NTNU

Norwegian University of
Science and Technology

Developing a laboratory cultivation protocol for local species of *Porphyra spp.*

Andreas Quale Lavik

MSc in Biology
Submission date: May 2016
Supervisor: Kjell Inge Reitan, IBI
Co-supervisor: Silje Forbord, SINTEF

Norwegian University of Science and Technology
Department of Biology

Acknowledgements

This thesis was written at the Department of Biology, NTNU, Trondheim 2016. All experiments were conducted at Sintef SeaLab and NTNU Centre of Fisheries and Aquaculture. The thesis was part of the project "Dyrking og foredling av makroalger av høy kvalitet til nye matprodukter", financed by the Regional Research Funds Vestlandet (project number: 245326) and Skattefunn (project number: 247546). The project is owned by Austevoll Seaweed Farm AS, and other partners are SINTEF Fisheries and Aquaculture (project leader), NTNU and Ørjan Johannesen. The thesis was written under supervision of Kjell Inge Reitan, NTNU, and Silje Forbord, SINTEF.

First I would like to thank my supervisors, Kjell Inge Reitan and Silje Forbord, for outstanding help, input, supervision and support. It could not have been done without, and your guidance and patience has been of crucial importance. I would also like to thank Tora Bardal for assistance in the laboratory, and for helpful tips, tricks and guidance in the use of equipment and software. I would also like to thank Dag Altin, for fruitful discussions and helpful suggestions on methodology and equipment use. Also a thank you goes to Andreas Hagemann at SINTEF, for guidance and friendship.

Another thank you goes to my fellow students at NTNU SeaLab, for creating a great environment, you are truly all outstanding people whom will be greatly missed. Also a special nod to Anna Rongved for support, encouragement and friendship, as well as her role as LAKS leader through the past year. Good job!

Trondheim, May 2016

Andreas Quale Lavik

Abstract

The cultivation of *Porphyra* for use in food has a long tradition in Asian countries, and with recent food trends and nutritional awareness, macroalgae has become a popular ingredient throughout the rest of the world as well. The Norwegian coast has several species of *Porphyra* suited for use in many ways, but harvesting wild material is time consuming and limited by few occurrences in the wild. Cultivation is thus the preferred option for future exploitation of the genus. This study is aimed at adapting existing cultivation techniques to local species and environmental factors, for indoor cultivation. A field search was performed to map local occurrences of the genus in the Trondheimsfjord, and a laboratory protocol was established for cultivation of collected material. Several variables were applied at different stages of the life cycle; stress for induction of spore release, treatment with germanium dioxide for decontamination, variations in light intensities and application of scallop shell as a substrate for growth. Also a method for quantification of conchochelis (sporophyte stage) growth were developed and applied.

The field search found that the genus is scarcely represented in local waters, with few and far between occurrences. The laboratory experiment proved that contaminants like diatoms is a challenge, but manageable with correct treatment. Tolerance levels for germanium dioxide were also established, and the experiment showed that the local species of *Porphyra* are well suited for indoor cultivation under the given conditions. The experiment was concluded when the life cycle was fulfilled, from wild collected gametophytes, through the conchochelis sporophyte stage, to growing gametophytes in the laboratory.

Sammendrag

Dyrking av *Porphyra* til bruk i mat har en lang tradisjon i Asiatiske land, og med nye trender og bevissthet rundt næringsinnhold har makroalger blitt en populær ingrediens i resten av verden og. Norskekysten har flere arter *Porphyra* som er egnet for mange typer bruk, men høsting av frittvoksende materiale er tidskonsumerende og begrenset av få forekomster. Dyrkning er dermed den foretrukne metoden for videre utnyttelse av slekten. Denne studien har som mål å tilpasse eksisterende dyrkningsteknikker til lokale arter og miljømessige faktorer, for innendørs dyrkning. Ett feltsøk ble gjennomført for å kartlegge lokale forekomster av slekten i Trondheimsfjorden, og en laboratoreprotokoll ble etablert for dyrkning av innsamlet materiale. Flere variabler ble anvendt i forskjellige stadier av livssyklusen; stress for å indusere sporeslipp, behandling med germanium dioxide for dekontaminering, variasjoner i lysintensitet og applisering av kamskjell som vekstsubstrat. I tillegg ble en metode for kvantifisering av conchochelis (sporofytt stadiet) vekst utviklet og anvendt.

Feltsøket fant at slekten er knapt representert i lokale farvann, med få forekomster med stor avstand i mellom. Laboratorieforsøket bevist at kontaminering av diatomer er en utfordring, men håndterbar med korrekt behandling. Toleransenivåer for germanium dioxide ble og etablert, og forsøket viste at lokale den lokale arten av *Porphyra* er godt egnet for innendørs kultivering under de gitte forhold. Forsøket endte da livssyklusen var fullført, fra frittvoksende gametofytter, gjennom conchochelis sporofytt stadiet, til voksende gametofytter i laboratoriet.

Table of contents

Acknowledgements 1
Abstract 2
Sammendrag 3
Table of contents 4
Glossary 6
1 Introduction 7
1.1 Taxonomy and distribution 7
1.1.1 Taxonomy 7
1.2.2 Distribution 8
1.2 Morphology and life cycle 9
1.2.1 Morphology 9
1.2.2 Life cycle 10
1.3 Cultivation of *Porphyra spp.* 12
1.3.2 *Porphyra* aquaculture 12
1.3.3 Nutritional value and uses for human consumption 13
1.4 Study aims and approach 14
2 Materials and methods 15
2.2 Field search for natural occurrences of *Porphyra spp.* in the Trondheimsfjord. 15
2.3 Collection of material 16
2.4 Spore isolation 19
2.5 Laboratory cultivation of *Porphyra spp.* life cycle 21
2.5.1 Variables for growth phases 25
2.6 Conchosporangium quantification 30
2.7 Decontamination – NaOCl, SSW washing and air drying 30
2.9 Statistics 31
3 Results 32
3.2 Field search for natural occurrences of *Porphyra spp.* in the Trondheimsfjord. 32
3.3 Collected material 33
3.4 Spore isolation 38
3.5 Laboratory cultivation of *Porphyra spp.* and growth variables 39
3.5.1 Induction of carpospore release and effects of stress 39
3.5.2 Conchochelis growth and induction of conchosporelation 40
3.5.3 Conchosporangium quantification and effect of GeO_2, light intensity and scallop shell 41
3.5.4 Gametophyte growth phase 48

3.6 Decontamination 49
4 Discussion 51
4.1 Field search 51
4.2 Collected material 52
4.3 Spore isolation 53
4.4 Laboratory cultivation of *Porphyra sp.* life cycle 53
4.5 Gametophyte growth phase 56
4.6 Decontamination 57
5 Recommendations and conclusion 58
5.1 Protocol for cultivation of local species of *Porphyra spp.* around the Trondheimsfjord: 58
5 References 60

Glossary

Monophyletic:
A group of organisms forming a clade consisting of an ancestral species, and all its descendants. Typically defined by a group of shared characteristics.

Polyphyletic:
A group characterized by sharing the same characteristics, but which has not been developed from a common ancestor. Similar traits developed unparalleled over time.

Carpospores:
Spores that germinate into the sporophyte conchochelis stage, developed in the gametophyte after fertilization.

Conchochelis:
The microscopic sporophyte phase of the *Porphyra* life cycle, occurring as filamentous elongated cells forming an intertwined mesh of red tissue.

Conchospores:
Spores developed in the conchochelis stage inside a filamentous differentiated structure, the conchosporangia, which is easily distinguishable from the rest of the conchochelis. Meiosis occurs when the conchospores germinates.

Dimorphic:
Two distinctly different forms, in the case of *Porphyra* life cycle it refers to the morphologically completely different conchochelis stage and blade phase.

Lanceolate:
Elongated and stretched shape.

Ovoid:
Rounded circular shape.

Monoecious:
Defined by a gametophyte carrying both male and female gametes within the same blade/structure.

Dioecious:
Defined by a gametophyte with gametes from only one sex, in other words two sexually separate plants.

1 Introduction

1.1 Taxonomy and distribution

1.1.1 Taxonomy

Rhodophyta, or red algae, is a large taxonomic group of eukaryotes with an estimated 2500-6000 species in about 680 genera (Woelkerling *et al.*, 1990) and one of the three major lineages of photosynthetic primary producing organisms. This loose estimate is associated with the extreme difficulties of accuracy in classification, partly because reliable species concepts barely exists within many genera (Gabrielson and Garbary, 1987) and the striking similarities in morphology and lack of distinct features makes it near impossible to distinguish many of the species without molecular analysis (Robba *et al.*, 2006). The genus *Porphyra* (Bangiales, Rhodophyta) is the most genetically diverse order of red algae (Ragan *et al.*, 1994) known today, with 268 species at present and 78 taxonomically currently accepted (Hawkes and Guiry, 2015). The order Bangiales has a monophyletic origin (Saunders and Hommersand, 2004), but investigations on the small-subunit rDNA within species of *Bangia* and *Porphyra*, proves these genera to be polyphyletic (Oliveira *et al.*, 1995). Five genera is currently known (*Porphyra, Bangia, Dione, Pseudobangia* and *Minerva)*, but analyses of the slowly evolving 18S rRNA gene indicates that there can be at least ten genera in total (Nelson *et al.*, 2006).

The initial morphological classification of the genera placed the filamentous species in *Bangia*, and the foliose bladed species within *Porphyra*, but with the recent advance in molecular studies the entire order is in continuous renewal (Saunders and Hommersand, 2004). The application of molecular techniques has proved important in expanding the taxonomic knowledge of the Bangiales, and has helped reveal many new ecotypes, cryptic species and genera (Blouin *et al.*, 2011). An example of the difficulties associated with classifications within the order is the relatively recent discovery by Kathleen Drew that *Conchochelis rosea* was in fact the conchochelis stage in the *Porphyra* life cycle (Drew, 1949). Previously believed to be its own species, this was a major breakthrough in control of the *Porphyra* life cycle, which earned her the name “Mother of the Sea” in Japan (Haines, 2001).

1.2.2 Distribution

Although species can be found ranging from Arctic and Antarctic waters to warm temperate and even tropical waters (Kapraun and Lemus, 1987), they are particularly well presented in boreal and cold temperate waters. *Porphyra* is abundant in the Atlantic, especially in the North Atlantic where there is evidence of speciation (Robba *et al.*, 2006) and one endemic species, *Porphyra dioica*, but it is unlikely that this is the area where the genus evolved. It was previously suggested that the Northern Hemisphere, with the North Pacific in particular, was the centre of diversity for the order, but recently many more taxa has been discovered in the Southern Hemisphere (Sutherland *et al.*, 2011). As well a recent study proposes that modern day Bangiales actually has a southern origin, centred around New Zealand (Broom *et al.*, 2003).The great diversity in life history strategies (Holmes and Brodie, 2004), and fossil records based on diagnostic cell division, indicates that the order occurred at least 1.2 billion years ago, making it the oldest taxonomically resolved eukaryote (Butterfield, 2000). Also, the filamentous *Bangia*-like *Bangiomorpha pubescens* appears to be the oldest known case of sexual reproduction (Butterfield, 2009, Butterfield, 2007). This ancient lineage opens up for several routes and times for its arrival to the North Atlantic, via the Tethys Sea, Panama Isthmus, Arctic Seaways and through the South Atlantic (Brodie *et al.*, 2008), and thus its global presence today.

The species thrive in shallow marine waters, predominantly in the intertidal littoral zone where they have adapted to temperature, desiccation and salinity extremes (Broom *et al.*, 2003), but also strictly subtidal species exists (Sutherland *et al.*, 2011). There are also some species that live in freshwater habitats, like *Bangia atropurpurea (Belcher, 1960).* Most are adapted to living epilithically on rocks, boulders, pebbles and bedrock, but many also attach to shells or other algae for a holdfast, and some live strictly as epiphytes. By example *Porphyra nereocystis* Anderson which lives it`s gametophyte stage in a symbiotic relationship at the upper stipe of the brown kelp *Nereocystis luetkeana*, and its conchochelis stage in barnacle shells (Dickson and Waaland, 1985).

1.2 Morphology and life cycle

1.2.1 Morphology

The general morphology of *Porphyra sensu lato* is considered to be simple for its dimorphic life history (Fuller and Gibor, 1984), however when studied at a cellular level it is complex in both morphology and physiology. The foliose gametophyte thallus is only one or two cell layers thick (Murakami and Packer, 1970) and has variable shapes, from narrow and lanceolate to broad ovoid shapes. The microscopic sporophyte phase known as conchochelis is filamentous with single cells forming intertwining threads, with a microscopic glycoproteinaceous structure called pit connections. These are not found in the gametophyte stage, and can be used to identify species (Bourne *et al.*, 1970, Pueschel and Cole, 1982). Although the thallus is quite uniform in cell structure and shape, and lacks distinctly visible features (Robba *et al.*, 2006), three differentiations are observed in most species; the cells of the holdfast, the vegetative cells and the sexually differentiated cells (Fuller and Gibor, 1984). These differentiated cells can occur along the entire blade length, and have species specific reactions to dissection, light conditions, temperature changes, enzyme treatment as well as having a varied wall composition and dividing potential (Fuller and Gibor, 1984). Over 90% of red algae species share some common components (Table 1.1) and the variety in proportions of chlorophyll α together with carotenoid pigments like phycoerythrin and phycocyanin gives the varied coloration in *Porphyra* thalli (Woelkerling *et al.*, 1990) from dark brown and black, to red, pink, violet or blue and even greenish. These pigments are commonly known as phycobilisomes, and are attached to the thylakoid membrane in the chloroplasts (Blouin *et al.*, 2011). The combination of characteristics in Table 1.1 does not occur in organisms not belonging to Rhodophyta, and might thus be considered unique traits of *Porphyra* (Woelkerling *et al.*, 1990).

Table 1.1: Unique traits of Rhodophyta (Woelkerling *et al.*, 1990).

Eukaryotic cells
No flagella
Food reserves stored as floridean starch (an α1-4, α1-6 linked glucan resembling amylopectin); storage in cytoplasm, not in chloroplasts
Phycoerythrin, phycocyanin and allophycocyanins
Chloroplasts with unstacked thylakoids
Chloroplasts lacking external endoplasmic reticulum

1.2.2 Life cycle

Porphyra has a heteromorphic life cycle which alternates between the haploid gametophyte foliose thallus, and the filamentous diploid sporophyte called conchochelis. Several modes of reproduction are exhibited by the various species of *Porphyra*, both sexual and asexual. Asexual reproduction is achieved by vegetative cells proliferating into archeospores which can directly germinate to form the thallus (Behura *et al.*, 2002). Recently some species of *Porphyra* has also been found to asexually reproduce by the creation of endosporangia and endospores, which grow in to thallus (Redmond *et al.*, 2014, Blouin *et al.*, 2011, Nelson and Knight, 2009, Nelson *et al.*, 1999, Hideo, 1961). The sexual reproduction is achieved after mature cells in the thallus differentiate into carpogonia in female individuals, depending on the species being either monoecious or dioecious, and other cells on the same or different thallus differentiate into male spermatangia (Behura *et al.*, 2002). The male gametes are products of mitotic division of blade cells, and are small and colourless, while the female gametes are larger and contain trichogynes or protrichogynes as a receptive extension into which the male nucleus can enter through a tube produced by the male gamete (Nelson *et al.*, 1999).

After fertilization between male and female gametes, the zygote undergoes mitotic division, and quickly matures into carpospores (Redmond *et al.*, 2014, Behura *et al.*, 2002). The arrangement of these cells within spore packets are species specific, but in most cases results in a square shaped division into four cells (Redmond *et al.*, 2014, Nelson *et al.*, 1999). It is these carpospores which then germinate unipolarly into the conchochelis phase of the life cycle. The conchochelis is usually found as a shell-boring thin red layer of microscopic filaments inside various shells or similar calciferous substrates (Redmond *et al.*, 2014, Blouin *et al.*, 2011, Behura *et al.*, 2002, Waaland *et al.*, 1990, Hideo, 1961) , but it can survive in most conditions and is not dependant on a specific substrate. After certain environmental conditions have been met, typically light period and temperature (Lindstrom *et al.*, 2007), the conchochelis start to form conchosporangia, which in turn produces conchospores. When released the conchospores then germinates to form the foliose blade phase, thus completing the life cycle. In many species meiosis occurs at the first cell division of the conchospore, creating a change in ploidy before the germination into the blade (Burzycki

and Waaland, 2009). The first division of the conchospore splits it into an upper and a lower cell, the second division creates two side-by-side cells of the upper one, and two cells on top of each other of the lower one. These then turn into blade cells and holdfast cells respectively (Mitman and Meer, 1994). Figure 1 shows a typical *Porphyra* life cycle (*Porphyra umbilicalis*), and an asexual reproduction pathway.

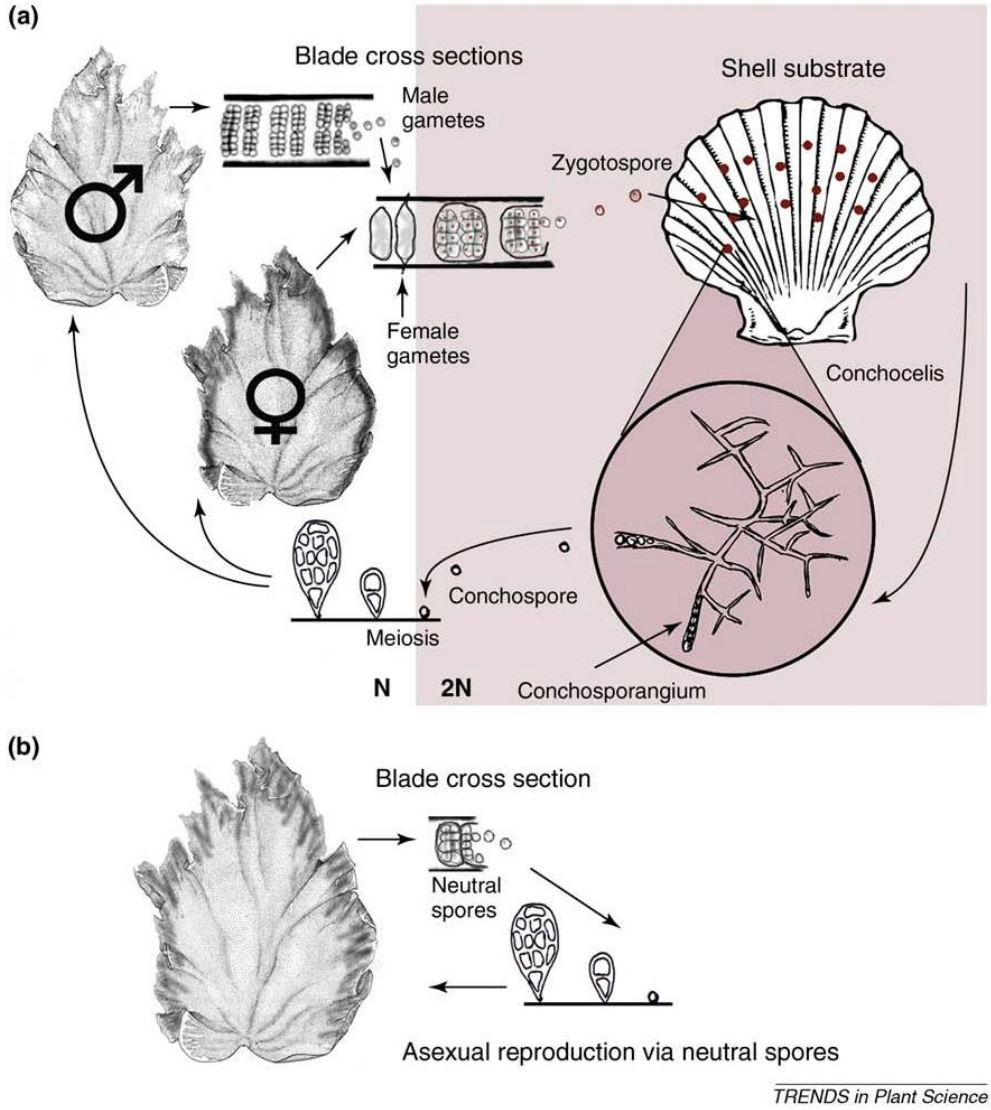

Figure 1: Typical *Porphyra* life cycle, with an example of *Porphyra umbilicalis*. The red cells in the scallop shells named zygotospores are equal to what this thesis describes as carpospores, a debated difference in nomenclature (Nelson *et al.*, 1999). (a) represents the sexual reproduction and (b) the asexual.

Porphyra umbilicalis is dioecious, which implicates that the male and female gametes are located in the same blade, and as with many other species of *Porphyra*, without any morphological difference distinguishable by eye. (Blouin *et al.*, 2011).

The life cycle cultivated *in situ* for this thesis is further described in chapter 2, Figure 2.5.

1.3 Cultivation of *Porphyra spp.*

1.3.2 *Porphyra* aquaculture

The first cultivation of *Porphyra* started long before modern aquaculture practices, and dates back to 960-1289AD in China and 1596-1616 in Japan (Blouin *et al.*, 2011). The technique was to enhance the natural seeding in coastal areas, by cleaning suitable natural surfaces and adding artificial substrates at the time the blades appeared in autumn. In Japan and Korea farmers placed clean bamboo stakes into soft sediment, and in China rocks were cleaned with lime to remove algae and invertebrates (Redmond *et al.*, 2014, Blouin *et al.*, 2011, Tseng *et al.*, 1981), to enable natural occurrences of conchospores to settle. It was not until Kathleen Drew`s discovery (Drew, 1949) of the *Porphyra* life cycle Japanese researchers managed to fully control the life history of *Porphyra* and develop modern large scale commercial cultivation (Redmond *et al.*, 2014, Blouin *et al.*, 2011, Yarish *et al.*, 1999).

Today *Porphyra* farming starts with conchochelis cultivated in large tanks on land, with a bottom layer of calciferous shells (or artificial calciferous substrate (Sahoo and Yarish, 2005)) it attaches to. The conchospore release is then controlled by changes in light and temperature, and then seeded onto floating nets made of synthetic fibre, effectively coating them in spores before transport to a nursery culture site in the sea for gametophyte development. The seeding is done either by placing floating nets in the tank on top of the shells, or by slowly rotating a large paddle wheel wound with net, into the culture. When placed in the sea there is various techniques to elevate the nets out of the water, either mechanically or by using variations in tide, to inhibit growth of fouling organisms. When the blades are 2 to 3mm long they are transferred to larger farm sites for further growth, before harvest approximately 40 days after seeding (Redmond *et al.*, 2014, Blouin *et al.*, 2011, Behura *et al.*, 2002, Hafting, 1999, Yarish *et al.*, 1999).

Porphyra spp. is one of the most valuable marine crops cultivated in the world today. In 2014 there was a total production of 1.8 million tons with a value of close to ~US$1 billion (FAO, 2016). This is a slight reduction from past years, with a height of ~US$1.6 billion value in 1994, although with one million tons produced that year (FAO, 2016). China, Japan and South Korea are the main areas of cultivation, with *P.yezoensis* and *P. Haitanensis* dominating the produced biomass (Blouin *et al.*, 2011). The algae is usually dried after harvest and used in a variety of ways in Asian cuisine, and in the latter years in the rest of the world as well, most notably as nori sheets in sushi. The future trend for worldwide production and consumption of *Porphyra*, and macroalgae in general, points toward increased demand due to a growing need for a more sustainable food production as well as new developments in utilization of bioactive components (Skjermo *et al.*, 2014).

1.3.3 Nutritional value and uses for human consumption

The popularity of *Porphyra* as a food supplement stems not only from its taste and texture, but also from its chemical composition and health benefits (Noda, 1993). Most species has high levels of calcium, sodium, potassium, iron and magnesium as well as vitamins A, B_{12}, C and E (Burtin, 2003). It is also one of the most protein rich genus of macroalgae, with some species reaching ~25-47% protein of dry weight, which is more than protein rich vegetables like soy bean (Taboada *et al.*, 2013, Blouin *et al.*, 2011, Burtin, 2003). For many of the species the make-up of these proteins are constituted by aspartic and glutamic acids, which has a strong effect on flavour development, with glutamic acid being the main component in the taste umami (MacArtain *et al.*, 2007). Another property of *Porphyra* is the phycoerythrines specific to Rhodophyta, which has been shown in recent studies to have antioxidant properties due to its biline being covalently linked to proteic chains (Burtin, 2003). Also the starch-based polysaccharide floridoside that is associated with *Porphyra* (MacArtain *et al.*, 2007) is proved to have health benefits for the immune system (Courtois *et al.*, 2008). Seaweeds may contain up to 2% of its dry weight as lipids as well, and much of these are made up of polyunsaturated fatty acids (PUFA) occurring in the form of omega-3 and omega-6 fatty acids (MacArtain *et al.*, 2007). Of total fatty acid content *Porphyra spp.* has up to 16.1% PUFAs, with 7.2% ω3 PUFAs and 7.97% ω6 PUFAs, in addition to 64.95% saturated fatty acids and 18.91% monounsaturated fatty acids (Sánchez-Machado *et al.*, 2004).

1.4 Study aims and approach

The aim of this study was to establish a cultivation protocol for control of the sexual life cycle of local species of *Porphyra sp.* based on wild material collected in the region, by adapting existing protocols for similar species to the environmental conditions attainable in the macroalgae laboratory at SINTEF SeaLab. This study was run within a project called “Dyrking og foredling av makroalger av høy kvalitet til nye matprodukter (NYMAT)” (Cultivation and processing of macroalgae of high quality for new food products), and the aim of the project is to cultivate and process macroalgae of high quality for use as food. It is a cooperation between Austevoll Seaweed Farm AS (ASF), SINTEF Fiskeri og Havbruk (SFH), Norsk senter for tang og tareteknologi (NSTTT) and NTNU, and financed by the Regional Research Funds Vestlandet (project number: 245326) and Skattefunn (project number: 247546).

The following sub-objectives were established:

- Evaluate the effect of four main variables in the different stages of growth; stress, decontamination by germanium dioxide, light regimes and scallop shells.

- Mapping the local occurrences of *Porphyra spp.* along the shoreline around Trondheim city, by examining five sampling sites over time.

- Finding an optimal combination of growth factors and decontamination strategies for further cultivation trials.

2 Materials and methods

For this thesis two experiments were carried out, one laboratory cultivation experiment at SINTEF SeaLab in Trondheim, and a field search for *Porphyra spp.* at four different sites in the Trondheimsfjord, between June and August 2015. The laboratory experiment was carried out with four different samplings of *Porphyra spp.,* collected from three different locations in the Sør-Trøndelag region, and one from Austevoll Seaweed Farm in the Hordaland region (Table 1). The aim of the cultivation experiment was to find a suitable method for cultivating the algae in a controlled laboratory environment. Also several methods for control of decontamination were investigated. Cultivation methods of *Porphyra spp.* suggested in The New England - Seaweed Culture Handbook (S. Redmond, 2014) was used as start conditions, with adaptations to local conditions and materials. The aim of the field search was to map the natural appearance of the genus in the local area.

2.2 Field search for natural occurrences of *Porphyra spp.* in the Trondheimsfjord.

The field search for Porphyra spp. was conducted between July and August 2015 at rocky intertidal shoreline around Trondheim city. Five locations were chosen based on earlier observations done by others (Kristine Steinhovden and Silje Forbord, SINTEF), and geographical qualities like freshwater runoff, roughness of coastline and topography. One location was emitted from the search ((5) Grilstadfjæra), due to construction work in the area making the location unsuited. The locations are shown in Figure 2.1.

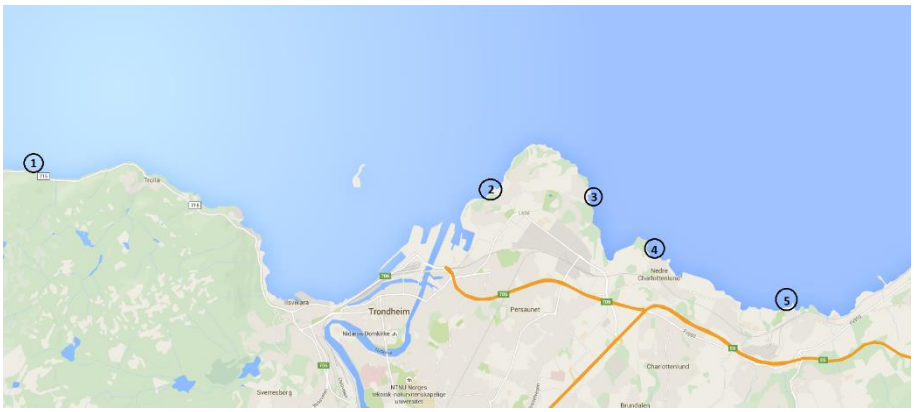

Figure 2.1: Map showing the search locations in the Trondheimsfjord. (1) Storsteinan (63°27'12.8"N 10°15'26.1"E), (2) Korsvika (63°27'04.9"N 10°26'08.9"E), (3) Ringvebukta (63°26'53.0"N 10°28'02.0"E), (4) Rotvollfjæra (63°26'17.6"N 10°29'43.3"E) and (5) Grilstadfjæra (63°25'55.7"N 10°32'22.9"E).

The method was based on searching a consistent stretch of upper littoral zone in the selected areas, and counting the numbers of visible individuals along the same stretch of shoreline over time. Knowing that the *Porphyra spp.* would dissolve through autumn, counting the occurrences in this period was chosen as a method for mapping the time window of gametophyte existence in the region. The actual search methods involved wading into 10-30cm deep waters looking for free floating individuals among rocks and other macroalgae, as well as lifting and searching inside communities of other macroalgae on the shoreline above sea level. The areas searched was heavily dominated by dense communities of *Ascophyllum nodosum*, *Fucus serratus* and *Fucus vesiculosus*, with some occurrences of other unidentified species in between them.

2.3 Collection of material

The seaweed used for the laboratory experiment were collected from three different locations in Sør-Trøndelag between May and June 2015 (Table 1, Figure 1-2). Also a sample of macroalgae (unidentified genus of different structure and coloration) was received from Austevoll Seaweed Farm located in Bakkasund outside Bergen (Table 2.1, Figure 2.3).

Table 2.1: Locations of collected wild material. The locations are showed on the map in Figure 1-4.

Coordinates	Location name	Number on maps
63°27'12.8"N 10°15'26.1"E	Storsteinan, Trondheimsfjorden	1
63°30'48.7"N 10°08'35.3"E	Gjellarvika, Trondheimsfjorden	2
63°41'59.6"N 8°52'00.7"E	Taraskjæret, Frøya	3
60°07'14.0"N 5°04'45.9"E	Austevoll Seaweed, Bakkasund	4

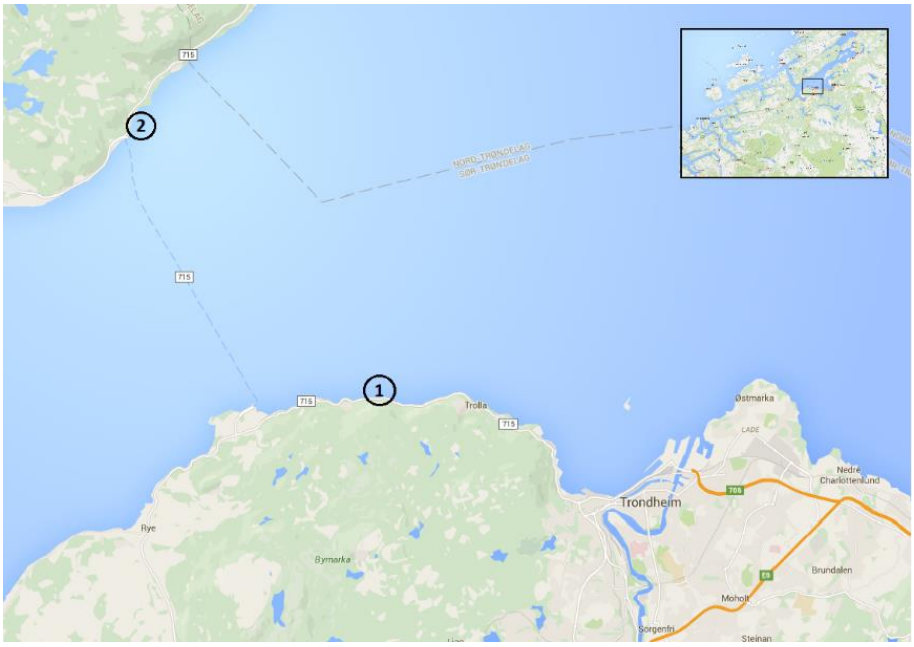

Figure 2.2: Map showing the locations of collected material in the Trondheimsfjord region. (1) Storsteinan in the Trondheimsfjord, collected by SCUBA divers in May 2015 in the sublittoral zone (depth 1-6m). (2) Gjellarvika in the Trondheimsfjord, collected by hand in June 2015 in the littoral zone at low tide. (Created with Google Maps).

Figure 2.3: Map showing the location of collected material in the Frøya region. (3) Taraskjæret, at the *Saccharina latissima* longlines deployed by SINTEF at Seaweed Energy Solutions AS location outside Frøya, collected by hand. (Created with Google Maps).

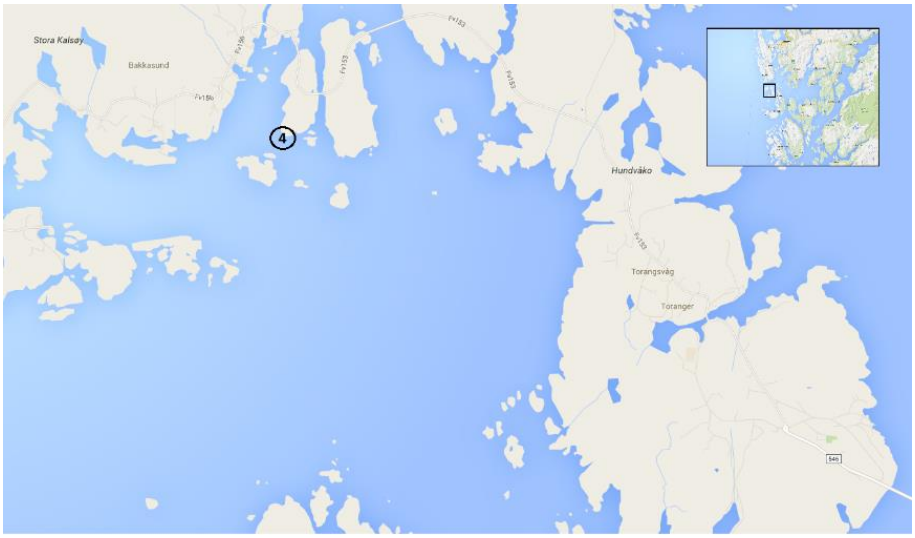

Figure 2.4: Map showing the location of the Austevoll Seaweed Farm and the sample received from (4) Bakkasund. Collected by hand at low tide. (Created with Google Maps).

The harvested samples were kept in humid zip-lock bags, or placed in plastic boxes with seawater, then immediately transferred to the macroalgae-lab at SINTEF SeaLab. The sample from Austevoll were sent by express mail in a styrofoam container. All material was kept in shallow tanks measuring 60cm (l) x 35cm(w) x17cm (d) with running seawater (1.5 L/min) pumped from 70m depth in the Trondheimsfjord (Photo 2.1). The water was sand filtered, particle filtered and treated with UV lighting before entering the lab. The macroalgae samples were kept in these tanks as backup for the experiment. The tanks were cleaned every 3rd day to inhibit diatom overgrowth.

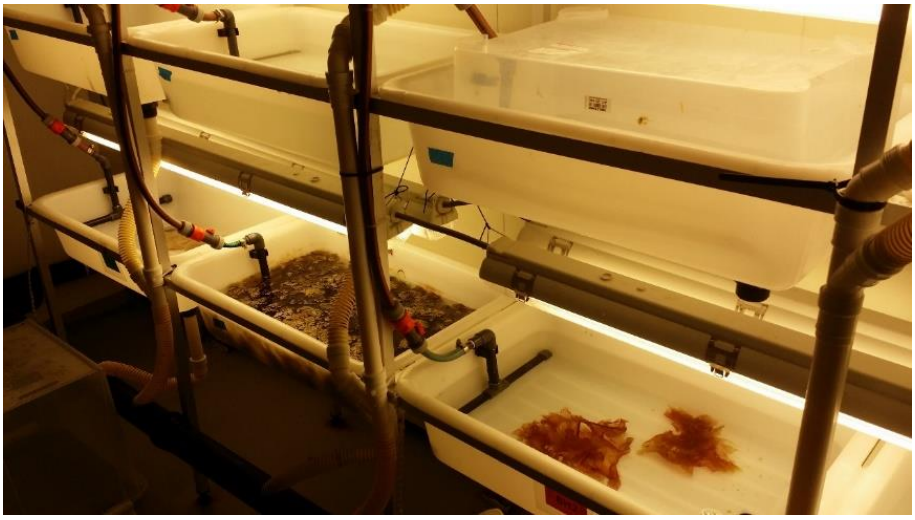

Photo 2.1: The setup for material storage in the laboratory, where the lower right is the samples from Storsteinan, while the tray in the bottom middle is the material received from Austevoll Seaweed Farm.

2.4 Spore isolation

Using a technique described in the New England Seaweed Culturing Manual (S. Redmond, 2014), several attempts were made for isolating single free floating carpospores for further growth. The technique involves melting a hand pipette made of glass over a bunzenburner, and stretching the tip. Then using the vacuum in the pipette to pick out single spores and applying them one by one to a petri dish each.

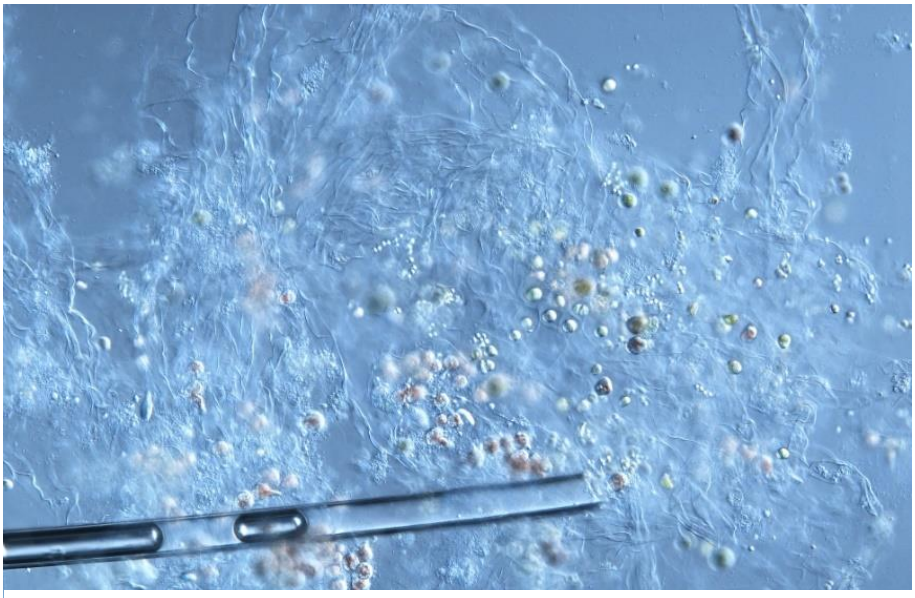

Photo 2.2: Elongated glass pipette tip to isolate spores, by creating suction with a finger at the end. The red circular dots are carpospores, accompanied by dead tissue released from the blades edge. Green dots are cells from the same area of dissolved tissue. 16x magnification.

After localizing spores in the raw material from Storsteinan, eight samples were isolated for cultivation. Spores were never fully free floating, but surrounded by dead tissue, diatoms and ciliates that were impossible to avoid with the pipette tip (Photo 2.2). They were then put in small petri dishes with 3mL Von Stosch growth medium (VS) (Sahoo and Yarish, 2005) and 1mL sterile seawater (SSW). The composition of the Von Stosch growth medium is shown in Table 2.2 and 2.3.

Table 2.2: Von Stosch growth medium recipe, 10mL of each stock solution is aseptically added to 940mL pasteurized filtered natural seawater (Sahoo and Yarish, 2005).

Component	*Stock solution* (g • L^{-1} dH_2O)	*Quanity used*	*Concentration in final medium*
$NaNO_3$	42.52	10mL	5.00×10^{-3}
$FeSO_4 \bullet 7H_2O$	0.28	10mL	1.00×10^{-5}
$MnCl_2 \bullet 4H_2O$	1.96	10mL	1.00×10^{-4}
$Na_2EDTA \bullet 2H_2O$	3.72	10mL	1.00×10^{-4}
Vitamins stock solution	(See below recipe)	10mL	----------

Table 2.3: Vitamins stock solution recipe, the thiamine • HCl is dissolved into 950mL of dH_2O, then 1mL of each of the two primary stock solutions is added (Sahoo and Yarish, 2005).

Component	*I° Stock solution* (g • L^{-1} dH_2O)	*Quanity used*	*Concentration in final medium*
Thiamine • HCl (vitamin B_1)	-----	200mg	5.93×10^{-6}
Biotin (vitamin H)	0.1	1mL	4.09×10^{-9}
Cyanocobalamin (vitamin B_{12})	0.2	1mL	1.48×10^{-9}

The temperature was kept at 10^{o} C and lights at a 12/12h light/dark light cycle with approximately 10µmol photons m^{-2} s^{-1}, measured exactly at the location of the tissue pieces. The growth media was changed every 14^{th} day by removing 2mL of the surface liquid, and replacing it with 1mL VS and 1mL of SSW.

After two months of growth, small pieces of healthy conchochelis were found in one of the eight petri dishes. The rest of the samples showed no sign of life. Twelve pieces of healthy conchochelis were put into multi-culture-dishes (FALCON Polystyrene Tissue Culture Plate, 6 Well), with pieces of autoclaved scallop shell (*Pecten maximus*) sized at approximately $1cm^2$. The wells contained increasing concentrations of germanium dioxide (GeO_2) solution made with 0.8946g GeO_2 pr. 200mL distilled freshwater. This GeO_2 solution was diluted with 1mL GeO_2 solution pr. 1000mL SSW and added with VS growth medium and SSW to the dishes. The concentrations are shown in Table 2.4.

Table 2.4: Wells in the multi-culture-dish and their corresponding concentrations of SSW, VS and GeO_2.

Well number	*mL SSW*	*mL Von Stosch*	*g. GeO_2*	*g. GeO_2 pr. mL liquid*
B1&B4	1	3	4.5×10^{-9}	1.1×10^{-9}
B2&B5	2	2	8.9×10^{-9}	2.2×10^{-9}
B3&B6	3	1	1.3×10^{-5}	1.3×10^{-5}

After two months of steady growth the conchochelis were moved to a new light regime, with approximately 50 µmol photons m^{-2} s^{-1} intensity. After another month of growth, the tufts of conchochelis was dried in a petri dish overnight, then delivered to NorBOL at NTNU Vitenskapsmuseet for DNA-barcoding. The classification was unsuccessful due to lack of genetic markers for the conchochelis stage of *Porphyra*.

2.5 Laboratory cultivation of *Porphyra spp.* life cycle

This experiment was set up according to suggestions in The New England - Seaweed Culture Handbook, chapter 6, Nori (S. Redmond, 2014). The experiment then followed four steps, with differing variables applied to each culture, see 2.5.1 for these. The four steps followed the life cycle of the algae, as depicted in Figure 2.5 and described below.

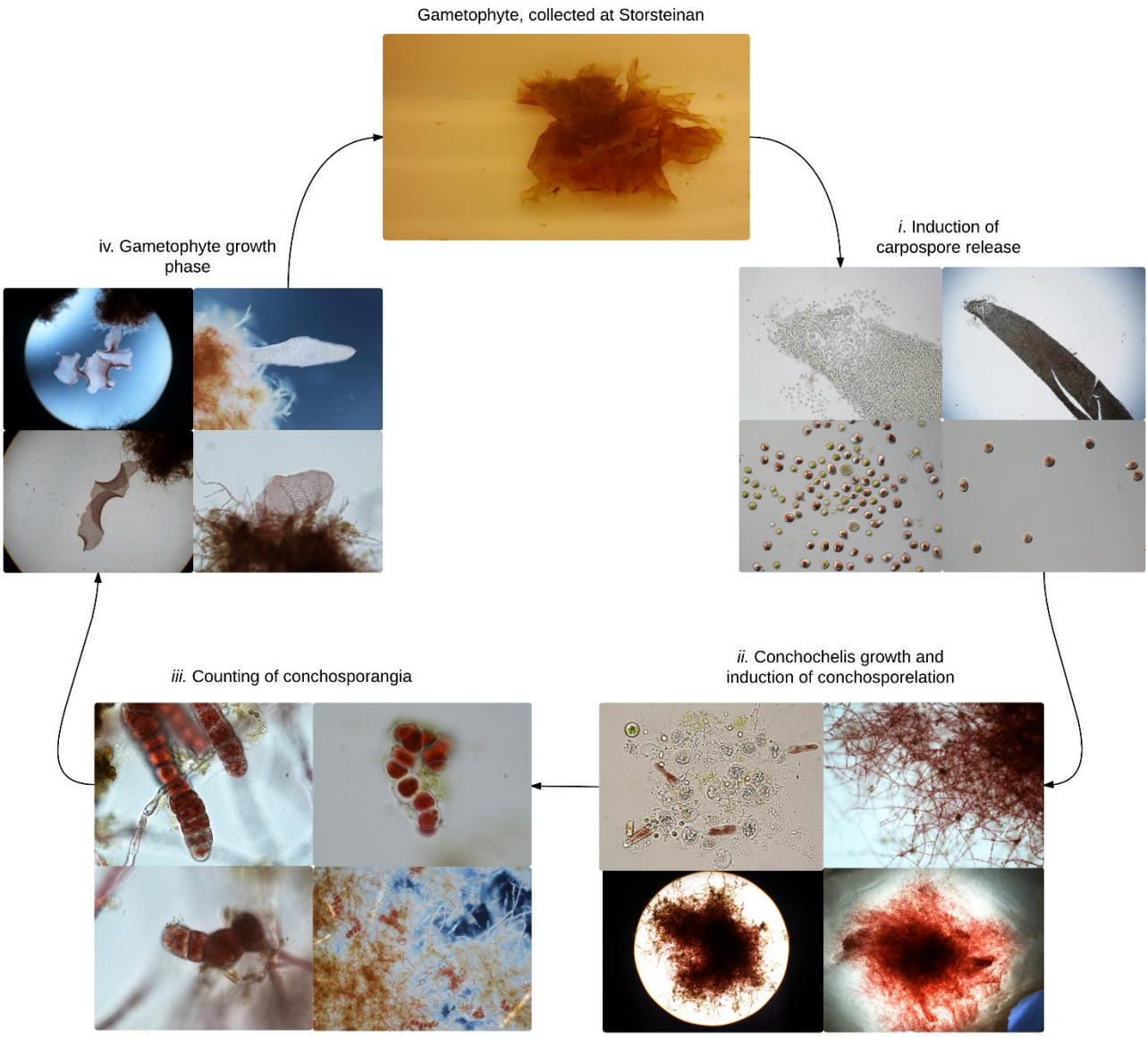

Figure 2.5: The life cycle of *Porphyra sp.* specimen collected from Storsteinan May 2015. The numeration corresponds to the four steps described in the section below. All photos, except top, were taken with microscope at magnifications ranging from 10x to 100,5x.

Top picture: The gametophyte collected at Storsteinan in May 2015, in the container at the macroalgae lab.

Photos i: Excised tissue pieces and free floating carpospores, 2,5x,10x and 100,5x magnifications.

Photos ii: Erupted carpospores at the upper left, the rest is conchochelis growth in multi-culture-dishes and scallop shell at the bottom right. 2,5x to 62x magnification.

Photos iii: Conchosporangia at 16x and 64x magnification.

Photos iv: Growing gametophytes at 2,5x magnification.

These are the four general steps the growth experiment followed.

i. Induction of carpospore release; pieces of tissue sized approximately 2mm^2 were excised from the edges and toward the center of the collected material, and moved to a multi-culture-dish with a volume of 5mL (Sigma-Aldrich, NUNC 12 well), containing 2mL VS and 2mL SSW.

The culture-dishes were kept at 10 °C and a 12h/12h light/dark light cycle with approximately 10 µmol photons m^{-2} s^{-1} light intensity (using Cool-white fluorescent lamps/tubes mounted 60cm above the tank) in the SINTEF algae lab. The light intensity was monitored using a Li-COR Underwater Quantum Sensor (Model Number: LI-192) measured with a LI-1400 Datalogger for the remainder for the experiment. The measurements were done as close to the tissue pieces as possible, and adjusted to the light permeability of the culture dishes (the lid removed approximately 10 µmol photons m^{-2} s^{-1}). The following two days the samples were checked with 2h intervals for spore release in the microscope (Zeiss Axioscope 2 plus, with a Nikon D5-U1 camera) at 16x magnification. Some pieces were stressed according to the suggestions in The New England Seaweed Culturing Handbook (S. Redmond, 2014) before placed in multi-culture-dishes for sporulation, see 2.5.1 for description.

ii. Conchochelis growth and induction of conchosporelation; after the spores successfully erupted into the conchochelis phase, the conchochelis were placed in new multi-culture-dishes (FALCON Polystyrene Tissue Culture Plate, 6 Well) with a volume of 15mL. These 6-well dishes were chosen since they fitted in the inverted microscope (NIKON Eclipse TE2000-S) for easier inspection, as well as having more volume for liquid. They were kept at 10 °C and a 12h/12h light/dark light cycle with approximately 10µmol light intensity and a gradient of GeO_2 and VS (see 2.5.1), until the appearance of the first conchosporangial filaments. The liquid content was changed every 14th day by removing 2mL of the surface liquid, and replacing it with respective ratios of VS and GeO_2 solution in the corresponding wells.

iii. Counting of conchosporangia; after the development of the first conchosporangia, these were counted as a measurement of development and growth. This was considered the best way to measure growth due to the fragile nature of the conchochelis. Conchochelis pieces were examined with microscope (Zeiss Axioscope 2 plus with a Nikon D5-U1 camera) at 10x magnification, and conchosporangia counted every second day (Photo 2.3). The conchochelis was placed at microscope slides (VWR cut color frosted, yellow) and covered by microscope cover-glass (24x50mm) to flatten the conchochelis for easier inspection. The flattening of the three dimensional structure inhibited vision in the central parts, so only the conchosporangia formed in the outer parts were counted initially. Since the process took about two hours, the samples were stored in a refrigerator with no light and 4 °C to minimize stress by temperature increase while being counted. The conchosporangial filaments were identified as two or more large circular cells surrounded by an outer membrane, as seen in Photo 2.3.

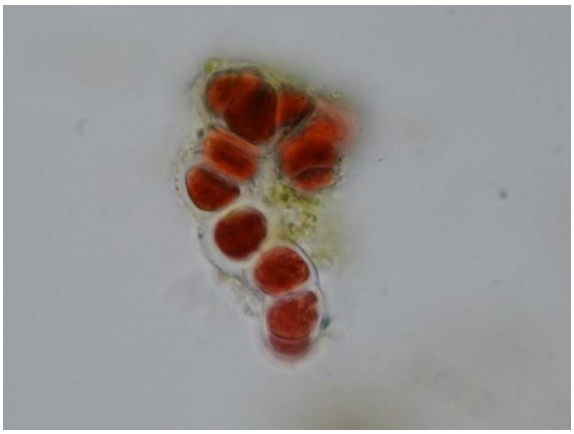

Photo 2.3: Conchosporangium containing 7-9 conchospores. 64x magnification

iv. Gametophyte growth phase; the conchochelis with conchospores that developed into gametophytes were moved to aerated Erlenmeyer flasks with a volume of 4L. The flasks contained 2L VS, 2L SSW and 1.25mL GeO_2 solution (0.8946g GeO2 pr. 200mL distilled freshwater), a concentration of 0.3125mL GeO_2 solution pr. 1000mL liquid. The light intensity was set at 100µmol photons m^{-2} s^{-1} at a 12h/12h day/night cycle in 10 °C for maximum growth.

Initially 80 pieces of tissue were taken from the material available, and split into five cultures that were set up without being stressed before cultivation (Table 2.5). See Table 2.6 for the cultures exposed to stress before cultivation.

Table 2.5: Locations collected, number of tissue pieces and names of cultures. Each tissue sample were given its own well in 6,12 and 16 well multi-culture-dishes. * = Storsteinan + Gjellarvika + Taraskjæret + Austevoll.

Location collected	Number of tissue pieces	Culture name
Storsteinan	12	TRHFJ IND2
Storsteinan	12	TRHFJ NOSTRESS
One from each location*	16	4 LOCATIONS NOSTRESS
Frøya	6	100GO FRØYA
Frøya	6	50GO FRØYA

2.5.1 Variables for growth phases

Four different variables were chosen to optimize the growth phases, A: Stress; for induction of carpospore release, B: GeO_2 and growth medium variations; to handle diatom contamination, C: Light; to see difference in growth ratios and D: Scallop shells for improved conchochelis growth conditions.

A: Stress

A total of 28 pieces of the excised fertile tissue from initially collected material were stressed. The pieces were put in moist cheesecloth (Kimberly-Clark Professional Medical Wipe) paper for 24h at 4 °C and no lighting in a refrigerator, and labeled STRESS. After 24h the tissue was rehydrated in SSW and put in a multi-culture-dish under approximately 25µmol photons m^{-2} s^{-1} light intensity for release of carpospores. The pieces of fertile tissue which developed carpospores that erupted into conchochelis were continuously split up to ensure further growth and survivability, and placed under different conditions. Table 2.6 shows an overview of the cultures exposed to stress.

Table 2.6: Locations collected, number of tissue pieces and names of cultures. Each tissue sample were given its own well in 12 and 16 well multi-culture-dishes. * = Storsteinan + Gjellarvika + Taraskjæret + Austevoll. These samples were exposed to the same stress factor.

Location collected	Number of tissue pieces	Culture name
Storsteinan	12	TRHFJ STRESS
One from each location*	16	4 LOCATIONS STRESS

B: GeO_2 and growth medium variations

After spores successfully erupted into the conchochelis phase, a gradient of GeO_2 was applied to hinder potential blooms of diatoms. Two different concentrations were used in the initial stage, with three duplicates of each type. One with 1mL GeO2 solution pr. 1000mL SSW(GO100) and one with 0.5mL GeO2 solution pr. 1000mL SSW(GO50). The concentrations of GeO_2 used at this point are the same as described in Table 2.4. Also the wells contained varied amounts of VS growth medium, as shown in Figure 2.6.

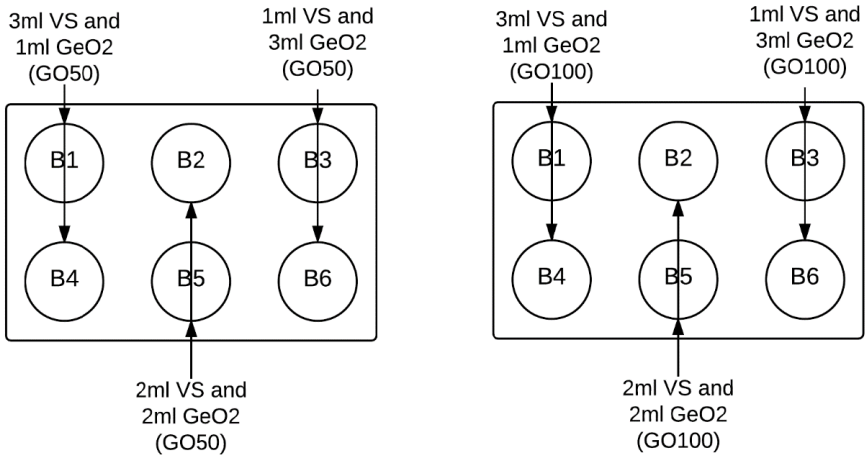

Figure 2.6: Overview of ratios of VS growth medium and GeO_2 concentration for the conchochelis growth phase, right after carpospore release. Three duplicates of each multi-culture-dish were made.

At this point all conchochelis derived from the material initially collected at Storsteinan, labeled TRHFJ IND2. Figure 2.7 shows the setup, timeline and duplicates of this culture, throughout the course of the experiment.

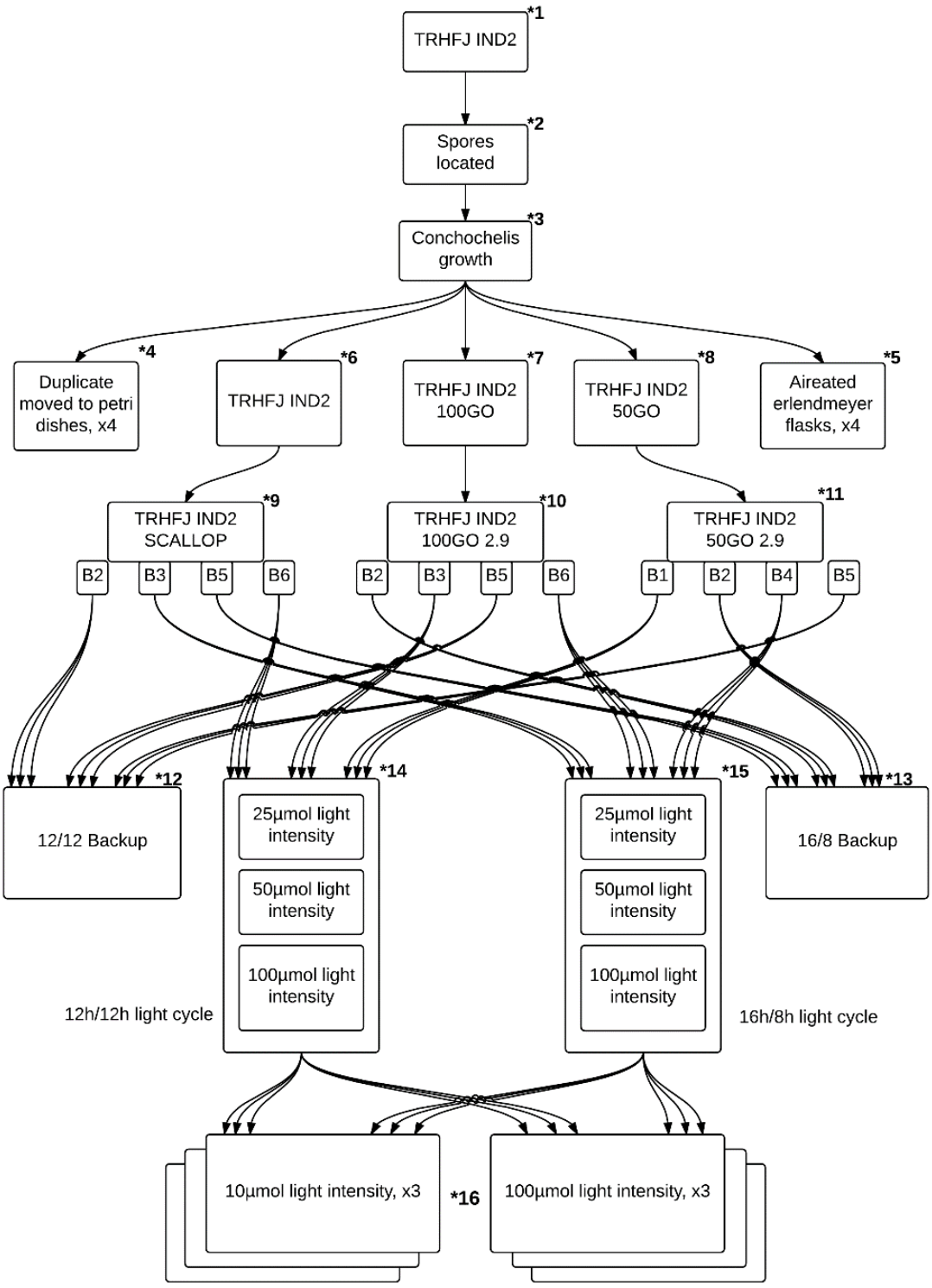

Figure 2.7: Flowchart showing the duplications of the most successful initial culture, TRHFJ IND2. The arrows indicates a split of conchochelis into new cultures. All steps and duplications after *3 are done during the conchochelis growth phase, to ensure maximum use and survivability of the available material. Note that the last step (*16) disregarded previous light intensities. The wells in the last step are described in Figure 2.8. It also served as the foundation for induction of conchosporangium development.

When the conchochelis were moved to a new light regime (from 10µmol to 25, 50 and 100µmol, as seen in Figure 2.7) for induction of conchosporelation, a diatom bloom halted the progress after three days. All conchochelis tufts was then moved to new culture dishes, with increased GeO_2 concentrations and new light regimes. Figure 2.8 shows the new setup, with GeO_2 ratios, light intensity and light cycles. GeO_2 is in mL of a solution made with 0.8946g GeO_2 pr. 200mL distilled freshwater. The wells also contained 2mL VS and 2mL SSW. All wells labeled B3 and B6 contained pieces of autoclaved scallop shell, sized approximately $1cm^2$. The conchochelis were distributed equally based on size and previous treatment history, disregarding the three days in varied light intensities.

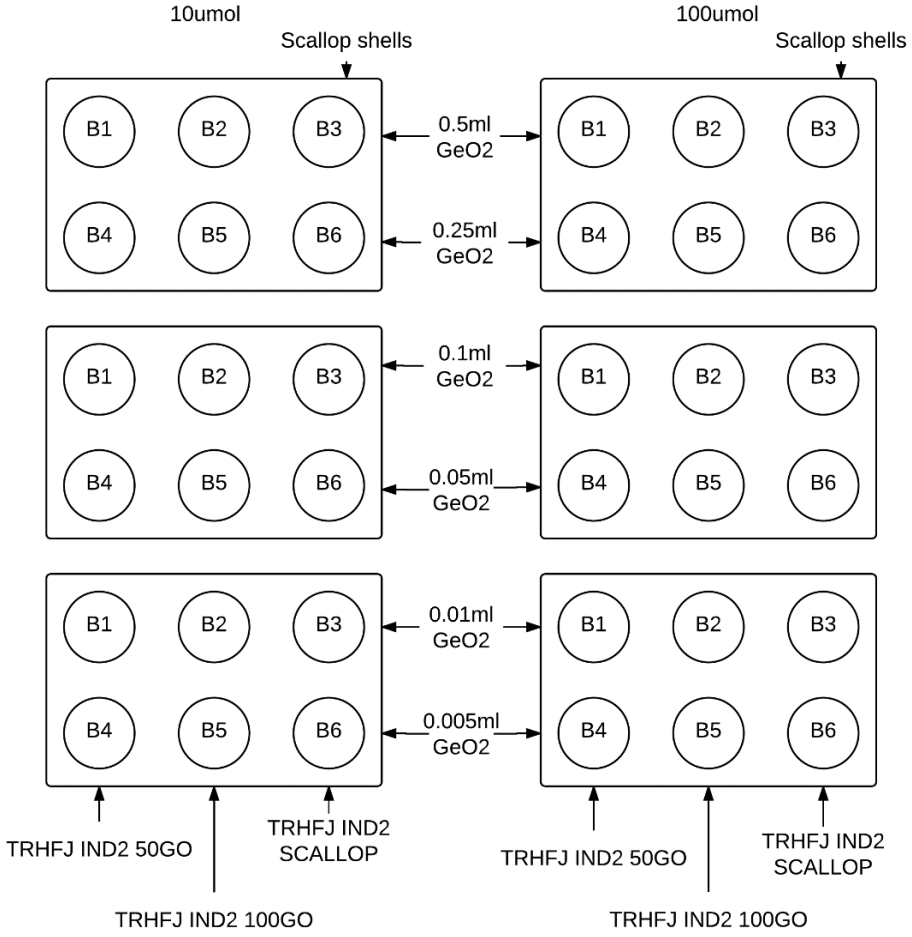

Figure 2.8: Flowchart showing distribution of conchochelis tufts at different ratios of GeO_2 and light intensities. Wells B3 and B6 contained small pieces of autoclaved scallop shell.

Table 2.7: Final concentrations of GeO_2 pr. mL of growth medium in each well, as described in Figure 2.8.

mL GeO_2 solution in each well	*mL VS and SSW*	*g. GeO_2 pr. mL liquid in each well*
0.5mL	4	2.23×10^{-3}
0.25mL	4	1.11×10^{-3}
0.1mL	4	4.47×10^{-7}
0.05mL	4	2.23×10^{-4}
0.01mL	4	4.47×10^{-8}
0.005mL	4	2.23×10^{-5}

C: Light

Following the formation of conchosporangial filaments, the tufts of conchochelis containing the highest amount of conchosporangia (avg. +-57.345) were split in three. Then divided under 25µmol, 50µmol and 100µmol photons m^{-2} s^{-1} light intensity to continue conchospore and filament development under differing light intensities. After a bloom of diatoms, the GeO_2 concentrations were increased (Figure 2.6), and the conchochelis were placed under two different light regimes only, approximately 10 µmol and 100 µmol at a 12h/12h light cycle.

D: Scallop shells

Pieces of autoclaved shell from the great scallop (*Pecten maximus*) were added to both aerated Erlenmeyer flasks and multi-culture-dishes during the experiment, to induce and enhance conchochelis growth. The pieces were made by breaking the shells inside a sterile container, then picking out the ones sized approximately $1cm^2$ and a clean white surface.

2.6 Conchosporangium quantification

Immediately following the first observations of conchosporangium in the conchochelis, they were examined in microscope and counted. Since the conchochelis tufts are so small, free floating, three dimensional and fragile, this was chosen as the most suited and practical method for measuring growth of tissue, and effect of decontamination by GeO_2. The dishes were moved from the algae lab, and set in a refrigerator while counted, to minimize stress. Once every week the growth medium and GeO_2 was replaced, and at this point the dishes were continuously kept in motion with a stirring plate (IKA Rocker 3D Digital at 50 rotations pr. minute) for the duration of the counting, to blend the liquids. The conchochelis were pressed in between microscope glass slides, and the conchosporangia visible at the edges of the conchochelis were counted. After 8 days of counting the conchochelis pieces were carefully torn open in the centre with tweezers, to make room for light in the central areas, as well as increasing visibility for counting.

2.7 Decontamination – NaOCl, SSW washing and air drying

In addition to the $GeO2_2$ treatment, several attempts at decontamination were tested to rid the cultures of epiphytes, ciliates and diatoms.

- NaOCl

12 individual pieces of conchochelis gathered from the IND2 TRHFJ culture was washed in SSW for five minutes, stirred continuously by hand. Then six of them were rinsed in a NaOCl solution of 0.033mL pr. 5mL of SSW for five minutes, and six in a NaOCl solution of 0.033mL pr. 15mL of SSW for five minutes (Rød, 2012). The dishes were then set at 10 °C and a 12/12h light/dark light cycle with approximately 25µmol light intensity for 24h before examined for carpospores.

- Washing with sterile seawater

12 pieces of conchochelis were taken from the the IND2 TRHFJ culture were stirred continuously in SSW for 5 minutes, then placed in a sentrifuge (Biofuge pico, Heraeus

Instruments) at 8000RPM for 2 minutes, and finally carefully wiped with cheesecloth to remove as much as possible of the diatoms. The pieces were then placed in a 6 well multi-culture-dish again for rehydration and examined for carpospores after 24h.

- Air drying

12 pieces of fertile tissue were excised from the material collected at Frøya, and rinsed in SSW for five minutes, then exposed to air drying in room temperature for 2h, 4h, 6h and 24h. Immediately following the drying period, the pieces were rehydrated in multi culture dishes and examined for carpospores after 24h.

2.9 Statistics

Statistical analyses were performed in SigmaPlot for Windows, version 13.0, with a significance level of $P \leq 0.05$. The effect of stress on carposporulation were made with a Brown-Forsythe Equal Variance t-test. The effects of GeO_2 and light treatments were analyzed with Mann-Whitney Rank Sum t-tests and Kruskall-Wallis one-way ANOVA. All graphs were made in SigmaPlot for Windows version 13.0 (Systat Software, Inc.,2015).

3 Results

3.2 Field search for natural occurrences of *Porphyra spp.* in the Trondheimsfjord.

The field search for occurrences of *Porphyra sp.* proved that the species is relatively rare in the region, and hard to locate. It seems that searching abilities and methodology affected the outcome of the search in the selected sites. The only location where *Porphyra spp.* were consistently localized was in the areas where swimming or deep wading was possible and performed (Korsvika in the end of August) in the subtidal zone (see Table 3.1). Finding the *Porphyra spp.* while the tide is low is very hard due to its tendency to stick to other fauna, rocks and pebbles when not under water, requiring a keen eye and a lot of time. At high tide the gametophyte is distinctly visible when attached to rocks, due to the characteristic blade with only two cell layers moving in the water. So the preferred method would be to use a diving suit and snorkeling along the shoreline to make the observations. Both Grilstadfjæra and Ringvebukta had poor conditions due to mud and unclear waters. Storsteinan could prove a useful location, but the observations were difficult here, due to a very densely crowded habitat in the upper littoral zone, with many larger species competing for resources. Widened searches covering up to 200m of shoreline yielded no findings at either Storsteinan or the two areas covered around Grilstadfjæra. Table 3.1 shows the results at each location for each search.

Table 3.1: Locations, date and results for the field search in the Trondheimsfjord.

	Korsvika (63°27'04.9"N 10°26'08.9"E)	**Storsteinan** (63°27'12.8"N 10°15'26.1"E)	**Rotvollfjæra** (63°26'17.6"N 10°29'43.3"E)	**Ringvebukta** (63°26'53.0"N 10°28'02.0"E)
18.08.2015	Found three samples within 20m shoreline, observed from a distance attached to rocks at approximately 0,5m to 1m depth.	Unable to locate *Porphyra spp.*	Unable to locate *Porphyra spp.*	Unable to locate *Porphyra spp.*
20.08.2015	Found five samples, along the exact same patch of 20m shoreline, between 0,5m and 1m depth.	Unable to locate *Porphyra spp.*	Found no suitable habitat or samples, due to construction and muddy waters.	Found two samples along 20m, after searching about 150m shoreline.
26.08.2015	Found two samples along the same stretch of 20m. Might have been the same as sampled earlier.	Unable to locate *Porphyra spp.*	No search.	Unable to locate *Porphyra spp.*
29.08.2015	Found six samples, along the same stretch of 20m shoreline. Same depth.	Unable to locate *Porphyra spp.*	Unable to locate *Porphyra spp.*	Unable to locate *Porphyra spp.*
14.09.2015	Found two samples, both close to shore and only visible from a distance.	No sign of *Porphyra spp.* while searching, despite improved technique.	Unable to locate *Porphyra spp.*	Unable to locate *Porphyra spp.*

3.3 Collected material

Material collected from Storsteinan (63°27'12.8"N 10°15'26.1"E) May 2015:

The material received from Storsteinan was by far the most successful for cultivation. After transport to the lab, 12 pieces of fertile edge tissue from the collected material was cut out and placed in its own well in a multi-culture-dish (labeled TRHFJ NOSTRESS). After 24 hours they were searched for carpospore presence. Only a few of the wells contained free floating spores, as they are seen in Photo 3.1, the rest were embedded in dead tissue. This made it impossible to estimate the amount of spores released pr. mm^2, because the spores would not move freely with the liquid, a necessity for counting them using the available Neubauer 0,1mm hemocytometer.

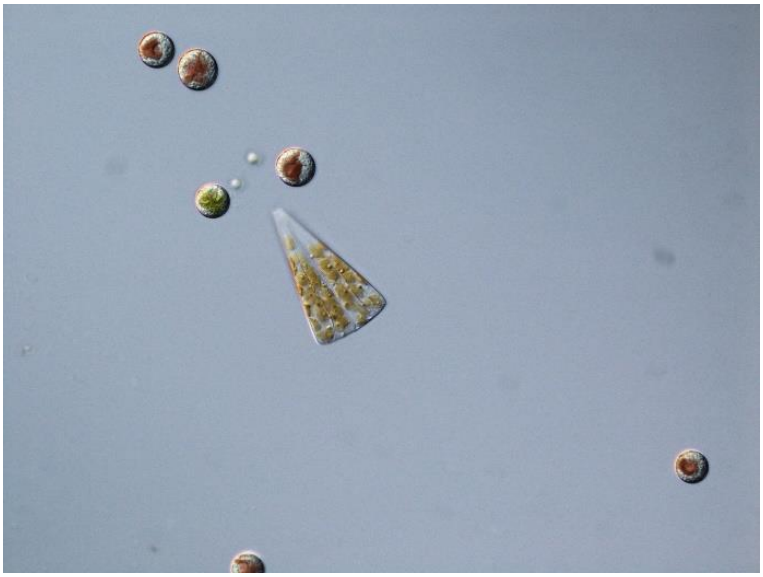

Photo 3.1: Free floating carpospores, released from the fertile edge of the tissue. The carpospores is approximately 5µm in diameter, here shown together with *Licmophora sp*., a benthic diatom commonly present in most samples. 64x magnification.

The carpospores were mostly released with dead tissue from the algae itself, which dissolved from the outer rim of the blade, toward the center. Photo 3.2 shows a gametophyte and a piece of fertile tissue, dissolving from the outer edge. Eventually the entire algae dissolved in the lab, something which happened to every collected specimen.

Photo 3.2: *Left*: Gametophyte structure floating in the macroalgae laboratory, with the dissolving edge visible as a green/yellow layer on its outer rim. *Upper right*: A piece of fertile tissue, cut towards the centre, from the edge of the dissolving gametophyte, approximately 2mmX6mm, showing release of carpospores and reduction of edge tissue. 2,5x magnification. *Lower right*: Same area of tissue, zoomed in at the tip. 10x magnification.

Small living green cells were released along with the carpospores inside the dead tissue as well, but these disappeared or dissolved in the culture after about 48h. Photo 3.3 shows the dissolved tissue and its content, exemplifying the difficulty of creating sterile cultures from this species, due to the high amount of contaminants in the surrounding tissue.

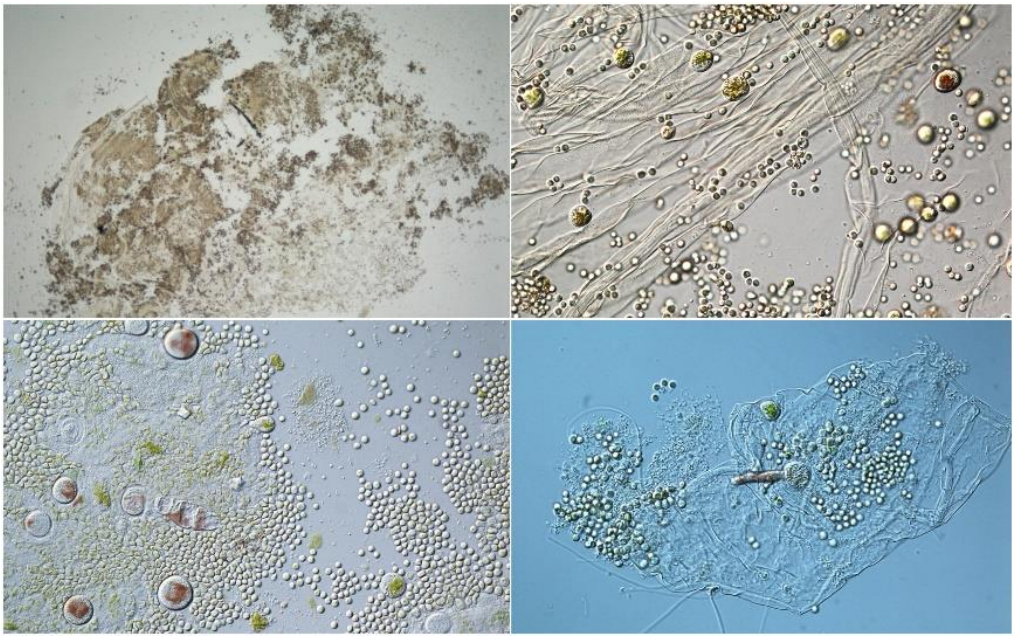

Photo 3.3: Photos showing different states of dissolved tissue and released carpospores.

Upper left: Dissolving tissue cut from the blade edge, green/yellow coloured and very fragile, with almost no consistent texture. 2,5x magnification. *Upper right*: One carpospore seen among strings of dead tissue and smaller living cells with a green colour. 40x magnification.

Bottom left: One erupted carpospore and several still intact, surrounded by many smaller cells and some dead tissue. 64x magnification. *Bottom right*: A single erupted carpospore, embedded in dead tissue. 40x magnification.

Material collected from Gjellarvika (63°30'48.7"N 10°08'35.3"E), Taraskjæret (63°41'59.6"N 8°52'00.7"E) and Bakkasund (60°07'14.0"N 5°04'45.9"E), May-June 2015:

The material collected from these sites had a far lower presence of carpospores when received, they also matured slower and had a lower density of spores. The material received from Austevoll Seaweed, collected at Bakkasund, was of a different species completely, and no spores were found in any of the samplings. Photo 3.4 shows a comparison of cell structure between samples from Bakkasund and Storsteinan.

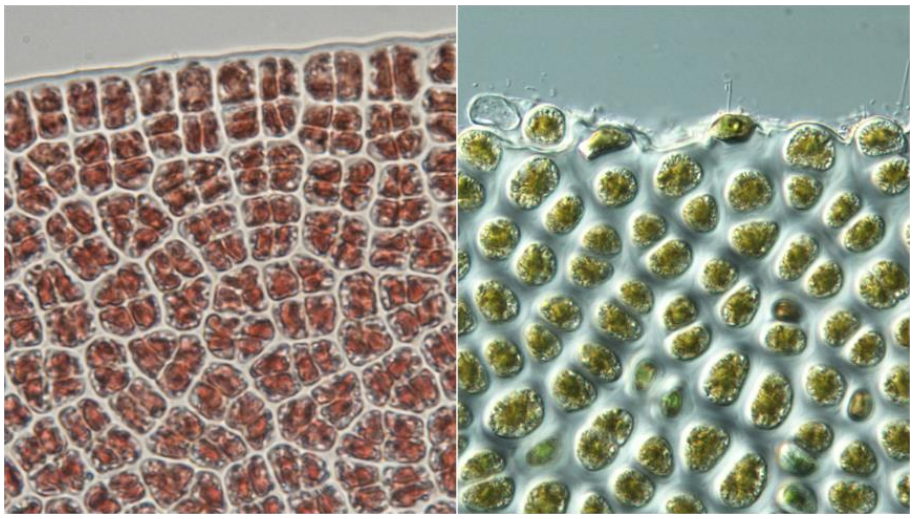

Photo 3.4: *Left*: Tissue edge in sample collected at Storsteinan, the square shape and four squared division shows that these are mature cells that will develop carpospores (S. Redmond, 2014). *Right*: The material received from Bakkasund had a different cellular structure. Fewer and more vertically stretched cells in two thick layers, green coloured. This never developed into any differentiating cells.

All the material from Bakkasund eventually deteriorated and was deemed unsuited for further trials. They were also covered with an unidentified epiphyte, shown in Photo 3.5, that grew quickly over a period of 6 days, eventually covering most of the tissue.

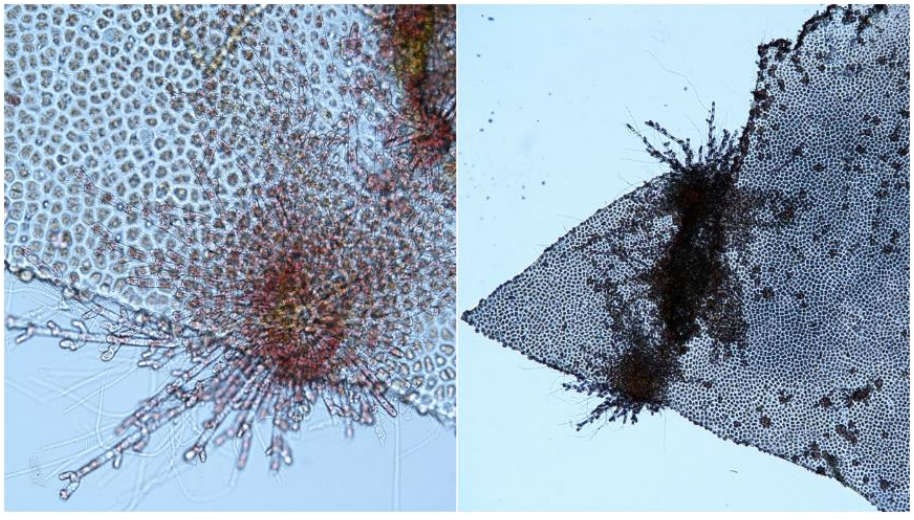

Photo 3.5: *Left*: Epiphyte growing at blade edge, 16x magnification. *Righ*t: Clusters of epiphytes. 4x magnification.

The material collected from Frøya and Gjellarvika did have some carpospores present in the edge tissue, but both samples were heavily overgrown with diatoms and unidentified epiphytes. The combination of contaminants, dissolving fertile tissue and low presence of carpospores made these samples unsuited for further cultivation. Photo 3.6 is of fertile tissue cut from the Frøya sample, with an epiphyte growing out of it, covering most of the tissue after 8 days of growth.

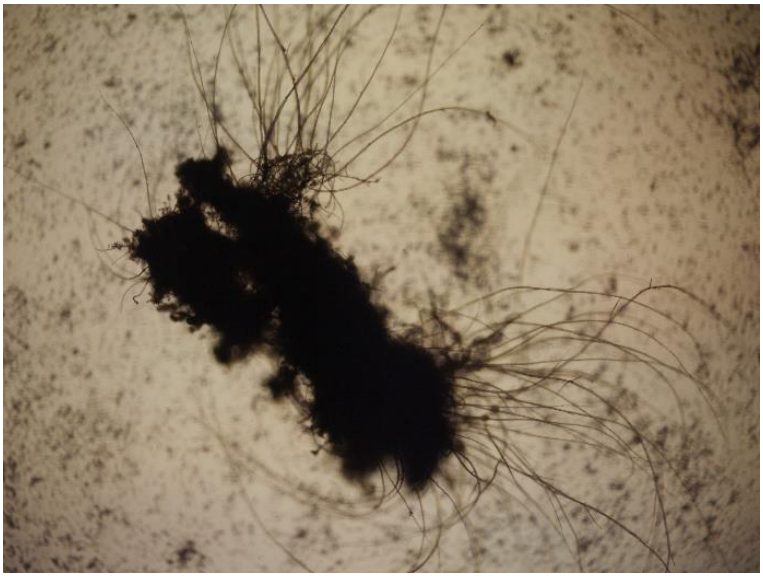

Photo 3.6: Unidentified epiphyte growing out of a previously fertile, 2mm x 4mm blade, edge collected at Frøya. 2,2x magnification.

The strong presence of diatoms these samples led to them eventually overshadowing and outcompeting the growth of conchochelis, as shown in the Photo 3.7. Each photo is of one well from the most successful Gjellarvika culture, that managed to develop healthy conchochelis growth. The conchochelis itself is also tightly packed with diatoms, inhibiting growth.

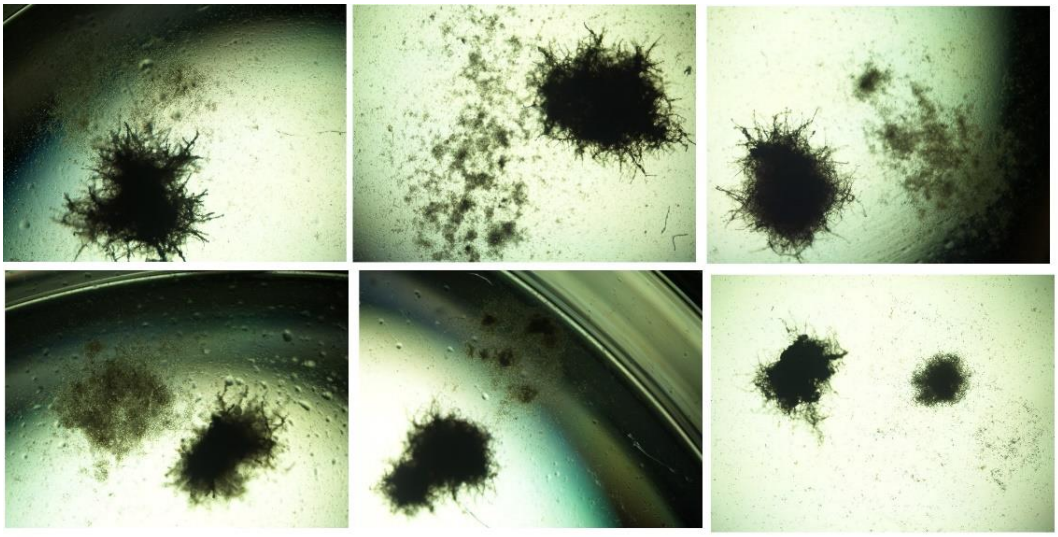

Photo 3.7: Healthy and growing conchochelis next to colonies of diatoms. The bigger black area is the conchochelis, while the more transparent parts is the largest colony of diatoms in the corresponding well. 2x magnification.

3.4 Spore isolation

Out of the eight petri dishes with isolated spores only one dish had carpospores develop into conchochelis, despite all being exposed to the same conditions. The rest of the spores died within 20 days of cultivation without ever erupting into conchochelis. There was a greatly reduced amount of diatoms in these cultures, indicating that even without fully isolating single spores, the method reduces contamination significantly. There was not performed sufficient trials to tell if the reduced diatom presence was a significant advantage over not having surrounding tissue enveloping the spores, which clearly gave a higher survival rate in the other experiments.

3.5 Laboratory cultivation of *Porphyra spp.* and growth variables

The growth experiment followed the stages described in 2.5, paragraphs 3.5.1, 3.5.2, 3.5.3 and 3.5.4 corresponds to the stages *i*,*ii*,*iii* and *iv*. The results are described together with the effects of the four growth variables; stress, GeO_2, light intensity and scallop shells.

3.5.1 Induction of carpospore release and effects of stress

After transport to the laboratory the collected material was searched for fertility in the edge tissue, by localization of carpospores or differentiating cell tissue. For the material collected at Storsteinan, 12 pieces of fertile tissue were exposed to stress factors (described in 2.5.1) and 24 were not stressed. The spore presence was categorized based on visual inspection, and arranged in three categories: "Many spores" = 20+ spores visible pr. mm^2 of tissue edge, "Few spores" = 1-20 spores visible pr. mm^2 of tissue edge and "No spores" = 0 spores visible pr. mm^2 of tissue edge. Figure 3.1 shows the amount of wells containing released carpospores for each 12-well-multi-culture dish created from the two specimen collected at Storsteinan, where each well held pieces of fertile tissue sized approximately $4mm^2$.

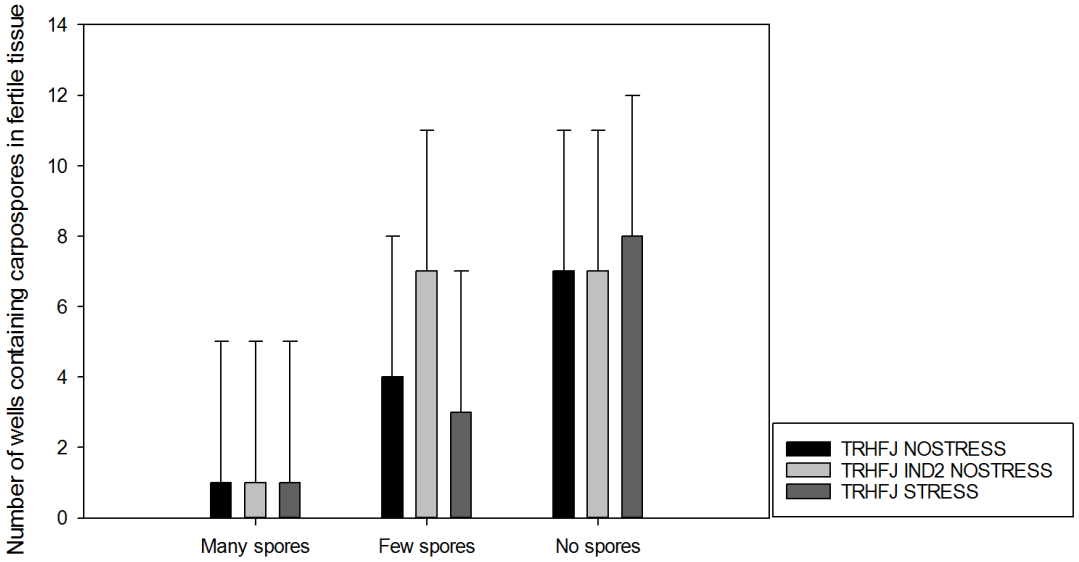

Figure 3.1: Number of wells pr. 12 well multi-culture-dish containing carpospores pr. mm^2 (n=12) with error bars expressing mean value of each sampling ±4SD. From cultures: TRHFJ NOSTRESS, TRHFJ IND2 NOSTRESS and TRHFJ STRESS.

A t-test for cultures TRHFJ NOSTRESS and TRHFJ STRESS shows no statistical significant difference between the two with P≤1,00, suggesting that the stress factor has no effect on the sporulation for these individuals. Comparing TRHFJ NOSTRESS and TRHFJ IND2 gives the same result with P≤1,00, yielding no significant difference between the two. This corresponds with visual observations and final result, where the unstressed cultures had a higher success rate.

3.5.2 Conchochelis growth and induction of conchosporelation

The conchochelis growth phase lasted up to 20 weeks, during which light adjustment and GeO_2 ratios were the most important growth factors (see 3.5.3). Light intensity was a strong factor for the growth ratio of the conchochelis, but also strongly enhanced diatom growth. Out of the 80 initial tissue pieces cut from the available material, only individuals from the culture labeled TRHFJ IND2 resulted in fulfilled life cycles. Setups similar to the one in 2.5.1, Figure 2.7, were performed for the other material as well, but due to the constant presence of diatoms, the ones from Frøya and Gjellarvika died during cultivation or dissolved in storage. The ones received from Bakkasund never reached conchochelis phase or released any spores.

There was a clear correlation between the amount of spores observed and excised in the initial pieces cut from the wild material, and the chances of successfully developing into healthy conchochelis. The wells containing tissue categorized with “High amount of spores”, 20+ carpospores pr. mm^2 of fertile tissue, survived the longest, had strongest growth as conchochelis and withstood diatom contamination best. In several cases the conchochelis grew well even when completely covered by diatoms (see 3.6), probably due to strong presence of carpospores in initially cut tissue. Diatoms covering both the exterior and interior of the conchochelis pieces was the case for most of the conchochelis grown, but in some cases the diatoms and the conchochelis split the well in half, effectively covering about 50% of the surface area each. In a duplicate made from the culture TRHFJ IND2 most wells were overgrown with diatoms at one point, with only 2/12 wells being visually categorized with good growth and low contamination. Still the conchochelis lived on for four months before the diatoms eventually outcompeted the conchochelis. As for the cultures which

successfully developed conchospores and eventually gametophytes, there was a strong correlation between GeO_2 ratios, light intensity and initial carpospore presence (see 3.5.3).

3.5.3 Conchosporangium quantification and effect of GeO_2, light intensity and scallop shell

The amount of filamentous conchosporangia produced by each piece of conchochelis was chosen as the most suited measure of growth, and these numbers serves as the most quantifiable result both in respect to growth and effect of disinfection treatment. Each conchochelis piece was counted every second day, for a total of 25 days. Due to the difficulties associated with flattening the conchochelis tufts and gaining vision of the conchosporangia, the first points of measurements are inconsistent. At the end of the counting period the conchochelis dissolved into very small pieces due to handling, and were difficult to distinguish by eye. This combined can be considered as drawbacks with the chosen method. As well the conchosporangia developed in clusters, as shown in Photo 3.8, and sometimes overlapping, thus making them difficult to distinguish.

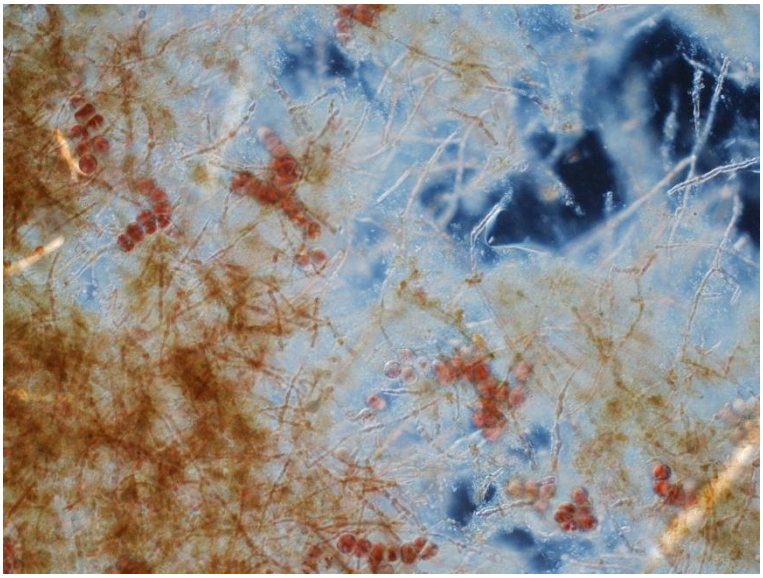

Photo 3.8: Clusters of conchosporangia among diatoms, conchochelis and dead tissue. 10x magnification.

Figure 3.2-3.7 shows the amount of conchosporangia counted in each well for each of the three cultures TRHFJ IND2 50GO, TRHFJ IND2 100GO and TRHFJ IND2 SCALLOP. The number of conchosporangia is plotted on the Y axis, against days of growth on the X axis, with each line representing a well in a multi-culture-dish, each with individual concentrations of GeO_2 and light intensity as described in Figure 2.8, 2.5.1. The first four figures are culture TRHFJ IND2 50GO at 10µmol and 100µmol, the next four are TRHFJ IND2 100GO at 10µmol and 100µmol, and the last are TRHFJ IND2 SCALLOP at 10µmol and 100µmol. The three highest and three lowest concentrations of GeO_2 are paired together.

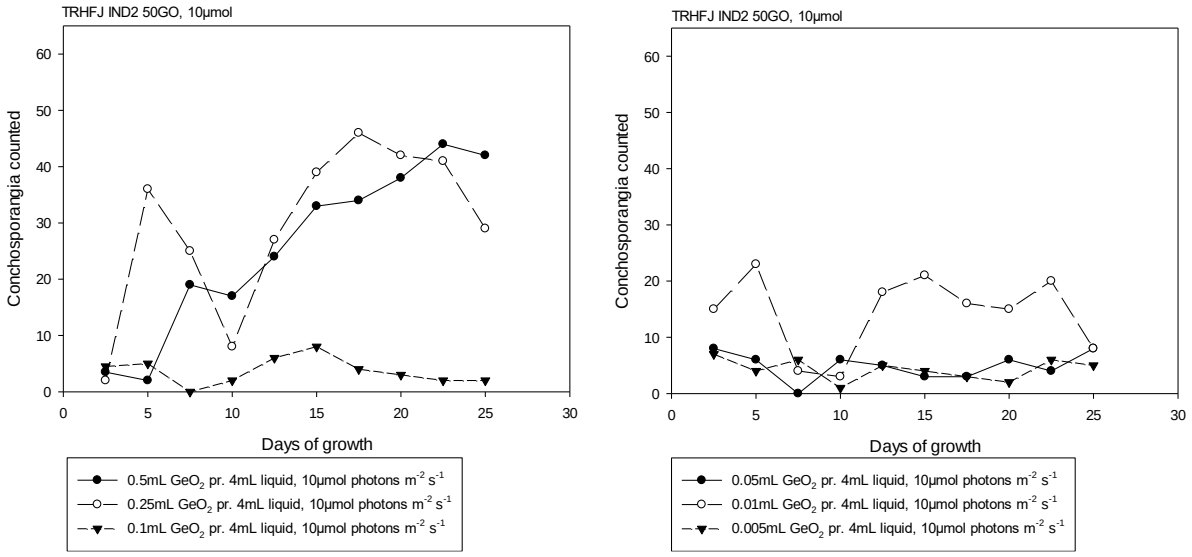

Figure 3.2: *Left*: Number of conchosporangia counted in the three highest concentrations of GeO_2 at 10µmol, for culture TRHFJ IND2 50GO. *Right*: Number of conchosporangia counted in the three lowest concentrations of GeO_2 at 10µmol, for culture TRHFJ IND2 50GO.

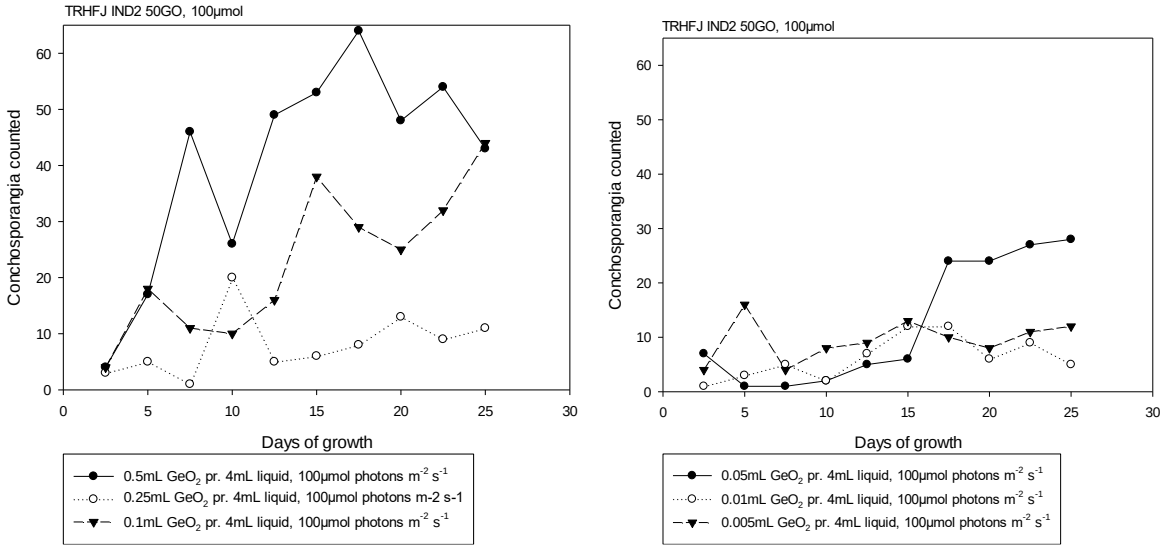

Figure 3.3: Left: Number of conchosporangia counted in the three highest concentrations of GeO_2 at 100µmol, for culture TRHFJ IND2 50GO. *Right*: Number of conchosporangia counted in the three lowest concentrations of GeO_2 at 100µmol, for culture TRHFJ IND2 50GO.

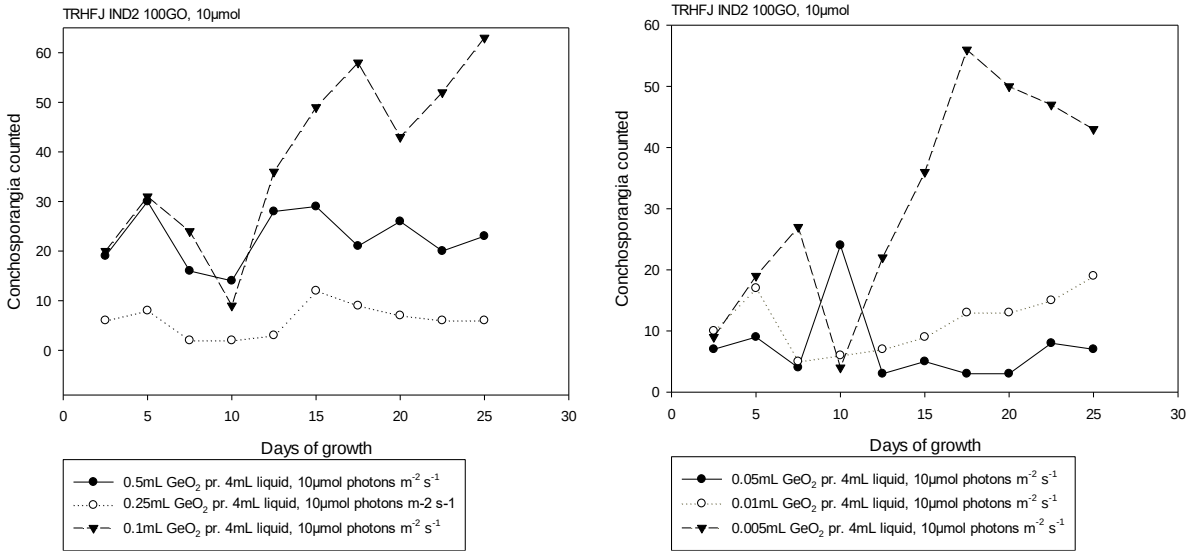

Figure 3.4: *Left*: Number of conchosporangia counted in the three highest concentrations of GeO_2 at 10µmol, for culture TRHFJ IND2 100GO. *Right*: Number of conchosporangia counted in the three lowest concentrations of GeO_2 at 10µmol, for culture TRHFJ IND2 100GO.

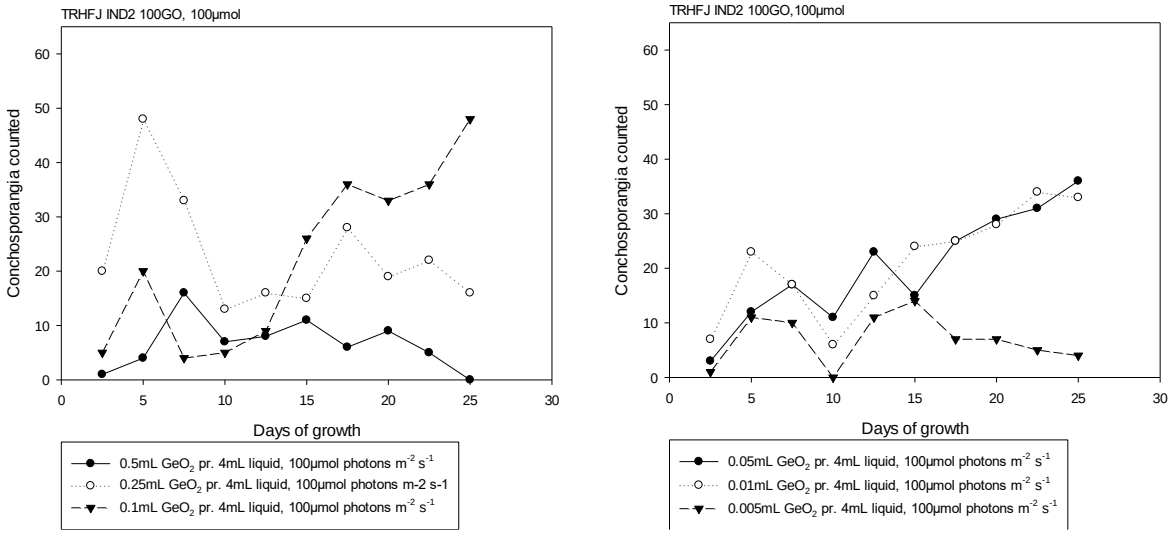

Figure 3.5: *Left*: Number of conchosporangia counted in the three highest concentrations of GeO_2 at 100µmol, for culture TRHFJ IND2 100GO. *Right*: Number of conchosporangia counted in the three lowest concentrations of GeO_2 at 100µmol, for culture TRHFJ IND2 100GO.

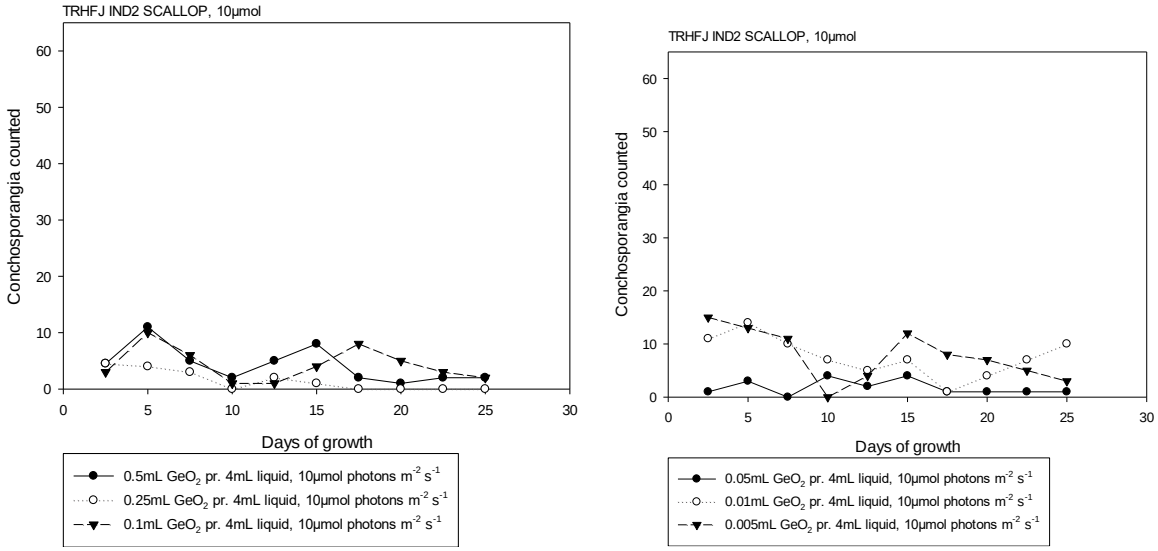

Figure 3.6: *Left*: Number of conchosporangia counted in the three highest concentrations of GeO_2 at 10µmol, for culture TRHFJ IND2 SCALLOP. *Right*: Number of conchosporangia counted in the three lowest concentrations of GeO_2 at 10µmol, for culture TRHFJ IND2 SCALLOP.

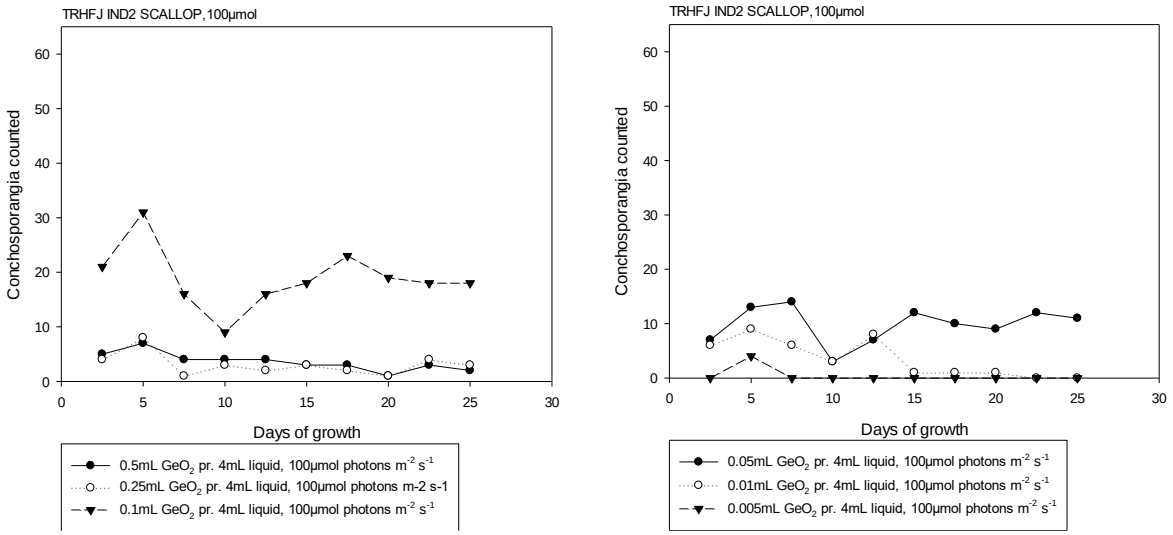

Figure 3.7: *Left*: Number of conchosporangia counted in the three highest concentrations of GeO_2 at 100µmol, for culture TRHFJ IND2 SCALLOP. *Right*: Number of conchosporangia counted in the three lowest concentrations of GeO_2 at 100µmol, for culture TRHFJ IND2 SCALLOP.

There is a clear negative correlation between the presence of scallop shells and development of conchosporangia, as can be seen from the low amounts detected in these cultures. None of the conchochelis that attached to the shell itself had any trace of conchosporangia. As well the conchochelis that attached had a different structure, with much thinner threads, growing tighter than the free floating ones. Photo 3.9 shows a piece of scallop shell with conchochelis attached to the surface.

Regarding the GeO_2 treatment there was generally a high amount of spores in the wells containing the higher concentrations, as seen in figures 3.2, 3.2 and 3.5 especially. But these matured into gametophytes much later (approximately two weeks) than the lowest concentrations, which developed more gametophytes earlier. Also towards the end of the counting period, the conchochelis in the higher concentrations started to bleach in the outer rim, and eventually after the experiment ended, the conchochelis in all wells with more than 4.47×10^{-8} gram of GeO_2 pr. mL liquid ended up dying. Although after successfully releasing conchospores which later germinated into gametophytes.

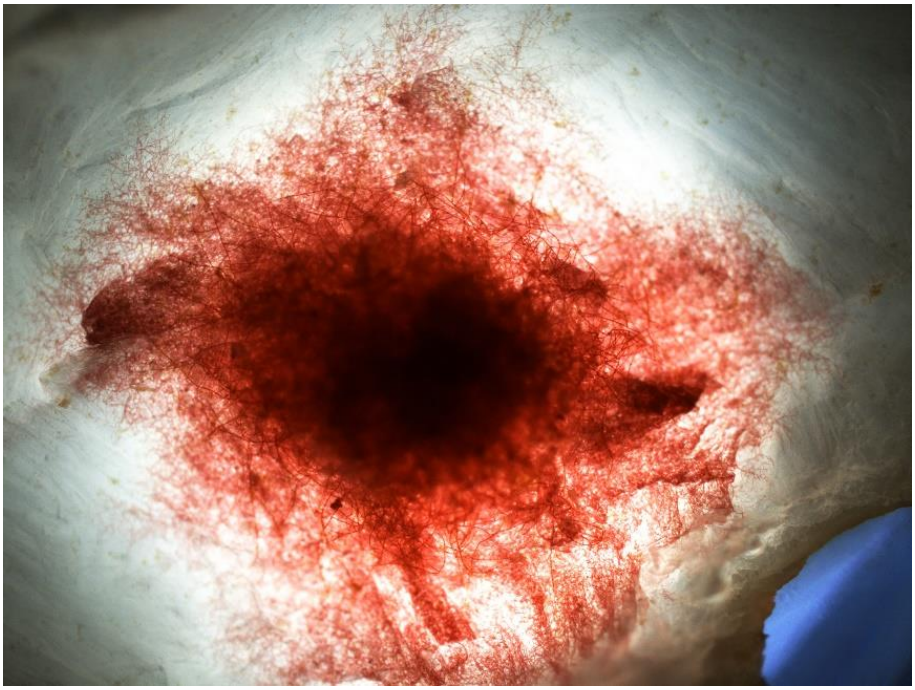

Photo 3.9: Piece of scallop shell with conchochelis growing at its inside surface. There was no sign of conchosporangia in any of the conchochelis that attached to shell substrate. 2,5x magnification.

When running a Mann-Whitney Rank Sum test on the highest and lowest GeO_2 treatment at 10µmol light intensity there is not a statistically significant difference (P = 0.332) between the median values. Testing on the same concentrations at 100µmol light intensity gives no statistically significant difference either (P = 0.055).

Table 3.2: 10µmol light intensity

Group	N	Missing	Median	25%	75%
0.005GO 10µmol	30	0	7.00	4.00	19.75
0.5GO 10µmol	30	0	18.00	4.25	28.25

Mann-Whitney U Statistic= 384.00

T = 849.000 n(small)= 30 n(big)= 30 (P = 0.332)

Table 3.3: 100µmol light intensity

Group	N	Missing	Median	25%	75%
0.005GO 100µmol	30	0	4.50	0.00	10.25
0.5GO 100µmol	30	0	6.50	3.750	30.25

Mann-Whitney U Statistic= 320.50

T = 785.500 n(small)= 30 n(big)= 30 (P = 0.055)

A Kruskal-Wallis One Way Analysis of Variance on all the groups gives H = 52.708 with 11 degrees of freedom and P = <0.001, which indicates a statistically significant difference between the groups. Since the there is no significant difference between the highest and lowest GeO_2 concentrations or lighting, but there are significant differences within the groups as a whole, one can assume that other factors are more important. The most likely factor is the amount of carpospores present in the initially cut tissue. The two groups with the largest difference in conchosporangia numbers are $0.1GeO_2$ 100 µmol (avg. +- 21.2666) and $0.05GeO_2$ 10µmol (avg. +-4.666), also indicating that GeO_2 is not a critical variable for development of conchosporangia. A Mann-Whitney Rank Sum test between the two gives P=<0.001, a statistically significant difference most likely not attributable to the small difference in GeO_2 concentration (4.473 X 10^{-7}g GeO_2 pr. mL liquid vs. 2.2365 X 10^{-4}g GeO_2 pr. mL liquid). A total of 4657 observations of conchosporangia were made, with an average of +-12.9361 conchosporangia in each culture at each point of measurement. Figure 3.8 shows total number of conchosporangia counted for each growth condition, with the ones grown at 10µmol on the left and 100µmol on the right. The mean value of total counted conchosporangia for 10µmol is 377.8 and for 100µmol it is 398.3, indicating that the light intensity is insignificant for conchosporangia development. On the other hand, only the cultures exposed to 100µmol light intensity developed gametophytes at the point the experiment ended. Only two weeks later did the 10µmol cultures develop gametophytes, see 4.X.X for more.

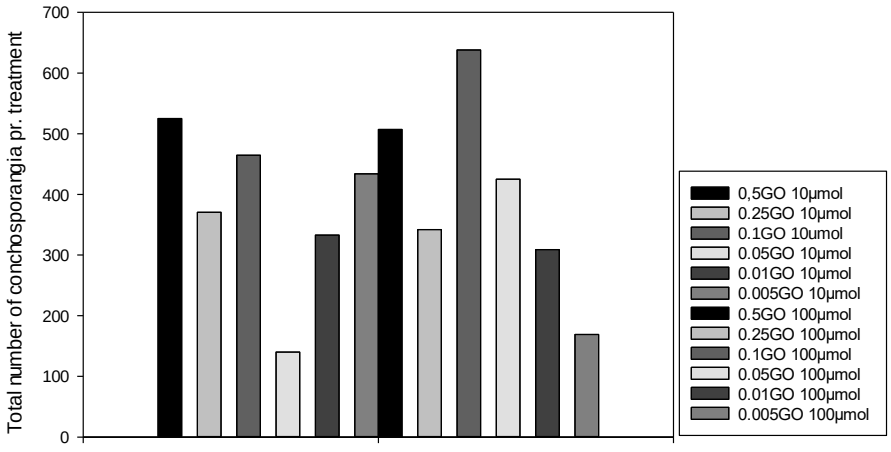

Figure 3.8: Total number of conchosporangium counted in each growth condition during the 25 days of counting. 10µmol light intensity on the left and 100µmol on the right. As one can see there is no clear trend towards which treatment is the most effective.

3.5.4 Gametophyte growth phase

The early gametophytes that was moved to Erlenmeyer flasks had a 100% survival rate, and all grew to the end of the experiment. They did however not grow fast at start, and reached a maximum of approximately 2cm^2 in size, with the smallest ones being about 2mm^2 in size. They did however grow faster once they had reached a certain size, and even though the experiment had to be discontinued before any measureable amount of biomass were achieved, they kept growing at a steady pace afterwards. See Photo 3.10 for size of the gametophytes after three weeks growth in Erlenmeyer flasks and Photo 3.11 for both conchochelis pieces and gametophytes free floating in the flasks.

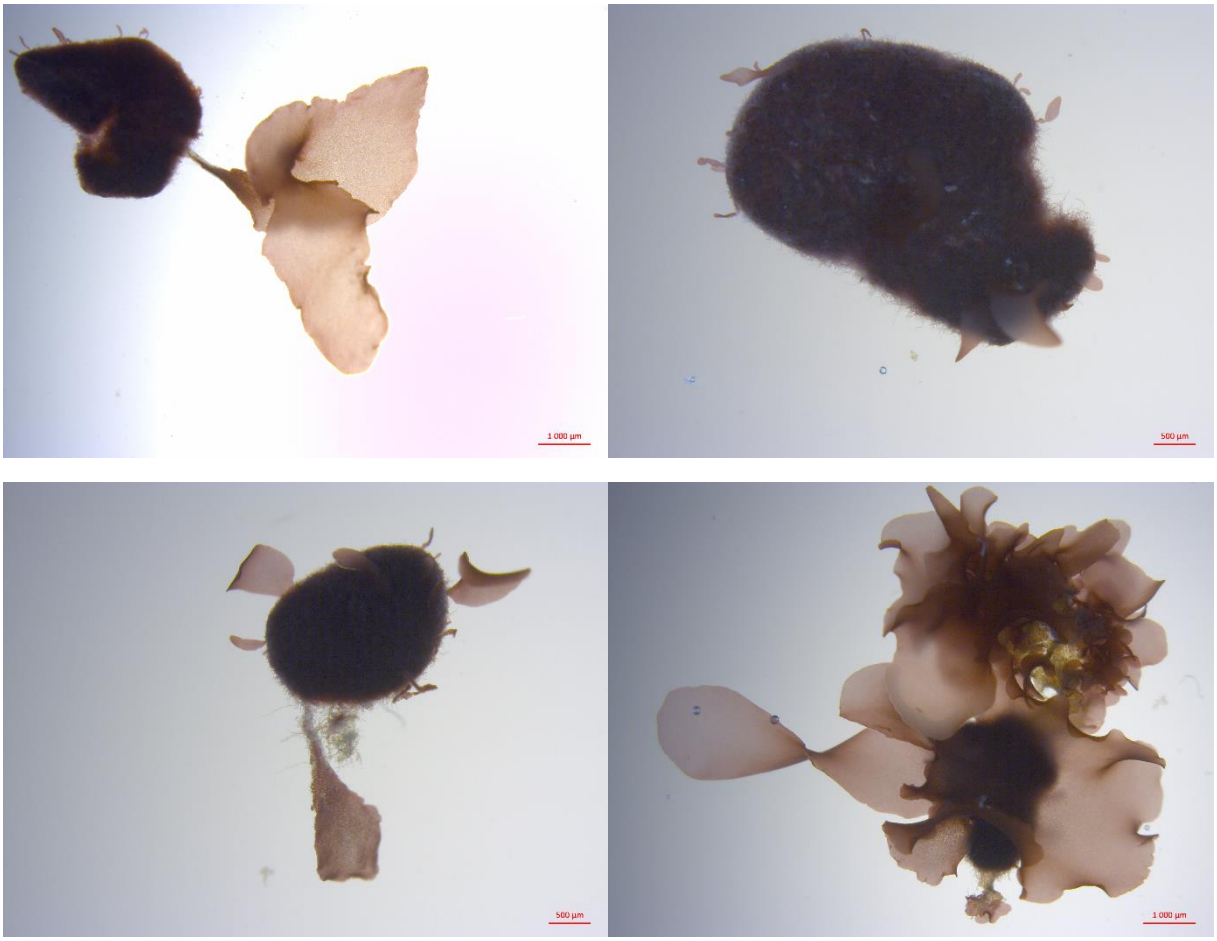

Photo 3.10: Four pieces of conchochelis with attached gametophytes growing out of the edges. 1.5x magnification.

Photo 3.11: Erlenmeyer flasks with conchochelis and a few gametophytes on the left, and growing gametophytes on the right.

3.6 Decontamination

The decontamination techniques applied before conchochelis growth (NaOCl, SSW rinsing and air drying) all proved lethal to the excised tissue. None of the 36 pieces exposed to the differing NaOCl solutions survived more than 24 hours. The rinsing and washing with SSW had no effect on removal of diatoms, since they stick very tightly to the conchochelis. Only a slight difference in diatom presence were detected by visual inspection. The pieces that were exposed to air drying also died within a short period, except the ones that were dried for only two hours, which survived for 2 days before dying.

Decontamination with GeO_2 is closely described in 3.5.3. The effect of this technique is arguably not important for development of conchosporangia, but can be considered necessary for growth of conchochelis. Every result shows that the conchosporangia has a high tolerance level for GeO_2, and high levels are recommended to avoid overgrowth of diatoms, see chapter 4 on this. Photo 3.12 shows threads of conchochelis completely covered in diatoms, which appeared after increasing light intensity from 10µmol to 25µmol, despite treatment with GeO_2. The conchochelis eventually outcompeted the diatoms and kept on growing, thanks to strong concentrations of GeO_2.

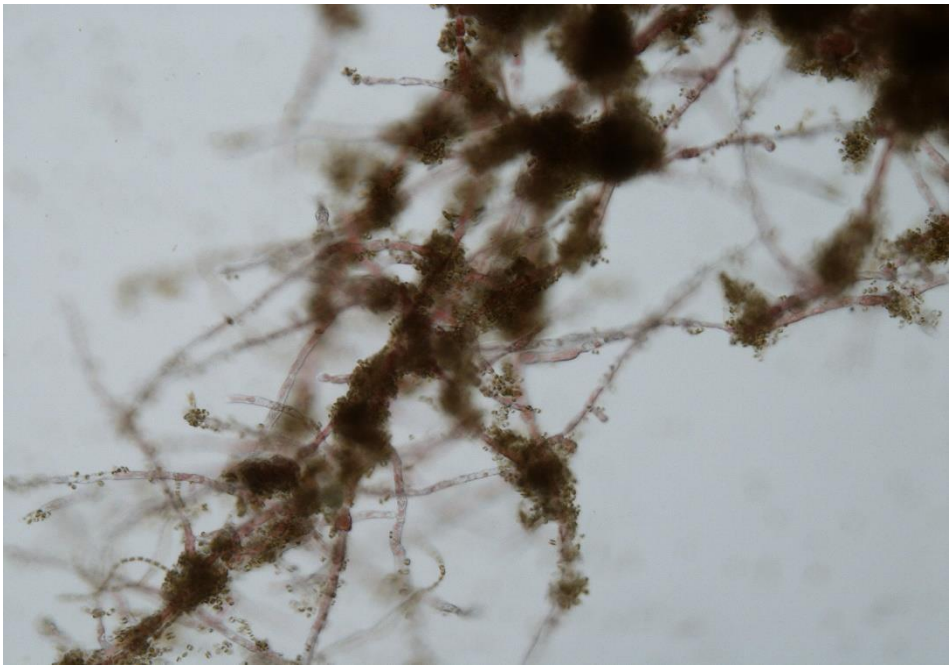

Photo 3.12: Example of tissue that survived even when completely covered by diatoms, a testament to the robustness of the conchochelis itself. The thin threads of red conchochelis is rarely visible beneath a thick layer of diatoms. 16x magnification.

4 Discussion

The study demonstrated that completing the life cycle of *Porphyra* by adaptation of existing techniques to local conditions is feasible, and served as a useful trial for mapping the major challenges associated with cultivation of the local species. The experiment provided sufficient variation to exclude techniques suggested by others, and enhancement of other. This includes suggestions for storage, carpospore release by stress, disinfection treatment with GeO_2, light intensities, growth mediums and basic laboratory techniques for spore isolation. A detailed overview of the life cycle was achieved, and a strong basis for future trials was established. As well the field search proved that local occurrences are relatively rare, but with some species variation across the areas searched.

4.1 Field search

The field search had far less occurrences of the algae than initially expected, the intertidal zone in all search areas are densely crowded communities of macroalgae, dominated by *Ascophyllum nodosum*, *Fucus serratus* and *Fucus vesiculosus,* with only a few and far between occurrences of *Palmaria palmata* and *Phycodrys rubens* (presumably floated ashore, due to being a subtidal species (Mathieson and Norall, 1975)). The thin and blade of *Porphyra,* combined with the density of species of similar coloration, makes the algae difficult to distinguish in between the other species. Thus subtidal searches were more successful, but difficult without equipment adapted to the cold waters of the Trondheimsfjord. But the algae can more easily be identified in the subtidal area, where its thin foliose blade has a distinct movement with the water masses compared to the heavier and thicker fauna occupying the same habitat. The coloration of the observed individuals is also of a much lighter pigmentation, almost translucent, and the size and shape of the blades are quite distinctively uncharacteristic, compared to surrounding fauna, making them easier to single out while floating in the water column. Correct speciation of the observed *Porphyra* is very difficult, as described in 1.2.1, but based on colorization (light brown vs. light red/pink) and general blade size and shape (Mortensen *et al.*, 2012), the observations made are likely of another species than the one cultured in this study. This is also supported by the fact that the cultivated species were collected by divers at approximately 2-3m depth, while

the observations were made just below the water line at low tide. In conclusion, diving equipment or dry suit and snorkeling gear, is recommended for mapping of local occurrences. As well there was a far better success rate in areas with rocky bottom and no to little presence of mud, sand or other "soft" substrates, thus such should be avoided.

4.2 Collected material

All samples collected had certain morphological variations distinguishable by eye. The ones from Gjellarvika were likely the same as observed in the field search, and based on visual inspection and comparison with pictures of documented species, most likely of the species *Porphyra purpurea (Mortensen et al., 2012)*. The specimen collected quickly deteriorated in the holding tank, also the entire tank was covered by diatoms within only a few days, nullifying attempts at cultivation. The sample received from Austevoll were of a different species entirely, with large dark green cells in a far thicker layer. Since this sample deteriorated quickly as well as having completely different morphology at both macroscopic and microscopic levels, further trials were disapproved. The specimens received from Taraskjæret and Storsteinan were likely of the same species, presumably *Wildemania amplissima* (recently *Porphyra amplissima (Sutherland et al., 2011)*), a strictly subtidal species (Pereira and Yarish, 2010). These did not dissolve at the same rate, and with cleaning every 3rd day, the tanks were kept free of diatoms. They did however dissolve in the fertile edge of the blade, where incisions were made to excise the carpospores. Due to most of the carpospores being released with and within surrounding dead tissue, the process of quantifying the amount of released spores were impossible using conventional methods (Neubauer 0,1mm hemocytometer), due to the hemocytometer`s dependency on free floating liquid, which where inhibited by the mucilageous tissue. The tissue released is likely a polysaccharide layer found between cells of *Wildemania amplissima*, a character considered a partial adaptation to intertidal stress (Blouin *et al.*, 2011). The release of carpospores within dead tissue might be a way of protecting the spores, as well as functioning as an element for adhesion to suited substrate. Personal observations also indicated that bacteria are more likely to enter and consume this material instead of the spores themselves. A clear recommendation for future storage of samples is thorough cleaning upon receiving them, by rinsing with sterile seawater. A temporary storage in a

non-flow-through tank containing GeO_2 might also help solve the problem with diatom overgrowth by killing the initial colonies that comes with the sample.

4.3 Spore isolation

The spore isolation techniques described in The New England Seaweed Culturing Manual (S. Redmond, 2014) and applied to the released spores from the sample collected at Storsteinan had varied success. The reduced amount of tissue surrounding the spores in these cultures might be the reason they did not survive, and is most likely the reason for the lack of diatoms. Which factor is the most beneficial for future cultivation trials is hard to tell with the relatively scarce material tested in this study, but further attempts at this isolation technique is probably advantageous.

4.4 Laboratory cultivation of *Porphyra sp.* life cycle

<u>Induction of carpospore release and effects of stress;</u>

The induction of carpospore release and effects of stress, described in 2.5 *i)* and 3.5.1, were based on techniques employed by S. Redmond and colleagues (Redmond *et al.*, 2014) and Varela-Alvarez and colleagues (Varela-Alvarez *et al.*, 2004). The stress factor did however show no significant improvement in release of spores, neither qualitatively or quantitatively, and can thus be considered excessive. Regular excision of fertile tissue with located carpospores and immersion in seawater should be sufficient for this species and growth conditions, as done in other experiments (Lindstrom *et al.*, 2007). It is worth noting that the time period during examination can be considered a stress factor. The selection of tissue at this point might however be considered of great importance, since the tissue pieces that were categorized with “Many spores” consistently survived far better than the others. The balance between size of tissue and amount of contaminants is naturally correlated, but with immediate or pre-treatment with GeO_2, this problem should be solved. All in all, a thorough examination of the selected tissue is recommended, as well as selection of tissue from all over the blade and not just one particular area.

Conchochelis growth and induction of conchosporelation;

The conchochelis growth phase were deeply troubled by diatom contamination, something that was strongly correlated with light intensities. Diatoms naturally grows faster than the conchochelis, but their growth is also inhibited by GeO_2, unlike the conchochelis. GeO_2 interferes with the process of silica frustule formation in diatoms, effectively inhibiting growth of new individuals (Shea and Chopin, 2007) even at extremely small concentrations. The effect of GeO_2 on macroalgae is poorly understood (Shea and Chopin, 2007), but red algae apparently has a high tolerance level for GeO_2, compared to other macroalgae (Rød, 2012, Shea and Chopin, 2007, Markham and Hagmeier, 1982). The conchochelis growth period can probably be shortened by increasing light intensities, but this obviously increases the risk of contaminations, as seen by the bloom occurring when light intensities were increased from 10µmol to 25µmol, see 2.5.1.

Conchosporangia quantification and effect of GeO_2, light intensity and scallop shell;

Several methods for measuring conchochelis growth were explored for this study. The fragile nature, three-dimensional structure and small size of the conchochelis made observations difficult. While photographic measurements of the conchochelis over time were tested, the method proved problematic due to conchochelis movement and unconcise placement within the culture dishes. Thus manual inspection and counting of germinating conchosporangia were deemed the best option for observing growth. The method chosen resulted in a flattening of the three-dimensional structure, thus inhibiting vision into the central parts. To overcome this, and also provide access to light in the central part, the conchochelis tufts was teared open after eight days of growth. This combined with handling every second day eventually resulted in the conchochelis dissolving into many smaller pieces, increasing light access and surface area, but also strongly effecting the numbers of conchosporangia visible for quantification. This had an effect on the observations made and led to discontinuous measurements not suited for exact determination of growth, a major drawback with the technique. For future employment of the same technique, tearing of the

conchochelis is recommended, or even a complete disintegration of the entire conchochelis structure, since growth can continue even with only a few cells connected together.

Despite inconsistency in early measurements, all cultures and variables experienced an increase in conchosporangia until the 22nd day of counting. After this peak the conchosporangia started disappearing, most likely due to release of conchospores, and few new were detected. This coincides with an experiment conducted by Varela-Alvarez and colleagues (Varela-Alvarez *et al.*, 2004), in which the same happened after 40 days of growth. That experiment did also show large variations of conchosporangia densities depending on temperature, with reduced numbers under lower temperatures. Since all stages of *Porphyra* life cycles closely correlates with temperature in most examined species (Green and Neefus, 2015, Lindstrom *et al.*, 2007, Varela-Alvarez *et al.*, 2004), it is natural to assume this also applies for this study. Thus a way of controlling temperature is recommended for future trials, which was not possible in this study.

GeO_2:

The application of GeO_2 to combat contamination by diatoms were admittedly underestimated at the beginning of this study. This did however lead to many trials with various combinations of GeO_2 concentrations, which in turn resulted in a reliable charting of tolerance levels. As mentioned, the higher concentrations (>= 4.47 X 10^{-8} g. GeO_2 pr. mL growth medium) all eventually killed the conchochelis, while all concentrations below this both exterminated diatoms and ensured healthy growth. The exact reason for conchochelis death with higher concentrations remains unknown. Nonetheless, concentrations below 4.47 X 10-8 g. GeO2 pr. mL growth medium is recommended.

Light:

The lack of differences in growth of conchochelis and development of conchosporangia between light intensities (the mean total number of conchosporangia for 10µmol; 377.8. For 100µmol;398.3, a neglectable difference of 20.5), contradicts several other experiments, which states that light regimes and temperature changes are the main driving force behind conchosporangia development (Green and Neefus, 2015, López-Vivas *et al.*, 2014, Redmond *et al.*, 2014, Lu and Yarish, 2011, Pereira and Yarish, 2010, Lindstrom *et al.*, 2007, Holmes

and Brodie, 2004, Yarish *et al.*, 1999). There might be several explanations for this, one being that both light regimes provided sufficient light for induction of development, and other factors catalysed the process (by example the continuous stress experienced by lab examinations every second day, which affected both light and temperature). Also local species might be adapted to different light regimes than those mentioned in other trials. Considering the northern latitude and long days in the Trondheimsfjord, they are likely exposed to far longer day/night cycles, but lower light intensities, thus other factors might be more important for this species.

Effect of scallop shell:

The conchochelis that bored into and grew inside the scallop shells provided, never developed conchosporangia or conchospores. This contradicts both traditional Japanese mass cultivation methods, as well as many experiments and trials for growth of *Porphyra* (Redmond *et al.*, 2014, Blouin *et al.*, 2011, Sahoo and Yarish, 2005, Behura *et al.*, 2002, Wikfors and Ohno, 2001, Hafting, 1999, Yarish *et al.*, 1999). However, it was established by Hideo Iwasaki already in 1961 (Hideo, 1961) that a calciferous substrate is not necessary for growth of conchochelis, and that liquid media with free floating carpospores is enough for development of healthy conchochelis. The fact that the settled carpospores did not develop conchospores, might be a matter of time. The structure of this conchochelis varied greatly from the free floating ones, with far thinner and longer stretched cells, and there is a possibility that conchospores would develop had the experiment continued.

4.5 Gametophyte growth phase

After the first gametophytes started appearing they were left for growth in the same medium for one week, before they were moved to Erlenmeyer flasks. Once they were moved here, with 100µmol light intensity, 50-50 VS and SSW and 0.31mL GeO_2 solution pr. L liquid, growth accelerated quickly. At the moment of writing the gametophytes are still growing, and more gametophytes are appearing every day. Approximate growth rate is hard to determine by eye, but within one week the larger gametophytes grew from about $1cm^2$ to

5cm^2. The continued fast growth and lack of contamination in the flasks, might be considered a success for the experiment, and suites as a foundation for further cultivation.

4.6 Decontamination

NaOCl:

The NaOCl treatment were applied according to a technique used for decontamination of *Saccharina latissima* sori, employed by Kaia Rød in her cultivation experiment (Rød, 2012). Although effective for *S. latissima*, it proved lethal at all concentrations (0.033mL pr. 5mL growth medium, and 0.033mL pr. 15mL growth medium) for *Porphyra*. All material exposed to NaOCl died, although the larger pieces of tissue that contained less dissolving material and more of the central red cells of the blade survived a bit longer (approximately 4-8h). In conclusion *Porphyra* has zero tolerance level for this method of disinfection.

Washing with sterile seawater:

The SSW washing methods provided only a slight reduction of diatom presence. The fact that the diatoms stuck to the conchochelis despite two minutes of 8000RPM in the sentrifuge, is a clear testament to the diatoms ability to cling to the conchochelis. It is worth noting that some samples washed in warm water experienced death of all diatoms, but this did not help at all in removing them from the culture.

Air-drying:

The subtidal species selected for cultivation apparently had very low tolerance for exposure to dry conditions. This is logical for several reasons, first of all its adaptation to constant water presence. As well salt concentrations is worth mentioning, as the drying continues the concentrations of salt increase, probably to a point where it eventually destroys the cells by loss of membrane integrity due to water loss (Blouin *et al.*, 2011). Although several species of *Porphyra* are adapted to water loss, it is clearly not the case for the species used in this trial.

5 Recommendations and conclusion

Although troubled by growth inhibiting contamination, the experiment ended in a fulfilled life cycle and a steady growth of biomass. In general, a stricter approach to decontamination is recommended, especially cleaning of collected material before storage, and application of GeO_2 in earlier phases of cultivation. The chosen method for growth proved successful, but more insight into the factors inducing conchosporelation is a necessity. The method for quantification of conchochelis growth and conchosporangia development is time consuming, but cheap and reliable if followed correctly, and thus recommended. It also predicted the end result with good accuracy, the cultures with the most conchosporangia developed more, and faster growing, gametophytes than the ones with lower numbers.

Following is a recommended protocol for further cultivation, which considers all factors experienced in this thesis. It is closely adapted to local species, and easily applicable with the given equipment and time used within this thesis.

5.1 Protocol for cultivation of local species of *Porphyra spp.* around the Trondheimsfjord:

1. Collection of wild material

Gametophytes should be moved to the laboratory in air-tight zip-lock bags as soon as possible, after rinsing in seawater. At the laboratory, thoroughly rinse the material with sterile seawater for 5 minutes, before examining the edge tissue. Look for carpospores using a microscope, these are square shaped cells divided in four and contained within the same cell pocket. Carefully excise pieces of this tissue, and place in sterile petri dishes containing 5mL VS growth medium and 5mL SSW. Apply 0.1mL GeO_2 solution (made of 0.8946g GeO_2 pr. 200mL distilled freshwater), and place under approximately 25µmol photons m^{-2} s^{-1} light intensity at a 12h night/day light cycle at 10°C.

2. Isolation of carpospores and conchochelis cultivation

Once spores are released from the material, one should attempt to isolate these with a pipette. One can melt a glass pipette over a bunzenburner and elongate the tip, then use a finger at the end to create vacuum before carefully extracting single spores. Place these in sterile and sealed petri dishes with 5mL VS, 5ml SSW and 0.1mL GeO2 solution. If unsuccessful at isolating spores, or spores are released with surrounding tissue, the entire piece of fertile tissue can be moved to the petri dish. Place at 10µmol photons m^{-2} s^{-1} light intensity at a 12h night/day light cycle and 10°C for conchochelis growth. Approximate growth period is two to three months. Change of liquid content is important, and should be done every 14^{th} day to avoid contamination and provide fresh growth medium.

3. Induction of conchospore development

The culture should be examined frequently to measure growth and health of the conchochelis, a bright red colour is desirable. After the first conchosporangia appear, filamentous structures containing large circular red cells in clusters, the light intensity should be increased to 25µmol photons m^{-2} s^{-1} light intensity at a 12h night/day light cycle. If they haven't appeared after two months of growth, increase light intensity and use a 16h day/ 8h night light cycle. Careful examination of development of conchosporangia is recommended, until the point they stop appearing in the conchochelis.

4. Gametophyte growth phase

Once the conchosporangia has stopped developing, or starts to disappear, the conchospores are being released. Move the conchochelis pieces to aerated Erlenmeier flasks with 2L VS growth medium, 2L SSW and 1.25mL GeO_2 solution. Place at 100-200µmol photons m^{-2} s^{-1} light intensity at a 12h night/day light cycle for gametophyte growth, until desired biomass is accumulated.

5 References

BEHURA, S., SAHOO, S. & SRIVASTAVA, V. K. 2002. *Porphyra*: the economic seaweed as a new experimental system. *Current Science,* 83**,** 1313-1316.

BELCHER, J. H. 1960. Culture studies of *Bangia atropurpurea*. *New Phytologist,* 59**,** 367-373.

BLOUIN, N. A., BRODIE, J. A., GROSSMAN, A. C., XU, P. & BRAWLEY, S. H. 2011. *Porphyra*: a marine crop shaped by stress. *Trends in Plant Science,* 16**,** 29-37.

BOURNE, V. L., CONWAY, E. & COLE, K. 1970. On the ultrastructure of pit connections in the conchocelis phase of the red alga Porphyra perforata. *Phycologia,* 9**,** 79-81.

BRODIE, J., MORTENSEN, A. M., RAMIREZ, M. E., RUSSEL, S. & RINKEL, B. 2008. Making the links: towards a global taxonomy for the red algal genus *Porphyra*(Bangiales, Rhodophyta). *Journal of Applied Phycology,* 20**,** 939-949.

BROOM, J. E. S., FARR, T. J. & NELSON, W. A. 2003. Phylogeny of the *Bangia* flora of New Zealand suggests a southern origin for *Porphyra* and *Bangia* (Bangiales, Rhodophyta). *Molecular phylogenetics and evolution,* 31**,** 1197-1207.

BURTIN, P. 2003. Nutritional value of seaweeds. *Electronic journal of Environmental, Agricultural and Food chemistry,* 2**,** 498-503.

BURZYCKI, G. M. & WAALAND, J. R. 2009. On the Position of Meiosis in the Life History of *Porphyra torta* (Rhodophyta). *Botanica Marina,* 30**,** 5-10.

BUTTERFIELD, N. J. 2000. *Bangiomorpha pubescens* n. gen., n. sp.: implications for the evolution of sex, multicellularity, and the Mesoproterozoic/Neoproterozoic radioation of eukaryotes. *Paleobiology,* 26**,** 386-404.

BUTTERFIELD, N. J. 2007. Macroevolution and macroecology through deep time. *Paleontology,* 50**,** 41-55.

BUTTERFIELD, N. J. 2009. Modes of pred-Ediacaran multicellularity. *Precambrian Research,* 173**,** 201-211.

COURTOIS, A., SIMON-COLIN, C., BOISSET, C., BERTHOU, C., DESLANDES, E., GUÉZENNEC, J. & BORDRON, A. 2008. Floridoside extracted from the red alga *Mastocarpus stellatus* is a potent activator of the classical complement pathway. *Marine Drugs,* 6.

DICKSON, L. G. & WAALAND, J. R. 1985. *Porphyra nereocystis*: A dual-daylength seaweed. *Planta,* 165**,** 548-553.

DREW, K. M. 1949. Conchochelis-phase in the life-history of *Porphyra umbilicalis*. *Nature,* 164**,** 748-749.

FAO. 2016. *FAO, Fisheries and Aquaculture Department Statistical query* [Online]. www.fao.org: FAO. Available: http://www.fao.org/figis/servlet/SQServlet?file=/work/FIGIS/prod/webapps/figis/temp/hqp_2528497931894868810.xml&outtype=html [Accessed 2016].

FULLER, M. P. & GIBOR, A. 1984. Developmental studies in *Porphyra*. Blade differentiation in *Porphyra perforata* as expressed by morphology, enzymatic digestion, and protoplast regeneration. *Journal of Phycology,* 20**,** 609-616.

GABRIELSON, P. W. & GARBARY, D. J. 1987. A Cladistic Analysis of Rhodophyta: Florideophycidean Orders *British Phycological Journal,* 22**,** 125-138.

GREEN, L. A. & NEEFUS, C. D. 2015. Effects of temperature, light level, and photoperiod on the physiology of *Porphyra umbilicalis* Kützing from the Northwest Atlantic, a candidate for aquaculture. *Journal of Applied Phycology,* 28**,** 1815-1826.

HAFTING, J. T. 1999. A novel technique for propagation of *Porphyra yezoensis* Ueda blades in suspension cultures via monospores. *Journal of Applied Phycology,* 11**,** 361-367.

HAINES, C. M. C. 2001. *International Women in Science: A Biographical Dictionary to 1950*, ABC-CLIO, Inc.

HAWKES, M. & GUIRY, M. D. 2015. *Porphyra C.Agardh, 1824, nom. cons.* [Online]. www.algaebase.org. Available: http://www.algaebase.org/search/genus/detail/?genus_id=W95431a857d41a2f8&-session=abv4:AC1F292C0cb9434335TUB59E595A [Accessed].

HIDEO, I. 1961. The life-cycle of *Porphyra tenera* in vitro. *The Biological Bulletin,* 121**,** 173-187.

HOLMES, M. J. & BRODIE, J. 2004. Morphology, seasonal phenology and observations on some aspects of the life history in culture of *Porphyra dioic*a (Bangiales, Rhodophyta) from Devon, UK *Phycologia,* 43**,** 176-188.

KAPRAUN, D. F. & LEMUS, A. J. 1987. Field and culture studies of *Porphyra spiralis var. amplifolia* Olivieira Filho et Coll (Bangiales, Rhodophyta) from Isla de Margarita, Venezuela. *Botanica Marina,* 30**,** 483-490.

LINDSTROM, S. C., CONITZ, J. M., HALL, S. & STEKOLL, M. S. 2007. Induction of conchospore release: ecotypic variation in northeast Pacific species of *Porphyra*. *Journal of Applied Phycology,* 20**,** 331-340.

LÓPEZ-VIVAS, J. M., RIOSMENA-RODRÍGUEZ, R., LLAVE, A. A. J.-G. D. L., PCHECO-RUÍZ, I. & YARISH, C. 2014. Growth and reproductive responses of the conchochelis phase of *Porphyra hollenbergii* (Bangiales, Rhodophyta) to light and temperature. *Journal of Applied Phycology,* 27**,** 1561-1570.

LU, S. & YARISH, C. 2011. Interaction of photoperiod and temperature in the development of conchochelis of *Porphyra purpurea* (Rhodophjyta: Bangiales). *Journal of Applied Phycology,* 23**,** 89-96.

MACARTAIN, P., GILL, C. I. R., BROOKS, M., CAMPBELL, R. & ROWLAND, I. R. 2007. Nutritional Value of Edible Seaweeds. *Nutrition Reviews***,** 535-543.

MARKHAM, J. W. & HAGMEIER, E. 1982. Observations on the effects of germanium dioxide on the growth of macro-algae and diatoms. *Phycologia,* 21**,** 125-130.

MATHIESON, A. C. & NORALL, T. L. 1975. Physiological studies of subtidal red algae. *Journal of Experimental Marine Biology and Ecology,* 20**,** 237-247.

MITMAN, G. G. & MEER, J. P. V. D. 1994. Meiosis, blade development, and sex determination in *Porphyra purpurea*(Rhodophyta). *Journal of Phycology,* 30**,** 147-159.

MORTENSEN, A. M., NEEFUS, C., NIELSEN, R., GUNNARSON, K., EGILSDÓTTIR, S., PEDERSEN, P. M. & BRODIE, J. 2012. New insights into the biodiversity and generic relationships of foliose Bangiales (Rhodophyta) in Iceland and the Faroe Islands. *European Journal of Phycology,* 47**,** 146-159.

MURAKAMI, S. & PACKER, L. 1970. Light-induced Changes in the Conformation and Configuration of the Thylakoid Membrane of Ulva and Porphyra Chloroplasts in Vivo. *Plant Physiology,* 45**,** 289-299.

NELSON, W. A., BRODIE, J. & GUIRY, M. D. 1999. Terminology used to describe reproduction and life history stages in the genus *Porphyra* (Bangiales, Rhodophyta). *Journal of Applied Phycology,* 11**,** 407-410.

NELSON, W. A., FARR, T. J. & BROOM, J. E. S. 2006. Phylogenetic relationships and generic concepts in the red order Bangiales: challenges ahead. *Phycologia,* 45**,** 249-259.

NELSON, W. A. & KNIGHT, G. A. 2009. Endosporangia - a new form of reproduction in the genus *Porphyra* (Bangiales, Rhodophyta). *Botanica Marina,* 37**,** 17-20.

NODA, H. 1993. Health benefits and nutritional properties of nori. *Journal of Applied Phycology,* 5**,** 255-258.

OLIVEIRA, M. C., KURNIAWAN, J., BIRD, C. J., RICE, E. L., MURPHY, C. A., SINGH, R. K., GUTELL, R. R. & RAGAN, M. A. 1995. A preliminary investigation of the order Bangiales (Bangiophycidae, Rhodophyta) based on sequences of nuclear small-subunit ribosomal RNA genes. *Phycological Research,* 43**,** 71-79.

PEREIRA, R. & YARISH, C. 2010. *The role of Porphyra in sustainable culture systems: Physiology and applications,* Springer Science, Springer.

PUESCHEL, C. M. & COLE, K. M. 1982. Rhodophycean Pit Plugs: An Ultrastructural Survey with Taxonomic Implications. *Botanical Society of America, Inc.,* 69**,** 703-720.

RAGAN, M. A., BIRD, C. J., RICE, E. L., GUTELL, R. R., MURPHY, C. A. & SINGH, R. K. 1994. A molecular phylogeny of the marine red algae (Rhodophyta) based on the nuclear small-subunit rRNA gene. *Proc. Natl. Acad. Sci. USA,* Vol. 91**,** 7276-7280.

REDMOND, S., GREEN, L., YARISH, C., KIM, J. & NEEFUS, C. 2014. New England seaweed culture handbook-nursery systems. Connecticut: University of Connecticut.

ROBBA, L., RUSSEL, S. J., BARKER, G. L. & BRODIE, J. 2006. Assesing the use of mitochondrial *cox1* marker for use in DNA barcoding of red algae. *American Journal of Botany,* 93**,** 1101-1108.

RØD, K. 2012. *Sori disinfection in cultivation of Saccharina latissima.* Msc, NTNU.

S. REDMOND, L. G., C. YARISH, , J. KIM, AND C. NEEFUS 2014. New England Seaweed Culture Handbook-Nursery Systems. *Connecticut Sea Grant,* CTSG-14-01**,** 92.

SAHOO, D. & YARISH, C. 2005. *"Mariculture of seaweeds" Phycological methods: Algal culturing techniques,* Phycological Society of America, Elsevier.

SÁNCHEZ-MACHADO, D. I., LÓPEZ-CERVANTES, J., LÓPEZ-HERNANDEZ, J. & PASEIRO-LOSADA, P. 2004. Fatty acids, total lipid, protein and ash contents of processed edible seaweeds. *Food Chemistry,* 85**,** 439-444.

SAUNDERS, G. W. & HOMMERSAND, M. H. 2004. Assessing red algal supraordinal diversity and taxonomy in the context of contemporary systematic data. *American Journal of Botany,* 91**,** 1494-1507.

SHEA, R. & CHOPIN, T. 2007. Effects of germanium dioxide, an inhibitor of diatom growth, on the microscopic laboratory cultivation stage of the kelp, *Laminaria saccharina*. *Journal of Applied Phycology,* 19**,** 27-32.

SKJERMO, J., AASEN, I. M., FORBORD, S., SOLBAKKEN, R., STEINHOVDEN, K. B. & HANDÅ, A. 2014. A new Norwegian bioeconomy based on cultivation and processing of seaweeds: Opportunities and R&D needs. *In:* SKJERMO, J. (ed.). SINTEF Fisheries and Aquaculture.

SUTHERLAND, J. E., LINDSTROM, S. C., NELSON, W. A. & BRODIE, J. 2011. A new look at an ancient order: Generic revision of the Bangiales (Rhodophyta). *Journal of Phycology,* 47**,** 1131-1151.

TABOADA, M. C., MILLÀN, R. & MIGUEZ, M. I. 2013. Nutritional value of the marine algae wakame (*Undaria pinnatifida)* and nori (*Porphyra purpurea*) as food supplements. *Journal of Applied Phycology,* 25**,** 1271-1276.

TSENG, C. K., LOBBA, C. S. & WYNNE, M. J. 1981. The biology of seaweeds. *Botanical Monographs,* 17**,** 680.

VARELA-ALVAREZ, E., STENGEL, D. B. & GUIRY, M. D. 2004. The use of image processing in assessing conchochelis growth and conchospore production in *Porphyra linearis*. *Phycologia,* 43**,** 282-287.

WAALAND, J. R., DICKSON, L. G. & DUFFIELD, E. C. S. 1990. Conchospore production and seasonal occurrence of some *Porphyra* species (Bangiales, Rhodophyta) in Washington State. *Hydrobiologia,* 204/205**,** 453-459.

WIKFORS, G. H. & OHNO, M. 2001. Impact of algal research in aquaculture. *Journal of Phycology,* 37**,** 968-974.

WOELKERLING, W. J., BROADWATER, S., COLE, K. M., COLEMAN, A. W., COOMANS, R. J., CRAIGIE, J. S., FREDERICQ, S., GABRIELSON, P. W. & GANTT, E. 1990. *Biology of the red algae,* Cambridge University Press, Press syndicate of the University of Cambridge.

YARISH, C., CHOPIN, T., WILKES, R., MATHIESON, A. C., FEI, X. G. & LU, S. 1999. Domestication of nori for Northeast America: the Asian experience. *Bulletin of the Aquaculture Assosiation of Canada,* 1**,** 11-17.