{-2} s^{-1}$). Nutrients were added (0.20 ml L^{-1} seawater) weekly to spike the nitrogen concentration to ~175 μM N (N/P = 25) using growth media F/2 + (Varicon Aqua Highcell F2P). After one month, the light:dark regime was changed to 8:16-h to simulate winter conditions and plants were kept under those conditions until start of experiments in October, January and February. Four experiments were set up to investigate the mentioned challenges. All experiments were performed at 10 °C with down-welling irradiance provided by pairs of fluorescent tubes (54 W, warm white, T5 Philips) and the distance to experimental tanks and neutral screens was used to adjust E_{PAR} (measured by use of a planar LiCOR sensor). Filtered (0.2–1 μm) and sterilized (150W-UV) natural seawater of 26–28 psu was used in all experiments.

2.1. Spore release (exp. 1)

A spore release experiment was conducted on the 10th of January 2017 testing two levels of water agitations (calm water vs. agitated water) and durations (1 vs. 3 h) to assess the difference in spore yield and spore release rate. Three plants (n = 3, 6–12 cm) were cleaned with 1 μm filtered-sterilized seawater using paper towels before they were left to partially dehydrate for 20 h between layers of paper towel in a dark room at 5 °C [26]. Subsequently, four sori pieces of each 1 cm^2 (corresponding to approx. 0.05 g FW) and similar appearance were cut from each plant, and divided in 12 glass beakers containing 50 ml clean seawater to make up triplicates of the four treatments. Randomly aligned beakers were exposed to irradiance of 25 E_{PAR} at 10 °C, according to [24,37]. Water agitation was mediated by air bubbles of 2.5 L air min^{-1} in half of the beakers whereas no aeration was applied in the other beakers. Agitation level was estimated prior to experiments by adjusting the airflow required to replace an equivalent volume of water per minute in a water-filled glass beaker submerged upside down into a larger beaker. After 1 or 3 h respectively, the spore yield was estimated by counting three subsamples of 5 ml for each replicate. Before subsampling, tissues were gently removed and the water in the beakers was stirred and aerated to obtain a well-mixed solution. Each subsample was pipetted into gridded well-plates and left to settle for 5 h at 5 °C. Raft counting of all subsamples was done using a 100 x magnification stereomicroscope.

2.2. Preference of spore settlement and seedling survival (exp. 2)

The effect of alginate coated twine and three levels of agitation on spore settlement and seedling survival was assessed during a 70 days experiment (8th of December 2016 to 17th of February 2017). Polypropylene 3-cordel twine (PP, Ø = 6 mm) was cut into 18 pieces of 10 cm and half were coated 24 h before the experiment with a ~1 mm thick layer of alginate glue and tested against non-coated twine (control). The glue was made by mixing 2 g of alginate powder (TYSA LN 80, Lot. no: 7152 NIM, EUROGUM A/S, Denmark) in 150 mL fresh water. Nine gently cleaned fertile fronds were divided between nine plastic beakers with 400 ml seawater together with both types of twine tilted towards the surface at a 20° angle. To enhance spore liberation and dispersal, the sori were fixed vertically in the beakers with constant agitation of water. Three levels of agitation were provided in triplicates by aeration of 0 L min^{-1}, 0.5 L min^{-1} and 2.5 L min^{-1}. The spore release phase ended after 22 days, where the sori were removed from the beakers and each pair of twine was transferred to clean beakers. From day 11 to day 70, the number of settled spores (per g FW) was counted weekly using a magnification microscope. From day 28, the density of seedlings was start counted. At high numbers of settled spores and seedlings, the average count on three subparts (1 cm) was used to estimate the density. The experimental conditions was 10 °C with a photoperiod of 10:14-h light:dark (35 E_{PAR}) to simulate spring growth season. Weekly water renewal was spiked to ~88 μM N by adding 0.4 ml of F/2 + growth medium in each beaker.

2.3. The effect of twine type and agitation on propagule re-attachment (exp. 3)

To optimize the spore use, the ability of released propagule aggregates to re-attach and grow was tested in experiment 3 and 4 (see 2.4). In exp. 3, propagule aggregates were macerated and used as seeding inoculum to test the effect of twine type and water agitation (Fig. 1F–G). On February 10 2017, two pieces of fertile sori (2 ∗ 4 cm) were excised and kept in a 0.5 L container with gently aeration and checked for released spores daily until February 17 where a spore aggregates were visible on the bottom. The aggregates were divided into two petri dishes (25 ml) and left to germinate for 30 days at 5 °C, 7 ± 2 E_{PAR} (10:14-h light:dark) with no nutrients added. Water was renewed twice during the period. After 30 days, the propagules were dislodged, poured into a glass beaker and macerated using a kitchen blender for one minute (Fig. 1G). The beaker was added 100 mL of clean seawater (total 150 mL) after which 15 mL of the propagule solution were added to each of nine open plastic beakers with a total water volume of 400 mL (0.2 μm filtered). The beakers contained a piece of either PP twine (10 cm, Ø = 6 mm) or polyvinyl Kuralon twine (10 cm, Ø = 2 mm). Three days before the experiment, all twines were washed and soaked in fresh water and left to dry before use. Each twine was placed in a vertical position in the beaker with approximately 20° angle to the light source. Winding the soft Kuralon twine three times around a cable tie secured its vertical position in the containers. Cultures were kept at 5 °C, 10:14-h light:dark regime of 30 ± 5 E_{PAR}. For the PP twine, two levels of agitation (0.5 L min^{-1} and 2.5 L min^{-1}) was applied in triplicates, whereas for the Kuralon twine agitation was only applied at one level (2.5 L min^{-1}). To evaluate the effect of treatment, the density of spores and seedlings was counted at day 3 (settlement) and at day 14 (attachment) using a stereomicroscope, as described in exp. 2.

2.4. Effect of salinity on growth of re-attached seedlings (exp. 4)

P. palmata seedlings were cultivated at four different salinities (15,

20, 25, and 30 psu) to assess the effect of salinity on early growth. The salinity range is realistic to simulate Danish inner surface waters in which seaweed cultivation takes place. The seeded twines were produced based on re-attachment of germinated propagules, as described in exp. 3. On November 23, a one-day-old spore aggregate was transferred to a petri dish and germinated until December 21st. After maceration, the solution was poured into an open plastic beaker containing 400 mL seawater and five pieces of PP twine (10 cm, Ø = 6 mm) in a 20° tilted position. The water was agitated by aeration (2.5 L air min^{-1}) to promote dispersal of propagules before settlement. After 14 days of cultivation, re-attachment of solitary and nested spores and tiny seedlings on all twines was confirmed under the stereomicroscope. Seedlings were then pre-cultivated at 26 ± 1 psu for 41 days, before transferred to different salinity treatments. Then, each twine was cultivated in separate beakers containing the different salinities for 57 days with weekly water renewal. The different salinities were obtained by adjusting natural filtered (0.2 µm) and sterilized seawater by MilliQ water or Red Sea Coral salt powder the day before use and was added nutrients to spike concentration to ~88 µM-N. During 2 month of culture, the seedling length was measured six times on the eight longest individuals from each beaker, according to [26].

2.5. Data analysis and statistics

The Shapiro-Wilk W test for normality of residuals and Levene test for homogeneity of variances and outliers was applied for all datasets using JMP 13 (SAS). The effects of spore release time and agitation on spore release yield (exp. 1) were compared by a two factor ANOVA (2fAN, α-level = 0.05) followed by a post hoc *t*-test (pooled error term). The effects of twine coating and agitation on final density (day 70) of tetraspores and seedlings (exp. 2) were analyzed by the Wilcoxon Test based on ranks followed by Steel-Dwass all pairs test due to non-independence of the pairwise cultured twines. The dataset were log (Y + 1) transformed to avoid zeroes, impose homoscedasticity but not normality. When pooled, datasets were compared by use of the 2-tailed signed-rank test. In exp. 3, the main factors (twine type and agitation) were unbalanced, but data came from a homoscedastic population. The mean density of re-attached propagules (spores and seedlings) on day 3 and day 14 was compared using the Wilcoxon Test based on ranks followed by Steel-Dwass All Pairs comparison test. In exp. 4, the eight longest seedlings on each twine were regarded as treatment replicates as no density-depended effect was assumed by use of tiny seedling sizes, even light availability, weekly water renewal and high nutrient concentration [26]. The effect of salinity on mean seedling length at the final date was assessed using one-way ANOVA followed by Tukey-Kramer HSD test on log-transformed data converted to micrometers (log(Y*1000)). When variables did not show significance, the data was pooled for further comparison (Student's *t*-test). All data are presented as mean ± SE if not stated otherwise.

3. Results

3.1. Spore release (exp. 1)

During the phase of spore release, water agitation mediated a significant effect (P = 0.0308) on the spore yield, whereas the effect of spore release time was not significant (P = 0.1051). The highest spore yield was found under the conditions of high agitation for 3 h and resulted in a spore yield of 67,906 ± 11,303 spores g FW^{-1} compared to 17,889 ± 3.652 spores g FW^{-1} in calm water for 1 h. No interaction between agitation level and spore release time was found (Fig. 2).

3.2. Preference of spore settlement and seedling survival (exp. 2)

To evaluate treatment effects, we compared only the final counts of the spore release phase (day 22) and after nursery (day 70), as this

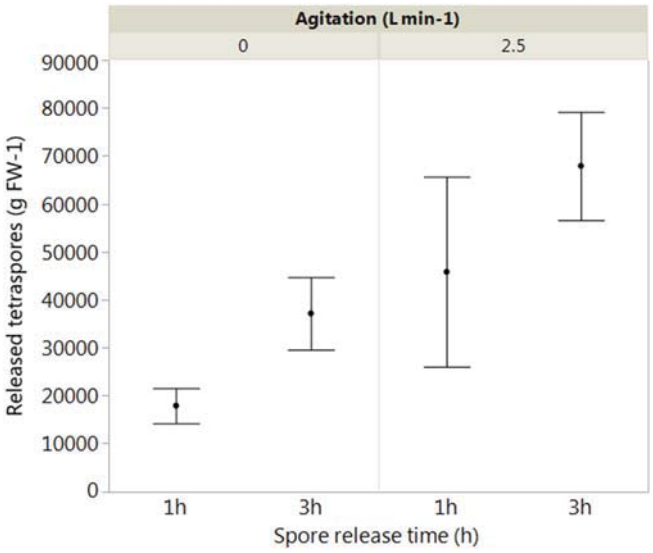

Fig. 2. Spore yield of *P. palmata* as a function of spore release time (1 and 3 h) and level of water agitation (0 L min^{-1} and 2.5 L min^{-1}). Data are presented as mean ± SE (n = 3).

represents two potential deployment dates and seedling density at deployment day has great effect on the final biomass harvest [24].

Within the first 22 days (spore release phase), spore settlement was evident on the pairwise-cultured twines at all agitation levels (Fig. 3). At day 22, the end of spore release phase, there was no significant differences between non-coated and coated twines for any of the agitation levels (ChiSq, P = 0.27). Data from both coating types were therefore pooled in the following analysis. The spore release rate was significantly higher in high-agitated cultures (P = 0.017) compared to semi-, and non-agitated cultures, evaluated by the amount of settled spores at day 22. Spores settled evenly in top and bottom of both types of twine (data not shown). Comparable with the spore release phase, no differences between coated and no-coated twine was found from day 22 and onwards (P = 0.51). Data from both coating types were therefore pooled in the following analysis. At day 70, the high-agitated cultures maintained a significantly higher spore density compared to the lower levels of agitation (P = 0.004), whereas the semi-, and non-agitated cultures were not significantly different. The density of settled spores in high-, semi-, and non-agitated cultures being 272 ± 75, 38 ± 26 and 2 ± 1 spores/twine, respectively.

In general, the seedling density increased in high-agitated cultures in contrast to lower agitated cultures (Fig. 3). Twine coating showed no significant effect on seedling densities after 70 days (P = 0.27). Thus, data from both coating types were pooled in the following analysis. Water agitation significantly affected the final seedling density (ChiSq, P = 0.0024; day 70), where the high-agitated cultures showed seedling densities of 141 ± 37 seedlings/twine compared to 11 ± 6 seedlings/twine in the semi-agitated cultures (P = 0.0224) and 3 ± 2 seedlings/twine in the non-agitated cultures (P = 0.0121) (Fig. 4).

3.3. Effect of twine type and agitation on propagule re-attachment (exp. 3)

The seeding material obtained by macerating the propagule aggregate following 30 days germination (propagule solution) contained both single and small clumps of spores and tiny seedlings (2–5 individuals) with a concentration of 37 spores/mL and 15 seedlings/mL of both solitary and nested propagules. The mean initial length of seedlings was 0.6 ± 0.2 mm (n = 10).

The settlement density of spores at day 3 (Fig. 5a) was similar between all treatment groups, despite of different agitation levels (28 ± 5 spores, pooled data). At day 14, the spore density was

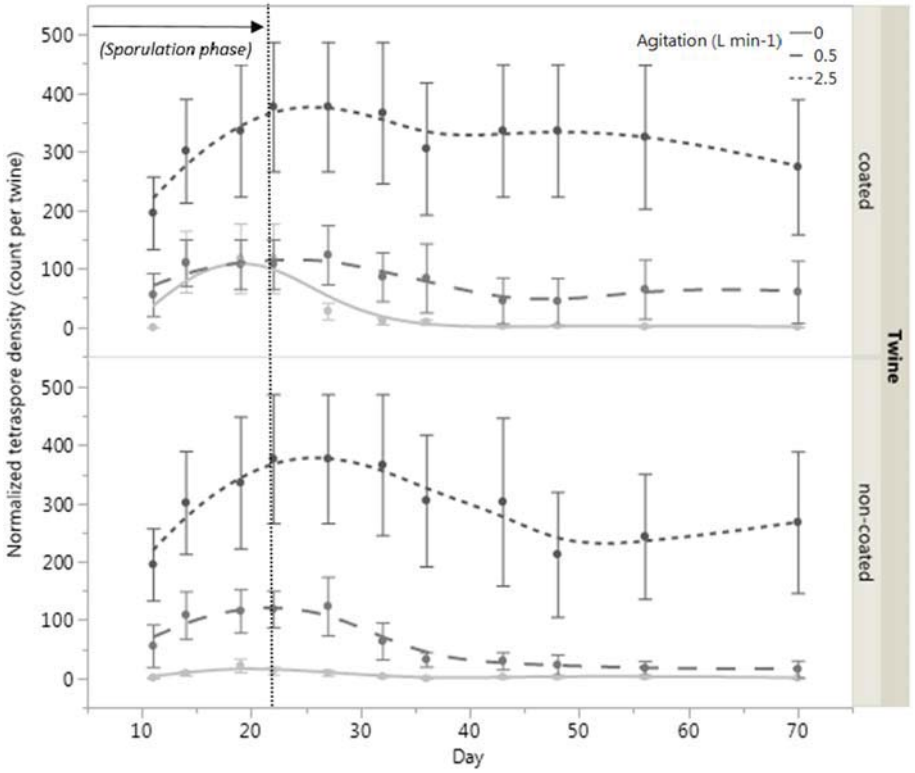

Fig. 3. Temporal development in spore densities on alginate coated and non-coated twine as effect of agitation. Pairs of the different twines were seeded in the same inoculation tanks. Sori were removed at day 22. Data points represent the mean count of spores ± SE (n = 3).

significantly lower on the Kuralon twine than on PP twine with same level of agitation (P = 0.016, students *t*-test) whereas the two agitation levels applied for the PP twine did not impose any differences in spore density.

Seedling density at day 3 differed significantly between the three groups (ChiSq, P = 0.0054), the low agitated group being significant lower than the two high-agitated groups, and the high-agitated groups being independent of twine type. High agitation promoted a settlement density of 58 ± 12 seedlings (pooled data, n = 6) compared to 10 ± 4 seedlings in the low-agitated PP group at day 3. Similar to day 3, the

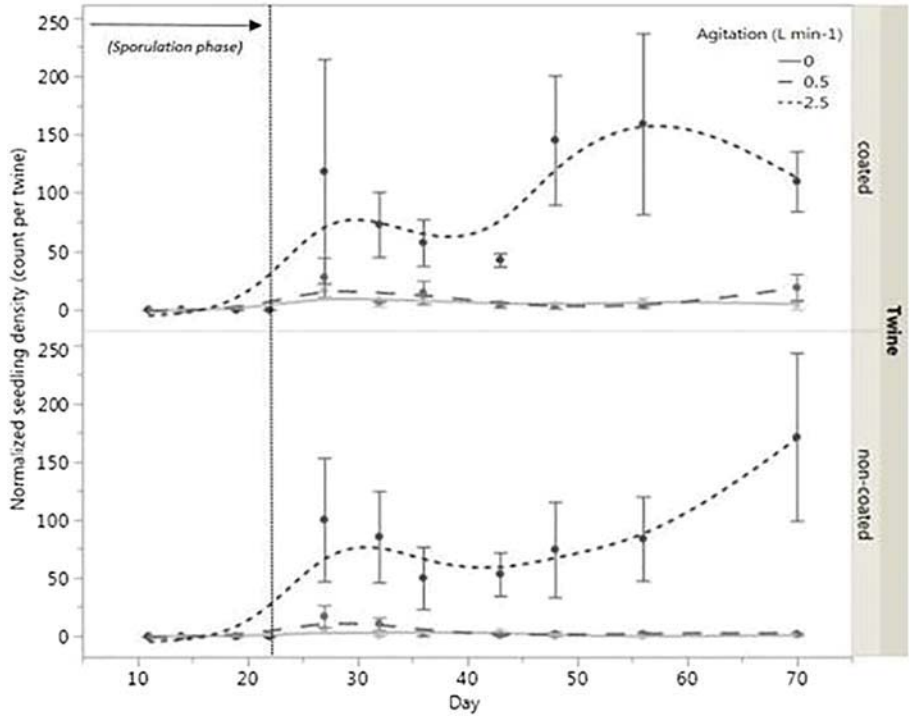

Fig. 4. Temporal development in seedling densities on alginate coated and non-coated twine as effect of agitation. Pairs of the different twines were seeded in the same inoculation tanks. Sori were removed at day 22. Data points represent the mean count of seedlings ± SE (n = 3).

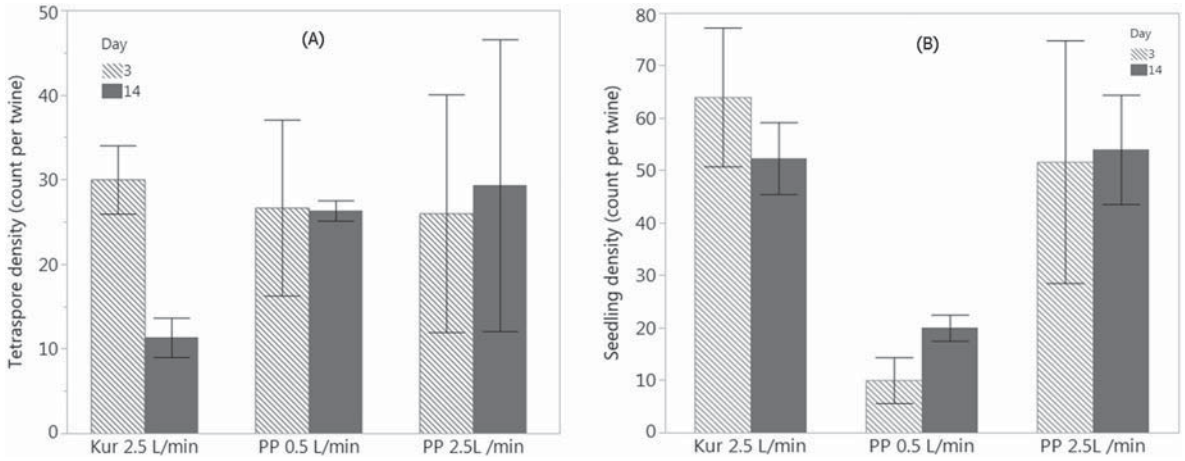

Fig. 5. Density of settled (day 3) and attached (day 14) *P. palmata* spores (A) and seedlings (B) obtained from inoculating two types of twine; Kuralon (Kur) and polypropylene (PP) in seeding tanks with a solution of germinated and macerated propagules. PP twine was cultured at two levels of water agitation by aeration of 0.5 L min^{-1} or 2.5 L min^{-1}, while Kur was cultured at 2.5 L min^{-1} only. Data represents mean ± SE (n = 3).

density of seedlings were significantly higher in both high-agitated groups compared to the low-agitated (P = 0.0053) at day 14, and high-agitated groups not being significantly different. The high-agitated groups showed a density of 53 ± 5 seedlings (pooled data, n = 6) compared to 20 ± 3 seedlings for the low-agitated group at day 14.

Combining counts of propagules in the inoculum with spore and seedling counts (Fig. 5), the success rate of reattachment (and survival) of settled spores and seedlings were estimated to 5% ((28 $spores_{ATTACHED}$ / 37 $spores/mL_{INOCULATION}$ * 15 mL) * 100%) and 39% ((58 $seedlings_{ATTACHED}$ / 10 $seedling/mL_{INOCULATION}$ * 15 mL) * 100%), respectively.

3.4. Re-attached propagules cultured in a salinity gradient (exp. 4)

The average seedling length increased significantly (P < 0.005) during the experiment in all salinity treatments (Fig. 6), the overall pattern being a continuous growth until the end of the experiment at salinities of 15 and 20 psu, whereas growth declined slightly at the end for cultures at 25 and 30 psu.

After 57 days of cultivation, the maximum average length of seedlings (n = 8) was significant (P = 0.0039) longer at 15 psu (2.0 ± 0.3 mm) and 20 psu (1.5 ± 0.4 mm) compared to the 30 psu culture (0.67 ± 0.54 mm), however not significantly differing from each other (Fig. 6). At 15 and 20 psu, the specific growth rate was 2.4% per day during ~2 months, using the equation ($Ln_{(L2)}$-$Ln_{(L1)}$/t2-t1), which is comparable to previous reports [14,26]. The mean seedling length of the 25 psu culture was significant smaller (P = 0.049) compared to 15 psu, but not the 20 psu culture (P = 0.59).

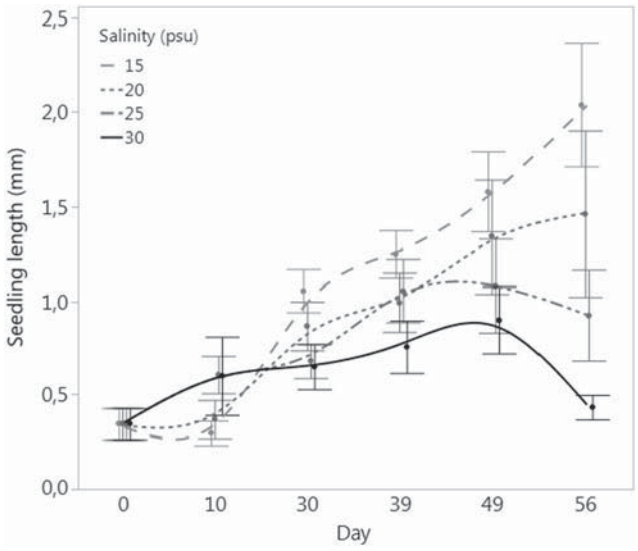

Fig. 6. Growth in seedling length (mm) measured weekly or biweekly on the eight longest seedlings, obtained by the re-attachment method, and cultured during 57 days on PP twine (Ø = 6 mm) at different salinities. Data points represents mean ± SE (n = 8).

4. Discussion

The main objective of this study was to investigate how modified conditions in each hatchery step, e.g. tetraspore release, settlement, and early stage growth can improve seedling production of *P. palmata*. In a stepwise approach, we have demonstrated a seeding method for *P. palmata* based on water agitation and maceration of germinated propagules for dispersal and re-attachment to cultivation substrate. The novel findings indicate a potent discoid adhesion capability of the young spores and seedlings after dislodgement which was enhanced by maintaining high water movement during inoculation. In comparison to protocols for cultivating other thalloid red macroalgae, such as *Gracilaria chilensis* [38] and *Sarcothalia crispata* [39], calm conditions are often described to allow tetra-, and carpospores to settle on horizontal surfaces. In addition, this method potentially shows a way for spore aggregates to be stored before maceration using low light and low temperature conditions (7 ± 2 PAR, 5 °C), which could be a mean of reduced risk of epiphyte fouling [14]. Based on collection of fertile sporophytes or by the suggested year-round manipulative induction of sori tissue [40] this method might be used to extend the seedling production season by control of the germination rate of tetraspores into seedlings. Using macerated propagules, the survival of settled spores and seedlings was estimated to 5% and 39% respectively, after 14 days of nursery cultivation. The lower survival of spores may however be an effect of spores germinating into seedlings during the 2 week nursery phase, which falsely affects the survival rate of spores negatively. Thus, as an alternative to the normal seeding method (Fig. 1A–D), which promotes a patchy spore density and a considerably spore loss during sporulation, the present maceration-method (Fig. 1E–H) suggests a way to increase spore utilization and a way to control seeding density.

4.1. Spore release

The spore release yield did not differ on a short-term basis (1 vs. 3 h, exp. 1), whereas a generally increasing spore density during the 22-day spore release phase in exp. 2 indicates an effect of long-term spore

release. This suggest a different strategy for hatchery spore release in *P. palmata* than for instance for brown macroalgae such as *S. latissima*, where a sufficient spores release is obtained within a short time-frame [41] and pinpoints an important consideration for *P. palmata* cultivation.

The high spore release yield of 67,906 ± 11,303 spores g FW^{-1} (corresponding to 22,635 spores g FW^{-1} h^{-1}) found in this study at high agitation (exp. 1) suggests that high-agitated water enhance sporangial rupture and thereby increase spore release. Also, the release rate found here was markedly higher than the estimated release rate of ~4 spores g FW^{-1} h^{-1} previously found by use of the gravity-based seeding method [24] where ~43.000 tetraspore released and settled during three days of sporulation.

Several factors affect the spore release of fertile *P. palmata*, such as season, geographical position and typography at collection site, which all affect the ripeness of sori [24,30]. This might explain the high variation in hatchery-based spore release yield of *P. palmata* in earlier studies of 5.000–60.000 spores g FW^{-1} (± 30.000) [20,23].

The positive effect of using relative strong water agitation during hatchery spore release, seeding and nursery phase might relate to the hydrodynamic forces found in the lower intertidal and sublittoral coastal waters which *P. palmata* inhabit. Similarly, the use of agitation enhanced the release of haploid spores in other macroalgae inhabiting coastal waters, such as *Alaria esculenta*, *Ulva lactuca* and *Porphyra umbilicalus* [10,42]. In these studies, water stirring also promoted spore-to-seedling germination.

4.2. Spore settlement and survival

In the next step of the hatchery phase, water agitation significantly enhanced spore settlement and final seedling density on twine when sori were left to inoculate for 22 days in the seeding tanks (Figs. 3–4), documenting the positive effect on seedling survival by water agitation. Also, non-agitated cultures resulted in poor spore and seedling dispersal on twines (exp. 2) when using sori in seeding. However, the application of even low level of water agitation (0.5 L min^{-1}) was sufficient to distribute germinated spores for re-attachment, whereas the re-attachment and survival of seedlings increased with higher level of water agitation (exp. 3) and reached a high survival rate (39%). This is in the high range of what has been found in other studies showing survival rates of 10% to 35% over the course of 7 days and 2 months during nursery phase, respectively [24,26]. Effectively, we suggest the use of high-level agitation for spore release and propagule dispersal to lower the amount of collected fertile sori required for hatchery seedling production.

4.3. Substrate coating

Several cultivation trials have documented the hatchery steps for cultivating *P. palmata* using the gravity-based seeding method [17,19,23,24,43], however, little improvement of spore-to-seedling viability has been shown. The use of coating on cultivation substrate is an emerging technology in hatchery seeding to lower the cost of hatchery operation [41]. For instance, mixing *S. latissima* gametophytes and tiny sporophytes into a binder solution containing soluble algal sugars and applying it on cultivation substrates has shown to prevent propagules to be washed off when deployed [42]. *P. palmata* is a common epiphyte on Laminariales stipes [25,44], indicating either a restricted distribution of spores by either spore dispersal or specific requirements for settlement and survival. In the present study, we tested a pure alginate mixture as a coating to simulate the encounter of kelp stipes seen in nature, to investigate potential settlement preferences of *P. palmata* tetraspores. Effects were however only minor and further investigations are needed to investigate e.g. whether mixing the macerated propagules into the binder before application, as done with *S. latissima* [42] would improve *P. palmata* settlement.

4.4. The effect of salinity on early seedling growth

P. palmata seedlings cultivated at constant low salinity (15 psu) for 57 days showed similar and even higher growth in length than higher salinities (up to 30 psu). Similar growth response has been found in other coastal macroalgae species; *Ulva intestinalis* (former *Enteromorpha intestinalis*) and *Fucus serratus* both showing the highest growth rate between 15 and 20 psu [45,46]. This indicates an opportunity to cultivate *P. palmata* in areas of Danish inner waters where a salinity of these levels often prevails.

5. Conclusions

To establish commercial *P. palmata* cultivation, a viable hatchery seedling production is crucial as tetraspores are short in supply, spore usage has currently been suboptimal and seedling density affects harvest yield. Based on this study, we suggest a new versatile strategy to optimize the use of *P. palmata* tetraspores based on germinated spore aggregates subjected to maceration to produce a solution of adhesive propagules showing the ability of discoid re-attachment on substrate. Besides, level of water agitation strongly affected the re-attachment of macerated propagules and led to increased spore release and spore distribution on twine. Future large-scale *P. palmata* hatcheries may benefit from this study, as a way to enhance spore-to-seedling efficiency by use of Germination, Maceration and Agitation (GMA) for enhanced efficiency in seeding production. The methods allows for extended season of seedling production by slowly germinating collected spore aggregates. To take *P. palmata* cultivation to the next level, more research in optimal use of the multiple and discrete sporulation events of *P. palmata* including control the slow germination and storing, is however still needed. Furthermore, to improve overall seedling density on cultivation substrates, future studies may investigate the optimal timing of a fertilization step to generate new sporophytes out of the female spores. Salinity responses of growing seedlings might also be important for future studies investigating site selection for cultivated *P. palmata* in brackish waters.

Declaration of interest

None.

Acknowledgements

We thank local scuba divers for collecting the *P. palmata* biomass and Kasper Lenda Andersen for tank maintenance in the hatchery. The study was funded by the TANG.NU-project funded by the Velux Foundation and by the Joint Doctoral Degree agreement between the Institute of Aquatic Resources (DTU Aqua) at Technical University of Denmark and the Norwegian University of Science and Technology (NTNU), Norway.

Statement of informed Consent, Human/Animal Rights: No conflicts, informed consent, human or animal rights applicable.

References

[1] O.G. Mouritsen, C. Dawczynski, L. Duelund, G. Jahreis, W. Vetter, M. Schröder, On the human consumption of the red seaweed dulse (Palmaria palmata (L.) Weber & Mohr), J. Appl. Phycol. 25 (2013) 1777–1791, https://doi.org/10.1007/s10811-013-0014-7.

[2] P.A. Harnedy, A. Soler-Vila, M.D. Edwards, R.J. FitzGerald, The effect of time and origin of harvest on the in vitro biological activity of Palmaria palmata protein hydrolysates, Food Res. Int. 62 (2014) 746–752, https://doi.org/10.1016/j.foodres.2014.04.035.

[3] P. Allsopp, W. Crowe, B. Bahar, P.A. Harnedy, E.S. Brown, S.S. Taylor, T.J. Smyth, A. Soler-Vila, P.J. Magee, C.I.R. Gill, C.R. Strain, V. Hegan, M. Devaney, J.M.W. Wallace, P. Cherry, R.J. FitzGerald, J.J. Strain, J.V. O'Doherty, E.M. McSorley, The effect of consuming Palmaria palmata-enriched bread on inflammatory markers, antioxidant status, lipid profile and thyroid function in a randomised placebo-controlled intervention trial in healthy adults, Eur. J. Nutr. 55

(2016) 1951–1962, https://doi.org/10.1007/s00394-015-1011-1.
[4] M. Bjarnadóttir, B.V. Aðalbjörnsson, A. Nilsson, R. Slizyte, M.Y. Roleda, G.Ó. Hreggviðsson, Ó.H. Friðjónsson, R. Jónsdóttir, Palmaria palmata as an alternative protein source: enzymatic protein extraction, amino acid composition, and nitrogen-to-protein conversion factor, J. Appl. Phycol. 30 (2018) 2061–2070, https://doi.org/10.1007/s10811-017-1351-8.
[5] The State of World Fisheries and Aquaculture, Food and Agriculture Organization of the United Nations, Fisheries and Aquaculture Department, Rome, 2014.
[6] A. Neori, A.M. Nobre, Relationship between trophic level and economics in aquaculture, Aquac. Econ. Manag. 16 (2012) 40–67, https://doi.org/10.1080/13657305.2012.649046.
[7] B. Grote, Bioremediation of aquaculture wastewater: evaluating the prospects of the red alga Palmaria palmata (Rhodophyta) for nitrogen uptake, J. Appl. Phycol. 28 (2016) 3075–3082, https://doi.org/10.1007/s10811-016-0848-x.
[8] N. Ye, H. Wang, G. Wang, Formation and early development of tetraspores of Gracilaria lemaneiformis (Gracilaria, Gracilariaceae) under laboratory conditions, Aquaculture 254 (2006) 219–226, https://doi.org/10.1016/j.aquaculture.2005.08.026.
[9] S.E. McNeill, R. Falshaw, Aquaculture of Sarcothalia atropurpurea tetraspores in New Zealand for carrageenan production, New Zeal. J. Mar. Freshw. Res. 51 (2017) 528–542, https://doi.org/10.1080/00288330.2017.1294085.
[10] N. Blouin, F. Xiugeng, J. Peng, C. Yarish, S.H. Brawley, Seeding nets with neutral spores of the red alga Porphyra umbilicalis (L.) Kützing for use in integrated multi-trophic aquaculture (IMTA), Aquaculture 270 (2007) 77–91, https://doi.org/10.1016/j.aquaculture.2007.03.002.
[11] M. Walsh, L. Watson, Part 1A: Market Analysis Towards the Further Development of Seaweed Aquaculture in Ireland, Dublin (2011).
[12] K.C. Morgan, J.L.C. Wright, F.J. Simpson, Review of chemical constituents of the red alga Palmaria palmata (dulse), Econ. Bot. 34 (1980) 27–50, https://doi.org/10.1007/BF02859553.
[13] R.S. Hagen Rødde, K.M. Vårum, B.A. Larsen, S.M. Myklestad, Seasonal and geographical variation in the chemical composition of the red alga Palmaria palmata (L.) Küntze, Bot. Mar. 47 (2004) 125–133.
[14] K.C. Morgan, F.J. Simpson, The cultivation of Palmaria palmata: effect of light intensity and temperature on growth and chemical composition, Bot. Mar. 24 (1981) 547–552, https://doi.org/10.1080/01619566709537448.
[15] N.C. Moroney, A.H. Wan, A. Soler-Vila, R.D. Fitzgerald, M.P. Johnson, J.P. Kerry, Inclusion of Palmaria palmata (red seaweed) in Atlantic salmon diets: effects on the quality, shelf-life parameters and sensory properties of fresh and cooked salmon fillets, J. Sci. Food Agric. 95 (2015) 897–905, https://doi.org/10.1002/jsfa.6753.
[16] A.H.L. Wan, A. Soler-Vila, D. O'Keeffe, P. Casburn, R. Fitzgerald, M.P. Johnson, The inclusion of Palmaria palmata macroalgae in Atlantic salmon (Salmo salar) diets: effects on growth, haematology, immunity and liver function, J. Appl. Phycol. 28 (2016) 3091–3100, https://doi.org/10.1007/s10811-016-0821-8.
[17] M.D. Edwards, M.J. Dring, Open-sea cultivation trial of the red alga, Palmaria palmata from seeded tetraspores in Strangford Lough, Northern Ireland, Aquaculture 317 (2011) 203–209, https://doi.org/10.1016/j.aquaculture.2011.04.007.
[18] B. Martínez, J.M. Rico, Seasonal variation of p content and major n pools in Palmaria palmata (rhodophyta), J. Phycol. 38 (2002) 1082–1089, https://doi.org/10.1046/j.1529-8817.2002.01217.x.
[19] J.C. Sanderson, M.J. Dring, K. Davidson, M.S. Kelly, Culture, yield and bioremediation potential of Palmaria palmata (Linnaeus) Weber & Mohr and Saccharina latissima (Linnaeus) C.E. Lane, C. Mayes, Druehl & G.W. Saunders adjacent to fish farm cages in northwest Scotland, Aquaculture 354–355 (2012) 128–135, https://doi.org/10.1016/j.aquaculture.2012.03.019.
[20] J.A. Manríquez-Hernández, J. Duston, D.J. Garbary, Effect of irradiance on bioremediation capacity of the red algae Chondrus crispus and Palmaria palmata, Aquac. Int. 24 (2016) 39–55, https://doi.org/10.1007/s10499-015-9907-6.
[21] M. Møller Nielsen, C. Paulino, J. Neiva, D. Krause-Jensen, A. Bruhn, E.A. Serrão, Genetic diversity of Saccharina latissima (Phaeophyceae) along a salinity gradient in the North Sea-Baltic Sea transition zone, J. Phycol. 52 (2016) 523–531, https://doi.org/10.1111/jpy.12428.
[22] L.M. Mortensen, Remediation of nutrient-rich, brackish fjord water through production of protein-rich kelp S. latissima and L. digitata, J. Appl. Phycol. 29 (2017) 3089–3096, https://doi.org/10.1007/s10811-017-1184-5.
[23] K. Browne, Mariculture of the Edible Red Alga, Palmaria palmata, Queen's University of Belfast, 2001.
[24] A. Werner, M. Dring, Cultivating Palmaria palmata, Dublin (2011).
[25] L. Irvine, M. Guiry (Ed.), Seaweeds of the British Isles, British Mu, London, 1983(1983).
[26] L. Le Gall, S. Pien, A.M. Rusig, Cultivation of Palmaria palmata (Palmariales, Rhodophyta) from isolated spores in semi-controlled conditions, Aquaculture 229 (2004) 181–191, https://doi.org/10.1016/S0044-8486(03)00390-9.
[27] A. Werner, M. Dring, Recommendations for Optimal Techniques for Obtaining Spores of Palmaria palmata, Settling and Maintaining Them Prior to Outplanting at Sea, Dublin (2011).
[28] C. Pueschel, Ultrastructure of tetrasporogenesis in Palmaria-palmata (Rhodophyte), J. Phycol. 15 (1979) 409–429.
[29] A.J. Hoffmann, The arrival of seaweed propagules at the shore: a review, Bot. Mar. 30 (1987) 151–165.
[30] J.C. Sanderson, Reducing the Environmental Impact of Sea-cage Fish Farming Through Cultivation of Seaweed, PhD Thesis (2006), pp. 1–399.
[31] A.R. Andersen (Ed.), Algal Culturing Techniques, Elsevier Academic Press, 2005.
[32] M.S. Foster, Regulation of algal community development in a Macrocystis pyrifera forest, Mar. Biol. 12 (1975) 331–342, https://doi.org/10.1007/BF00388990.
[33] T.A. Norton, Dispersal by macroalgae, Br. Phycol. J. 27 (1992) 293–301.
[34] M.N. Clayton, Propagules of marine macroalgae: structure and development, Br. Phycol. J. 27 (1992) 219–232, https://doi.org/10.1080/00071619200650231.
[35] R.L. Fletcher, M.E. Callow, The settlement, attachment and establishment of marine algal spores, Br. Phycol. J. 27 (1992) 303–329, https://doi.org/10.1080/00071619200650281.
[36] A.D. Boney, Mucilage sheaths of spores of red algae, J. Mar. Biol. Ass. UK. 55 (1975) 511–518.
[37] S.J. Pang, K. Lüning, Tank cultivation of the red alga Palmaria palmata: year-round induction of tetrasporangia, tetraspore release in darkness and mass cultivation of vegetative thalli, Aquaculture 252 (2006) 20–30, https://doi.org/10.1016/j.aquaculture.2005.11.046.
[38] K. Alveal, H. Romo, C. Werlinger, E.C. Oliveira, Mass cultivation of the agar-producing alga Gracilaria chilensis (Rhodophyta) from spores, Aquaculture 148 (1997) 77–83, https://doi.org/10.1016/S0044-8486(96)01415-9.
[39] M. Avila, E. Ask, B. Rudolph, M. Nuñez, R. Norambuena, Economic feasibility of Sarcothalia (Gigartinales, Rhodophyta) cultivation, Hydrobiologia 398/399 (1999) 435–442, https://doi.org/10.1007/978-94-011-4449-0_52.
[40] E.A. Titlyanov, T.V. Titlyanova, P. Kadel, K. Lüning, New methods of obtaining plantlets and tetraspores from fragments and cell aggregates of meristematic and submeristematic tissue of the red alga Palmaria palmata, J. Exp. Mar. Bio. Ecol. 339 (2006) 55–64, https://doi.org/10.1016/j.jembe.2006.07.014.
[41] P.D. Kerrison, M.S. Stanley, A.D. Hughes, Textile substrate seeding of Saccharina latissima sporophytes using a binder: an effective method for the aquaculture of kelp, Algal Res. 33 (2018) 352–357, https://doi.org/10.1016/j.algal.2018.06.005.
[42] R. Gordon, S.H. Brawley, Effects of water motion on propagule release from algae with complex life histories, Mar. Biol. 145 (2004) 21–29, https://doi.org/10.1007/s00227-004-1305-y.
[43] B. Martínez, R.M. Viejo, J.M. Rico, R.H. Rødde, V.A. Faes, J. Oliveros, D. Álvarez, Open sea cultivation of Palmaria palmata (Rhodophyta) on the northern Spanish coast, Aquaculture 254 (2006) 376–387, https://doi.org/10.1016/j.aquaculture.2005.10.025.
[44] B. Santelices, Patterns of reproduction, dispersal and recruitment in seaweeds, Oceanogr. Mar. Biol. Annu. Rev. 28 (1990) 177–276.
[45] I. Martins, J.M. Oliveira, M.R. Flindt, J.C. Marques, The effect of salinity on the growth rate of the macroalgae Enteromorpha intestinalis (Chlorophyta) in the Mondego estuary (west Portugal), Acta Oecol. 20 (1999) 259–265, https://doi.org/10.1016/S1146-609X(99)00140-X.
[46] F.J.L. Gordillo, M.J. Dring, G. Savidge, Nitrate and phosphate uptake characteristics of three species of brown algae cultured at low salinity, Mar. Ecol. Prog. Ser. 234 (2002) 111–118 https://www.int-res.com/abstracts/meps/v234/p111-118/.

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Paper 3: New seeding methods for efficient hatchery production

Journal of Applied Phycology
https://doi.org/10.1007/s10811-019-01998-0

23RD INTERNATIONAL SEAWEED SYMPOSIUM, JEJU

New hatchery methods for efficient spore use and seedling production of *Palmaria palmata* (dulse)

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Received: 20 August 2019 / Revised and accepted: 11 November 2019

Abstract
Palmaria palmata (dulse) is a high valued rhodophyte; nevertheless, its hatchery methods are underdeveloped. New hatchery methods are required to improve the spore use efficiency and seeding quality, which are important benchmarks for a viable cultivation. This study investigated a method using vertical seeding tanks (exp. 1), hemispherical agitation and flow-through conditions to improve spore dispersal on net substrates. Tanks were inoculated with different amounts of sori tissue, which sporulated in three consecutive seeding periods. The results demonstrated significant effect of seeding period where 5–15 g FW sori could be used to seed three nets (~ 126 m rope) over the course of 9 days providing a density up to 10 seedlings cm^{-1} after 32 days. The effluent spores were collected in detaining tanks and germinated into a propagule mix of female and male gametophytes. The propagule mix was efficient as a secondary seeding inoculum, as propagules were able to reattach to substrates up to 39 days after their release as spores (exp. 2). Adding male gametes to the propagule mix and spore seeded ropes was tested as a relevant hatchery step to activate female gametophytes and significantly resulted in more than a doubling of seedlings (exp. 3). This study present new methods and strategies to improve spore use efficiency and to obtain an equal spore dispersal on net substrates for hatchery production of *P. palmata*.

Keywords Rhodophyta · Dulse cultivation · Spore dispersal · Fertilization · Hatchery · Tetraspores · Reattachment · Spore seeding

Introduction

The rhodophyte *Palmaria palmata* (L.) F. Weber and D. Mohr has traditionally been used for human consumption with records dating back to the ninth century (Mouritsen et al. 2013). The emerging evidence of its umami flavor (Mouritsen et al. 2013), bioactive and health properties of protein hydrolysates (Harnedy et al. 2014; Admassu et al. 2018), water extracts (Lee et al. 2017), and biorefined compounds (Schiener et al. 2017) has renewed interest in the use of *P. palmata*, resulting in increased focus on its cultivation. *P. palmata* can be cultivated in tanks using vegetative growth (Morgan and Simpson 1981; Pang and Lüning 2004; Matos et al. 2006; Corey et al. 2014), but the prospects of cultivating the species from tetraspores have lately received increasing attention in Europe (Edwards and Dring 2011; Werner and Dring 2011; Sanderson et al. 2012). However, despite studies founding the essential understanding of the life cycle (van der Meer and Chen 1979; van der Meer and Todd 1980) and cultivating *P. palmata* from spores (Browne 2001; Sanderson 2006; Werner and Dring 2011; Grandorf Bak 2019; Schmedes et al. 2019), the methodology for large-scale hatchery production in Europe is still in its infancy.

Essentially, the use of spores for cultivation of *P. palmata* is based on three steps: (1) the collection or induction of sori, (2) the release and dispersal of spores, and (3) the spore attachment and growth, and it is the efficiency and success of these three steps that determines the usefulness of a given hatchery protocol. As the steps are interlinked, the initial handling of sori, which represent only 8–10% of the total frond area during the peak season of fertility (Werner and Dring 2011), is crucially important. Currently, the most commonly used hatchery protocol to produce *P. palmata* seeded substrates is a flat tank using a 1:1 areal coverage of substrate with sori and a 3-day spore release duration. However, this method requires a large amount of sori and, furthermore, results in poor dispersal and high

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Published online: 09 December 2019

mortality (60–90%) of the spores (Werner and Dring 2011). Also, by the using this method, it is often observed that a considerable amount of the spores settle on tank surfaces instead of the intended cultivation substrate (personal observation).

Taking into account these results and observations, it is clear that there is a bottleneck in spore use efficiency within *P. palmata* hatcheries. This particular matter was addressed in a recent study suggesting a GMA-seeding method (germination, maceration, and agitation methods) as an alternative seeding method for *P. palmata* (Schmedes et al. 2019). This study found that germinated propagules (i.e., a mix of spores and seedlings) of *P. palmata* were able to establish discoid reattachment to a substrate after forced de-attachment and maceration, resulting in high settlement efficiency as well as a high dispersal of the propagules. However, the extensive use of this seeding method is still unknown. Besides the use of this seeding strategy, the use of male gametes of *P. palmata* to fertilize female gametophytes has been suggested as a potential hatchery step to optimize the hatchery production (Mine and Tatewaki 1994; Le Gall et al. 2004; Schmedes et al. 2019). When using sori for spore-seeding, a mixture of male and female spores develops after attachment; however, only the males will develop into a harvestable thallus unless a fertilization step, enabling zygote formation in the female gametophyte, is included. Theoretically, this would double the seedling density, as the crustose-like female gametophyte will develop into a sporophytic thallus after the zygote. Yet, this is still uninvestigated under relevant hatchery conditions.

Because of the continued challenges in producing *P. palmata* sporophytes and gametophytes, this present study investigated hatchery methods and strategies based on Schmedes et al. (2019) to further improve the utilization of spores for cultivation of *P. palmata* on substrates. Five strategies to improve the hatchery protocol were tested: (1) a vertical seeding tank in flow-through condition agitated with air bubbles to promote dispersal of spores; (2) the effect of different amount of sori during the seeding phase; (3) the effect of using the same sori material in three consecutive seeding periods on the spore germination and seedling density; (4) the use of detained spores as a propagule seeding inoculum, based on the GMA method; (5) The effect of fertilization on the number of attached seedlings under relevant hatchery conditions.

Materials and methods

Three experiments were set up to test the five improvements in hatchery techniques described above. Experiment 1 investigated a new flow-through seeding system for improved seeding dispersal, additionally using different sori amounts in consecutive seeding periods as a means to improve sori use. This experiment addressed the first three steps of hatchery improvements. Experiment 2 covered step four, optimizing the use of detained spores, and finally, experiment 3 exploited the opportunity to include fertilization as a means to improve spore use, addressing step five of hatchery improvement techniques.

Experiment 1: spore seeding and sori use

Nine conical tanks (polyethylene, 30 L) were set up as seeding tanks in a parallel flow-through system with a water flow of 0.5 L min^{-1} (Fig. 1a) in a 10 °C cold room. Air bubbles from the bottom (1.2 L min^{-1}), bubbled through the sori package near the surface (Fig. 1c), generated a hemispherical circulation of the water allowing the spores to disperse and aerated the tank volume. Water for the entire system was circulated from a reservoir tank with a circulation pump and exchanged (10% of total volume) on a daily basis. No nutrients were added. From each seeding tank, an outlet, placed diagonal to the inlet, led the effluent water directly into a 2-L spore-detaining tank (SDT) (Fig. 1b). Here, effluent spores aggregated on the bottom, while water was led out in the top and back to the reservoir tank through a nylon filter (15 μm) for UV treatment (60 W). Spores detained in the SDTs were used in experiment 2.

Twenty-seven pieces of net (polypropylene, 0.25 × 1.40 m, ø = 5-7 mm) were prepared as cultivation substrate by soaking them in lukewarm tap water a month before, then kept in clean seawater for 7 days before use. At the experimental start, the nets were submerged in the seeding tanks as a two-layered net spiral (Fig. 1b).

Fertile *Palmaria palmata* tetrasporophytes and male gametophytes were collected January 4, 2019 in the intertidal zone near Fornæs light house, Denmark (56.443534 N, 10.958985 E) and kept in running seawater (4 °C) in dim natural light (~15 μmol photons m^{-2} s^{-1}, PAR). On 10 January, 218 fertile fronds were rinsed in 0.2 μm filtered and sterilized seawater (Fig. 2a) before being desiccated for 20 h at 5 °C in darkness. The presence of ripe sporangia was verified by inspection (Fig. 2b). Triplicates of three groups of sori amount (5, 10, or 15 g FW; fresh weight) were prepared as sori packages by placing the tissue between two layers of plastic net (15 × 15 cm; Fig. 2c).

The experiment started January 11, 2019 by adding a net and a sori package (3 replicates of 3 sori amounts) to each of the nine tanks. The seeding ran over a course of 9 days, keeping the same sori package in the tanks for all 9 days but exchanging the nets with new ones every third day to test the potential of consecutive seeding periods (days 0–3, days 3–6, days 6–9) using the same sori. During seeding, the surface irradiance was 15–22 μmol photons m^{-2} s^{-1}, 10:14 h L:D. At the end of each consecutive seeding period, the nine nets were labeled and transferred to nursery tanks containing 300 L enriched seawater (0.2 μm filtered), 10% strength of

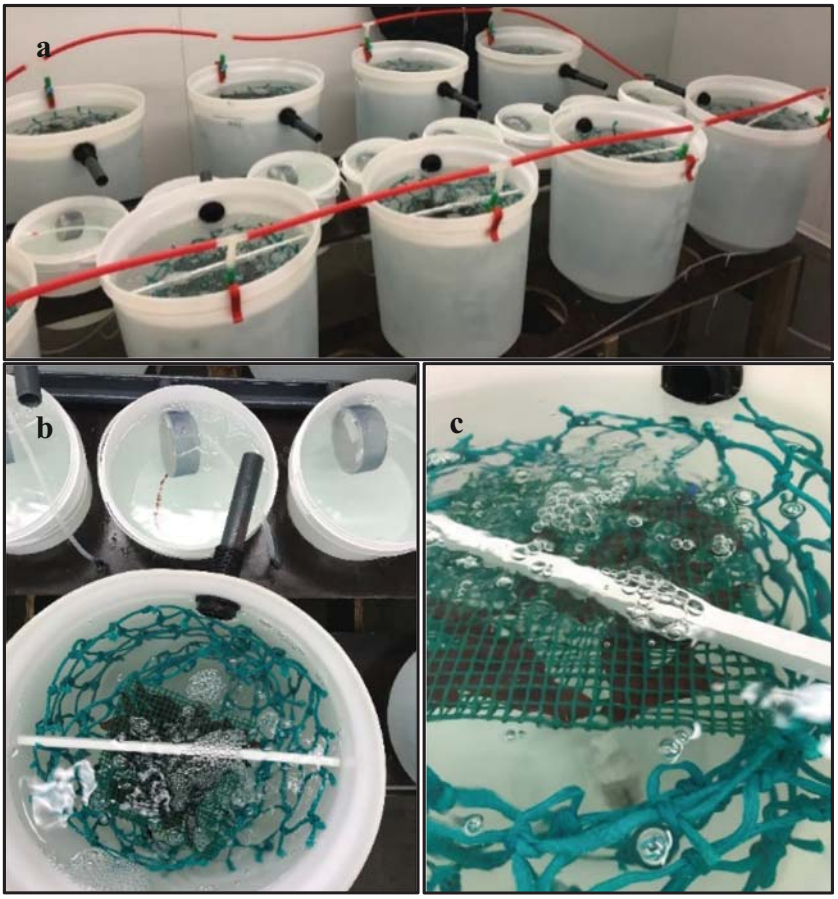


Fig. 1 **a** Parallel flow-through setup of conical seeding tanks (30 L) used for experiment 1. **b** The sori packages were fixed centrally above the net spirals and 2 cm below water surface. Effluent spores were detained in spore-detaining tanks (SDTs). **c** Aeration (1.2 L min^{-1}) from the bottom provided hemispherical water circulation and dispersal of released spores

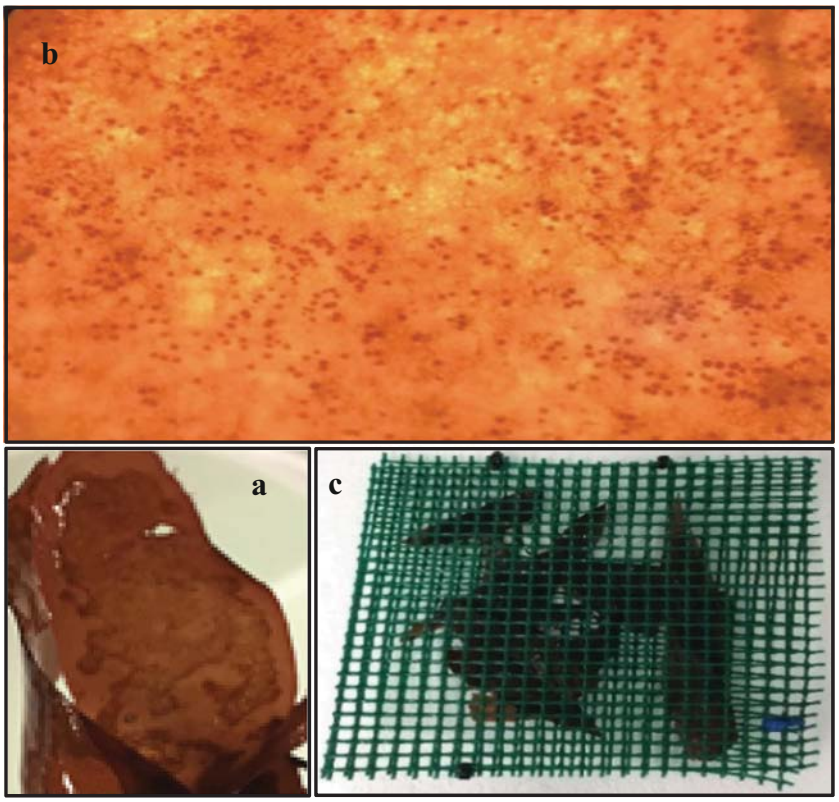


Fig. 2 **a** Fertile tetrasporophytic fronds were cleaned and the presence of dark-red sporangia were validated (**b**). **c** Sori packages (5, 10, or 15 g FW) were prepared by placing the sori between two layers of green plastic mesh ($n = 3$)

Varicon Aqua Cell-Hi F2P, a F/2 nutrient medium with vitamins based on Guillard and Ryther (1962). Air stones provided water agitation in the tanks after 3 days. Irradiance was 35 μmol photons m^{-2} s^{-1} (10:14 h L:D) but raised to 50 μmol photons m^{-2} s^{-1} from week 2.

Data acquisition

The net-seeding efficiency using sori in the flow-through system was calculated by Eq. 1:

$$Net\ seeding\ efficiency = \frac{\#spores\ on\ net}{\#Total\ spores(net + detained)} \times 100 \quad (1)$$

This was carried out by counting the number of attached spores and seedlings on a 4-cm net subsample for each of the 9 nets ($n = 3$) 3 days after each seeding period. Additionally, the nets were counted at days 19 and 32 after each of the consecutive seeding periods to compare spore and seedling density within and between groups over time. The subsamples were taken from the same position regarding the inlet and outlet of the tanks on each net spiral. To verify an even spore dispersal on the entire net, additional subsamples were taken from the 10 g sori batch in the first seeding period (days 0–3). From each net ($n = 3$), three subsamples were taken from the bottom and top part of the net ($n = 9$) and tested for unequal variance using Levene's test. Similarly, by counting spores on subsamples ($n = 21$) from all around one net spiral in the same vertical level, the spore dispersal was assessed. By verification, we extrapolated the spore and seedling densities for each counting day and calculated the germination success by Eq. 2:

$$\text{Germination } (\%) = \frac{\#\text{Seedlings}}{\#\text{Total propagules (spores + seedlings)}} \times 100 \quad (2)$$

The total number of detained spores in each SDT after a seeding event (Fig. 3a) was estimated based on subsample counting, by the following process: (1) A homogenous spore mix (Fig. 3b) was prepared by reducing the SDT volume, dislodging the spore aggregates and macerating the solution for 30 s with a kitchen blender. (2) A 10-mL subsample was transferred to a gridded Petri dishes (Fig. 3c) and added 20 mL enriched (10% F/2) seawater. (3) The spores were kept at 5 °C and 5 ± 2 μmol photons m^{-2} s^{-1} (10:14 h L:D) for 3 days before five random fields (each 0.0035 mm^2) were photographed and counted. The total number of spores was estimated by factor multiplying the mean count of subsamples (STD_{VOLUME} 10 mL^{-1} subsample and Petri dish area 0.0035 mm^{-2}).

Experiment 2: mixed propagules as seeding inoculum

In experiment 2, the propagules germinated from detained spores from experiment 1 were tested as seeding inoculum, according to the GMA method (Schmedes et al. 2019). After subsampling for Petri dish cultures in experiment 1, the rest of the macerated spore solution from the last seeding period (days 6–9) was poured into a plexiglas containers (5 L), settled on the bottom (Fig. 4a) and cultivated at 20 μmol photons m^{-2} s^{-1} (adjusted to 5 ± 2 μmol photons m^{-2} s^{-1}, after 10 days) and 5 °C. After 1 day, enriched seawater and germanium-oxide (10% F/2 + 1 mg L^{-1} GeO) was added and after 6 days, an air stone was put for aeration. The detained spores germinated into a mixture of propagules (i.e., spores and gametophytes) at these conditions before use.

Twenty-seven days after the initiation of days 6–9 seeding period, some of the propagules on the bottom were dislodged (Fig. 4b), suspended in 400 mL enriched (10% F/2) seawater, and macerated to break propagule aggregates (Fig. 4c) according to Schmedes et al. (2019). The propagules were photographed before and after the maceration treatment (Fig. 4d, e). A total of 20 mL of the propagule mix was added to each of 10 beakers ($n = 10$) containing 400 mL enriched (10% F/2) seawater and a piece of rope (10 cm) standing upright in the beaker to test the ability of the propagules to reattach. The rope was made by untangling several net meshes and cut and cut into pieces. Immediately, 1 mL subsamples were withdrawn and the concentration of macerated propagules was estimated to 259 ± 8 spores mL^{-1} and 422 ± 13 seedlings mL^{-1} (mean ± SE, $n =$

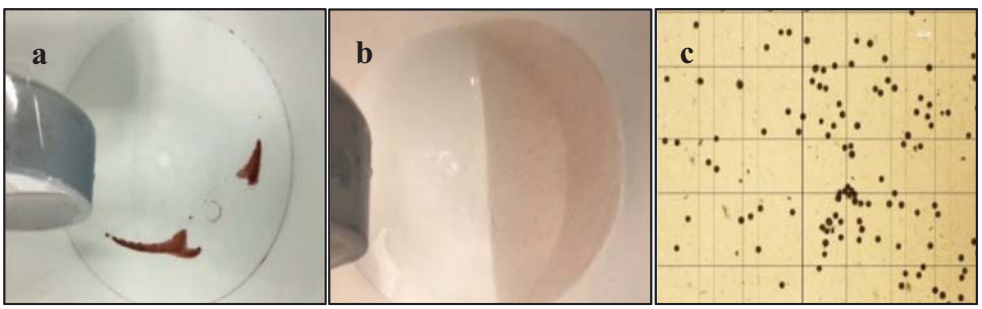


Fig. 3 **a** Dark-red aggregates of spores on the bottom of spore-detaining tanks (STDs) accumulated during sporulation. **b** A well-mixed spore solution was obtained by dislodging the aggregates and macerating them. **c** The number spores were counted in five random fields (each of 0.0035 mm^2) and used as a basis to estimate the total amount of detained spores

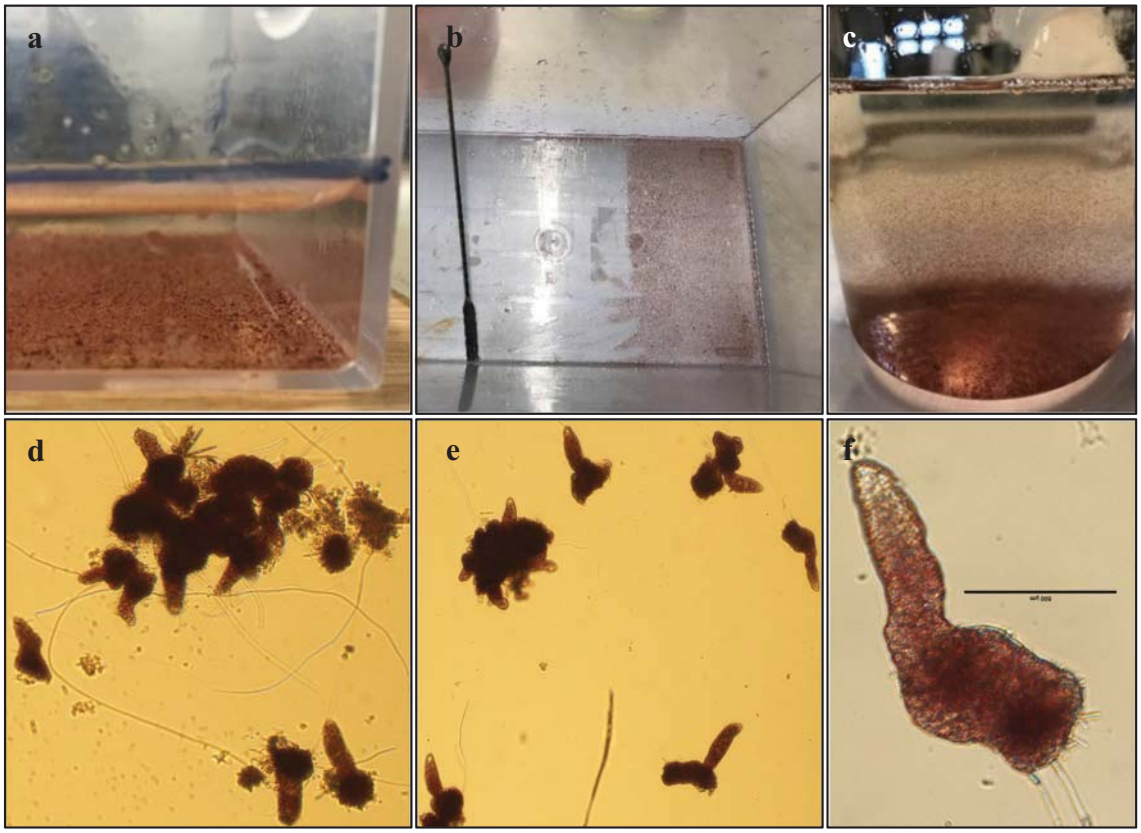


Fig. 4 **a** Detained and macerated spores of *P. palmata* at the bottom of a 5-L Plexiglas tank. **b** Plexiglas tank after dislodgement of propagules on the left side of the tank. **c** Propagule mix after dislodgement and maceration. The solution was inspected before (**d**) and after (**e**) the maceration treatment. **f** Single individual seedling displaying hair-like proliferations from the basal disc area. Scale bar represent 500 μm

4). The beakers were constantly agitated from the bottom via air bubbles (2.5 L min^{-1}) to promote the dispersal of the propagules.

After 3 days inoculation ("agitation period days 0–2"), the maximum density of attached spores and seedlings was estimated by counting the three densest subparts (0.5 cm) for each rope, after which the ropes were transferred to 20 L tanks at the similar conditions until they were counted again at day 10. After transferring the ropes pieces out of the beakers, the propagule mix that maintained in the beakers under same conditions until day 10, where concentration (mean of technical replicate ± SE) of spores (237 ± 7) and seedlings (394 ± 9) was similar to the starting concentration (two-sampled *t* tests: $t(4)$, $p = 0.086$, $p = 0.131$, respectively). Then, new pieces of rope were added for 3 days inoculation to see how long after the dislodgement and maceration the propagules would be able to reattach ("agitation period days 10–12"). Also, at day 10, new beakers ($n = 10$) were set up under same conditions using freshly dislodged and macerated propagules to see if the age of the propagules (27 vs. 37 days) at dislodgement affected the ability to reattach. The concentration of propagules (250 ± 20 spores and 396 ± 22 seedlings) was similar (two-sampled *t* tests: $t(4)$, $p = 0.584$, $p = 0.341$) with the other start concentrations.

Experiment 3: including fertilization in hatchery

The effect of fertilizing female gametophytes was tested by adding male gametes to propagules cultured in petri dishes and spore-seeded ropes cultured in beakers. A solution of propagules (349 ± 26 propagules mL^{-1}, $n = 9$, technical replicates) was prepared from the same detained spore batch as used in experiment 2 by dislodging and macerating after 11 days of germination. A total of 15 mL propagule solution was then poured into each of 18 petri dishes and filled with additional 10 mL enriched (10% F/2) seawater and kept in 15 ± 3 μmol photons m^{-2} s^{-1} PAR (12:12 h L:D) irradiance at 5 °C.

At day 1, a solution of male gametes was prepared and 15 mL of this solution was added to nine petri dishes, containing the propagule solution. Nine other dishes received 15 mL seawater (10% F/2) as a control. The male gamete solution was prepared by desiccating (3 h, dark at 5 °C) thirty fertile male gametophytic fronds (22.4 g FW; see Fig. 5a, b) before being rehydrated (1 h, 5 μmol photons m^{-2} s^{-1} PAR) in an agitated volume of 400 mL seawater + 0.4 mL GeO (1 g L^{-1}). The male gametes (Ø ~5 μm) were visible using high magnification (Fig. 5c, d). The concentration of gametes was estimated to be ~ 1000 gametes mL^{-1}, using a Neubauer cell counting chamber. After adding, the cultures were mixed on

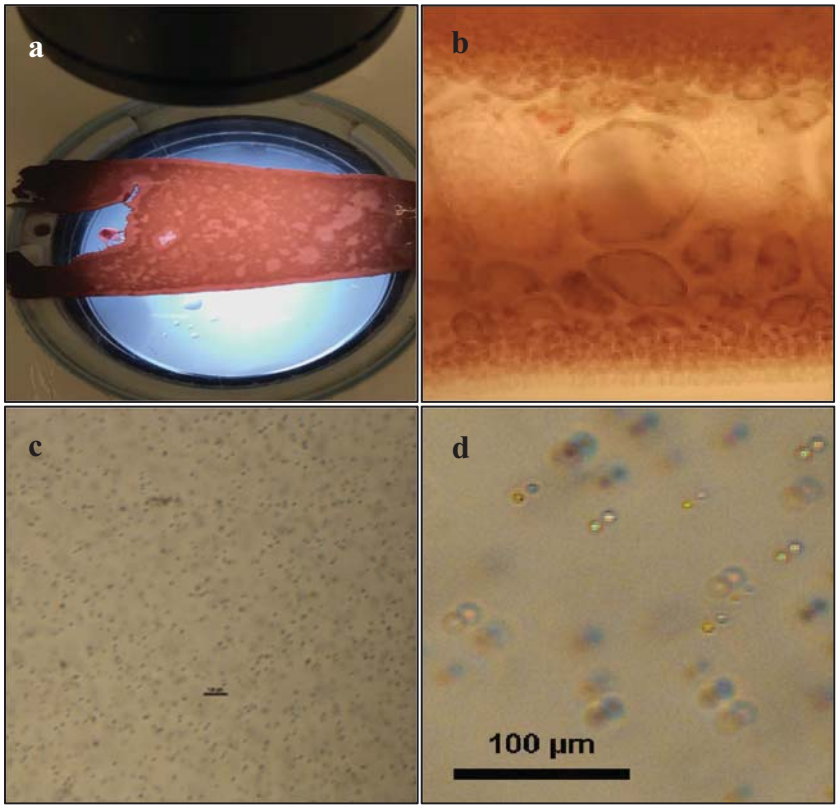


Fig. 5 **a** Fertile male gametophytes inspected by stereomicroscopy. **b** Cross-section microscopy was used to verify reproductive appearance of the males. **c**, **d** Spherical male gamete released in a solution

a stirring table for 30 min at 160 rpm. At day 4 and 6, the petri dish volumes were exchanged with new additions of male gamete solution (5 mL, ~ 10^6 gametes mL^{-1}).

For each petri dish, spores and seedlings were counted in five random fields (each 0.0035 mm^2) at days 0, 3, 10 and 19 and the number of seedlings was adjusted to the spore count of previous counting day.

In addition, we assessed the effect of fertilization on the seeded rope pieces (exp. 2). This was carried out in beakers (1.8 L) kept at 5 °C, in which 4 cm pieces of seeded rope were tumbled by air bubble agitation ($n = 11$). The pieces were excised from "days 3–6 and 10 g sori" nets (exp. 1) and nursed for 16 days at 10 °C, before being transferred to the beakers. This was followed by adding 40 mL solution containing male gametes to the beakers, while a control group was added the same enriched seawater (10% F/2) with no gametes ($n = 11$). The number of spores and seedlings was counted at days 1, 3, 6, and 12, and the seedling number was presented as relative to spore number.

Statistics

For all data sets, Shapiro-Wilk test was used to check normality and Levene test was used to check variance homogeneity. The analysis was carried out using SAS, JMP 13, using a significance level of 0.05. When sufficient, data were log-transformed to ensure homogeneity of variance and normal distributed residuals and ANOVAs were conducted to compare the main effects. In case of none normal distribution or homogeneity of variance, the Wilcoxon two-sample test was used. All data are given as mean ± standard error (SE), unless stated otherwise.

Experiment 1 In a factorial design, nets were manipulated to be in one of nine groups forming the combination of consecutive seeding periods (3 levels; days 0–3, days 3–6, days 6–9) and sori amount (3 levels; 5, 10, 15 g FW), and 2-way ANOVA, including the interaction term, using log-transformed data was conducted to compare effects on spore and seedling numbers attached to the nets, the number of detained spores in STDs, the spore-seeding efficiency, as well as the germination success (%) between all groups, followed by Tukey's HSD post hoc test. The Wilcoxon two-sample test was used to assess for any significant change in mean densities of spores and seedlings across the counting days within each factorial group.

Experiment 2 Datasets were log-transformed to ensure normal distribution, but spore counts did not obey homogeneity of variance (Levene, $p < 0.0208$). Hence, the Wilcoxon test was used to assess for significant difference in mean settlement density at each count day.

Experiment 3 For the petri dish cultures, the effect of adding male gametes on the number of seedlings adjusted to the previous spore count was compared using a two-tailed Student's *t* test ($n = 11$). For the rope cultures, the univariate repeated measures ANOVA was used to test effect of the between factor (two levels; male gametes vs. control) and the within factors (time; four levels) on the adjusted number of seedlings. Datasets were normal distributed and displayed equal variance after log-transformation. The Mauchly's sphericity test: χ^2 (5) = 8.3513, $p = 0.1379$ allowed to report the p value of the F test.

Results

Experiment 1: spore seeding and sori use

Spores settled on all nets during the consecutive seeding periods and for all amount of sori used (Fig. 6a–c). Spores were present on all counting days, while seedlings (Fig. 6d–f) were only present from counting day 19 and onwards. On counting day 3, the number of spores attached to the nets was significantly affected by seeding periods (2-way ANOVA: $F_{2,8} = 7.2461$, $p = 0.0049$), the second (Fig. 6b) and third (Fig. 6c) seeding periods (days 3–6 and days 6–9) showing similar and significant higher spore densities (Tukey's: $p = 0.0036$) than the first seeding period (days 0–3) (Fig. 6a). Also, the sori amount used for seeding, significantly affected the number of attached spores (2-way ANOVA: $F_{2,8} = 4.0989$, $p = 0.0341$), showing significant difference between high and low sori amounts (Tukey's, $p = 0.0109$). On counting day 19, the number of spores (Fig. 6a–c) and seedlings (Fig. 6d–f) as well as the spore germination success (Fig. 6g–i) was similar for all groups (2-way ANOVA: $p > 0.5606$). On counting day 32, the spore settlement was still similar across all groups (2-way ANOVA: $p > 0.1179$) (Fig. 6a–c), whereas the number of seedlings (Fig. 6d–f) was significant affected by seeding period (2-way ANOVA: $F_{2,8} = 6.1718$, $p = 0.0091$), showing similar seedling density in second and third period (Tukey's, $p = 0.8110$), both significantly higher than the first seeding period (Tukey's, $p = 0.0105$, $p = 0.0383$, respectively). At day 32, the average seedling density was 36 ± 8 seedlings per 4 cm. However, the germination success (Fig. 6g–i) up to 50–80% turned out to be similar between seeding periods (2-way ANOVA: $F_{2,8} = 3.0829$, $p = 0.0706$) and sori groups (2-way ANOVA: $F_{2,8} = 0.1873$, $p = 0.83$). Within each seeding period (Fig. 6a–c), the spore count decreased significantly over time (chi-squared: χ^2 (9) = 10.14, $p = 0.0063$), whereas the increase in seedling density was only significantly on nets produced in second (Mann-Whitney two-sample test: $p = 0.0030$) and third ($p = 0.0295$) seeding periods.

The additional rope pieces sampled to assess whether settlement was homogeneous dispersed across the entire net, showed that this was the case. Both spores and seedlings showed equal variance between top and bottom of the nets (spores: $p = 0.17$, seedlings: $p = 0.40$) and means (Mann-Whitney two-sample test: $p = 0.40$). Furthermore, the densities showed equal variance horizontally around the net spiral (spores: $p = 0.23$, seedlings: $p = 0.33$). Hence, the number of spores settled on rope pieces cut from the nets was extrapolated to whole nets (Fig. 7a–c) and summed with the number of detained spores (Fig. 7d–f) to calculate the total number of released spores. This number was then used to estimate a net seeding efficiency of the system (Fig. 7g–i) by Eq. 1. The net

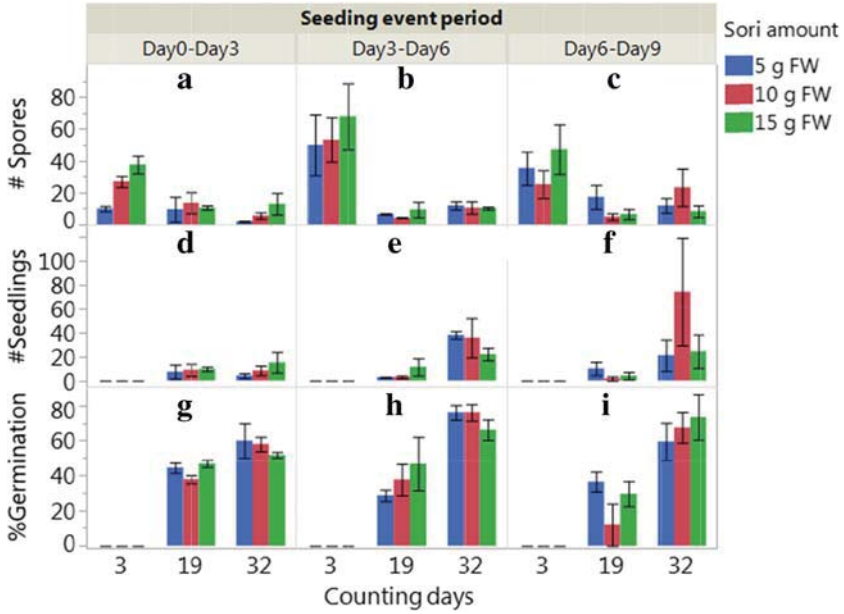


Fig. 6 The number of spores (**a**–**c**) and seedlings (**d**–**f**) attached to the nets and the spore germination percentage (**g**–**i**). 4 cm net pieces were counted on days 3, 19, and 32 after each of the three consecutive periods (days 0–3, days 3–6, days 6–9) with the use of different sori amounts (5, 10, 15 g). Data presented as mean ± SE, $n = 3$

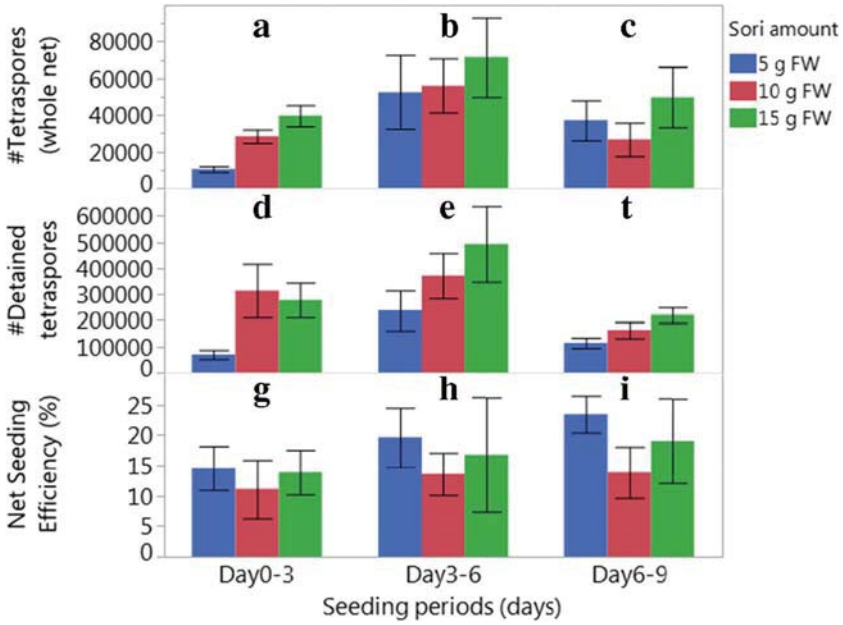


Fig. 7 **a**–**i** The extrapolated number of spores attached to the nets and **d**–**f** the total number of detained spores collected in the spore-detaining tanks (SDTs) as a function of three consecutive seeding periods and the use of three different amounts of sori (5, 10, 15 g FW). **g**–**i** Net seeding efficiency, calculated from Eq. 1. Data presented as mean ± SE, $n = 3$

seeding efficiency of the agitated, flow-through seeding tank system using sori packages was in average 16 ± 1.6% (Fig. 7g–i) and not affected by either seeding period or sori amount (2w-an: $p > 0.28$). This indicates that app. 84% of the released tetraspores were washed out and detained (Fig. 7d–f).

Effluent spores aggregated as red plumages on the bottom of all the SDTs, and were visible within 3 h during the first seeding period (days 0–3). The number of detained spores from the flow-through system (Fig. 7d–f) was significantly affected by seeding period (2-way ANOVA: $F_{2,8} = 5.66$, $p = 0.0124$), where second period provided higher detained spore yield compared to the third period (Tukey's: $p = 0.0117$) and similar spore yield as first period ($p = 0.6593$). The amount of sori used also affected the number of detained spores significantly (2-way ANOVA: $F_{2,8} = 5.07$, $p = 0.0178$) where 15 g sori provided higher yield compared to 5 g sori (Tukey's: $p = 0.0175$). In total, the amount of released spores (detained + attached to nets) from the three different sori packages during 9 days of sporulation was significantly higher for the 15 g sori group (1,300,563 ± 71,639), compared to the 10 g sori (959,697 ± 69,618) and the 5 g sori group (522,230 ± 61,869) spores.

Experiment 2: mixed propagules as seeding inoculum

On counting day 3, the number of reattached spores (Fig. 8a vs. b) and seedlings (Fig. 8c vs. d) was significantly higher, when the inoculum was dislodged at days 27–29 compared to days 37–39 (Mann-Whitney two-sample test

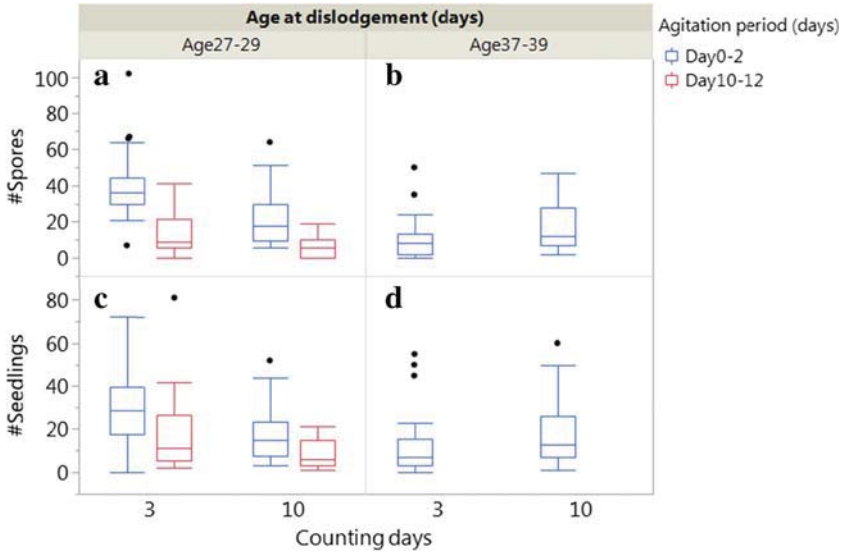


Fig. 8 Attached spores (**a**, **b**) and seedlings (**c**, **d**) on rope pieces seeded with macerated propagule solution at days 3 and 10 and as a function of age at dislodgement (days 27–29 vs. days 37–39) and agitation period (days 0–2 vs. days 10–12). Data is presented as outlier box plots (1st, 3rd quartile whiskers) based on three subpart counts on each of ten pieces of rope ($n = 10$, $N = 30$)

(10), $p < 0.0001$). Furthermore, the number of reattached spores and seedlings on ropes exposed to different agitation periods (days 0–2 vs. days 10–12) was significantly different ($p < 0.0001$, $p = 0.0012$, respectively). After nursing these ropes for additional 7 days (counting day 10), the effect of agitation period on the density of spores and seedlings remained significant ($p = 0.0002$, $p = 0.0009$). In contrast, the effect of inoculum age at dislodgement was insignificant on the spore density ($p = 0.0697$) and seedling density ($p = 0.9938$).

Experiment 3: including fertilization in hatchery

After 19 days in petri dish culture, the adjusted seedling number was 50.25% higher (Fig. 9a) with male gametes solution added, compared to the control (two-tailed Student's t test: $t(10)$, $p < 0.0001$), whereas no significant differences were found at the other days ($p > 0.98$), except day 3, where the control group showed a slightly higher number of seedlings ($p = 0.0042$).

The effect of adding male gametes to the rope cultures (Fig. 9b) was significant from day 3 (MANOVA: $F_{1,19} = 121.445$, $p < 0.0001$) with higher number of seedlings at all counting days (253.2% at day 12). Yet, the effect of time was insignificant on the number of seedlings (MANOVA: $F_{3,17} = 0.5462$, $p = 0.6573$) but caused a significant interaction term of gamete addition × time: $F_{3,17} = 4.0842$, $p = 0.0235$, because of the higher seedling count after day 0.

Discussion

The results of this study demonstrate effective methods and strategies in the pursuit of optimizing the hatchery production of *P. palmata* by using vertical seeding tanks, consecutive use of sori packages and agitation during the seeding phase. The strategy of using a secondary seeding inoculum of germinated propagules, based on collecting the effluent spores, obviously increase the spore use efficiency. With presented seeding system, 9 nets were spore-seeded, with an equivalent linear length of ~ 126 m of rope, using as little as 5 g FW sori, which result in an average of 9 seedling cm^{-1} after 32 days. The use of spores for seeding substrates is prospective for larger scale cultivation (Browne 2001; Edwards 2007; Werner and Dring 2011). The latter argued that an initial seeding density of spores cm^{-1} is required to obtain a final seedling density of ~ 6–8 seedlings cm^{-1} as a mortality rate of spores of 60–80% took place. On top, only male gametophytes developed a thallus, representing 50% of the total amount of spores. The initial spore density encountered in the present study (exp. 1) on the nets was lower than 100 spores cm^{-1}; however, we estimated a spore germination success (reciprocal to mortality) of 50–80% on the nets similar to what was achieved in a previous study (Le Gall et al. 2004). This indicates that the seeding and nursery conditions presented here were good, though it might be overestimated, as we were not able to count the dying spores. In contrast, we observed a germination success of only 8–14% in petri dishes cultured in stagnant seawater under the same conditions (unpublished work), which is in the range of previous report (Edwards 2007; Edwards and Dring 2011). By using higher amounts of sori in the seeding phase of net, the nets showed higher spore density after 3 days, but decreased to similar levels for all sori amount used, after 32 days of nursery, in line with previous findings (Edwards 2007; Werner and Dring 2011). In present study, the average seedling density after 32 days of nursery was not significantly affected by the sori amounts tested. Besides testing efficient ways to handle sori tissue for optimal spore yield, it is important that future hatchery trials consider the sori-to-substrate density, as high spore density seem to even out during nursery; thus, the sori could have been used more efficient. A current hatchery protocol for *P. palmata* found that a sori-to-substrate ratio of 150 g FW sori 84 m^{-1} substrate was required to secure sufficient seedling density, which converts to ~ 130 kg FW fronds to seed one long-line of 100 m (Werner and Dring 2011). In comparison, several kilometers of substrate can be seeded with motile zoospores of *Saccharina latissima* by using 150 g sori, due to the multifold number of biflagellate

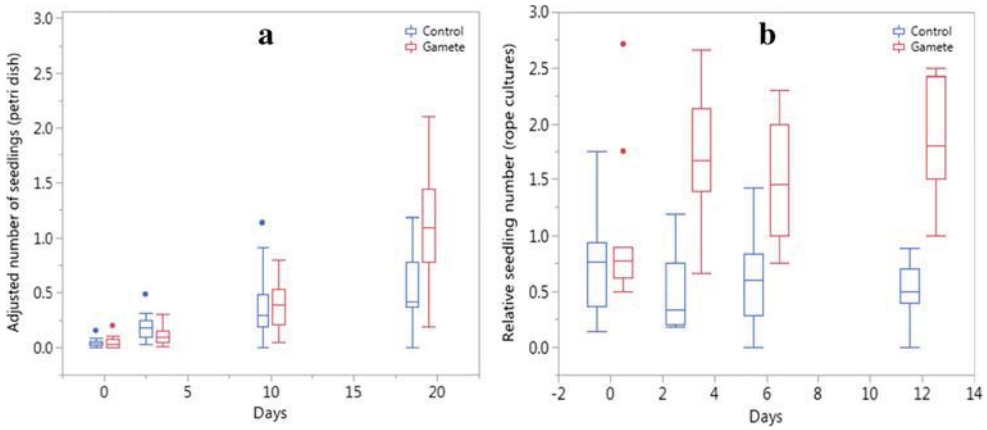


Fig. 9 **a** The normalized seedling count in petri dish cultures (n = 10) of macerated *P. palmata* propagules as an effect of adding male gametes. **b** The normalized seedling count on spore-seeded rope cultures (n = 11) as an effect of adding male gametes. Data is presented as outlier box plots (1st, 3rd quartile whiskers)

zoospores released within 1 h and their capability of high dispersal (personal observation). We suggest that a lower amount of sori is sufficient for seeding, while considering the sori-to-substrate ratio.

Three times the amount of seeded substrate was produced by using three consecutive seeding periods, compared to the conventional 3-day seeding phase. The highest seedling density after 32 days for nets seeding was found in the second (~ 8 seedlings cm^{-1} and third seeing period (~ 10 seedlings cm^{-1}). In the first seeding period (days 0–3), our tank setup provided a spore-seeding efficiency of 16%, meaning that ~ 84% of the released tetraspores were detained in the down-stream detaining tanks. The amount of detained spores was highest after the second seeding period (days 3–6). Even 38 days after finalizing the last seeding period, we observed red tetraspore aggregates in some of the spore-detaining tanks. This observation supports previous findings, where sori was observed to release for 21 days (Schmedes et al. 2019) to 40 days (Wood 2018).

Several bottlenecks have been identified by using the current hatchery protocol for *P. palmata* for large-scale cultivation—a protocol where sori is placed above the cultivation substrates (1:1 areal coverage) in horizontal tanks, which impose a high sori requirement. Facing other challenges when hatching rhodophytes, such as seasonal variation in spore availability (Kain 1986; Le Gall et al. 2004), a relative low spore release yield (Edwards 2007), poor spore dispersal before settlement (Edwards and Dring 2011), and low survival of spores (Sanderson 2006; Werner and Dring 2011), all impose a low spore use efficiency and little control of seedling quality. Overall, an even spore dispersal and good seedling density was found in this study using a flow-through system and relative high aeration, agitating the water. Nevertheless, further investigations of the previous mentioned challenges are highly relevant to optimize before commercial hatcheries can be established. Hence, the recently developed GMA method (Schmedes et al. 2019) to improve the spore efficiency for hatching *P. palmata* was investigated. This method was applied by dislodging and maceration of detained spores, which then germinated into propagules, which again were macerated to break aggregates of spores and tiny seedlings into single and small groups of spores and seedlings, before used as a secondary seeding inoculum. The level of water agitation of 2.5 L min^{-1} dispersed the propagules, yet, did not compromise the establishment of a discoid reattachment on the ropes (exp. 2). Results demonstrated that the reattachment was negatively affected by the biological age at dislodgement and amount of days in agitation, which adds knowledge to the extent of which macerated *P. palmata* propagules can be used as seeding inoculum. This is in agreement with findings in other red seaweed species, such as *Chondracanthus chamissoi* (Gigartinales), which forms a substrate reattachment by the production of secondary attachment discs (Sáez et al. 2008). The thallus fragments showed a decreasing reattachment probability over time (Fonck et al. 2008). Also, the red seaweed *Gelidium chilense* (Montagne) formed bundles of rhizoids in agitated water (Santelices and Varela 1994), while absent in *Gelidium coulteri* cultured in stagnant water conditions (Macler and West 1987). In comparison, a direct seeding method for cultivating the brown macroalgae species *S. latissima*, as a way to optimize hatchery duration and costs, is currently used by the Hortimare Company, as a seeding technique for commercial cultivation in Europe. Here, tiny germinated and activated sporophytes are applied to substrates and establish firm attachment with their developing haptera organs, with a potential benefit of using a glue (Kerrison et al. 2018). Whether the propagule seeding method of *P. palmata* would benefit by using glue as a means to maximize seedling density is of high interest due to the high commercial value of the biomass.

The inclusion of a fertilization step proved to be highly relevant to increase the number of seedlings in the hatchery production, by releasing male gametes and adding this male gamete solution to the female gametophytes that are developing their trichogynes (Mine and Tatewaki 1994; Le Gall et al. 2004). This resulted in at least a doubling of the number of seedlings on spore-seeded rope and suggests that male gametes can be added 10–22 days after seeding substrates to increase the overall spore use efficiency of *P. palmata*.

Conclusions

A new method is reported here to handle fertile sori of *P. palmata* for efficient release and dispersal of tetraspores using sori packages in vertical flow-through tanks. This achieves a sufficient seedling density ~ 9 seedlings cm^{-1} rope. Consecutive use of sori greatly improves the spore use efficiency and was found to have a positive effect on seeding quality, even for different sori amounts used in the sporulation phase. The spore inoculation success (net-seeding efficiency ~16%) was relative low; however, the ~ 84% effluent spores was used in propagule inoculation by using the GMA method. Hence, to increase the use of spores for efficient inoculation, the importance of addressing following parameters to further improve the spore use efficiency for seeding *P. palmata* is needed. The following are of importance, i.e., tank typology, water motion for optimal spore dispersal and settlement, intermittent batch conditions to increase net seeding efficiency, substrate-to-volume density, sori position, and flow-through rate. It was demonstrated that effluent spores from the SDTs were suited as a secondary seeding inoculum, using the germinate-macerate-agitate seeding method (Schmedes et al. 2019).

Acknowledgments We sincerely thank Pascal David Alain Barreau and Kasper Lenda Andersen for their technical help installing the experimental setup, as well as for system maintenance and data collection.

Funding information The study was funded by the Joint Doctoral Degree agreement between the National Institute of Aquatic Resources (DTU Aqua) at Technical University of Denmark and the Norwegian University of Science and Technology (NTNU), Norway, as well as the Tang.nu project (under Grant Agreement No. 13744, Velux Foundation) and the MacroSea project, Grant no. 254883, funded by the Research Council of Norway.

Compliance with ethical standards

Informed consent of the document with no conflict of interest. We did not conduct research on human or animals.

References

Admassu H, Gasmalla MAA, Yang R, Zhao W (2018) Bioactive peptides derived from seaweed protein and their health benefits : antihypertensive, antioxidant, and antidiabetic properties. J Food Sci 83:6–16

Browne KL (2001) Mariculture of the edible red alga, *Palmaria palmata*. PhD thesis, Queen's University of Belfast

Corey P, Kim JK, Duston J, Garbary DJ (2014) Growth and nutrient uptake by *Palmaria palmata* integrated with Atlantic halibut in a land-based aquaculture system. Algae 29:35–45

Edwards MD (2007) The cultivation of the edible red alga, *Palmaria palmata* for aquaculture, PhD thesis, The Queen's University of Belfast

Edwards MD, Dring MJ (2011) Open-Sea cultivation trial of the red alga, *Palmaria palmata* from seeded tetraspores in Strangford Lough, Northern Ireland. Aquaculture 317:203–209

Fonck E, Martínez R, Vásquez J, Bulboa C (2008) Factors that affect the re-attachment of *Chondracanthus chamissoi* (Rhodophyta, Gigartinales) thalli. J Appl Phycol 20:311–314

Grandorf Bak U (2019) Seaweed cultivation in the Faroe Islands: an investigation of the biochemical composition of selected macroalgal species, optimised seeding technics, and open-ocean cultivation methods from a commercial perspective. PhD thesis, Technical University of Denmark

Guillard RRL, Ryther JH (1962) Studies on marine planktonic diatoms I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. Can J Microbiol 8:229–239

Harnedy PA, Soler-Vila A, Edwards MD, FitzGerald RJ (2014) The effect of time and origin of harvest on the in vitro biological activity of *Palmaria palmata* protein hydrolysates. Food Res Int 62:746–752

Kain JM (1986) Plant size and reproductive phenology of six species of Rhodophyta in subtidal Isle of Man. Br Phycol J 21:129–138

Kerrison PD, Stanley MS, Hughes AD (2018) Textile substrate seeding of *Saccharina latissima* sporophytes using a binder: an effective method for the aquaculture of kelp. Algal Res 33:352–357

Le Gall L, Pien S, Rusig AM (2004) Cultivation of *Palmaria palmata* (Palmariales, Rhodophyta) from isolated spores in semi-controlled conditions. Aquaculture 229:181–191

Lee D, Mizuho N, Yutaka S, Hiroki S (2017) Anti-inflammatory effects of dulse (*Palmaria palmata*) resulting from the simultaneous water-extraction of phycobiliproteins and chlorophyll *a*. Food Res Int 100: 514–521

Macler BA, West JA (1987) Life history and physiology of the red alga, *Gelidium coulteri*, in unialgal culture. Aquaculture 61:281–293

Matos J, Costa S, Rodrigues A, Pereira R, Pinto IS (2006) Experimental integrated aquaculture of fish and red seaweeds in Northen Portugal. Aquaculture 252:31–42

Mine I, Tatewaki M (1994) Attachment and fusion of gametes during fertilization of *Palmaria sp*. (Rhodophyta). J Phycol 30:55–66

Morgan KC, Simpson FJ (1981) The cultivation of *Palmaria palmata*: effect of light intensity and temperature on growth and chemical composition. Bot Mar 24:547–552

Mouritsen OG, Dawczynski C, Duelund L, Jahreis G, Vetter W, Schröder M (2013) On the human consumption of the red seaweed dulse (*Palmaria palmata* (L.) Weber & Mohr). J Appl Phycol 25:1777–1791

Pang S, Lüning K (2004) Tank cultivation of the red alga *Palmaria palmata*: effects of intermittent light on growth rate, yield and growth kinetics. J Appl Phycol 16:93–99

Sáez F, Macchiavello J, Fonck E, Bulboa C (2008) The role of the secondary attachment disc in the vegetative propagation of *Chondracanthus chamissoi* (Gigartinales; Rhodophyta). Aquat Bot 89:63–65

Sanderson JC (2006) Reducing the environmental impact of sea-cage fish farming through cultivation of seaweed. PhD thesis, UHI Millenium Institute & Scottish Association of Marine Science (SAMS)

Sanderson JC, Dring MJ, Davidson K, Kelly MS (2012) Culture, yield and bioremediation potential of *Palmaria palmata* (Linnaeus) Weber & Mohr and *Saccharina latissima* (Linnaeus) C.E. Lane, C. Mayes, Druehl & G.W. Saunders adjacent to fish farm cages in Northwest Scotland. Aquaculture 354–355:128–135

Santelices B, Varela D (1994) Abiotic control of reattachment in *Gelidium chilense* (Montagne) Santelices & Montalva (Gelidiales; Rhodophyta). J Exp Mar Biol Ecol 177:145–155

Schiener P, Zhao S, Theodoridou K, Carey M, Mooney-McAuley K, Greenwell C (2017) The nutritional aspects of biorefined *Saccharina latissima*, *Ascophyllum nodosum* and *Palmaria palmata*. Biomass Convers Biorefin 7:221–235

Schmedes PS, Nielsen MM, Petersen JK (2019) Improved *Palmaria palmata* hatchery methods for tetraspore release, even settlement and high seedling survival using strong water agitation and macerated propagules. Algal Res 40. https://doi.org/10.1016/j.algal.2019.101494

van der Meer JP, Chen LCM (1979) Evidence for sexual reproduction in the red algae *Palmaria palmata* and *Halosaccion ramentaceum*. Can J Bot 57:2452–2459

van der Meer JP, Todd ER (1980) The life history of *Palmaria palmata* in culture. A new type for the Rhodophyta. Can J Bot 58:1250–1256

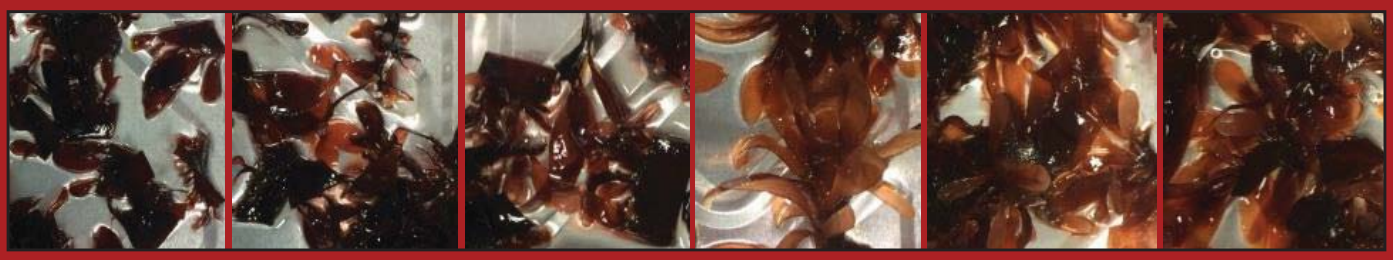
Werner A, Dring MJ (2011) Aquculture explained: cultivating *Palmaria palmata*. Irish Sea Fisheries Board, report no. 27 - project PBA/SW/07/001. Dublin

Wood E (2018) Cultivation of *Palmaria palmata*: spore release, vegetative propagation and decontamination techniques. Scottish Associaiton for Marine Science (SAMS) and University of the Highlands and Islands

Paper 4: Growth and productivity in land-based cultivation of *P. palmata*

After 6 weeks of low nutrient (120 µM-NO_3) pulse cultivation

10 PAR | 30 PAR | 80 PAR | 150 PAR | 200 PAR | 280 PAR



Followed by 2 weeks cultivation with one high nutrient pulse (1200 µM-N)

Journal of Applied Phycology

Productivity and growth rate in Palmaria palmata affected by salinity, irradiance and nutrient availability – the use of nutrient pulses and interventional cultivation

--Manuscript Draft--

Manuscript Number:	JAPH-D-20-00033
Full Title:	Productivity and growth rate in Palmaria palmata affected by salinity, irradiance and nutrient availability – the use of nutrient pulses and interventional cultivation
Article Type:	Original Research
Keywords:	Seedstock; RAS seaweed; nutrient pulses; N-starvation; N-removal; brackish salinity
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Funding Information:	Norges Forskningsråd (254883) — Not applicable
	Velux Fonden (DK) (13744) — Not applicable
	Norges Teknisk-Naturvitenskapelige Universitet — Not applicable
	Danmarks Tekniske Universitet — Mr Peter Søndergaard Schmedes
Abstract:	Land-based cultivation of the rhodophyte Palmaria palmata is promising for high productivity and nutrient mitigation, yet the cultivation strategy and the knowledge of the effect of various environmental factors is incomplete. In a two-phased cultivation trial, marginal proliferations were used as seedstock to test the impact of irradiance (10-280 µmol photons m -2 s -1 PAR) by consecutive nutrient phases using pulse additions (10% vs. 100% F/2+) on specific growth rate (SGR) and productivity (exp.1). The effect of salinity (15-35‰) and nutrient concentration (10 vs. 100% F/2+) on frond growth was investigated (exp.2). The SGR peaked at 200 µmol photons m -2 s -1 PAR in both nutrient phases with a peak of 6.86±0.4% d -1 (mean±SE, n=3). Above 80 µmol photons m -2 s -1 PAR, thalli turned pale green after 3 weeks at low nutrient. Shifting to a high nutrient cultivation, thalli recovered its red color after ten days, even at 280 µmol photons m -2 s -1 PAR and significantly upshifted SGR dry matter (DM), nitrogen (N), phosphorous (P) and ash content by 79.3, 56.0, 27.3, and 16.4%, respectively. Peak productivity of DM (1.17 g DM m -2 d -1), carbon (C) (406.41 mg C m -2 d -1), N (20.61 mg N m -2 d -1), and P (2.06 mg P m -2 d -1) coincided SGR. Salinity significantly affected SGR of P. palmata , and peaked at 15‰. This study highlights the use of marginal proliferations seedstock and nutrient pulses for biomass propagation of P. palmata and suggests a boosted N removal while avoiding epiphytes using intervention practice.

DTU Aqua

15 January 2020

Dear Editors,

We are submitting an original research article titled “Productivity and growth rate in *Palmaria palmata* affected by salinity, irradiance and nutrient availability – the use of nutrient pulses and interventional cultivation” to be considered for publication in the Journal of Applied Phycology. The manuscript has not been previously published, in whole or in part, and it is not under consideration by any other journal. This submission is approved by both authors. If accepted, the manuscript will not be published elsewhere in the same form, in English or in any other language, without the written consent of the publisher.

The present study documents a new strategy to boost the nitrogen removal in land-based cultivation of the commercial valued rhodophyte *Palmaria palmata*. We found that marginal shoot proliferations are suitable seedstock with a potential for large-scale cultivation. We investigated the use of a relative long pre-cultivation phase using intermittent batch conditions and adding low nutrient pulses to obtain a sub-lethal N-starvation of the seedstock. This was done in a gradient of irradiance levels to identify the irradiance level for saturated and optimal growth and to find the optimal level of N-starvation. The N-starved material was used as a highly N-extractive seedstock in a consecutive high nutrient cultivation and the results demonstrate a substantial effect on the N removal rate. This two-phased cultivation strategy potential serve as an effective method to increase algal N removal in fish farm effluent while avoiding growth of epiphytes. Furthermore, in a factorial design growth trial using young fronds from the same wild population, we found an optimal growth rate at lowest salinity tested (15‰, parts per thousand), which was surprising, yet indicates a potential adaptation in *P. palmata* populations in the Inner Danish waters, which is a brackish saline estuary.

We also discuss further optimization of land-based cultivation of *P. palmata* regarding utilization of nutrient rich system water from Recirculated Aquaculture Systems, which we found have the option to control the salinity level over the course pf a year. We believe this paper augments current knowledge on cultivation of *P. palmata*, and would be appropriate for the readers of Journal of Applied Phycology.

With best regards,

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Productivity and growth rate in *Palmaria palmata* affected by salinity, irradiance and nutrient availability – the use of nutrient pulses and interventional cultivation

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Abstract

Land-based cultivation of the rhodophyte *Palmaria palmata* is promising for high productivity and nutrient mitigation, yet the cultivation strategy and the knowledge of the effect of various environmental factors is incomplete. In a two-phased cultivation trial, marginal proliferations were used as seedstock to test the impact of irradiance (10-280 µmol photons m^{-2} s^{-1} PAR) by consecutive nutrient phases using pulse additions (10% vs. 100 % F/2+) on specific growth rate (SGR) and productivity (exp.1). The effect of salinity (15-35‰) and nutrient concentration (10 vs. 100 % F/2+) on frond growth was investigated (exp.2). The SGR peaked at 200 µmol photons m^{-2} s^{-1} PAR in both nutrient phases with a peak of 6.86±0.4 % d^{-1} (mean±SE, n=3). Above 80 µmol photons m^{-2} s^{-1} PAR, thalli turned pale green after 3 weeks at low nutrient. Shifting to a high nutrient cultivation, thalli recovered its red color after ten days, even at 280 µmol photons m^{-2} s^{-1} PAR and significantly upshifted SGR dry matter (DM), nitrogen (N), phosphorous (P) and ash content by 79.3, 56.0, 27.3, and 16.4%, respectively. Peak productivity of DM (1.17 g DM m^{-2} d^{-1}), carbon (C) (406.41 mg C m^{-2} d^{-1}), N (20.61 mg N m^{-2} d^{-1}), and P (2.06 mg P m^{-2} d^{-1}) coincided with SGR. Salinity significantly affected SGR of *P. palmata*, and peaked at 15‰. This study highlights the use of marginal proliferations seedstock and nutrient pulses for biomass propagation of *P. palmata* and suggests a boosted N removal while avoiding epiphytes using intervention practice.

Key words: Seedstock; RAS seaweed; nutrient pulses; N-starvation; N-removal; brackish salinity

Introduction

The rhodophyte seaweed *Palmaria palmata* (L.) F. Weber & D. Mohr is highly valued in cosmetics and nutritious as food, snack and aquaculture feed supplement (Morgan et al. 1980a; Holdt and Kraan 2011; Mouritsen et al. 2013; Moroney et al. 2015). Currently, the *P. palmata* supply is sourced by hand harvesting wild populations which is time consuming and raise increasing concerns on sustainability of wild seaweed populations (Ugarte and Sharp 2001; Monagail et al. 2017). The increasing demand has promoted more than two decades of research in the cultivation of *P. palmata* to secure a reliable biomass supply (Martínez et al. 2006; Werner and Dring 2011; Schmedes and Nielsen 2019), reimbursed by the potential application of the bioactive hydrolysates derived from the species (Harnedy et al.

2014; Beaulieu et al. 2016). Despite several studies investigating methods for both open-sea and land-based cultivation of *P. palmata*, a production strategy to improve nitrogen removal in land-based cultivation is incomplete. In open-sea cultivation, based on propagating spore-seeded substrates with sporelings into a harvestable biomass (Alveal et al. 1997; Browne 2001), the environmental parameters varies naturally, which determine growth, but offers little control of biomass quality (Hurd and Harrison 2001; Hafting et al. 2012). Here, sporelings may experience harmful levels of irradiance, nutrient deficiency, stressful fluctuations in salinity (Montaque and Ley 1993) and may be prone to epi-fouling (Faes and Viejo 2003; Martínez and Rico 2008; Edwards and Dring 2011).

In contrast, land-based cultivation serves as a sustainable and scalable method to produce large quantities of biomass of desired quality sourcing nutrient rich seawater e.g. from a fish production (Haglund and Pedersen 1992; Kim et al. 2013). Here, full control of environmental factors, such as light, nutrients and salinity, is optional (Morgan and Simpson 1981ab; Hafting et al., 2012; Schmedes et al. 2019). Manipulation of these external factors in land-based cultivation holds the potential of a year-round production targeting specific valuable molecules (Parjikolaei et al. 2013), and could additionally secure a high production of a clean and marketable biomass as crucial for the application of cultivated seaweeds (Abreu et al. 2001; Neori et al. 2004).

Furthermore, the awareness of nutrient recycling by algae cultivation have promoted the concept of integrated multi-trophic aquaculture (IMTA), e.g., the co-cultivation of nutrient releasing fish and nutrient extractive seaweeds (Neori et al. 1996; DeBoer and Ryther 1977; Chopin et al. 1999; Troell et al. 2009). High nutrient availability in IMTA cultivation is beneficial as it leads to increased seaweed yield, pigmentation, and light-stress tolerance (Neish et al. 1977; Morgan and Simpson 1981b; Sanderson et al. 2012; Zhao et al. 2017). With the high market value of *P. palmata* and its high nutrient requirement, this species is a promising candidate for IMTA cultivation. Several studies have addressed the importance of scale and stocking density in culture systems on productivity and N removal (Pereira and Yarish 2006; Sanderson et al. 2012; Kim et al. 2013; Corey et al. 2014; Manriquez-Hernandez 2016). However, when *P. palmata* is cultivated in close proximity to net pen salmon farms (constant high nutrient concentration) studies find that tissue quality is compromised, e.g., the outer layer is not clean due to epi-fouling of ephemeral algae and detritus settlement on the seaweed (Sanderson 2006; Werner and Dring 2011). In contrast, pulse additions of nitrate or ammonium in tank trials show high productivity of *P. palmata* while avoiding or reducing growth of epiphytic ephemeral algae (Neish et al. 1977; Morgan and Simpson 1981b) in line with the nutrient control of the seasonal growth in several ephemeral algae Pedersen and Borum 1996). In a deducible manner, Mortensen (2017) suggest to move open-sea cultivation rigs with *Saccharina latissima* from nutrient poor to nutrient rich locations before harvest as a method to avoid epi-fouling and increase the tissue quality (protein content) within 1-2 weeks. Using lower light intensities, obtained by shading land-based tanks or submersion of cultivation rigs to deeper water depths could also be a way to avoid epi-fouling and increase tissue quality of cultivated *P. palmata*.

Apical frond tips of *P. palmata* are meristematic and often used as vegetative seedstock in land-based cultivation to propagate biomass and for nitrogen (N) removal (Matos 2006; Kim et al 2013; Corey 2014; Manríquez-Hernandez et al. 2016). However, it requires a high amount of broodstock fronds to produce this seedstock material, which may be a bottleneck for its large-scaled cultivation. Instead, tiny meristematic proliferating shoots, identified as dwarf-like proliferations and cell aggregates in the frond cortex layer is suggested as a source for large quantities of seedstock for

mass cultivation (Titlyanov et al. 2006). In addition, meristematic proliferations along the edge of older fronds is observed in *P. palmata* (Faes and Viejo 2003).

The present study aims to investigate the potential of cultivating *P. palmata* by the use of marginal proliferations as seedstock for land-based cultivation focusing on light saturation, optimal growth rates and productivity of dry matter (DM), carbon (C), nitrogen (N), phosphorus (P) and ash. The study assesses the use of a nutrient interventional practice, e.g., applying a consecutive cultivation phase of low (pre-cultivation) followed by a high nutrient availability, as a means to avoid epiphytes and to boost N removal. Finally, we investigate the combined effect of nutrient and salinity on *P. palmata* growth, as an identification of the cultivation potential of *P. palmata* in systems where these factors are in play. In a sea-based perspective, this could be in areas with stratified water column with high saline nutrient rich waters at the bottom and low saline nutrient poor waters on top. In a land-based situation, this could be in an IMTA situation using nutrient rich system water from fish farming, as indicated by fig. 1 showing an example of how salinity in system water from a a Recirculated Aquaculture System (RAS) fish farm can vary over time.

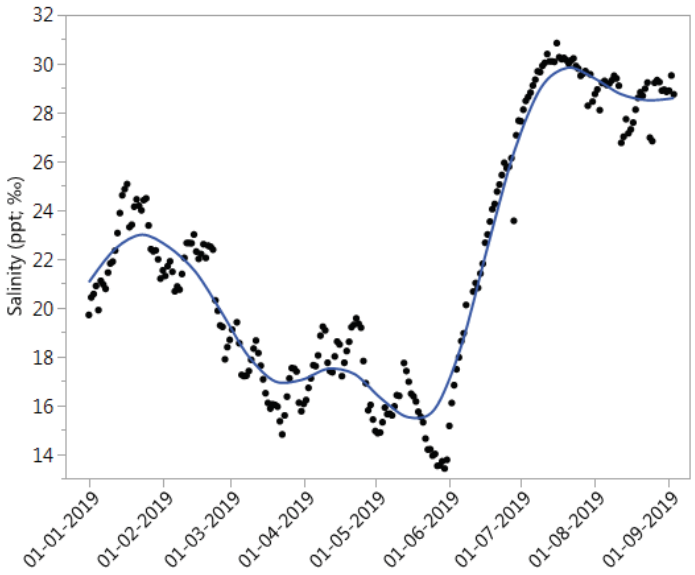

Figure 1. Example of the salinity in system, water from a land-based RAS salmon farm in Denmark. Data delivered by Atlantic Sapphire, Denmark.

Materials and methods

Vegetative fronds of *P. palmata* was collected January 12, 2018 (exp.1) and March 23, 2017 (exp.2) from an intertidal population at Fornæs light house (N 56.444096, E 10.958792) at 3-4 meters depth. At this location, the intertidal habitat is characterizing by yearly fluctuations in salinity (15-27.5‰) with an average of ~20‰. Collected fronds were acclimated by ramping temperature 1° C d^{-1} reaching 10 °C and cultured in filtered (0.2 µm) and UV treated local seawater (25-27‰) spiked to ~88.2 µM-NO_3 by adding 10% of full strength vitamin-containing F/2+ growth media with N/P ratio ~33 (Varicon Aqua Highcell F2P), based on Guillard and Ryther (1962).

Exp.1: Using marginal proliferations as vegetative seedstock - the effect of irradiance and nutrient pulses on growth and tissue contents

A nutrient intervention study without control treatment (quasi-experimental design) was conducted to assess the effect of irradiance on growth, productivity and tissue contents by low nutrient pulse pre-cultivation followed by a single high nutrient addition. Initially, the seedstock was prepared by cutting 1440 tissue fragments (0.5*0.5-1 cm) from the marginal edge of thirty adult fronds all possessing 2-7 proliferations of 1-5 mm length. The fragments were randomly dividing into 18 groups (each of 80 fragments) to constitute triplicates (n=3) of six light irradiance exposures (10, 30, 80, 150, 200, and 280 µmol photons m^{-2} s^{-1} PAR). The light intensity was measured perpendicular to the light source from the middle of each culture flask (in air) using a planar LI-COR quantum sensor measuring 400-700 nm irradiance. The initial and final stocking density was 3.2-3.5±0.1-0.2 g fresh weight (FW) L^{-1} and 7.1-7.5±0.2-0.9 g FW L^{-1} (mean±SE, n=18) respectively for each nutrient phase, which was in the range of the density used by Morgan and Simpson (1981a). The tissues were agitated in 1 L flat-bottom DURAN flasks with aeration. Weekly, during 6 weeks, a low nutrient pulse of 120 µM-NO_3 was added to fresh seawater and the biomass was weighed. This low nutrient addition treatment was assumed to imply nutrient starvation of the tissue (Neish et al. 1977; Morgan and Simpson, 1981b). After the 6 weeks pre-cultivation, a 2 weeks period of high nutrient pulse cultivation was initiated after reducing biomass density to 3.5±0.2 (mean±SE, n=18). Excess biomass was stored (-18 °C) and used for analysis of DM, C, N, P, and ash content. A single nutrient pulse of 1200 µM-NO_3 was added to each flask and the FW was noted every third day. Temperature during the full experiment was 10 °C. The specific growth rate (SGR), expressed in % d^{-1} (FW/FW) was used to assess the effect of irradiance and nutrient level. The SGR was estimated using equation 1 (DeBoer et al. 1978):

$$SGR\ \% = \frac{ln(FW_{t2}) - ln(FW_{t1})}{t_2 - t_1} * 100\%$$

, where FW_{t2} and FW_{t1} are the fresh weight at days t_1 and t_2, respectively.

The light saturation of growth (I_{SAT}) was estimated, according to Andersen et al. (2013) as the intercept between the initial slope between 10-80 µmol photons m^{-2} s^{-1} PAR and the irradiance level of highest growth performance (n=3).

Water nutrient concentration and tissue color validation

Water samples (5 mL) were taken before and after water renewal in the final week of the low nutrient pulse cultivation and every third day during the high nutrient pulse cultivation. After syringe filtration (0.2 µm, Teflon) water samples were stored frozen (-18 °C) until concentrations of nitrate (NO_3) and orthophosphate (PO_4) were analyzed by ultraviolet spectrophotometry SmartChem200 discrete analyzer (AMS Alliance) to assess the water nutrient availability (Hansen and Koroleff, 1999). The tissue fragments were photographed and were visually inspected to validate pigmentation recovery after incidence of bleaching, i.e. loss of red color during the consecutive low and high nutrient pulse cultivation.

Analysis of tissue contents: DM, N, P, and Ash

The DM content (DM/FW * 100%; %DM) was determined after drying tissue samples to constant weights at 60° C. The C and N contents were analyzed using a Vario El Cube analyzer and expressed as the percentage of DM (%C, %N). The tissue P content (%P) was analyzed using a Shimadzu UV-160 spectrophotometer at 880 nm (Koroleff, 1983). The ash content (%Ash) was determined using a muffle furnace 530 °C for 18 hours (Larsen 1987).

Productivity (DM, C, N, and P) and doubling time

The area specific productivity of DM on a system basis was calculated from the high nutrient addition cultivation as below, modifying the equation according to Kim et al. (2007):

$$Productivity\ g\ DM\ m^{-2}d^{-1} = (FW_{t2} * \frac{DM\%_{avr}}{100}) - (FW_{t1} * \frac{DM\%_{avr}}{100}) * \frac{1}{t_2 - t_1} * \frac{1}{area}$$

, using the FW at final day (t_2) and initial day (t_1) and the average dry matter content between t_2 and t_1 ($DM\%_{avr}$) assuming a linear increment in %DM from t_1 to t_2. The area of each experimental unit was 0.0625 m^2.

The C, N and P productivity on dry matter basis (mg m^{-2} d^{-1}) was calculated by multiplying the average level of %C, %N, and %P between t_1 and t_2, assuming constant increments over time. By verification of constant SGR across days, equate doubling time (T_2, days) was used to estimate the difference in productivity between cultivation conditions.

Exp.2: The effect of salinity and nutrient availability on frond growth

In a factorial design, the effect of nutrient concentration (100% vs. 10% F/2+) and salinity (15, 25, 35‰) on SGR was tested based on weekly individual wet weight increments during 8 weeks. Single fronds (4.4-4.7 cm, n=15) kept in six independent tanks (5 L) aerated by an air stone were cultivated at 10 °C and exposed to an irradiance of 20 µmol photons m^{-2} s^{-1} PAR. The different salinities were obtained by adjusting the seawater with MilliQ water or Red Sea Coral. Weekly, the fronds were weighed individually and the tank salinity was validated by use of a digital salinity meter before water renewal. The SGR was estimated by use of eq. 1.

Statistics

In all cases, the null hypothesis was rejected at the significance level of 0.05. The effect of irradiance (6 levels), nutrient strength (2 levels) and salinity (3 levels) on SGR as well as tissue contents (%DM, %C, %N, %P, and %Ash) were analyzed using general linear regression models (GLM using standard least squares) to assess for interaction effects. Using the final and initial FW (n=3), the SGR was normal distributed (Shapiro Wilks: p>0.0644), and obeyed homogeneity of variance (Levene: p>0.1631), except data in exp.1 that displayed unequal variance (Levene: p = 0.033). The difference in variance of mean SGR between groups were assessed using the Wilcoxon test (exp.1) and the all pairs

Tukey-Kramer HSD post hoc (exp.2). The effect of irradiance on productivity in the high nutrient cultivation treatment was analyzed fitting polynomial regressions and the differences in variance of means analyzed using the Wilcoxon test. In exp.2, the statistical analysis was based on using the technical replicates (individual fronds). Data is presented as the means±SE and pooled when insignificant different between treatments. All statistics and graphs were carried out in JMP 14 Pro, SAS.

Results

Exp. 1. The marginal proliferations on tissue fragments grew during all 8 weeks in both nutrient phases and SGR curves (fig.2) displayed a second-degree polynomial shape in both nutrient phases, saturating at 114 (low) and 178 (high) µmol photons m^{-2} s^{-1} PAR. Irradiance and nutrient availability both affected SGR positively ($F_{3,32}$=31.64, p<0.0001, R^2; 0.74) with a significant interaction term ($F_{1,1}$ = 4.1, p=0.0435), indicating a different upshift in SGR with increasing light at high nutrient availability compared to low nutrient availability. At the high nutrient phase, SGR peaked at 150-280 µmol photons m^{-2} s^{-1} PAR with an average SGR of 6.1±0.4 % d^{-1} (n=9), whereas the highest SGR was found at 200-280 µmol photons m^{-2} s^{-1} PAR at the low nutrient phase averaging to 3.9±0.2 % d^{-1} (n=6) (fig. 2).

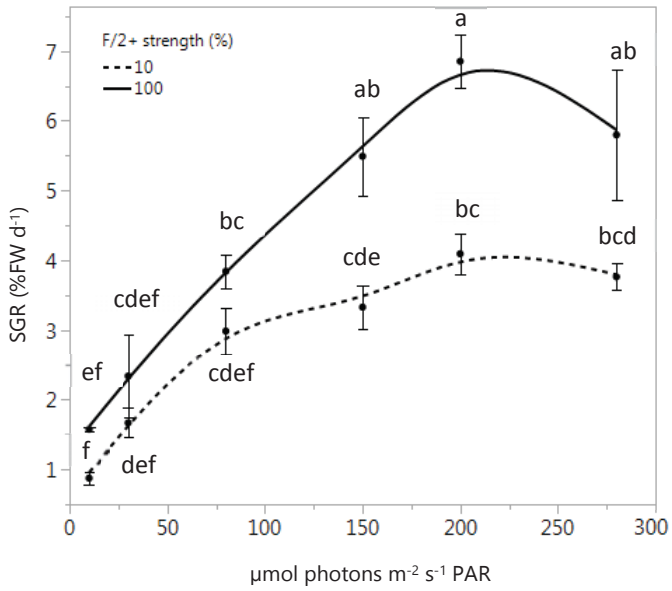

Figure 2. Mean specific growth rate (% FW day^{-1}) during 2-6 weeks in *P. palmata* exposed to different irradiance levels (10-280 µmol photons m^{-2} s^{-1} PAR (16:8 h) at 10 °C. In a consecutive manner, fronds were first cultivated using low nutrient pulse additions (10 % F/2+ ~120 µM-NO_3 for 6 weeks) followed by 2 weeks where one high nutrient pulse (100% F/2+ ~1200 µM-NO_3) was added. Data points represent the mean±SE (n=3). SGR´s with different lettering are significant different (Tukey-Kramer HSD all pairs).

Nutrient concentrations and tissue color

At the low nutrient pulse cultivation, NO_3 and PO_4 in the water exhausted after one week of cultivation (data not shown), whereas at high nutrient cultivation, the concentrations of NO_3 and PO_4 depleted after 10 and 3 days respectively at irradiance levels >150 µmol photons m^{-2} s^{-1} PAR (fig. 3A-B).

At low nutrient cultivation, the thalli appeared pale green after 3 weeks (fig. 4, top) and some frond tips lost color and turned white at light exposures of 150-280 µmol photons m^{-2} s^{-1} PAR. However, the color of thalli fully recovered within ten days of cultivation after single high nutrient addition (fig. 4, bottom). The SGR was lower during the first three days, compared to day 7 and day 10 (two-tailed t test(6): p=0.0005), indicated by the curve thickness (fig.3A, B).

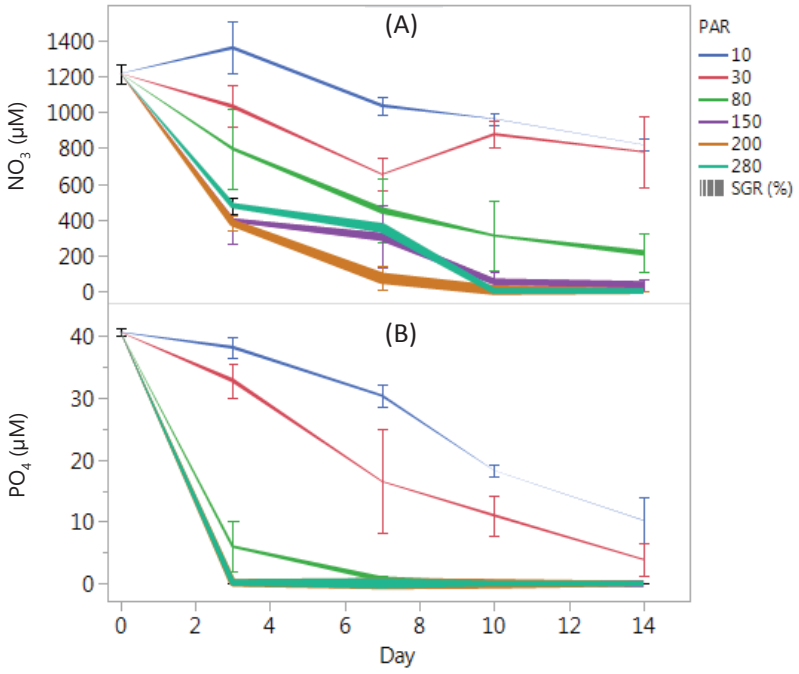

Figure 3. Concentrations of NO_3 and PO_4 (µM) during the high nutrient cultivation. Concentrations of NO_3 and PO_4 depleted after 3 and 10 days respectively >150 µmol photons m^{-2} s^{-1} PAR. Curve thickness indicates value of SGR (% FW d^{-1}). Data represents mean±SE (n=3).

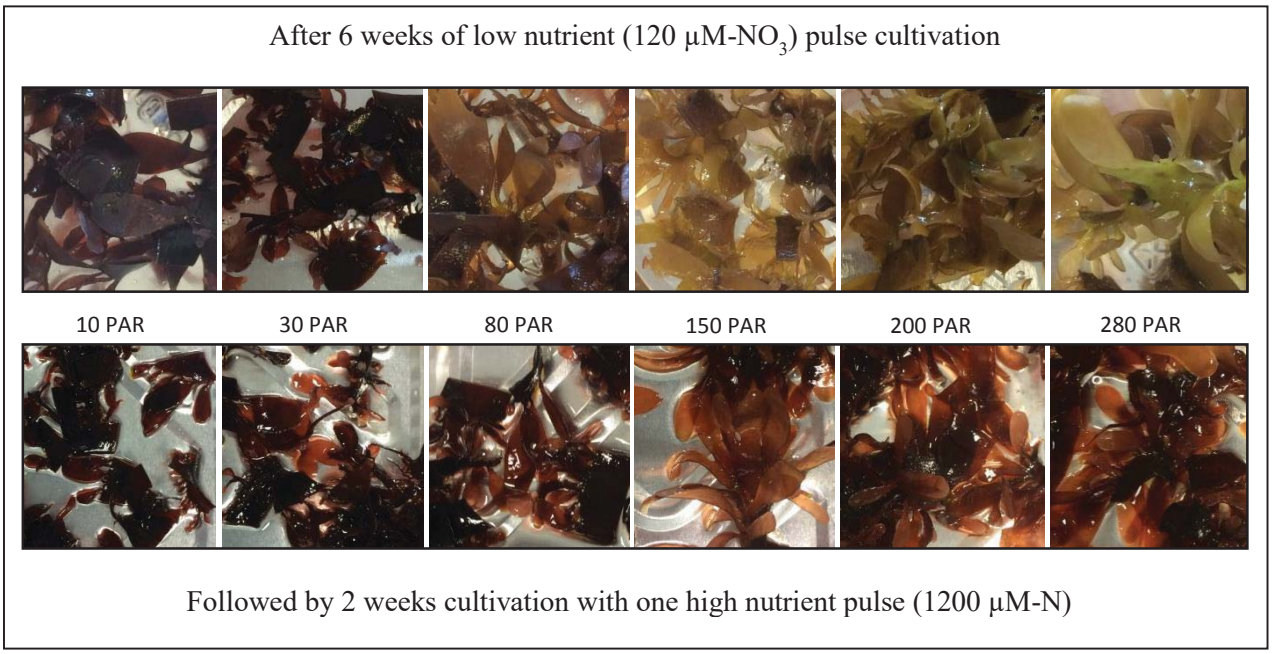

Figure 4. Top) The color of new grown thalli after 4 weeks of cultivation using low nutrient pulse additions showed that thalli turned pale green (top) >150 µmol photons m^{-2} s^{-1} PAR, some with white tips. Bottom) Followed by 2 weeks of high nutrient cultivation, the red color recovered after ten days even at highest irradiance exposure (280 µmol photons m^{-2} s^{-1} PAR).

Tissue contents

The %DM remained similar across irradiances during low nutrient cultivation ($F_{1, 16}$=1.01, p=0.3998), while a decrease in %DM was observed when growing new thalli using high nutrient pulse cultivation (table 1). The shift from low to high nutrient pulse addition resulted in a 79.3% increase in %DM across all irradiances (GLM; $F_{3,32}$=101.8, p<0.0001, R^2; 0.90). The %C remained similar across the different irradiance levels (GLM: $F_{3,32}$=2.8, p=0.0628, R^2; 0.20), whereas high nutrient availability affected %C negatively (p=0.0224) (table 1). The %N content was negatively affected by irradiance, while high nutrient pulse cultivation significantly elevated %N by 56% across irradiances (p=0.0051, R^2; 0.76) with a 109% increase of %N at 200 µmol photons m^{-2} s^{-1} PAR. During low nutrient pulse cultivation, the 10-30 µmol photons m^{-2} s^{-1} PAR exposures resulted in 3.1±0.2 %N (n=6), which was higher than at 80-280 µmol photons m^{-2} s^{-1} PAR (1.4±0.1 %N, n=12) (Wilcoxon(3): p<0.0268). Following the high nutrient pulse, the %N was similar at 10-150 µmol photons m^{-2} s^{-1} PAR with a peak of 4.1±0.2 %N (n=3) at lowest irradiance, which was higher than 2.6 ± 0.1 %N (n=9) obtained at 150-280 µmol photons m^{-2} s^{-1} PAR (Wilcoxon (3): p<0.0257). The P content was negatively affected by irradiance (GLM; $F_{3,32}$=6.7, p=0.0012, R^2; 0.38), while high nutrient cultivation enhanced %P by 27.3% (p=0.0178) across irradiances with no interaction effects (p=0.2759). The %P content was higher at 10-30 µmol photons m^{-2} s^{-1} PAR compared to 150-280 µmol photons m^{-2} s^{-1} PAR (Wilcoxon (3): p<0.0195) using low nutrient cultivation. After 2 weeks of high nutrient treatment the %P was similar at all irradiances (Wilcoxon(3): p=0.18). The %Ash was positive affected by nutrient availability (GLM: $F_{3,32}$=17.13, p<0.0001, R^2; 0.61), while the different irradiances did not exert a significant effect (p=0.1897). Whilst the %Ash content was higher at 10-30 µmol photons m^{-2} s^{-1} PAR compared to the other irradiances (Wilcoxon(3): p = 0.0232) within low nutrient treatment, the %Ash was highest at 200 PAR compared

to 30 and 80 µmol photons m^{-2} s^{-1} PAR using the high nutrient pulse (Wilcoxon(3): $p<0.0393$). Thus, the shift from low to high nutrient pulse addition resulted in a 16.4% increase in %Ash across all irradiances and caused a significant interaction term ($F_{1,1}=25.18$, $p<0.0001$).

PAR	F/2+	%DM	%C	%N	%P	%Ash
10	10%	10.4 ± 0.3	33.8 ± 2.0	3.4 ± 0.0	0.3 ± 0.0	23.5 ± 0.5
	100%	21.5 ± 1.8	34.9 ± 1.1	4.1 ± 0.2	0.2 ± 0.0	21.0 ± 0.3
30	10%	9.9 ± 0.2	35.4 ± 1.2	2.7 ± 0.4	0.3 ± 0.0	21.5 ± 0.8
	100%	20.6 ± 0.9	33.4 ± 0.9	3.6 ± 0.2	0.4 ± 0.1	20.4 ± 1.2
80	10%	10.9 ± 0.7	38.6 ± 1.8	1.8 ± 0.2	0.2 ± 0.0	16.9 ± 0.9
	100%	19.8 ± 0.2	35.2 ± 0.7	3.0 ± 0.4	0.4 ± 0.0	20.0 ± 0.1
150	10%	11.4 ± 0.5	36.4 ± 1.2	1.5 ± 0.0	0.2 ± 0.0	15.9 ± 0.4
	100%	17.6 ± 0.9	33.5 ± 1.4	2.9 ± 0.4	0.3 ± 0.0	22.6 ± 1.0
200	10%	10.1 ± 0.2	35.7 ± 0.7	1.2 ± 0.0	0.1 ± 0.0	16.8 ± 0.6
	100%	16.2 ± 0.4	33.3 ± 2.0	2.3 ± 0.0	0.2 ± 0.0	23.6 ± 0.3
280	10%	10.9 ± 0.4	33.4 ± 0.5	1.2 ± 0.2	0.2 ± 0.0	17.5 ± 0.7
	100%	18.1 ± 1.3	32.2 ± 0.4	2.6 ± 0.2	0.2 ± 0.0	23.0 ± 0.3
Effects	Irradiance	None/Neg	None	Neg	Neg	None
	Nutrient	Pos	Neg	Pos	Pos	Pos

Table 1. The relative content of dry matter content (%DM), carbon (%C), nitrogen (%N), phosphorous (%P), and ash (%Ash) on dry matter basis in *P. palmata* after cultivation at low nutrient pulses (6 weeks) followed by high nutrient pulse cultivation (2 weeks). Data represent the mean±SE (n=3). At the bottom, statistical significant effects of irradiance and nutrients on %DM, %C, %N, %P, and %Ash are indicated by "None" if no significant effect were found and by "Pos" or "Neg" if effects were found to be positive or negative, respectively.

The tissue C:N ratio (FW:FW) showed a linear fit (R^2 >0.83) when plotted against SGR (fig. 5A, B) and reached 29±0.9 (n=3) using low nutrient pulses. Here, growth exerted a higher rate of change in tissue C:N compared to high nutrient pulse, causing a significant interaction of nutrient availability and SGR (GLM: $p<0.0001$). Likewise, the rate of change in %N and %P was fastest at low nutrient additions compared to high nutrient addition (fig. 5C, D). The N:P ratio (g/g) in new thalli was estimated ~10 (data not shown), suggesting a continued N-limitation after the high nutrient pulse cultivation. A decreasing %Ash was associated with the increasing SGR at low nutrient treatment (fig. 6A) while opposite at high nutrient addition (fig. 5B).

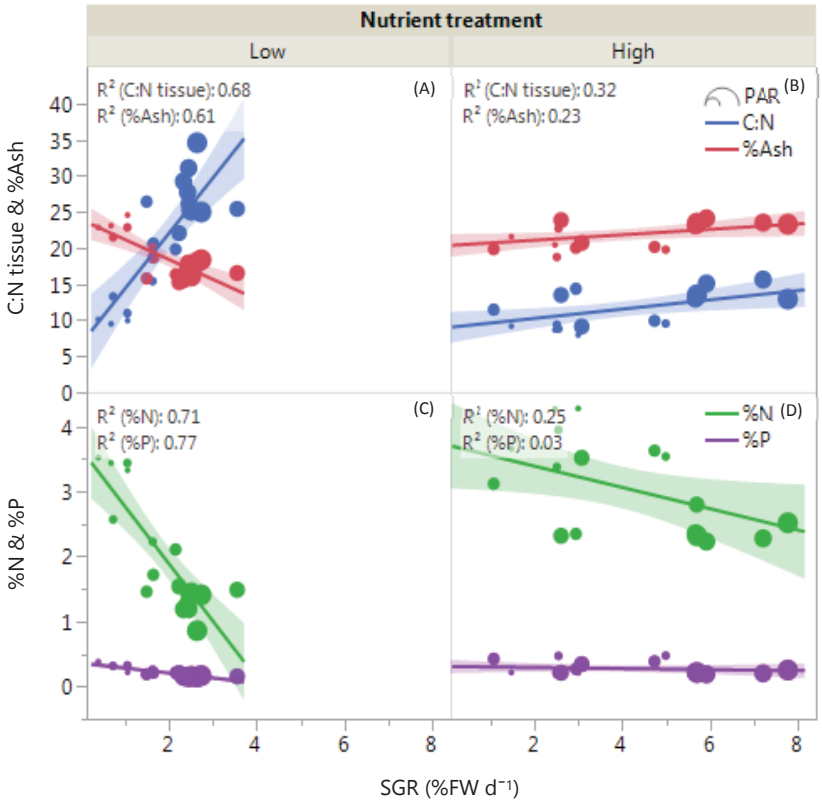

Figure 5. Linear correlations between tissue C:N ratio (w/w), %Ash, %N, and %P and the SGR% obtained in exp.1. Data point size indicate irradiance level (µmol photons m^{-2} s^{-1} PAR) and represent single values.

Productivity in DM, C, N, and P

Generally, irradiance significantly stimulated the productivity of DM, C, N, and P on dry weight basis using a high nutrient addition (fig. 6) which fitted polynomials of second degrees ($F_{2,15}$>34.41, p<0.0001, R^2>0.82). Verifying a steady state SGR across days ($F_{1,1}$: p>0.4951), the fastest doubling (T_2) of biomass was 11 days using 200 µmol photons m^{-2} s^{-1} PAR at high nutrient treatment. This was four times faster than T_2 at 10 µmol photons m^{-2} s^{-1} PAR. Productivity peaked at 200 µmol photons m^{-2} s^{-1} PAR (1.117±0.101 g DM m^{-2} d^{-1}) and was in average 0.949±0.09 g DM m^{-2} d^{-1} at 150-280 PAR (fig. 6A). The peak productivity of 406.4±37.8 mg C, 20.6±1.6 mg N, and 2.1±0.1 mg P m^{-2} d^{-1} (fig. 6B-D) was 14, 6.5, and 9.2 times the yields obtained at 10 µmol photons m^{-2} s^{-1} PAR. Across exposures of 150-280 µmol photons m^{-2} s^{-1} PAR the average productivities was 322.49±31.17 mg C, 17.87±1.08 mg N, 1.75±0.11 mg P m^{-2} d^{-1}.

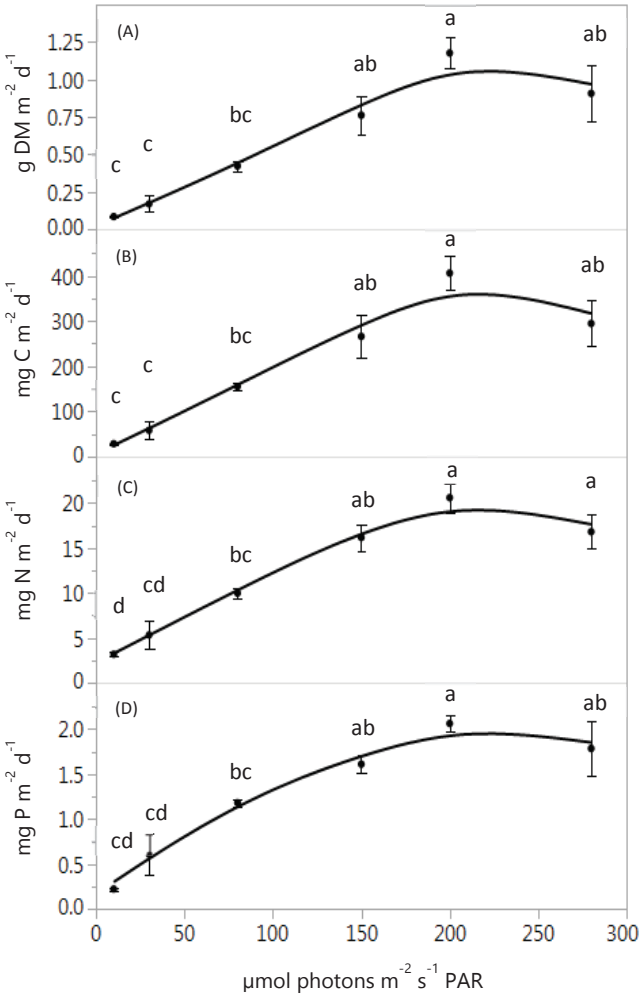

Figure 6. Daily productivity rates in DM, C, N, and P in *P. palmata* at different irradiance levels based on 2 weeks of cultivation at high nutrient conditions after an initial phase at low nutrient conditions for 5 weeks. Data represent the mean±SE (n=3).

Exp.2. The effect of salinity

The individual fronds grew during all weeks in each of the six combination of salinity and nutrient level. The linear regression model of SGR as a function of salinity and nutrient ($F_{2,86}$=13.37, p=0.0466, R^2; 0.32) identified a negative effect of salinity ($F_{1,1}$=31.76, p<0.0001) and a positive effect of nutrient availability ($F_{1,1}$=8.4, p=0.0047) with no interaction effects (p=0.99) (fig. 7). The highest SGR of 2.9±0.2 % d^{-1} (n=15) was found at 15‰ and 100% F/2+ (Tukey(15): p<0.0107). At 10% nutrient strength, the SGR was also higher when cultured at 15‰ compared to both 25‰ and 35‰ (Tukey(15): p<0.0050) (fig. 7). At 25-35‰ the fronds showed similar SGR at both 10% and 100% nutrient availability (Tukey(15): p>0.22).

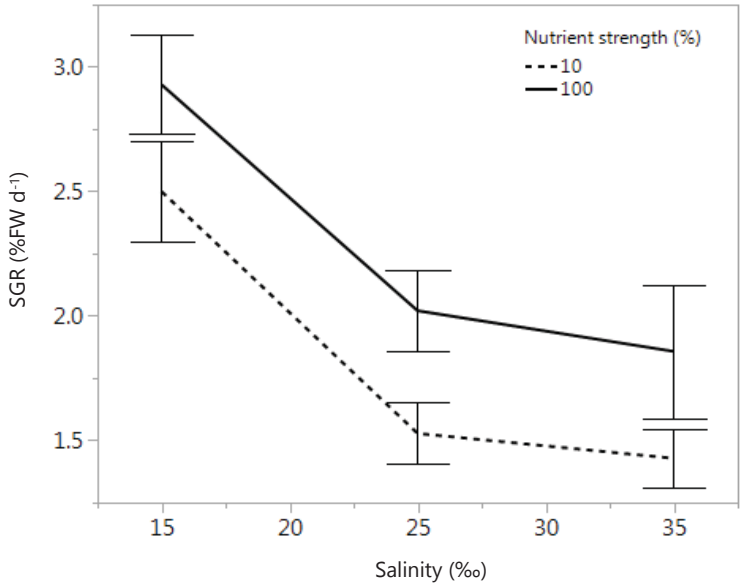

Figure 7. The specific growth rate (SGR, % FW d^{-1}) in *P. palmata* cultured in a factorial combination of two nutrient availabilities (10 vs. 100 % F/2+) and three salinities (15, 25, 35‰) and exposed to 20 µmol photons m^{-2} s^{-1} PAR (16:8 h). Data values represent the mean±SE (n=15).

Discussion

Growth rates and N dynamics

The present study demonstrates that the use of tiny marginal proliferations as vegetative seedstock for mass cultivation sourced by isolating shoots along the frond edge is suitable and grow into intact new fronds. This adds to the previous work on techniques for mass propagation of *P. palmata* (Titlyanov et al. 2006) and supports the primary reason to use young tissue seedstock, as nutrient pulse additions exert the highest growth response compared to older fronds in situ (Morgan and Simpson, 1981b) and in vivo (Martínez and Rico 2008). In the present study, six weeks of low nutrient pulse cultivation promoted a peak SGR of 3.54±0.17 % d^{-1} at 80-280 µmol photons m^{-2} s^{-1} PAR. By this, the new thalli turned pale green, while tissue C:N reached ~29. This suggests N-limitation of growth by the exhaustion of external

nutrients and a depletion of internal N-storage, causing catabolism of phycobilliproteins (Morgan & Simpson 1981a,b; Martinez and Rico 2002). Following the depletion of tissue N-storage at low nutrient pulse additions, the %N in thalli elevated after a single high nutrient pulse addition. Concomitantly, the SGR elevated significantly to 6.8±0.5 % d^{-1} at 150-280 µmol photons m^{-2} s^{-1} PAR with a peak of 8.5±0.6 % d^{-1}. The concentration of water NO_3 depleted during the first three days where the SGR appeared to slow down during this initial high nutrient uptake at optimal irradiance, which is in accordance to the uptake-growth dynamic described for a green algae (Elrifi and Turpin 1986). The red color of thalli recovered within ten days, indicating a synthesis of phycobilliproteins (Laycock, 1979). Like in other perennial rhodophytes, *P. palmata* displays an adaptive nutrient uptake strategy by synthesizing and accumulating phycoerythrin at nutrient replete conditions, which serve as a protein-N storage to sustain growth in periods of low ambient concentrations by mobilizing this reserve (Chapman and Cragie 1977; Bird et al. 1985; Pedersen and Borum 1996). On short term, the nutrient uptake rate of perennial seaweeds slows down imposed by negative feedback control of the storage-N pools (Hanisak 1990; McGlathery et al. 1996; Harrison & Hurd, 2001). In contrast, ephemeral epiphytic algae thrive at constant high nutrient availability, thus the growth of perennials are not competitive to ephemeral species, which impose a challenge in cultivation of perennial seaweeds (Morgan and Simpson, 1981b). Therefore, a biphasic cultivation strategy to impose a high dynamic range of the N pools is advantageous and paramount for perennials to exert a high N removal potential while avoiding epiphytes. This was one of the founding idea behind the use of consecutive cultivation (quasi-experimental design) where the use of intermittent batch conditions exhaust external nutrients and depletes internal nutrient pools, which is preventive against ephemeral algae blooms (Neish et al. 1977; Morgan and Simpson 1981b). Hence, by this condition, pre-cultured thalli, which are N-starved, will serve as suitable input material using consecutive high nutrient additions, as the seedstock will display a high nutrient uptake. Without the consecutive shift from low to high nutrient pulse cultivation we speculate that thalli grown >150 µmol photons m^{-2} s^{-1} PAR would photo-chemically deteriorate the biomass at extended cultivation (Chapman and Cragie 1977; Martinez and Rico 2002). Whilst nutrient availability and irradiance level is easy to control in land-based cultivation, it is different in open-sea cultivation of *P. palmata*, where escalating photochemical bleaching can deteriorate the biomass before harvest (Werner and Dring 2011). Table 2 shows relevant studies on tank-based cultivation of *P. palmata* by summarizing the SGR, DM yield and N removal rates.

The severe to moderate nutrient limitation in the present study explains why the SGR is the lower range of previous studies (1.1-20 % d^{-1}) (table 2), where higher level of SGR was obtained using either flow-through or frequent nutrient repletion's (Morgan and Simpson 1981b; Sagert and Schubert 2000; Corey et al. 2014).

Yield (g DM m^{-2} d^{-1})	N removal (mg N m^{-2} d^{-1})	SGR (% d^{-1})	Culture details	Remarks	Reference
1.18±0.10	20.61±1.62	6.85±0.38	14 days, 10 °C, L:D=16:8, 200 µmol photons m-2 s^{-1} PAR, 4.8 g FW L^{-1}	Batch. Single nutrient pulse addition of 1200 µM-NO_3. Warm fluorescent light	Present study
0.91±0.19	16.82±1.91	5.8±0.93	14 days, 10 °C, L:D=16:8, 280 µmol photons m-2 s^{-1} PAR, 4.4 g FW L^{-1}		Present study
2.7		5	34 days 10 °C, 42 µmol photons m-2 s^{-1} PAR	Weekly pruning. Ft. Cold fluorescent light (19 ly d-1)	Morgan and Simpson (1981a)
		7.8	10 °C, L:D=16:8, 117 µmol photons m^{-2} s^{-1} PAR	Light exposure of 53 ly d^{-1}	Morgan and Simpson (1981a)
18		7.6	34 days, 10 °C 191 µmol photons m-2 s^{-1} PAR, 3.6 g FW L^{-1} (50 g m^{-2})	Light exposure of 86 ly d^{-1}	Morgan and Simpson (1981a)
1.2		3.9*	35 days, 10 °C, 12 µmol photons m^{-2} s^{-1} PAR	500 µM-NO_3 additions four/wk (Ft). Weekly pruning. Cold fluorescent light (0.09 ly min-1).	Morgan and Simpson (1981b)
28			10-15 °C	Excessive nutrient availability	Morgan et al. (1980b)
40.3±12.8	1350-2740		28 days, 17-21 °C, 10 g FW L^{-1} (5 kg m^{-2})	N removal from cascade system of 3 tanks in Ft setup. Fish farm effluent (140 L h^{-1})	Matos et al. (2006)
1.51	40*	7.4	14 days, 6 °C, L:D=16:8, 140 µmol photons m^{-2} s^{-1} PAR, 1-30 g FW L^{-1} (0.2-0.6 kg m^{-2})	Ft fish farm effluent (50 L tanks). Excessive nutrients. Cold fluorescent light	Kim et al. (2013)
0.86		4.6	16 °C		Kim et al. (2013)
	7.36*	8-9	14 days, 10 °C, L:D=16:8, 125 µmol photons m^{-2} s^{-1} PAR, 2 g FW L^{-1}	1 L (batch). NO3 + NH4 additions 2 times/wk (300 µM-N), von Stosch	Corey et al. (2013)
0.004*	0.56	-2.2-1.1	180 days, 0.4-8.5 °C, L:D=16:8, 100-600 µmol photons m^{-2} s^{-1} PAR, 2.4 g FW L^{-1} (3 kg m^{-2})	Flow-through using fish farm effluent. Natural + fluorescent light	Corey et al. (2014)
	1.76 ± 0.59	0.64	30 days, 10 °C, L:D=16:8, 149 µmol photons m^{-2} s^{-1} PAR, 7 g FW L^{-1}	Batch conditions (> 2856 µM-NO3)	Trembley-Gratton et al. (2018)

Table 2: A literature comparison of previous reported land-based productivities in *P. palmata*. Abbreviation: Ft; flow-through conditions. Asterisk: * values estimated by calculation based on reported parameter levels. Present productivity in DM and N removal rates is in the mid-lower range of previous findings. One reported N productivity rate was extremely high (Matos et al. 2006).

Eventually, our results suggest that growth and productivity of *P. palmata* is optimal at 200 µmol photons m^{-2} s^{-1} PAR at 10° C. The saturation of SGR (I_{SAT}) was estimated to 114 and 173 µmol photons m^{-2} s^{-1} PAR using either low or high nutrient concentration. These results are in the high range and comparable to previous findings, where *P. palmata* saturated growth at 117-140 and maximal growth was found up to 289 µmol photons m^{-2} s^{-1} PAR (Morgan and Simpson 1981b; Sagert and Schubert 2000).

Productivity and nutrient removal

The growth in fresh weight reflected an increased dry-weight based productivity of DM, C, N, and P, peaking at 150-280 µmol photons m^{-2} s^{-1} PAR. In contrast, irradiance negatively affected the relative contents of %DM, %N, %P in both nutrient treatments, suggesting an accumulation of water in new cell divisions. This result is in accordance to a previous study, showing that irradiance stimulate growth of new algae tissue and caused a reduction in the nutrient content (Manríquez-Hernández et al. 2016), but in contrast to the findings in Morgan and Simpson (1981a). The positive effect of the shifting to high nutrient availability led to an increase in %DM accompanied by an increase in %Ash, while %C remained stable. The DM productivity of 1.17 g DM m^{-2} d^{-1} found in the present study is lower than previous reporting (1.20-40.2 g DM m^{-2} d^{-1}, table 2). Here, maximum values up to 40.20 g DM m^{-2} d^{-1} is obtained by cultivating apical tips using weekly re-stocking and flow-through condition or frequent nutrient pulses >500 µM-NO_3 four times per week (Morgan and Simpson 1981a; Matos et al. 2006).

While *P. palmata* is NH_4-sensitive, it displays an uptake affinity for NH_4 as N source at non-toxic levels, yet studies show increased growth when NO_3 is the N source (Morgan and Simpson 1981a; Corey et al. 2013). Noticeably, the entire culture system, accounting the aeration and biofilms, may to a larger extend contribute to N removal than the algae tissue alone (Caines et al. 2014), as found by Matos et al. (2006), where 1350-2450 mg N m^{-2} d^{-1} was removed in a cascading tank setup at semi scale. The present small-scale culture system provided a maximal mean N removal at highest growth rates, similarly to Corey et al. (2014), who found a substantial lower N productivity. The peak N productivity of the present study was 20.61 mg N m^{-2} d^{-1} on system basis and in the mid-high range of previous studies, except for study using a cascading tank setup and substantial higher biomass density (Matos et al. 2006). Scaling of a cultivation system, optimized biomass densities, timing of nutrient repletion as well as the selection of species is important to maximize the nutrient mitigation by land-based seaweed cultivation.

Like in the coastal cultivation of agarophytes *Kappaphycus sp.*, a practice of multiple harvests and restocking of new meristematic seedstock would likely optimize the productivity and maintain highest growth rates (Johnson and Gopakumar, 2011). Eventually, by completing four harvests and restocking, each of 11 days, at optimal conditions, e.g., use of 200 µmol photons m^{-2} s^{-1} PAR and repletion of nutrients after N-starvation, the accumulated DM, N, and P harvest yields would be 4 times the yields provided by one production cycle at 10 µmol photons m^{-2} s^{-1} PAR (T_2 of 44 days).

Salinity

The fronds cultivated at the 15‰ showed higher SGR compared to fronds cultured at 25-35‰. This is supportive to a previous study, where *P. palmata* sporelings showed enhanced SGR when cultivated at 15-20‰ compared to 25-30‰ (Schmedes et al. 2019). The mentioned study used biological material sampled from the same shallow water population as this study, and results might indicate an adaption of this population to prevailing brackish salinity, as indicated by the broad salinity tolerance and high SGR at low salinities. This potential adaptation might comprehend the great parts of inner Danish waters, where low and fluctuating salinies are common. In support, other intertidal macroalgae species are able to grow in salinities of 1-12‰ if pre-cultured at higher salinities, which suggests a high salinity tolerance of intertidal species (Khfaji and Norton, 1979). Decreasing salinity further than 15‰ is expected to be stressful, yet light

and salinity stress tolerance is higher when algae tissue are nutrient replete (Zhao et al. 2017). This is in accordance with findings of light-induced stress at nutrient deplete status (fig. 4) and tolerance to low salinity conditions (fig. 7) in the present study and might add to an explanation on why tissue bleaching is common in sea-based cultivation especially in areas where surface water stratifies and becomes N-depleted.

Hence, the idea of cultivating marine algae in low salinity regimes, for instance by coupling the cultivation to a land-based facility to enable a utilization of released nutrients is highly relevant for *P. palmata*, (DeBusk et al. 1986; Haglund and Pedersen 1992; Yarish et al. 2002; Corey et al. 2013). The high nutrient availability during summer months sourced from a land-based facility could potentially sustain a high productivity and N removal rates of well-nourished *P. palmata* (Matos et al. 2006), however, conditioning that temperature can be kept below upper optimal limit of 15° C (Morgan and Simpson 1981b).

Conclusions and perspectives

Environmental factors, such as irradiance, salinity and nutrients show pronounced season fluctuations, leading to limiting and excessive availabilities, which control seaweed growth, nutrient uptake, ultimately determining the biomass yield and quality (DeBoer and Ryther 1978; Martins et al 1999; Hurd and Harrison 2001). In present study, the controlled levels of irradiance and nutrient concentration imposed limitation of growth during a pre-cultivation phase and provided N-starved thalli displaying sub-lethal photochemical bleaching. In the consecutive cultivation phase where a single high nutrient pulse was added, the growth and N removal using tiny *P. palmata* vegetative shoots was boosted due to the severe N-starvation in the pre-cultivation.

The present results confirm previous observations that well-nourished *P. palmata* is obtained by low growth rates or conversely by replete nutrient conditions and add knowledge on how to optimize the N removal in controlled environments using a consecutive cultivation strategy (Morgan & Simpson 1981a, b). The use of a consecutive cultivation protocol with a pre-cultivation at N-starving conditions followed by a high nutrient pulse provides a highly N-extractive seedstock suited for a boosted N removal. Applying intermittent batch conditions, serve as a method to alternate nutrient concentration (deplete-replete) and control epiphyte growth. In perspective, by use of frequent water renewals to avoid limitation of micronutrients and monitoring of dissolved N and P concentrations to set the time for a secondary high nutrient pulse addition would potentially improve the N removal in a cultivation strategy, where nutrient removal is of interest – for instance by coupling land-based salmon RAS and seaweed cultivation. In addition, local adaption of *P. palmata* to low salinity regimes implies an option for N removal at brackish conditions.

Acknowledgement

This study was funded by the Joint Doctoral Degree agreement between the National Institute of Aquatic Resources (DTU Aqua) at Technical University of Denmark and the Norwegian University of Science and Technology (NTNU), Norway, as well as the Tang.nu project (under Grant Agreement No. 13744, Velux Foundation) and the MacroSea project, Grant no. 254883, funded by the Research Council of Norway.

References

Abreu MA, Pereira R, Buschmann AH, Sousa-Pinto I (2011) Nitrogen uptake responses of Gracilaria vermiculophylla (Ohmi) Papenfuss under combined and single addition of nitrate and ammonium. J Exp Mar Biol Ecol 407:190–199.

Alveal K, Romo H, Werlinger C, Oliveira EC (1997) Mass cultivation ofthe agar-producing alga Gracilaria chilensis (Rhodophyta) from spores. Aquaculture 148:77–83.

Andersen GS, Pedersen MF, Nielsen SL (2013) Temperature acclimation and heat tolerance of photosynthesis in Norwegian Saccharina latissima (Laminariales, Phaeophycea) J Phycol 49:689–700.

Beaulieu L, Sirois M, Tamigneaux É (2016) Evaluation of the in vitro biological activity of protein hydrolysates of the edible red alga, Palmaria palmata (dulse) harvested from the Gaspe coast and cul- tivated in tanks. J Appl Phycol 28:3101–3115.

Bird KT, Habig C, DeBusk T (1982) Nitrogen allocation and storage patterns in Gracilaria tikvahiae (Rhodophyta). Journal of Phycology 18:334-338.

Browne KL (2001) Mariculture of the edible red alga Palmaria palmata. PhD thesis, Queen's University of Belfast, UK. 186 pp.

Caines S, Manríquez-Hernández JA, Duston J, Corey P, Garbary DJ (2014) Intermittent aeration affects the bioremediation potential of two red algae cultured in finfish effluent. J Appl Phycol 26:2173-2181

Chapman ARO, Craigie JS (1977) Seasonal growth in Laminaria longicruris: relations with dissolved inorganic nutrients and internal reserves of nitrogen. Mar Biol 40:197-205.

Chopin T, Yarish C, Wilkes R, Belyea E, Lu S, Mathieson A (1999) Developing Porphyra/salmon integrated aquaculture for bioremediation and diversification of the aquaculture industry. J App Phycol 11:463–472.

DeBoer JA, Ryther JH (1977) Potential yields from a waste- recycling algal mariculture system. In: Krauss, R.W. (Ed.), The Marine Plant Biomass of the Pacific Northwest Coast. Oregon State University Press, pp. 231–248.

DeBoer JA, Guigli HJ, Israel TL, D'Elia CF (1978) Nutritional studies of two red algae. I. Growth rate as a function of nitrogen source and concentration. J Phycol 14:261–266.

DeBusk TA, Blakeslee M, Ryther JH (1986) Studies on the outdoor cultivation of Ulva lactuca L. Bot Mar 29:381–386

Edwards MD, Dring MJ (2011) Open sea cultivation trails of the red alga, Palmaria palmata from seeded tetraspores in Strangford Laugh, Northern Ireland. Aquaculture 317:203–209.

Elrifi IR, Turpin DH (1986) Nitrate and ammonium induced photosynthetic suppression in N-limited Selenastrum minutum. Plant Physiol 81:273–9.

Faes VA, Viejo RM (2003) Structure and dynamics of a population of Palmaria palmata (Rhodophyta) in northern Spain. J Phycol 39:1038–1049.

Guillard RR, Ryther JH (1962) Studies on marine planktonic diatoms. I. Cyclotella nana Hustedt and Detonula confervacea (cleve) Gran. Can J Microbiol 8:229–239.

Hafting JT, Critchley AT, Scott MLC, Hubley A, Archibald AF (2012) On-land cultivation of functional seaweed products for human usage. J Appl Phycol 24:385–392.

Haglund K, Pedersen M (1992) Growth of the red alga Gracilaria tenuistipitata in brackish water in Sweden. Growth, nutrient uptake, co-cultivation with rainbow trout and epiphyte control. J Appl Phycol 5:271–284

Hanisak, MD (1990) The use of Gracilaria tikvahiae (Gracilariales, Rhodophyta) as a model system to understand the nitrogen nutrition of cultured seaweeds. Dev Hydrobiol 58:79–87.

Hansen, HP, Koroleff F (1999) Determination of nutrients, in Methods of Seawater Analysis, 3rd ed., edited by K. Grasshoff, K. Kremling, and M. Ehrhardt, pp. 159–228, Wiley-VCH, Weinheim, Germany, doi:10.1002/9783527613984.ch10.

Harrison PJ, Hurd CL (2001) Nutrient physiology of seaweeds: application of concepts to aquaculture. Cah Biol Mar 42:71–82.

Harnedy PA, Soler-Vila A, Edwards MD, FitzGerald RJ (2014) The effect of time and origin of harvest on the in vitro biological activity of Palmaria palmata protein hydrolysates. Food Res Int 62:746–752

Holdt SL, Kraan S (2011) Bioactive compounds in seaweed: functional food applications and legislation. J Appl Phycol 23(3):543–597
Johnson B, Gopakumar G (2011) Farming of the seaweed Kappaphycus alvarezii in Tamil Nadu coast - status and constraints. Marine Fisheries Information Service T&E Ser., No. 208.

Khfaji AK, Norton TA (1979) The effects of salinity on the distribution of Fucus ceranoides. Estuar Coast Mar Sci 8:433–439

Kim JK, Duston J, Corey P, Garary DJ (2013) Marine finfish effluent bioremediation: effects on stocking density and tem- perature on nitrogen removal capacity of Chondrus crispus and Palmaria palmata (Rhodophyta). Aquaculture 414–415:210–216.

Koroleff F (1983) Determination of phosphorus, in: Grasshoff, K. pp. 125–131.

Larsen B (1978) Brown seaweeds: analysis of ash, fibre, iodine and mannitol. In: Hellebust, J.A, Craigie, J.S. (Eds.), Handbook of Phycological Methods. Cambridge University Press, Cambridge, UK, pp. 181–189.

Laycock MV, Mclnnes AG, Morgan KC (1979) D-homocysteic acid in Palmaria palmata. Phytochemistry 18: 1220.

Manríquez-Hernández JA, Duston J, Garbary DJ (2016) Effect of irradiance on bioremediation capacity of the red algae Chondrus crispus and Palmaria palmata. Aquacult Int 24:39–55.

Martínez B, Rico JM (2002) Seasonal variation of nutrient content and major N pools in Palmaria palmata (Rhodophyta). J Phycol 38:1082–9.

Martínez B, Viejo RM, Rico JM, Rødde RH, Faes VA, Oliveros J, Álvarez D (2006) Open sea cultivation of Palmaria palmata (Rhodophyta) on the northern Spanish coast. Aquaculture 254:376–87.

Martínez B, Rico JM (2008) Changes in nutrient content of Palmaria palmata in response to variable light and upwelling in Northern Spain. J Phycol 44:50-59.

Martins I, Oliveira JM, Flindt MR, Marques JC (1999) The effect of salinity on the growth rate of the macroalgae Enteromorpha intestinalis (Chlorophyta) in the Mondego estuary (west Portugal). Acta Oecologica 20(4):259−265.

Matos J, Costa S, Rodrigues A, Pereira R, Sousa-Pinto I (2006) Experimental integrated aquaculture of fish and red seaweeds in Northern Portugal. Aquaculture 252:31–42.

McGlathery KJ, Pedersen MF (1999) The effect of growth irradiance on the coupling of carbon and nitrogen metabolism in Chaetomorpha linum (Chlorophyta). J Phycol 35:721–731.

Monagail MM, Cornish L, Morrison L, Araújo R, Critchley AT (2017) Sustainable harvesting of wild seaweed resources. Eur J Phycol 52:4, 371-390.

Montague CL, Ley JA (1993) A possible effect of salinity fluctuation on abundance of benthic vegetation and associated fauna in northeastern Florida Bay. Estuaries(16)-4:703-717.

Morgan KC, Wright JLC, Simpson FJ (1980a) Review of the chemical constituents of the red alga Palmaria palmata (dulse). Econ Bot 34:27–50.

Morgan KC, Shacklock PF, Simpson FJ (1980b) Some aspects of the culture of Palmaria palmata in greenhouse tanks. Bot Mar 23:765-770.

Morgan K, Simpson F (1981a) Cultivation of Palmaria (Rhodymenia) palmata: effect of high concentrations of nitrate and ammonium on growth and nitrogen uptake. Aquat Bot 11:167–171.

Morgan KC, Simpson FJ (1981b) The cultivation of Palmaria palmata. Effect of light intensity and temperature on growth and chemical composition. Bot Mar 24: 547–552.

Mortensen LM (2017) Remediation of nutrient-rich, brackish fjord water through production of protein-rich kelp *S. latissima* and *L. digitata*. J Appl Phycol 29:3089–3096.

Mouritsen OG, Dawczynski C, Duelund L, Jahreis G, Vetter W, Schröder M (2013) On the human consumption of the red seaweed dulse (Palmaria palmata (L.) Weber & Mohr. J App Phycol 25:1777–1791.

Neish AC, Shacklock PF, Fox CH, Simpson FJ (1977) The cultivation of Chondrus crispus. Factors affecting growth under greenhouse conditions. Can J Bot 55:2263-2271.

Neori A, Krom MD, Ellner SP, Boyd CE, Popper D, Rabinovitch R, Davidson PJ, Dvir O, Zuber D, Ucko M, Angel D, Gordin H (1996) Seaweed biofilters as regulators of water quality in integrated fish-seaweed culture units. Aquaculture 14:183–199.

Neori A, Chopin T, Troell M, Buschmann AH, Kraemer GP, Halling C, Shpigel M, Yarish C (2004) Integrated aquaculture: rationale, evolution and state of the art emphasizing seaweed biofiltration in modern mariculture. Aquaculture 231:361–391.

Parjikolaei BR, Kloster L, Bruhn A, Rasmussen MB, Fretté XC, Christensen KV (2013) Effect oflight quality and nitrogen availabil- ity on the biomass production and pigment content of Palmaria palmata (Rhodophyta). Chem Eng Trans 32:967–972.

Pedersen MF, Borum J (1996) Nutrient control of algal growth in estuarine waters. Nutrient limitation and the importance of nitrogen requirements and nitrogen storage among phytoplankton and species of macroalgae. Mar Ecol Prog Ser 142:261-272.

Pereira R, Yarish C, Pinto IS (2006) The influence of stocking density, light and temperature on the growth, production and nutrient removal capacity of Porphyra dioica (Bangiales, Rhodophyta). Aquaculture 252:66-78.

Sagert S, Schubert H (2000) Acclimation of Palmaria palmata (Rhodophyta) to light intensity: comparison between artificial and natural light fields. J Phycol 36:1119–1128

Sanderson JC (2006) Reducing the environmental impacts of sea-cage fish farming through cultivation of seaweed. PhD Thesis, pp. 339. Open University, Scottish Association for Marine Sciences, Oban.

Sanderson JC, Dring MJ, Davidson K, Kelly MS (2012) Culture, yield and bioremediation potential of Palmaria palmata (Lin- naeus) Weber & Mohr and Saccharina latissima (Linnaeus) C.E. Lane, C. Mayes, Druehl & G.W. Saunders adjacent to fish farm cages in northwest Scotland. Aquaculture 354–355: 128– 135.

Schmedes PS, Nielsen MM (2019) New hatchery methods for efficient spore use and seedling production of Palmaria palmata (dulse). J Appl Phycol 1-11 doi: 10.1007/s10811-019-01998-0

Schmedes PS, Nielsen MM, Petersen JK (2019) Improved Palmaria palmata hatchery methods for tetraspore release, even settlement and high seedling survival using strong water agitation and macerated propagules. Algal Res 40. doi.org/10.1016/j.algal.2019.101494

Titlyanov EA, Titlyanova TV, Kadel P, Lüning K (2006) New methods of obtaining planlets and tetraspores of fragments and cell aggregates of meristematic and submeristematic tissue of the red alga Palmaria palmata. J Exp Mar Biol Ecol 339:55–64.

Trembley-Gratton A, Boussin JC, Tamigneaux É, Vandenberg GW, Le Francois NR (2018) Bioremediation efficiency of Palmaria palmata and Ulva lactuca for use in a fully recirculated cold-seawater naturalistic exhibit: effect of high NO3 and PO4 concentrations and temperature on growth and nutrient uptake. J Appl Phycol 30:1295-1304

Troell, M, Joyce A, Chopin T, Neori A, Buschmann AH, Fang JG (2009) Ecological engineering in aquaculture — potential for integrated multi-trophic aquaculture (IMTA) in marine offshore systems. Aquaculture 297:1–9.

Ugarte RA, Sharp G (2001) A new approach to seaweed management in Eastern Canada: the case of Ascophyllum nodosum. Cahiers de Biologie Marine, 42:63–70.

Yarish C, He P, Carmona R, Liu S, Kraemer G, Neefus C, Chopin T, Nardi G, Curtis J, Lonergan S (2002) The Aquaculture of Porphyra Leucosticta (Rhodophyta) for an Integrated Finfish/Seaweed Recirculating Aquaculture System in an Urban Application. J Phycol 38:39

Van der Meer JP, Todd ER (1980) The life history of Palmaria palmata in culture. A new type for the Rhodophyta. Can J Bot 58:1250–1256.

Werner A, Dring MJ (2011) Cultivating Palmaria palmata. 'Aquaculture Explained' Series No. 27 Bord Iascaigh Mhara (BIM). 76 pp.

Zhao LS, Li K, Wang QM, Song XY, Su HN, Xie BB, Zhang XY, Huang F, Chen XL, Zhou BC, Zhang YZ (2017) Nitrogen Starvation Impacts the Photosynthetic Performance of Porphyridium cruentum as Revealed by Chlorophyll a Fluorescence. Sci Rep 17;7(1):8542. doi: 10.1038/s41598-017-08428-6

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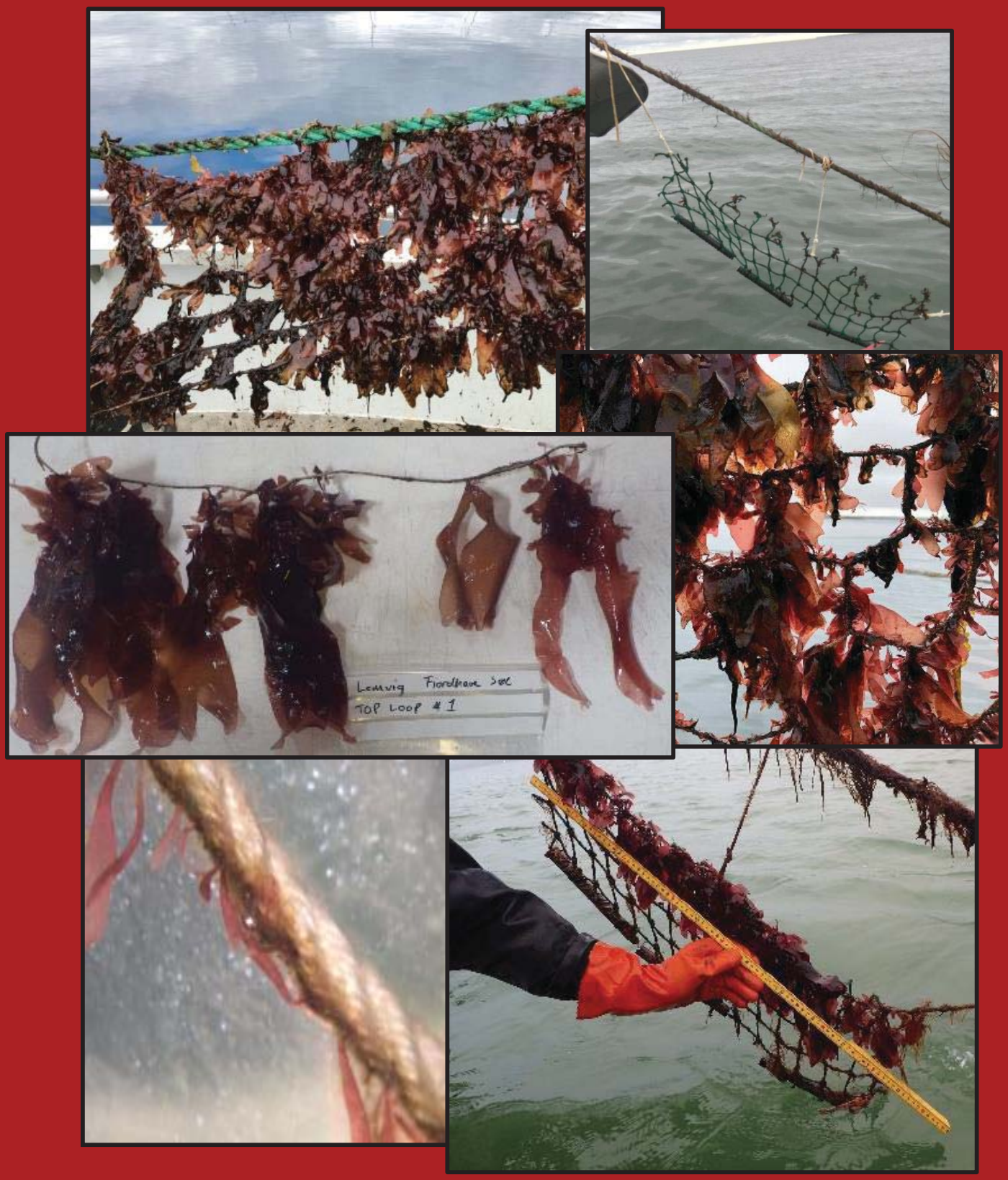
Paper 5: Investigating seabased grow out of *P. palmata*



Seabased cultivation of *Palmaria palmata* - the effect of seeding method, substrate type, deployment configuration, and farm location

(Manuscript draft)

Peter S. Schmedes and Mette Møller Nielsen

Abstract: Current cultivation methods for *P. palmata* (dulse) are insufficient for a feasible production at sea, mainly due to low hatchery efficiency. Low spore use efficiency implies a high spore donor requirement and emphasizes the need for optimization of hatchery and cultivation methods to improve the harvest yield. The present study investigated three hatchery cases to optimize the sea-based grow out yield by using two nursery durations; short or long periods (0.7-1.5 vs. 8-11 months), and three different substrates (kuralon twine, polypropylene (PP) rope and nets) seeded by two different methods (spore vs. propagule seedstock). Seeded materials were cultivated at different farms in inner Danish waters to compare growth. In the first case, spore-seeded kuralon and PP rope were nursed for either short or long time before deployed at three sites in Danish waters. The best growth was obtained at the farm site with the highest degree or exposure and water renewal (Nissum) by attaching kuralon twine on a carrier rope after a long nursery phase, and resulted in a biomass yield (287±33 g FW m^{-1}). In the second case, nets nursed for a short nursery phase was unsuccessful due to epi-fouling, whereas the use of a long nursery (10 months) resulted in larger seedlings at deployment and resulted in better growth. Furthermore, growth on horizontal nets was better than on kuralon. Case 3 investigates for the first time the temporal extend of using germinated propagules as dulse seedstock inoculum and assess the growth potential using 39 days old vs. 240 days old propagules for seeding. Only 39 days old propagules reattached and were eventually deployed at sea. Propagule-seeded ropes with long nursing sustained a length growth in range of the spore-seeded nets.

This study demonstrates that horizontal deployment configuration of nets and propagule-seeded ropes holds a strong potential for improving grow-out yield of *P. palmata*. Furthermore, the study emphasizes the importance of deployment time, seedling size at deployment, cultivation depth, cultivation site, optimal deployment configuration for maximizing dulse production. This study provides valuable insight into improving the hatchery efficiency and cultivation yield of *P. palmata*.

Key words: Seaweed yield, dulse cultivation, spore, hatchery seeding, propagules.

Introduction

Timing the seedling production in seaweed hatcheries is crucial for the quality of seeded substrates and optimal harvest in sea-based cultivation (Browne 2001, Edwards 2007, Werner and Dring 2011). Cultivation of *Palmaria palmata* (dulse) relies on seeding substrates with tetraspores and recently its cultivation has attracted increased focus due to the high prize on its biomass and outstripping of natural populations by wild harvesting (Walsh and Watson 2011, Wood 2018, Poul Erik from Gourmettang.dk, *pers. comm. 2019*). In North Atlantic (NA) waters, the temporal overlap in access to tetraspores from wild fertile tetrasporophytes and the out planting season for propagating the biomass restrict the cultivation season and may lead to a reduced harvest yield (Werner and Dring 2011, *Paper 1*). Previous successful cultivation trials stress that an initial spore density ≈100 spores cm^{-1} cultivation substrate is required for successful grow out. Obtaining this seeding density, using the current hatchery method, is based on placing sori above net substrates horizontally in seeding tanks and use of batch conditions (Werner and Dring 2011). However, the method requires about 130

kg fresh weight (FW) fertile sporophytic biomass to deliver enough spores to seed one longline of 100 m with 70 nets. This corresponds to 2.6 t FW fertile plants for seeding one hectare (equivalent to ~115 km rope ha^{-1} and 5.74 km rope per 100 m longline) and emphasizes a low efficiency in spore use. In hatchery, sporulation results in patchy settlement distributions (0-500 spores cm^{-1}) using calm water conditions (Le gall et al. 2004, Werner and Dring 2011) and escalating spore mortality reduce the density to 10-50 seedlings cm^{-1} after the initial two weeks germination. Consequently, the seedling density after nursery growth varies tremendously. A density of 10-20 seedling cm^{-1} is suggested a minimum quality (Sanderson 2006, Werner and Dring 2011, 2013). Maintaining higher spore survival is achieved by earlier transfer to larger tanks and by often water renewal (Le Gall et al. 2004) or by repeated spore washing before they settle (Pang and Lüning 2006), though these methods might not be applicable in a commercial hatchery.

Having both the hatchery seeding and the out-planting of substrates within the same season, in pursuit of a high and clean biomass harvest, the tiny seedling size at deployment restricts the cultivation success due to fouling. Settlement of debris, growth of epiphytes and filtering organisms cover the seedlings, which impose shading and poor access to resources (increased boundary layer), potentially inhibit or delay growth to an extent that the final yield of the seaweed is substantially reduced and emphasize the main challenge dulse cultivation (Werner and Dring 2011). After settling, tetraspores need 1-3 months to grow into seedlings of 0.5-0.8 cm, which is the recommended seedling size at deployment to reduce the negative effects of fouling (Werner and Dring 2013). A previous study found that for seedlings <1 cm of *Sarcothalia crispate*, another commercial cold-water rhodophyte, epi-fouling inhibited the seedling growth, emphasizing the importance of local farming experience (Avila et al 1999, McNeill and Falshaw 2017). In essence, high growth rate after deployment is paramount for a feasible seaweed cultivation to obtain successful yield and ensure palatable appearance, demanded for many of its applications (Abreu et al. 2011, Paper 4). Farming at sea holds the potential for high area-specific productivity (Mann 1973) by cultivating seaweeds in three dimensions (fig.1) using different substrate types and deployment configurations, yet this potential depends on the light transmission and water body type (Sanderson, 2006, Bruhn et al. 2016, Bak 2019).

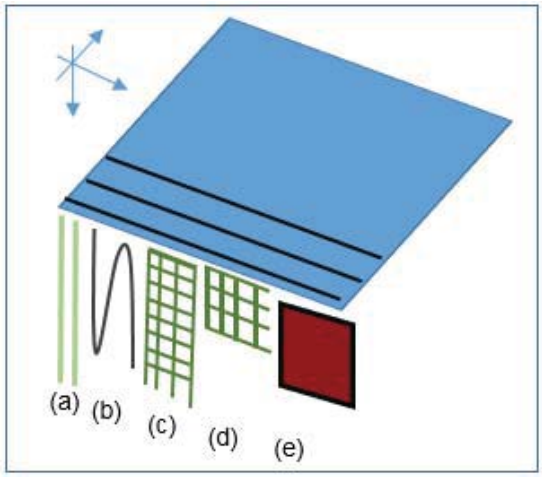


Figure 1. Deployment configuration of substrates using a suspended longline (black line) for three-dimensional farming. (a) single vertical dropper lines, (b) continuous loops, e.g., a kuralon twine coiled on a thicker carrier line, (c) vertical net, (d) horizontal net and (d) solid textile sheet by AT~SEA Technologies, Belgium.

A previous study cultivating dulse in NA waters found substantial lower biomass yield at the deepest part of the single vertical deployed dropper lines after one growth season, which the author attributed light limitation through the water column (Sanderson 2006). This depth-depended biomass growth is also evident when using net substrates aiming to maximize the amount of rope substrate per meter longline (Werner and Dring 2011; Sanderson et al. 2012). Yet, the knowledge about the interaction of net deployment configuration and cultivation depth on the evenness of biomass distribution is incomplete and only one study mentioned the positive effect of out-planting larger dulse seedlings using a long nursery phase (Werner and Dring 2011), similar to the over-summering nursery used in the Chinese kelp industry (Su et al. 2017). A long nursey phase allow the development of dulse seedlings that are robust and tolerate higher irradiance exposure than sporelings (fig. 10, *Thesis*). Using a short nursey phase, a previous lab study showed that an irradiance of 50 µmol photons m^{-2} s^{-1} PAR within the first 12 weeks impairs growth and is potentially lethal for germinating dulse seedlings (Edwards and Ding 2011). While irradiance often exceed this level at water surface, such spore-derived seedlings would also be light limited in a few meters below sea level (bsl) in water bodies exhibiting a low secchi-depth (Z_S) of < 2.5 m (depth for 10% of surface irradiance available), which is normal for the eutrophic Limfjord in Denmark. As of such, only 5 µmol photons m^{-2} s^{-1} PAR would be available at a depth (z) of 2.5 m, using the equation $E_{(Z)} = E_{(Zo)} * e^{-Z*Kd}$ and the equation $K_d = 2.3/Z_S$ (Markager and Fossing 2014) to estimate the K_d value, which suggests light limited growth condition. In contrast, when seedlings develop thicker thallus they require 140-178 PAR for saturation of growth and tolerate up to 280 PAR (12:12 h light) for 6 weeks (Schubert and Sagert 2000, Paper 4). This is supportive to the early suggestions of an optimal cultivation depth of 1-2 m bsl (Browne 2001) for cultivating dulse and supports the finding of uneven biomass distribution on nets extending 3 meters down into the water column (Sanderson 2006; Edwards and Dring 2011) where seedlings at the deeper part was light limited. Late in the growth season, light intensity becomes excessive and potentially causing deterioration of the biomass (Werner and Dring 2011). This demands investigation in net deployment configurations for optimal utilization of the cultivation depth and areal-specific biomass yield.

The high nutrient status of inner Danish waters indicates a great potential for seaweed cultivation (Nielsen 2015, Seghetta et al. 2016, OSPAR 2008). The inner Danish water receive a substantial amount of nutrients from the Baltic Sea as well as from agricultural runoff. Danish coastal waters varies greatly in depth, exposure, salinity, residence times (TR) and annual temperature fluctuations (Conley et al. 2000, Hofmeister et al. 2009). The Limfjorden (fig. 2A-B) is a shallow sound with a high amount of upland run-off, where different bays and inlets varies in salinities and RTs. As a whole, the Limfjorden has a high RT up to 225 days, considering the time required for half of the water volume to be exchanged (Janus Larsen, Aarhus University, *pers. comm., Feb 2020*). The Limfjorden is highly eutrophic with relative high mean chlorophyll concentration and resuspension, which implies a high light extinction coefficient, e.g., low transmission of light. In comparison, the inner Danish waters are generally more exposed, show lower RT and great variation in salinity (fig. 2C). Eventually, adequate light intensity and temperature, as well as incidence of fouling settlement, of for instance filtering organisms, restricts seabased seaweed cultivating in Danish waters which ought to take place between

autumn and late spring (Nielsen 2015, Bruhn et al 2016). Hence, the challenge of exploiting the inner Danish waters for optimal biomass yield demands investigations of cultivation strategies.

Because of the mentioned challenges, the present study investigates the growth and biomass yield using three hatchery cases based on recently suggested seeding methods (Paper 3) and the apparent benefit of deploying over-summered seedlings by cultivation at several farm sites in inner Danish waters.

Material and methods

Three different types of substrate (twine, rope, and net) were seeded with dulse in three *hatchery cases* by use of two different seedstocks (newly released spores vs. germinated propagules), according to Paper 4. The seeded material were kept in nursey for either short (0.7-1.5 months) or long (9-11 months) time before being deployed using different deployment configurations (single vertical dropper lines, continuous loops, and nets) (fig.1). The substrates were cultivated at different longline farms in one of two growth seasons (2017-2018 and 2019-2020) at various farm sites in Denmark (fig. 2A).

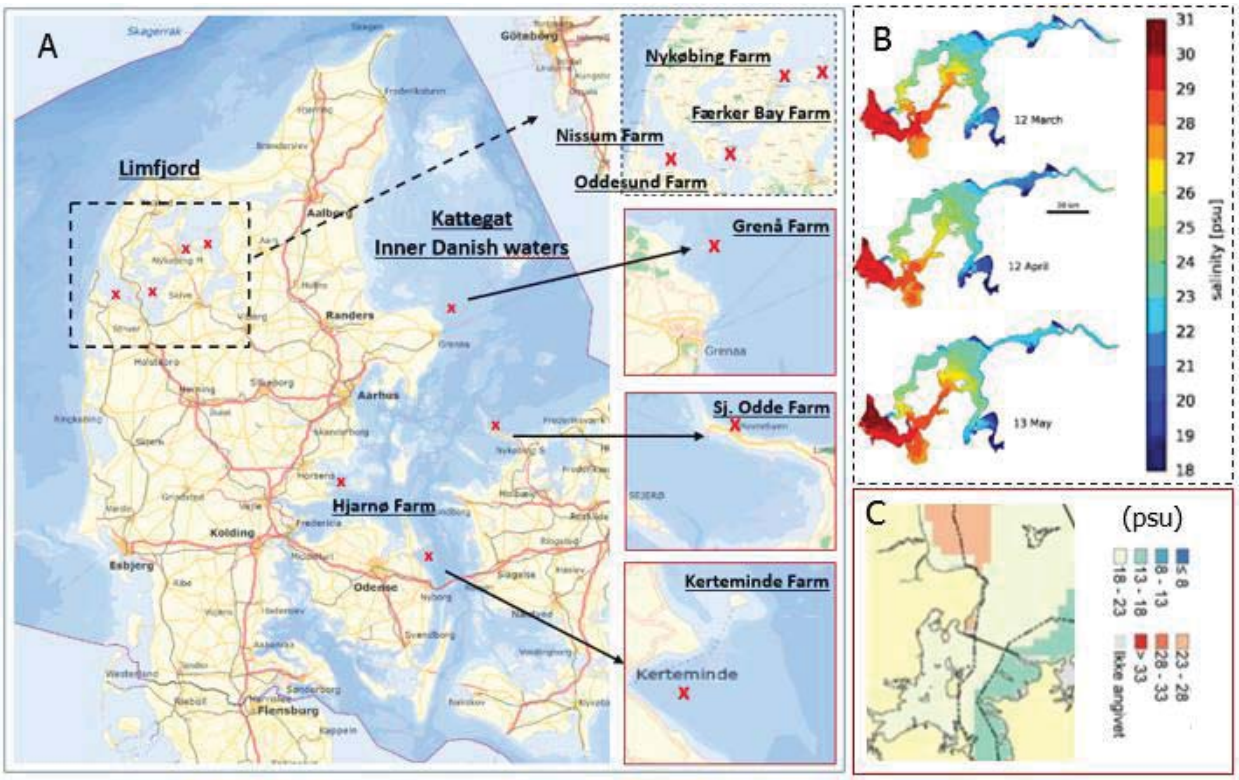


Figure 2. A: Map of Denmark showing the cultivation sites used for growth trials performed in 2017-2018 and 2019-2020 and the modelled salinity distribution (Hofmeister et al. 2009) in the Limfjord at three date in spring (B) and in the mean surface salinity (C) in the central Kattegat (Danish Hydrological Institute, DHI). *(Map credit: the Geodatastyrelse, Denmark)*.

In the Limfjorden (fig.2A), the farm sites had a 3-5 m water depth and they differed in a west-east manner mainly based on their exposure, water currents and salinity (fig. 2B). Salinity data used in this study, was obtained from the nearest station of the National Monitoring Programme (NOVANA) and Danish Hydrological

Institute (Boderskov et al. *in prep*) and calculated as an average of the entire the water column in the period 2017-2020.

The Nissum Farm (3 m water depth) is a highly exposed site with low currents and a mean salinity of 30 psu, followed by the highly exposed Oddesund Farm (5 m water depth) with moderate currents. The Nykøbing Farm (5 m) is moderately exposed with a mean salinity of 28 psu and with low currents while the Færker Bay farm (5 m) is moderately exposed with low currents and a mean salinity of 26 psu. In Kattegat, the Grenå Farm (12 m) was the most exposed site with highest currents of all farms and a mean salinity of 20 psu (13-29 psu in range) (fig.2C). The Hjarnø farm (8 m) is highly exposed with moderate currents and a salinity of 22 psu. The Sj. Odde Farm (1.5 m) is a small pilot test site accessible from the shore and is highly exposed and displays low currents and salinity (13-18 psu). The nearby Rørvig pilot-scale farm is poorly exposed site with moderate currents and displays a mean salinity of 19 psu. The Kerteminde Farm (4 m) has a salinity of 13-18 psu and is moderately exposed and has high water currents.

Light data. In Nissum, irradiance was monitored (averaging every 10 minute) at two different water depths by use of Odessey PAR loggers. In Færker Bay, the amount of lux was recorded by use of two HOBO loggers (every 30 minute) at 0.5 m and 2.5 m bsl. The lux was transformed to photoactive radiation (µmol photons m^{-2} s^{-1} PAR, 400-700 nm) by multiplying factor 0.023, based on a web based horticulture lux converter. For other stations, the amount of PAR available at relevant depths $E(z)$ was computed based on monitored surface irradiance and the use of the Z_S derived from NOVANA database (Boderskov, *in prep.*).

Hatchery case 1. Two types of twisted rope (Polypropylene (PP), kuralon) were spore-seeded using 50 fertile *P. palmata* fronds in a 400 L inoculation tank during 20 days, according to Paper 2. Twelve PVC cylinders containing 6 m PP rope (Ø=6 mm) were placed horizontally, while two PVC cylinders containing 40 meters of kuralon twine (Ø=2 mm) were fixed vertically in the same tank. Aeration from the bottom provided constant water agitation for spore dispersal, according to the "tumble method" (Sanderson 2006). After 29 days in nursery at 10 °C and 15 µmol photons m^{-2} s^{-1} (12:12 h light:dark) seedling length on the kuralon was smaller than on PP ropes (~1 mm), likely due to the vertical orientation towards the light source. Both seeded materials were deployed as vertical single droppers (n=5) January 26, 2017 at the Færker Bay ("spring 2017") with a seedling density of 10±3 spores cm^{-1} kuralon and 8±1 spores cm^{-1} PP (mean±SE, n=3). Growth at different cultivation depths (1.5, 2.5, 3.5 m bsl) was monitored by measuring the five longest fronds at each sampling event. After 11 months in nursery (November), seedling length had reach ~ 0.5 cm in length and seeded material from the same batch were deployed ("fall 2017") and growth assessed at 1.5 vs. 3.5 m at Nissum Farm and Færker Bay, and at 0.5 vs. 2.5 meters water depth (Hjarnø Farm).

In addition, about 17.5 m seeded kuralon (11 months nursed) was deployed as continues loops in the Nissum Farm, by coiling the kuralon twine around a carrier line. Loop were hanging at 1.5-3-5 meters of depth with a section of 30 cm between each loops, where the kuralon was parallel to the sea surface.

Hatchery case 2. Net pieces (0.25 * 1.40 meter) made of multi-braided PP twine (Ø=5 mm) with a mesh size of 4 cm were estimated to contain an equivalent of 42 m linear meter rope. Nets were placed as nets-spirals

(0.25 m in height) submerged in vertical flow-through bubbles tanks and spore-seeded in early January 2019 (Paper 3). After 20 days in nursery, five nets were deployed in horizontal configuration, with the long side parallel to and 1 m below sea surface at the Nykøbing farm to assess growth rate of seedlings (<1 mm) in an eutrophic water body at the beginning of the growth season. The rest of the batch remained in nursey for additional nine months of over-summering nursery growth at 15 µmol photons m^{-2} s^{-1} PAR and deployed (n=3-5) in late October 2019 at all farms sites, except Færker Bay and Hjarnø (fig. 2A). At late deployment, seedlings had reached a size range of 0.5-5 cm depending on the position on the nets. At the Grenå Farm, nets were deployed in both vertical and horizontal configuration (n=5) (fig.1C-D). In a vertical configuration, the nets extended 1.4 meters in the water column, which extrapolates to 6.28 km rope per longline (100 m longline). In a horizontal configuration, the net extended 0.25 meters below the longline extrapolating to 1.05 km rope per longline. Noting the position (top/bottom) for the longest fronds measured on the nets as well as the biomass distribution of the final harvest was used to evaluate the effect of light availability.

Hatchery case 3. Pieces of rope (15 cm) of braided PP were seeded using discoid reattachment of propagules (Paper 3) using either young propagules (29 days) or old propagules (240 days) as seedstock. Up until use, propagules were kept for nine months at a L:D regime of 12:12 h at 15 µmol photons m^{-2} s^{-1} PAR, adjusted to 7 PAR after 2 weeks at 5 °C and with gentle aeration. Some bottles containing the propagules for 240 days were exposed to either white or red light to assess the effect on quality and reattachment capability. No nutrients were added the first 60 days, then 10 % F/2+ medium was added at water renewal every month.

Seeding at day 29 was done by detaching and macerating attached propagules into a seeding mix, of which 20 mL poured into separate seeding tanks (400 mL) containing a piece of near-vertical orientated rope and agitated for three days. Afterwards, the pieces of rope (15 cm, n=15) were fixed vertically in a 20 L tank with air agitation and cultured for 8 months at 5 °C, 30 µmol photons m^{-2} s^{-1} PAR, 10 % F/2+. By this, reattached seedlings reached a size range of 1-4 cm on the ropes. Likewise, after 240 days (delayed use), the propagule seedstocks (red and white light exposed) was used as inoculum to seed additional rope pieces (n=10) subsequently nursed for a month. However, it was obvious after three and ten days that the propagules did not reattachment to rope substrates and thus, these were never deployed.

Deploying the propagules-seeded ropes from the first inoculation (29 days old) in Nykøbing Farm, Nissum Farm, and Grenå Farm in late October 2019, allowed a comparison between farms. Propagule-seeded ropes (n=5) were inserting perpendicularly in a carrier line with 30 cm spacing , which was suspended at 1.5 m depth. A substrate density of 500 m rope per longline is estimated using such configuration, and extrapolates to 10 km rope ha^{-1} (100 m longline, 20 lines ha^{-1}).

Deployments from hatchery case 1 were monitored in spring 2017 at Færker Farm (26/01/2017, 29/03/2017, 09/05/2017, 22/05/2017) and in autumn 2018 at Nissum Farm (19/9/2017, 13/02/2018, 04/04/2018, 04/05/2018), Færker (19/09/2017, 01/02/2018, 22/03/2018, 17/04/2018, 09/05/2018) and at Hjarnø farm (21/09/2017, 15/12/2017, 27/03/2018, 07/06/2018). The deployments from hatchery case 2 and 3 was monitored at Nykøbing and Nissum farm (03/10/2019, 05/11/2019, 03/02/2020), and at Kerteminde Farm (12/10/2019, 01/11/2019, 25/01/2020), Oddesund Farm (01/11/2019, 28/11/2019, 28/01/2020) Sj. Odde Farm

(5/11/2019, 31/01/2020). The maximal length of fronds on each deployed unit (n=5, N=25) was measured at different cultivation depths. At the final monitoring (NOT YET), the biomass yield was evaluated by bringing back rope pieces to the lab for drained fresh weight measurement. These measures were used to evaluate the biomass yield (g FW m^{-1} seaweed line) between the different deployment depths and farms.

Statistics (still for consideration before final submission)

Case1: Within farms, the welch´s anova was used to compare the final mean frond length between cultivation depths after confirming the assumption of normality (Shapiro-Wilk's test) and homogeneity of variance (Levene test).

Case 2 and 3 (in consideration): The density of seedlings and seedling length are expected to influence on the biomass yield and is tested in a 2-way ANCOVA (Farm site * cultivation depth) OR a linear regression model: Biomass yield = Sampling date + Frond length + Seedling density + Cultivation depth.

Results

Field growth 2017-2018 (Farms: Færker Bay, Hjarnø, Nissum) – Hatchery case 1

The spring 2017 deployment in Færker Bay resulted only in visible frond growth on the PP droppers during the season, likely caused by more fouling on kuralon twine on carrier rope compared to the PP rope as well as smaller seedlings on the kuralon. Both on May 9 and May 22, the maximum mean frond length at 2.5 m was significant higher than at 1.5m and 3.5 m depth (1-way ANOVA:$p<0.0272$, n=5) (Fig. 3A). The harvest yield was 27±10 g FW m^{-1} seaweed line (mean±SE, n=3).

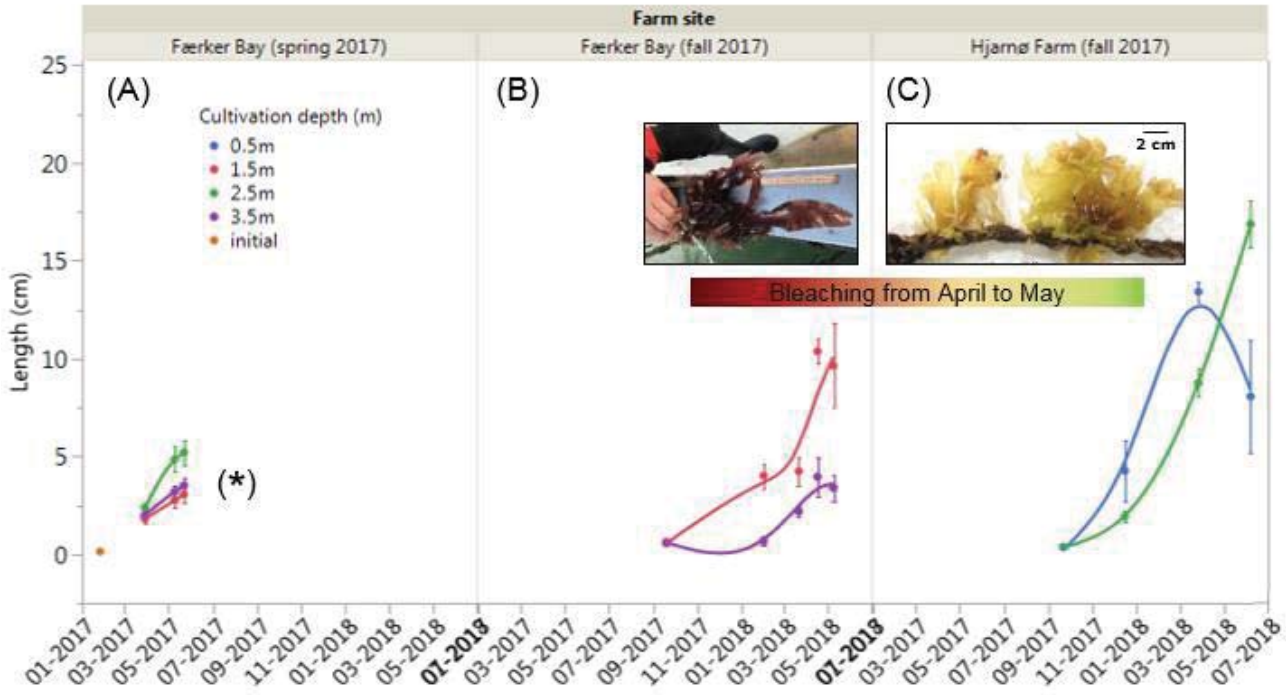


Figure 3. Seasonal frond growth (cm) in longline cultivated *P. palmata* monitored at different cultivation depths (0.5, 1.5, 2.5, 3.5 m) and different farm sites. A (spring 2017): Growth on PP droppers in Færker Bay after nursing for one month. B (Fall 2017): Growth on kuralon droppers deployed in Færker Bay and Hjarnø Farm after 11 months nursery. C: Growth on kuralon droppers deployed in Hjarnø Farm after 11 months nursey. Frond length reduced at shallow cultivation depth (0.5m) at Hjarnø Farm due to light-induced bleaching of thalli. Asterisk: only dropper lines of PP showed growth in Færker Bay (spring 2017) while kuralon dropper lines was used in B-C. Points represent the mean±SE, n=3-5.

The recorded photoactive radiation (PAR) in Færker Bay spring 2017 showed that the average irradiance at both 0.5 and 2.5 m depth (solid and dashed curves, fig 4.A) was well below 50 µmol photons m^{-2} s^{-1} PAR (dotted line, fig. 4A) during the first month after out planting. However, several incidence of >50 PAR was noted for the shallow depth in this period (fig. 4A). Exceeding 50 µmol photons m^{-2} s^{-1} PAR is found potential lethal for germinating dulse seedlings within their first 12 weeks (Edwards and Dring 2011). In the late part of the growth season (fig. 4B), numerous incidence of PAR irradiance was measured to exceed the I_{SAT} for well-nourished dulse (178 µmol photons m^{-2} s^{-1}) level at both that the shallow cultivation depth (0.5 m) and to a minor extend at deeper cultivation depth (2.5 m) (fig. 4B).

These light data in combination, supports the results that the best growth was at 2.5 meters depth (fig. 3A), whereas growth was reduced at deeper water, where light might have been a limiting factor (fig. 4B), and at more shallow water, where light levels might have been harmful in the early period after deployment (fig. 4A).

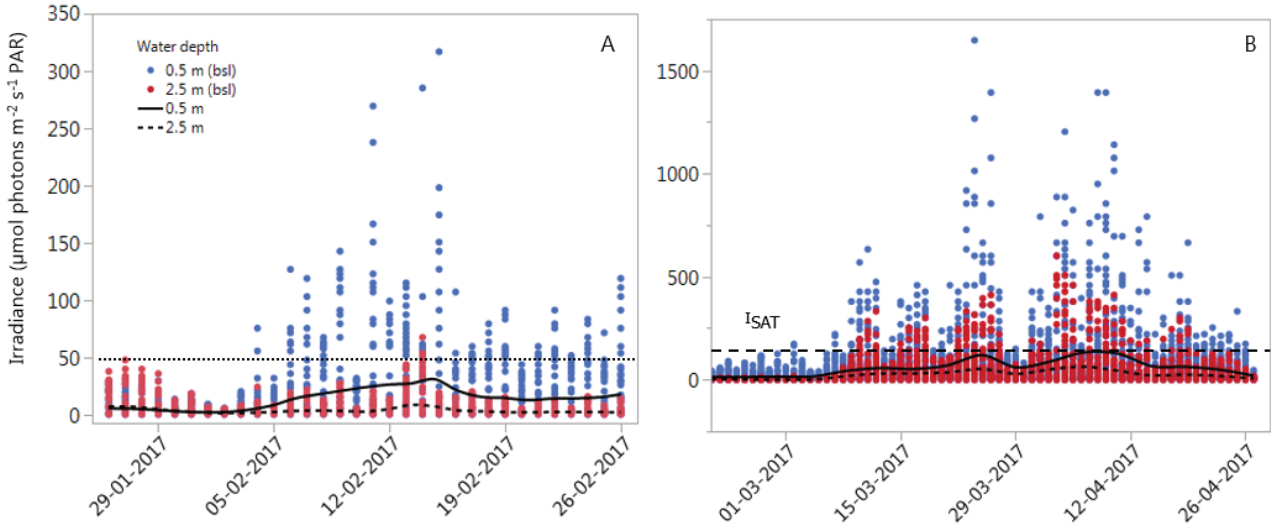


Figure 4. Photoactive radiation (PAR) at two different depths (0.5m and 2.5m bsl) in Færker Bay in spring 2017 during the first month after deployment from January 26 to February 26, 2017 (A) and during the rest of the growth season (B). Each data point represent the integrated amount of PAR (each 30 min).

In the second growth trial (deployment in fall 2017, fig. 3B-C), fronds grew on all deployed droppers in Færker Bay and in Hjarnø Farm. In Færker Bay (fig. 3B), the mean frond length of 9 cm at 1.5 m cultivation depth was longer, yet not significantly, than the fronds grown at the 3.5 m (Welch´s$_{1,2.4}$ = 7.4:p=0.0907, n=3). Until late March, the mean frond growth at Hjarnø Farm (fig. 3C) was longer at 0.5 m compared to the fronds at 2.5 m depth (Welch´s$_{1, 6.9}$ = 28.3:p=0.0011). However, in June, the final monitoring of Hjarnø Farm reveled that fewer fronds were present on the droppers and the ones that were left suffered from bleaching (picture fig. 3). Hence, the lower number of fronds imposed a higher deviation of the mean frond length, which caused the difference between the two depths to be insignificant (Welch´s$_{1, 2.7}$ = 8:p=0.0732). At 0.5 m the fronds length had reduced to 8.1±2.8 cm (mean±SE, n=3), while fronds at 2.5 m depth had grown into 16.9±1.4 cm (mean±SE, n=5). In Færker Bay, the harvest yield after 8 months at sea was 27±10 g FW m^{-1} seaweed line (mean±SE, n=3). In Hjarnø Farm, the harvest yield after 9 months at sea was 199±49 g FW m^{-1} seaweed line (mean±SE, n=3).

In Nissum farm, the autumn deployed kuralon droppers (fig. 5A) showed significant longer fronds (22.5±1.9 cm, n=3) and less fouling was observed at the cultivation depth 1.5 m compared to the PP droppers (2.9±0.4 cm, n=2) (Welch´s$_{1, 15.8}$ = 19.3:p=0.0005).

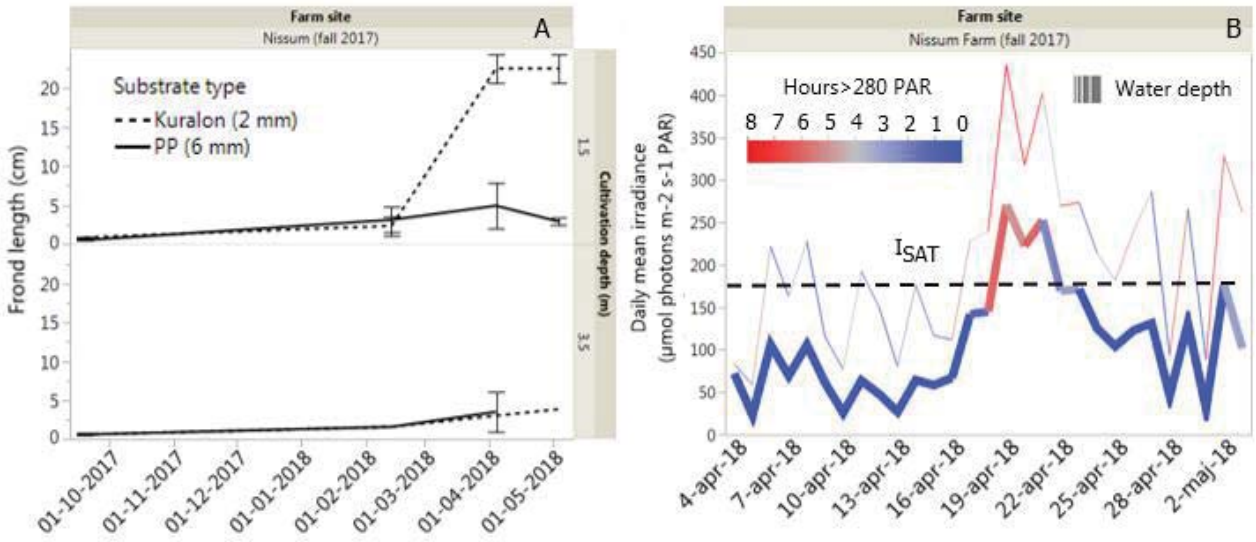


Figure 5. A: Seasonal frond length in longline cultivated dulse deployed after 11 months nursey phase and monitored at two cultivation depths (1.5 and 3.5 m) during the growth season September 2017 to May 2018 at Nissum Farm. Data points represent the mean±SE (n=3). B: The curves show the daily mean irradiance (PAR) measured at 1.5m and 3.5 m bsl and the color-coding indicate the amount of hours where irradiance exceed a level of 280 PAR, which starts to impair dulse growth rate.

The top part of the loop configuration (fig. 1B) displayed similar frond length as the dropper configuration (21.6±0.9 cm, n=3) (fig 1A) (data not shown). The maximum biomass yield amounted to 289±33 g m^{-1} twine (mean±SE, n=3) and was registered on the top horizontal part of the loops at a depth of 1.5 m. At the cultivation depth of 3.5 m below sea surface, both substrates showed reduced frond growth (fig. 5A). Unfortunately, this poor growth might be a result of physical contact with the seafloor at low water level, which was associated at rough winter weather.

The amount of measured light (µmol photons m^{-s} s^{-1} PAR) available at 3.5 meters depth at Nissum farm, also suggests that growth is strongly light limited during spring season 2018 at this depth (fig. 5B). During spring, these seedlings were 12-16 months old and thallus was thicker than spore-seedlings. The growth saturation point for thick-leafed seedlings was found to be 178 µmol photons m^{-2} s^{-1} PAR (Paper 4). By inspecting fig. 5B, the thick curve (3.5 m cultivation depth) is in 80 % of all the days below the 178 PAR growth light saturation point (dashed line). The red-blue bar color-coding reveals the amount of hours per day where irradiance exceed 280 µmol photons m^{-2} s^{-1} PAR, which by mentioned authors was found to impair growth rate at long-term exposures. According to this, mid-April was stressful; however, the following days of lower irradiance seems to mitigate any stress, as fronds did not bleach.

Field growth 2019-2020 (Grenå, Nykøbing, Oddesund, Nissum, Sj. Odde, Rørvig, Kerteminde) – hatchery case 2 and 3.

The deployment of seeded nets February 1st 2019 after 0.7 month of nursery growth at Nykøbing Farm resulted in total coverage of hydroid fouling and no visual seedling growth was noted after 4 months in May. This trial was abandoned yet, tiny dark-red seedlings remained attached on the ropes underneath the dense hydroid cover. In contrast, the over-summered spore-seeded nets (fig.6 A-B) and propagule-seeded ropes (fig. 6C) provided robust seedlings, which showed good increments in frond length during the early growth season.

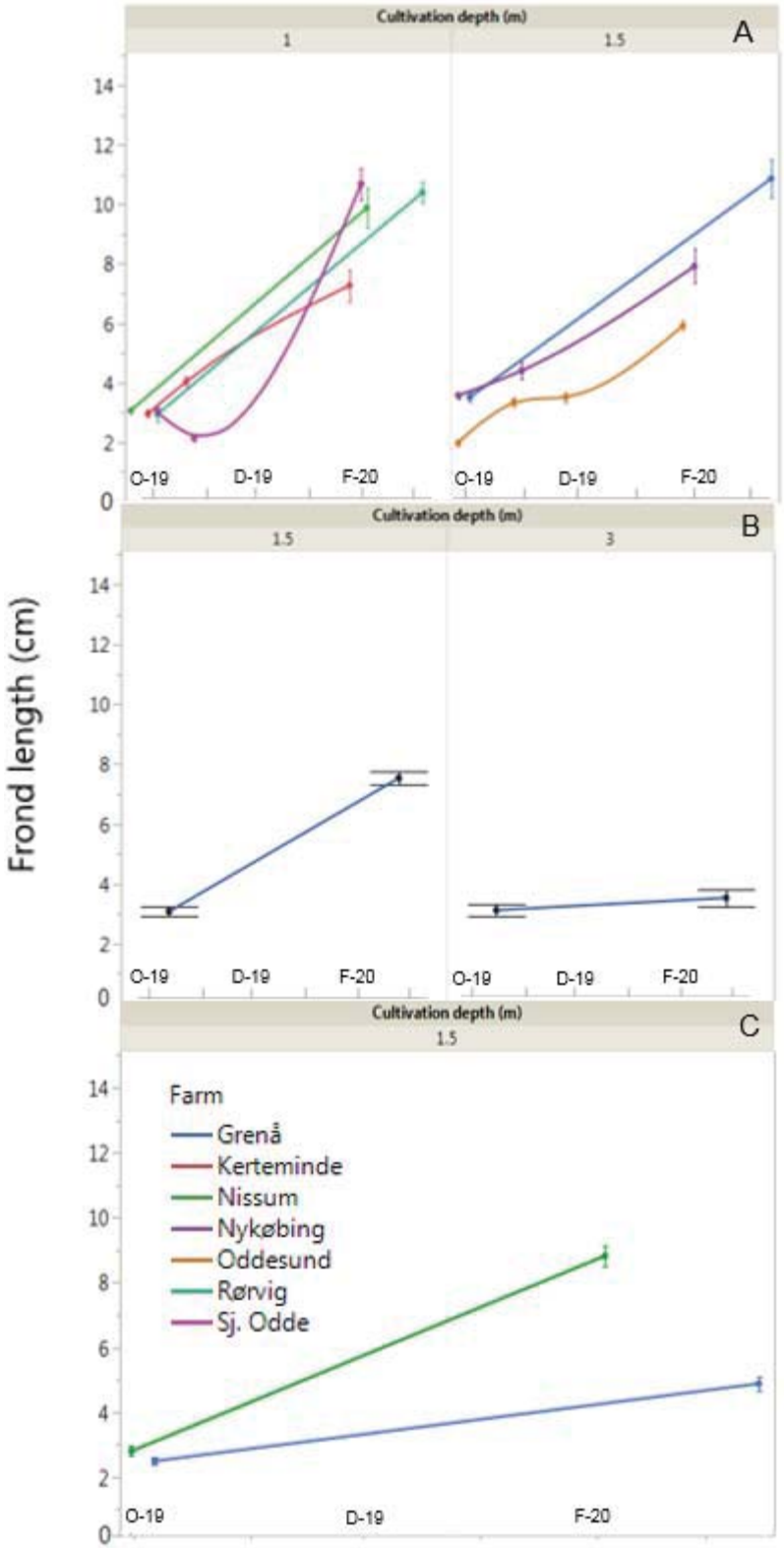


Fig. 6. Seasonal development in dulse frond length from October 2019 to March 2020 at different farms in inner Danish waters. Substrates were spore-seeded nets using horizontally (A) or vertically (B) deployment configuration or pieces of propagule-seeded ropes inserted in a horizontal deployed carrier line (C).

Seeded horizontal nets showed no major differences between farm sites (fig. 6A) all having greater mean length of fronds after the same amount of time at sea (4 months) in the early part of growth season (February) compared to long-nursed kuralon twine even at the best site in the Limfjord (fig. 5A). However, the frond size at Grenå might be underestimating, as nets were lowered additional 1-2 meters at an unknown time during late winter, due to insufficient buoyancy. The frond size on the vertical nets was slightly smaller than on the horizontal nets, and growth was in highly reduced with depth (fig. 6B). Propagule seeded ropes grew well, with better growth at the Nissum farm site as compared

to Grenå (fig. 6C). Macerated propagules were unable to establish discoid reattachment after 240 days. This attributes their large individual size and potential clumpy formation due to coalescence (fig. 7). Red light exposure provided a much cleaner (less green microalgae) propagule seedstock (fig. 7A-C) than the white light after 240 days of culture (fig. 7D). Both light qualities sustained good spore germination with red/white inoculum contained 31/60 ± 0.5/13 germinated spores (females) and 180/251±32/5 sprouting seedlings (males) mL^{-1}, respectively.

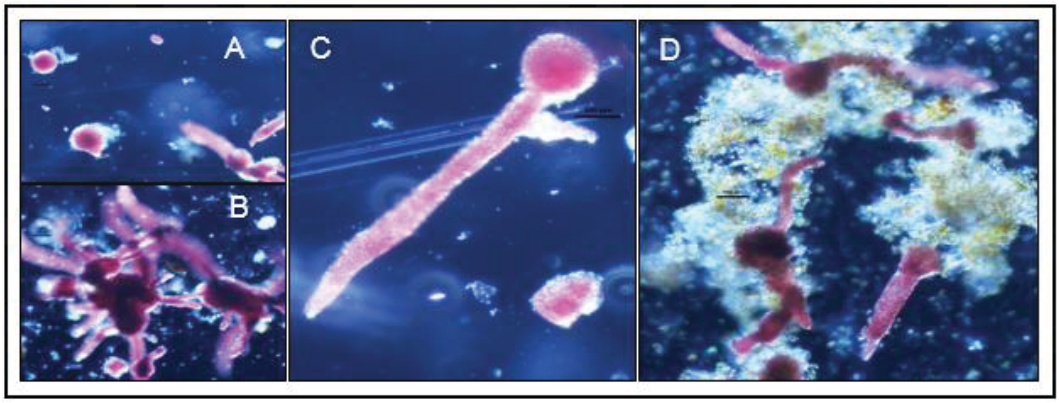


Figure 7. From hatchery case 3 a long nursey phase (240 days) was tested by culturing propagules at either white or red light. After maceration ´, none of the propagules showed to establish discoid reattachment to rope substrates. A-C: male seedlings develops in aggregates, with potential coalescence, which might be unsuited for reattachment due to their larger size relative to discoid strength or simply debris clogging the basal discs adhesive mechanism.

Results indicate the importance of frond size at deployment, as seedlings on nets and twine were about 3 cm and 0.5 cm, respectively, thus a greater initial size promotes increments in frond length. For estimation of potential biomass yield per hectare the choice of substrate type is potentially the most importance factor, as the net substrate ought to provide a high density compared to single vertical droppers.

Discussion (preliminary)

The present study demonstrates for the first time successful cultivation of dulse (*P. palmata*) in several parts of the inner Danish waters based on two seeding methods with prospects for an efficient hatchery production of seeded materials. The initial seedling density of the twine, rope, and nets were 5-15 cm^{-1} and with 40-80 spores and 30-60 male seedlings cm^{-1} on the propagule seeded ropes, which is lower than the previous stated optimal seeding density ≈100 spores cm^{-1}. Hence, this study documents that good hatchery quality (seedling length, density) is essential. The timing of deployment, nursery duration and farm location have a significant effect on the growth and potential biomass yield. By early deployment (tiny seedlings), the cultivation was unsuccessful in the eutrophic Limfjord, which suggest a seedling size of >0.5 cm after long nursey is required for success. In contrast, Dion and Delépine (1981) found high growth rate of *P. palmata* sporelings after short hatchery duration by cultivation in Atlantic Oceanic waters. Even in oceanic clear waters, the growth of dulse may halt at a size of only 1-5 cm reaching summer time (Bak 2019), which might be attributed to their adapted

endogenous rhythm in growth or a matter of insufficient nutrient availability (Hurd and Harrison 2001, Faes and Viejo 2003). By insertion of bigger fronds (vegetative propagation), growth up to 30-40 cm within 4-5 months and higher biomass yield is obtained (Sanderson 2006, Bak 2019) however this method is yet unsuited for large-scale cultivation in Europe. This seems to suggest that the seedling size at deployment and farm site is important for fronds to reach a good size at the beginning of the growth season. The present study found a beneficial effect on growth by deployment during autumn instead of spring, which is in accordance to previous trials (Sanderson 2006, Werner and Dring 2011). However, by deployment in autumn or spring, the growth of tiny spore-derived seedlings may suffer from excessive light intensity shortly after deployment (fig. 4A) and late in the growth season (fig. 4B, fig. 5B) as well as be light limited depending on the cultivation depth (fig. 6B), which emphasizes the need for local knowledge on water conditions. The cultivation effort is crucially halted by early incidence of biofouling in eutrophic water bodies (central Limfjord) using short nursey periods and spring deployment, which collectively stress the importance of longer nursing periods to improve cultivation success and gaining higher biomass yields in those areas.

The preliminary observations on cultivating different seeded substrates obtained from different hatchery setups, seems to suggest that growth, potential biomass yield and biomass evenness is favored by using nets in horizontal configuration compared to vertical nets, single droppers or loops of continues twine on a carrier line. The advantage of seeding and cultivating nets is likely an optimal combination of suitable substrate surface and a structure providing a high amount of rope per unit of space. Nets deployed in vertical configuration seems to be light limited (fig. 6B). This was reinforced by an undesired lowering of the longline until early March, which effectively put the nets at 3 m bsl (horizontal) and 3-4.5 m bsl at Grenå Farm and suggest that the optimal cultivation depth for nets is 1-2 meters during the winter season. The biomass density on nets and propagule-seeded ropes seems similar (g/linear meter, *data not shown*), while longer fronds on the nets compared to the propagule-seeded ropes suggests that the denser seeding obtained on the ropes could be lowered without reducing the potential yield. By a reduction in propagule seedstock concentration, a higher seeding efficiency is possible. The highest biomass yield of 289±33 g m^{-1} was obtained solely from the part closest to the sea surface (1.5 m bsl) and is in the lower range of previous studies using single droppers (Sanderson 2006, Werner and Dring 2011, Sanderson et al. 2012). A kuralon twine imposes a low utilization of the top water column, compared to a net in either vertical or horizontal configuration. Hence, we expect a conservative yield improvement of 5-10 times per meter longline compared to the reported yield in Danish waters (≈1.4-2.9 kg FW m^{-1}), by utilizing the upper 1.5 meters at exposed sites in the eutrophic Limfjord (fig. 3B), and by adjusting the cultivation depth during growth season in the mesotrophic water type such as Hjarnø (fig. 3C). The smaller frond size obtained on the propagule-seeded ropes, suggests that the higher seedling density obtained in the hatchery is impairing growth rate (self-shading), in comparison to the lower seedling density on nets cultivated at the same farm, which shows longer fronds. Likely, the amount of available light reduces by increasing seedling density along with increasing water depth, emphasizing an optimal seedling density is below 30-60 cm^{-1}.

Incidence of tissue bleaching restricts the cultivation season and the timing of harvest ought to ensure high commercial value of the dulse biomass, but cuts off biomass growth potential. The present study found that

cultivation in June resulted in severe bleaching of fronds cultivated at 0.5 m and moderate bleaching at 2.5 meters depths at the Hjarnø farm situated in the mesotrophic part of the inner Danish waters (fig. 3C). By harvesting one months earlier (May), fronds did not bleach at the Nissum Farm in the western part of the Limfjord, and suggest that harvest time determine biomass quality. At this location, even with several incidence of excessive irradiance (fig. 5B) the fronds did not bleach, likely because of sufficient recovery periods during nighttime and following days of adequate light exposure. However, by the seasonal increase in daily mean irradiance, cultivated fronds near sea surface will accumulate stress, reinforced by increasing nutrient depletion of tissue and increasing ambient temperatures and bleaching will probably occur at an even lower irradiance. Dulse was found to cope with short-term lab exposures of up to 1600 PAR (Pang and Lüning 2004) under laboratory conditions, while long-term tolerance of high irradiance is depended on high nutrient status (Zhao et al. 2017, Paper 4), which can be achieved by co-cultivation with sea-based fish farming (Sanderson 2006).

Conclusion and perspectives

In dulse cultivation, it remains a challenge to obtain large-sized seedlings ready for autumn deployment within the same season as performing the hatchery. Present study demonstrated for the first time the use the cultivation of substrates seeded by different hatchery methods (spore and propagules) with the advantage of using shifted hatchery and out-planting seasons in Danish waters. A long nursery duration seems as a prerequisite to optimize seedling size before deployment and obtain a higher yield than by cultivation within one season. This study indicates that dulse cultivation yield is optimal by cultivating net substrates in the upper water column. Optimization of dulse grow out request more research in the production tetraspores early in the year or by use of germinated propagules (“GMA-seeding”) to advance on the production of good quality seedlings and eventually lowers the production costs. The unsuccessful attempt to prolong hatchery seeding with 240 days (all-year potential) request more research of GMA-seeding as a new alternative seeding method. High density substrates, like nets, seems advantageous compared to single lines regarding yield per unit area relying on 3D farming, which is demanded to minimize the marine surface occupation.

Acknowledgements

Thanks to the technical staff at Danish Shellfish Centre (DTU AQUA), the seaweed company DanskTang Aps, and volunteers from sea Gardens in Nykøbing Mors and Kerteminde helping with the data collection at the farms.

References

Abreu MA, Pereira R, Buschmann AH, Sousa-Pinto I (2011) Nitrogen uptake responses of *Gracilaria vermiculophylla* (Ohmi) Papenfuss under combined and single addition of nitrate and ammonium. J Exp Mar Biol Ecol 407: 190–199.

Avila M, Ask E, Rudolph B, Nuñez M, Norambuena R (1999) Economic feasibility of Sarcothalia (Gigartinales, Rhodophyta) cultivation. Hydrobiologia 398/399: 435-442.

Boderskov T, Macleod A, Nielsen MM, Rasmussen MB, Pycke B, Groenendal B, Balsby TJS, Bruhn A (in. prep.) Cultivation of Sugar kelp in Danish waters.

Bruhn A, Tørring DB, Thomsen M, Canal-Vergés P, Nielsen MM, Rasmussen MB, Eybye KL, Larsen MM, Balsby TJS, Petersen JK (2016) Impact of environmental conditions on biomass yield, quality, and bio-mitigation capacity of *Saccharina latissima*. AEI 8: 619-636.

Browne KL (2001) Mariculture of the edible red alga Palmaria palmata. PhD thesis, Queen's University of Belfast, UK. 186 pp.

Conley DJ, Kaas H, Møhlenberg F, Rasmussen B (2000) Characteristics of Danish Estuaries. Estuaries 23: 820–837.

Dion, P., Delépine, R., 1981. Studies of the development of *Palmaria palmata* Rhodophyceae using in situ controlled cultures. Proc Int Seaweed Symp 10, 265–270.

Edwards MD, Dring MJ (2011) Open-Sea cultivation trial of the red alga, *Palmaria palmata* from seeded tetraspores in Strangford Lough, Northern Ireland. Aquaculture 317:203–209.

Hofmeister R, Burchard H, Bolding K (2009) A three-dimensional model study on processes of stratification and destratification in the Limfjord. Continental Shelf Research 29(11):1515-1524.

Kim JK, Duston J, Corey P, Garary DJ (2013) Marine finfish effluent bioremediation: effects on stocking density and temperature on nitrogen removal capacity of *Chondrus crispus* and *Palmaria palmata* (Rhodophyta). Aquaculture 414– 415:210–216.

Le Gall L, Pien S, Rusig AM (2004) Cultivation of *Palmaria palmata* (Palmariales, Rhodophyta) from isolated spores in semi-controlled conditions*. Aquaculture*, 229(1–4): 181–191.

Mann KH (1973). Seaweeds - their productivity and strategy for growth. Science, 182, 975- 981.

Morgan KC, Simpson FJ (1981b) The cultivation of *Palmaria palmata*. Effect of light intensity and temperature on growth and chemical composition. Bot Mar 24: 547–552.

Markager S, Fossing H (2014) Lyssvækkelse – technical report M06, DCE – National Center for Environment and Energy (Aarhus University).

McNeill SE, Falshaw R (2017) Aquaculture of *Sarcothalia atropurpurea* tetraspores in New Zealand for carrageenan production, New Zeal J Mar Freshw Res 51: 528–542.

OSPAR (2008): Eutrophication status of the OSPAR maritime area. Second OSPAR integrated report. OSPAR Commission, London, Publication 372/2008.

Pang, S., and Lüning, K., 2006. Tank cultivation of the red alga *Palmaria palmata*: Year-round induction of tetrasporangia, tetraspore release in darkness and mass cultivation of vegetative thalli. *Aquaculture*, 252(1): 20–30.

Seghetta M, Tørring D, Bruhn A, Thomsen M (2016) Bioextraction potential of seaweed in Denmark — An instrument for circular nutrient management. Science of The Total Environment 563-564:513-529.

Sanderson JC (2006) Reducing the environmental impact of sea-cage fish farming through cultivation of seaweed. PhD thesis, UHI Millenium Institute & Scottish Association of Marine Science (SAMS).

Sanderson JC, Dring MJ, Davidson K, Kelly MS (2012) Culture, yield and bioremediation potential of Palmaria palmata (Linnaeus) Weber & Mohr and Saccharina latissima (Linnaeus) C.E. Lane, C. Mayes, Druehl & G.W. Saunders adjacent to fish farm cages in northwest Scotland. Aquaculture 354-355:128-135.

Sanderson, JC (2015) Maximising production of *Palmaria palmata* (Linnaeus) Weber & Mohr, 1805. MASTS Report.

Su L, Pang SJ, Shan TF, Li X (2017) Large-scale hatchery of the kelp Saccharina japonica: a case study experience at Lvshun in northern China. J Appl Phycol 29: 3003-3013.

Bak UG (2019) An investigation of the biochemical composition of selected macroalgal species, optimized seeding techniques, and open-ocean cultivation methods from a commercial perspective. Industrial PhD thesis. Technical University of Denmark.

Walsh M, Watson L (2011) A market analysis towards the further development of seaweed aquaculture in Ireland. *Irish Sea Fisheries Board*. Report - Project No. PBA/SW/07/001(01).

Werner A, Dring MJ (2011) Aquaculture explained: cultivating *Palmaria palmata*. Irish Sea Fisheries Board, report no. 27 - project PBA/SW/07/001. Dublin.

Werner, A., Dring MJ (2013) Recommendations for optimal ongrowing and harvesting techniques for *Palmaria palmata* in different Irish sites with indications of yield. *Irish Sea Fisheries Board.* Report - PBA/SW/07/001.

Wood E (2018) Cultivation of *Palmaria palmata*: spore release, vegetative propagation and decontamination techniques. Scottish Associaiton for Marine Science (SAMS) and University of the Highlands and Islands.

Zhao LS, Li K, Wang QM, Song XY, Su HN, Xie BB, Zhang XY, Huang F, Chen XL, Zhou BC, Zhang YZ (2017) Nitrogen Starvation Impacts the Photosynthetic Performance of *Porphyridium cruentum* as Revealed by Chlorophyll a Fluorescence. Sci Rep 17(1): 8542.