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Optimising cultivation method for the conchocelis stage of Industrial *Porphyra* cultivation (Rhodophyta, Bangiales)

- Towards the development of an RGB-based image analysing method to improve the estimation of conchocelis growth in Industrial *Porphyra* cultivation

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Bodø, June 2021

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Abstract

Nori (*Porphyra spp*., *Pyropia spp*.) is among the most valuable cultivated marine crops, and the end products is mainly used for sushi making, a traditional Asian cuisine. In cultivation, the microscopic life stage of these algae, also known as conchocelis, is usually grown on bivalve shells. Although this cultivation process is well establish in Asian countries, there is room for increased production efficiency. In industrial production, the growth of conchocelis is usually measured through qualitative observations based on change in colour. However, the development of a quantitative method to measuring growth may increase efficiency during cultivation.

This study aims to investigate whether RGB-based image analysis, using R-, G-, and B values from images of conchocelis, can be used as a quantitative method to measure conchocelis growth for industrial cultivation purposes. Thus, images of 40 shells with conchocelis growth taken regularly over a period of 74 days were examined. Furthermore, since chlorophyll *a* content can be used as a measure for algae growth, the relationship between R, G, B and chlorophyll *a* was investigated as a control. Thus, 15 shells with conchocelis growth collected on day 58 of the experiment were examined. Additionally, an observational control was preformed, in which growth development was evaluated based on colour.

The present study indicates that R-, G-, and B values from images of conchocelis can measure conchocelis growth. The study further indicate that some RGB features are likely to measure growth better than others. A relationship between R, G, B and chlorophyll *a* was not detected. Although, if suggested improvements to experimental design are implemented such a relationship may be demonstrated. If so, this method will be the preferred control, compared to the additional observational control used in the present study.

The overall findings demonstrated in this study suggest RGB-based image analysis to have the potential to serve as a low-cost, non-invasive and low labour intensive method for conchocelis growth assessment, and thereby has the potential to increasing efficiency in industrial production.

Sammendrag

Nori (*Porphyra spp., Pyropia spp.)* er blant de mest verdifulle dyrkede marine algene på verdensbasis, og sluttproduktet blir i all hovedsak brukt til å lage sushi, en rett tilknyttet det tradisjonelle asiatiske kjøkken. Det mikroskopiske livsstadiet for disse algene, også kjent som conchocelis, dyrkes vanligvis på muslinger. Selv om denne dyrkningsmetoden er godt innarbeidet i asiatiske land er det rom for effektivisering i denne delen av produksjonen. I industriell produksjon er vekst hos conchocelis vanligvis målt gjennom kvalitative observasjoner av fargeendring. Utviklingen av en kvantitativ metode for måling av vekst vil imidlertid kunne øke effektiviteten i produksjonen.

Denne studien har som formål å undersøke om RGB-basert bildeanalyser, da gjennom R-, G-, og B verdier fra bilder av conchocelis, kan nyttes som en kvantitativ metode for å måle conchocelis-vekst i industriell produksjon. Dette ble undersøkt for bilder av 40 skjell med conchocelis-vekst, innsamlet over en periode på 74 dager. Videre, siden mengden klorofyll *a* kan nyttes som et mål på algevekst, ble forholdet mellom R, G, B og klorofyll *a* undersøkt som en kontroll. Dette ble undersøkt for 15 skjell med conchocelis-vekst innsamlet på dag 58 i eksperimentet. I tillegg til denne kontrollen, ble det gjennomført en observasjonskontroll der vekst ble målt på bakgrunn av farge.

Denne studien indikerer at R-, G-, og B verdier fra bilder av conchocelis kan måle conchocelis-vekst. Studien indikerer ytterligere at noen RGB-funksjoner trolig måler vekst bedre enn andre. En relasjon mellom R, G, B og klorofyll *a* ble ikke funnet. Om foreslåtte endringer til eksperiment-design blir gjennomfør kan imidlertid en slik relasjon muligens demonstreres. I så tilfellet, vil denne metoden være en foretrukket kontroll, sett opp mot observasjonene nyttet som kontroll i dette studiet.

De samlede funnene i denne studien indikerer at RGB-basert bildeanalyse har et potensial som en økonomisk, ikke-ødeleggende og lite arbeidskrevende metode for å måle conchocelis-vekst, og har sådan potensial for å øke effektiviteten i den industrielle produksjonen.

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Glossary

(General terminology)

Monoecious = Male- and female reproductive organ are found in the same individual

Dioecious = Male- and female reproductive organ are found in different individuals

Heteromorphic = Appears in different shapes and sizes at different stages of its life cycle

Epilithic = Growing on the surface of rocks or other hard substrata

Epiphytic = Growing on the surface of other plants or algae

Mitosis = Cell division in which the original chromosome number is maintained (normal cell cycle)

Meiosis = Cell division that ultimately results in four cells with only one copy of each chromosome (sexual reproduction)

Haploid = A cell or an organism having a single set of chromosomes (n). A gamete or an asexually reproducing organism is haploid

Diploid = A cell or an organism having paired chromosomes (2n), where one set is inherited from each parent (maternal and paternal parent). A vegetative cell or a sexually reproducing organism is diploid

Gamete = A haploid reproductive cell. Female gametes are called egg cells or ova and male gametes are called sperm

(Porphyra specific terminology)

Zygotospores/zygotosporangia = Proposed to replace the terms carpospore and carposporangia in Bangiales (M. Guiry, 1990)

Foliose/blade phase = The macroscopic gametophyte phase of *Porphyra*, assumed to be haploid (Nelson, Brodie, & Guiry, 1999)

Conchocelis phase = The microscopic sporophyte phase of *Porphyra*, generally assumed to be diploid (Nelson et al., 1999)

1 Introduction

1.1 *Porphyra/Pyropia* production and utilisation

Cultivation of nori (*Porphyra spp., Pyropia spp.)* is the fifth-largest industry in light of aquaculture production of aquatic algae (FAO, 2020). The high market value of this crop makes it among the most valuable cultivated marine crops in the world, with total global sales exceeding 1.5 billion US$ (Levine & Sahoo, 2010). In 2018 the production accounted for 2.8 million tons in live weight (FAO, 2020). The major producing countries are China, Japan and the Republic of Korea (FAO, 2020), accounting for 99.8% of the total world production (Cho & Rhee, 2019). The primary species used in cultivation are *Pyropia yezoensis* (Ueda) M.S. Hwang & H.G. Choi and *Pyropia tenera (*Kjellmann) N. Kikuchi, M. Miyata, M.S. Hwang & H.G. Choi*,* whereby the end product is mainly used as food. Nori is the Japanese name for these algae genera and the name normally used by consumers. They can, however, also be referred to by their many other names, *e.g.* laver (UK, USA, Canada), purple laver (Britain, Ireland), kim (Korea), zicai (China) or karengo *(New Zealand)* (Levine & Sahoo, 2010). The food product Nori is made out of dried *Porphyra*- or *Pyropia* blades processed into thin sheets usually used to wrap sushi rolls. It is commonly used in various traditional Asian cousins, and for many Asian countries, it plays an essential role in the populations diet. However, the growing global awareness of the food products and the health-promoting substances found within nori algae has resulted in increased global consumer demand (Cho & Rhee, 2019). The food value lies primarily in the high level of proteins, vitamins and minerals these algae holds (Blouin et al., 2011). The protein content of nori may range from 25–50% (Levine & Sahoo, 2010). For instance, the commonly cultivated species *P. yezoensis* can reach a protein content of 44% in dry weight (Noda, 1993). In regard to vitamins, nori contains high levels of the essential vitamins C and B (*e.g.* B_{12}). By comparison, the quantities of vitamin C are higher than that found in, *e.g.* oranges (Levine & Sahoo, 2010). Vitamin B_{12} is unique to nori as this vitamin is not found in other eatable seaweeds. Additionally, the exceptional quantities makes it an excellent sea vegetable to include in vegan diets (Cho & Rhee, 2019). Nori also contains a high antioxidant concentration (Zhang et al., 2010), although further research is needed to fully confirm the health benefits from the antioxidant composition (Cho & Rhee, 2019). In addition

to the value nori provide in foods, the pigment r-phycoerythrin is commercially used as a fluorescent “tag” in immunofluorescent diagnostics, flow cytometry, and antibody labelling (Levine & Sahoo, 2010). Furthermore, many species of these algae have a high uptake of dissolved nutrients, *e.g.* different nitrogen- and phosphorus sources, making it an interesting candidate for Integrated Multi-trophic Aquaculture (IMTA) systems (Knoop, Griffin, & Barrento, 2019; Pereira, Kraemer, Yarish, & Sousa-Pinto, 2008; Xiao et al., 2017). Given the high market price, the increasing global demand and the specific properties found within nori algae, there has been increasing attention to investigating non-Asian species, *e.g.* Atlantic species. However, due to the complex lifecycle, the cultivation of non-Asian species has proven difficult and is still in need of further research (Knoop et al., 2019).

1.2 *Porphyra/Pyropia* biology and aquaculture

Porphyra C. Agardh and *Pyropia* J. Agardh, belong to the diverse order Bangiales in the division Rhodophyta, or red algae. Based on the recent revision of this order, some taxa previously organised in the genera *Porphyra* are now recognised to belong to other genera, such as *Pyropia (G. M. Guiry, In Guiry, & Guiry, 2020a, 2020d; Sutherland et al., 2011)*. Therefore, although the classical term *Porphyra* is commonly used in the literature describing the present or historical cultivation techniques of these algae, both genera will, in this cases, be referred to in the present study. Furthermore, in cases where the term *Porphyra* is used on its own, the statement may be true for both *Porphyra*- and *Pyropia* species. According to the most recent taxonomic treatment adopted by Yoon et al. (2017), twelve genera is currently recognised in the Bangialis order with approximately 130 species. They are distributed worldwide, ranging from the Arctic- to Antarctic waters to warm temperate and even tropical seas. However, the centre of diversity is found within the North Pacific. *Porphyra*- and *Pyropia* species thrives in shallow marine environments, predominantly within the littoral zone, in which they are well adapted to the extreme variations this habitat holds. Most species are epilithic and grow on rocks, boulders, pebbles and bedrock in addition to shells or other hard substrates. However, some are strictly epiphytes, growing on the surface of other algae or plants (Broom et al., 2002; Graham, Graham, & Wilcox, 2009; G. M. Guiry et al., 2020a).

1.2.1 Life cycle of *Porphyra*

Porphyra genus has a heteromorphic alternation of generations, meaning that it appears in different shapes and sizes at different life cycle stages. It alters between a haploid gametophytic foliose blade phase (male/female) and a diploid microscopic filamentous sporophyte phase, known as the conchocelis phase. Conchocelis bore into shells or other substrata, and when mature, they produce conchospores that are released into the water column and germinate to form new blades (Figure 1) (Blouin et al., 2011; G. M. Guiry et al., 2020a; Redmond, Green, Yarish, Kim, & Neefus, 2014).

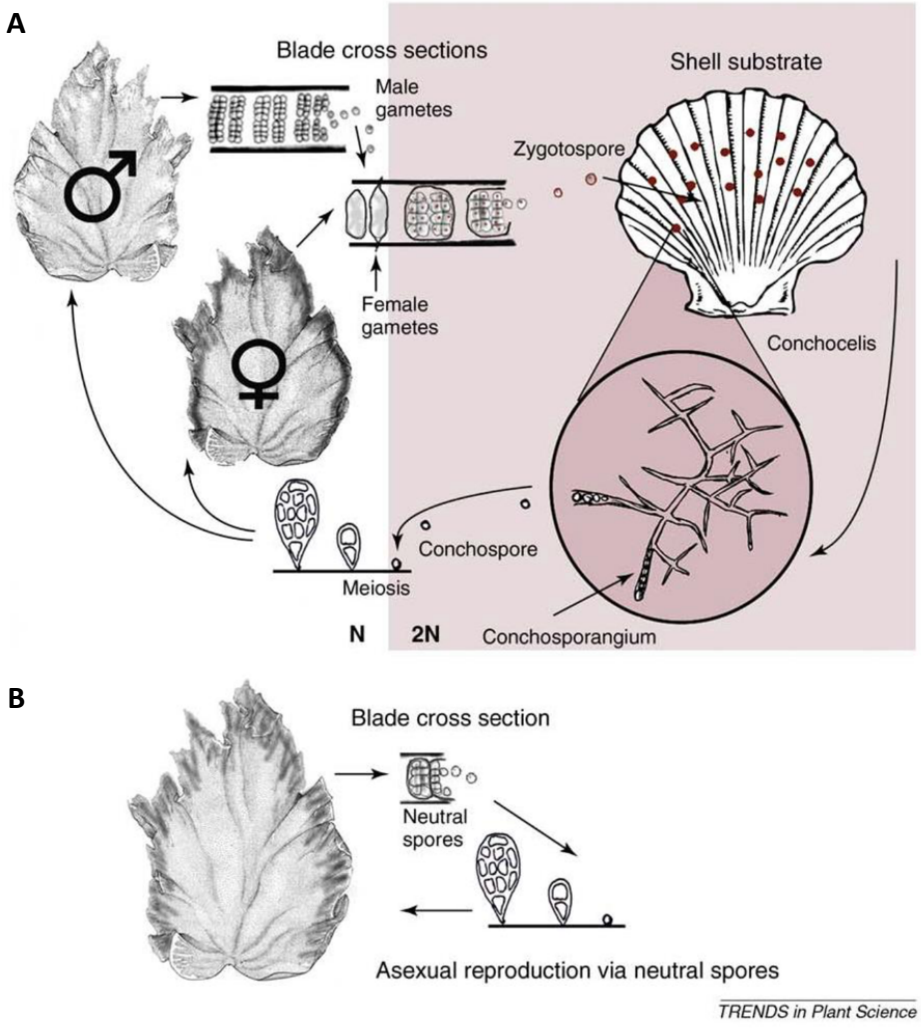

Figure 1 Life cycle of *Porphyra Umbilicalis* Kützing

(A) Sexual reproduction phase and **(B)** asexual reproduction phase by natural spores (Blouin, Brodie, Grossman, Xu, & Brawley, 2011).

Both sexual (n) and asexual (2n) reproduction are exhibited by the various species of *Porphyra (Nelson et al., 1999; Sahoo, Tang, & Yarish, 2002)*, and the diversity within reproductive modes is remarkable (Nelson et al., 1999).

In sexual reproduction (Figure 1 A), fertilised eggs from the haploid gametophyte undergo cell division through mitosis resulting in a zygotosporangium with mature zygotospores (G. M. Guiry et al., 2020a; Redmond et al., 2014; Sahoo et al., 2002). After fertilization, mature zygotospores are released into the water column. When settled on shells or similar calcareous substrata, these mature zygotospores usually germinate unipolarly to form filamentous conchocelis; the diploid sporophyte phase of *Porphyra (G. M. Guiry et al., 2020a; Mitman, 1991; Sahoo et al., 2002)*. Conchocelis bore into the calcareous substrata and can be visualised as a red, brown or pink colouration on the shell or substrata. It can also survive on a non-calcareous substrate where it appears epilithic (Mitman, 1991; Redmond et al., 2014). When conditions are favourable, conchocelis develop into mature conchosporangium branches where cell division through meiosis occurs, resulting in haploid conchospores being released into the water (Redmond et al., 2014; Sahoo et al., 2002). When conchospores settle on various substrata, typically rocks (epilithic) but also other algae (epiphytic), they germinate into new haploid gametophytes, thus fulfilling the life cycle (G. M. Guiry et al., 2020a; Redmond et al., 2014).

There is a high diversity within asexual reproductive modes in the *Porphyra* genus (Nelson et al., 1999), but overall, it occurs through asexual blade phase spores such as agamospores or natural spores (Figure 1 B) or various differentiations of vegetative cells, endosporangia or endospores (Nelson et al., 1999; Sahoo et al., 2002).

1.2.2 The beginning of *Porphyra/Pyropia* aquaculture

Cultivation of *Porph*yra and *Pyropia* species began long before the modern aquaculture industry. The early state of cultivation was implemented by fishermen that, over time, accumulated knowledge of the approximate time and place of *Porphyra/Pyropia* blade appearance in nature. Based on this knowledge, they found that the annual harvest could be increased by simply increasing the area of appropriate hard substrata in the shoreline for *Porphyra/Pyropia* blades to settle on (Blouin et al., 2011). In the late 16th and early 17th

century, this knowledge was further developed, and more professional practises for cultivation started to evolve in Japan, Korea and China. Thereof the "Bamboo-Hibi planting" technique in Japan and Korea, where bamboo stakes were placed into soft sediment in shallow areas along the shore. Another practise started to evolve in China, known as the Chinese "cleaning" technique, where lime was used to remove epiphytes from rocks previous to *Porphyra/Pyropia* blade appearance (Blouin et al., 2011; Sahoo & Yarish, 2005). However, as the life cycle was not yet understood, large annual fluctuations in *Porphyra/Pyropia* blade appearance made cultivation unpredictable (Blouin et al., 2011). In 1949, Kathleen Drew discovered that *Conchocelis rosea* is, in fact, the sporophyte phase (hence "Conchocelis") of *Porphyra* rather than being a separate species (Drew, 1949). This discovery provided a deeper understanding of the *Porphyra* life cycle. Thus the development of mass cultivation techniques was made possible. Together with the rapid progress in controlling the life cycle of *Porphyra* (Hence, also *Pyropia*) for commercial cultivation that followed, this discovery marked the beginning of the *Porphyra/Pyropia* aquaculture industry (Blouin et al., 2011). Japanese and Chinese researchers constituted an essential role in this rapid development (FAO, 2009), although a parallel effort was made in Korea (Blouin et al., 2011). The key aspects of the new industry were within; (1) development of greenhouses for conchocelis cultivation on shell substrate, (2) seeding of conchospores onto nets, outplanting of nets into the sea, (3) harvesting of *Porphyra/Pyropia* blades from nets, (4) and development of specific equipment for making nori sheets (Blouin et al., 2011).

1.2.3 *Porphyra/Pyropia* cultivation today

The methods currently used to cultivate *Porphyra* or *Pyropia* are similar among countries with minor modifications associated with adaptations to local growing conditions and the different local traditional practises used among farmers. In Asia, the complex process of cultivating conchocelis is well managed. The conchocelis cultivation starts in mid-spring (March/April), corresponding to the appearance in nature. Calcareous shells are traditionally used as substrata for the conchocelis to grow on. That being said, there has been an increasing use of artificial substrata. In Japan, for instance, vinyl films covered with calcite granules are used to allow algal settlement. Calcareous shells or artificial shells are organised in the bottom of shallow tanks and covered by 20–30cm of seawater (Figure 2 A). Zygotospores from fertile

Porphyra- or *Pyropia* gametophytes are introduced into the tanks to settle on the corresponding substrate. This introduction can be accomplished by introducing chopped material of fertile gametophytes into the tanks directly, which then are removed after spore settlement on shells. An alternative method used is by introducing only a suspension of zygotospores into the tanks. The release of zygotospores can be artificially induced by exposing the gametophytes to stress in the form of either air drying or grinding. Zygotospores evolve into filamentous conchocelis that bore into the shell substratum (Figure 2 B). During cultivation, the shells may be organised to hang vertically in the water or kept at the bottom of the tanks (Sahoo et al., 2002; Sahoo & Yarish, 2005). Regulation of growth during the conchocelis phase of the cultivation is a complex process where temperature and light are carefully regulated throughout the cultivation period.

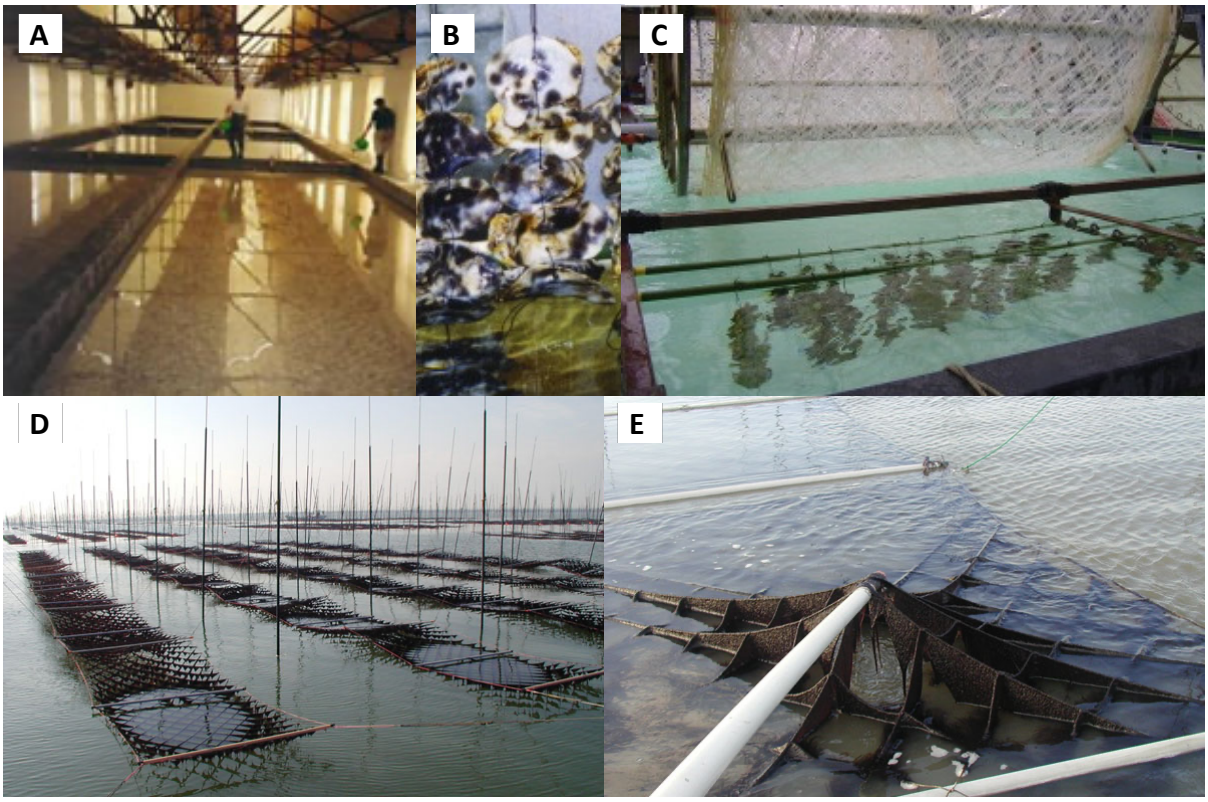

Figure 2 Examples from *Porphyra/Pyropia* cultivation.

(A) Cultivation facility for conchocelis. Conchocelis growing on shells organised in shallow basins (FAO, 2009).
(B) Conchocelis *P. yezoensis* can be seen as dark patches growing on oyster shells, organised to hang vertically in a basin (Blouin et al., 2011).
(C) Seeding of conchocelis onto nori nets. Net is wound onto a rotating paddle wheel and submerged into the waterbody, where shells hang vertically in the basin (Blouin et al., 2011).
(D, E) Cultivation of *Porphyra/Pyropia* gametophytes out in the sea. Ariake Sea, Saga, Japan (Shun Gate., 2015).

Overall, conchocelis is cultivated during gradually raising temperatures, and sporulation is induced through decreasing temperatures. (FAO, 2009). The mass discharge of conchospores can be promoted in different ways*, e.g.* stirring through compressed air bubbles, low-temperature seawater, or exposure to fresh seawater and vitamin B_{12}. Further on in the cultivation, conchospores are seeded onto nets (Figure 2 C). A standard procedure for net seeding includes assembling shells with conchocelis within tanks, either in mash bags or hanged vertically in the waterbody. Seeding nets are then attached to a rotating paddle wheel that is submerged into the water. This system allows conchospores to float to the surface and attach to the seeding net. In Asia, this procedure is typically done in autumn when water temperatures are 23–24°C. After seeding, nets are placed out in the sea for nursery cultivation and carefully monitored for blade development (Figure 1 D, E) (Sahoo et al., 2002; Sahoo & Yarish, 2005).

1.3 An area for innovation within the existing cultivation method

To this day, no simple precise methods for quantitative measurements of conchocelis growth within industrial *Porphyra* (or *Pyropia*) cultivation exists. Commonly, the growth development of conchocelis is regulated through visual observations, whereby the change in colouration of shells is used to indicate the level of conchocelis growth. These observations are often made as rough estimates by farmers. Observations may often be supported by microscopy sampling, whereby growth on shells is investigated under a microscope. However, this latter method is time-consuming and labour intensive (Personal communication, C. Bruckner).

Environmental conditions such as light and temperature are carefully regulated to optimise conchocelis growth. Measures of growth development throughout the cultivation provide an estimate of timing, *e.g.* the optimal timing for regulation of environmental conditions, and predictability, to make estimations for later cultivations steps*, e.g.* to estimate the conchospore release or to estimate the amount of seeding nets needed in nursery cultivation of gametophytes into the sea (FAO, 2009). The existing practices for evaluating conchocelis growth provide a rough estimate of the growth development. The development of a quantitative method for a more accurate and objective estimation of conchocelis growth would, however, provide a higher level of control. Such a method may therefore serve to increase efficiency at the early stages of industrial *Porphyra* production.

1.3.1 Pigmentation and colour in red algae, emphasising on *Porphyra spp*.

Although a wide range of pigments is found in red algae; chlorophylls, phycobiliproteins and carotenoids, the phycobiliproteins are the primary antenna pigments (Dumay & Morançais, 2016). They are water-soluble pigment-protein complexes that in red algae are arranged to form phycobilisomes (Figure 3). The phycobiliprotein phycoerythrin is the main pigment responsible for the red colouration present in most red algae (Graham et al., 2009). In *Porphyra*, as conchocelis grow on shell substrate, the phycobiliproteins in the conchcocelis may causes the shell to alter colouration from natural white to a lighter- or darker shade of red. This colouration can be visualised as light pink, red, brown or even blackish in high phycoerythrin consentrations. Phycobiliproteins, together with carotenoids, are accessory pigments. This means that they pass the absorbed energy to chlorophyll. Chlorophylls are the major pigments, particularly chlorophyll *a*, transmitting the energy to photosystem II (PS II) reaction centre where the energy reaction of photosynthesis occur (Dumay & Morançais, 2016).

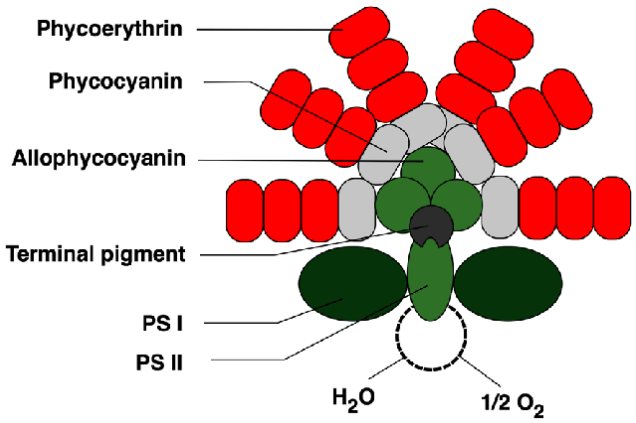

Figure 3 Schematic structure of pigments in Phycobilisome

The phycobiliproteins phycoerythrin, phycocyanin and allophycocyanin capture light energy at their respective wavelength and transfer this energy efficiently to the terminal pigment chlorophyll a. The energy is then transmitting to the photosystem II (PS II) reaction centre, where the energy reaction of photosynthesis occurs. The Phycobilisome, or the antenna complex, is anchored to the thylakoid membrane within the plastid of the cell (Alam, 2019).

The content of light harvesting pigments can be adjusted as a physiological response to environmental changes. For the conchocelis stage of *Porphyra*, limited amount of research has investigated how environmental changes affect the content of different light harvesting pigments. Lin and Stekoll (2011) have found that the content of phycobiliprotein are affected by irradiance, nutrients and culture duration.

1.3.2 RGB-based image analysis

This alteration in the colour shade caused by conchcoelis growth and the phycobiliproteins that conchcoelis holds may be detectable through RGB-based image analysing methods. Image based methods to quantify growth development in industrial production is widely investigated in related fields of study, *e.g.* agriculture (Chen et al., 2020; Hu, Zhang, Sun, & Zhang, 2013; Kawashima & Nakatani, 1998; Mohan & Gupta, 2019). The red, green and blue (RGB) colour model is the most commonly used colour representation for digital colour images (Chen et al., 2020), and several studies have suggested this model to successfully measure the chlorophyll content in higher plants (Hu et al., 2013; Mohan & Gupta, 2019; Yadav, Ibaraki, & Gupta, 2010). The motivation for this research focus has been towards developing a none destructive method that is low labour intensive and time-consuming to evaluate plant growth and health during cultivation (Hu et al., 2013; Kawashima & Nakatani, 1998; Rigon, Capuani, Fernandes, & Guimarães, 2016). However, to the extent of my knowledge, no other research has investigated the use of an image analysing method based on RGB features to measure growth in conchocelis.

The RGB colour model provides information based on the colour intensity of the primary colours red (R), green (G) and blue (B) within an image. In this model, numbers ranging from 0–255 are used to express the intensity of R-, G- and B light within images, thereby allowing for a numeric presentation of the colours that the image holds. The value zero equals no light and the value 255 states full light intensity. Pure red light will therefore have the numeric value R = 255, G = 0, B = 0. The same system accounts for pure green light (R=0, G=255, B=0) and pure blue light (R=0, G=0, B=255). When R, G and B are all zero (0,0,0), this value equals pure black light, and when R, G and B are all at 255 (255,255,255), pure white light is shown (Değirmenci, 2017).

1.4 Study aims and objectives

The work included in the present study contributes to the first step towards developing a quantitative method of measuring conchocelis growth to increase efficiency within the early stages of industrial *Porphyra* cultivation. *Porphyra dioica* and *Porphyra purpurea* conchocelis growing on bivalve shells successfully alter the colour of the shell material from white to red, pink or brown shaded.

The present study aims to investigate if R-, G-, and B values acquired from conchocelis images could be used as a non-invasive and low labour technique to quantify conchocelis growth for industrial *Porphyra* cultivation purposes. Quantification of conchocelis growth, even as a rather rough estimate, may increase efficiency at the early stages of industrial *Porphyra* production.

The following sub-objectives were formulated:

- Test if the alteration in R-, G- and B values measured by RGB-based image analysis is a suitable method for conchocelis growth assessment
- Study the possible correlation between chlorophyll *a* content in conchocelis and R-, G- and B values measured by RGB-based image analysis of conchocelis.

2 Material and Methods

2.1 Sample collection and preparation

Bivalve shell material of the species *Arctica islandica* Linnaeus was collected from sandy beaches within Bodø and Gildeskål municipality, Norway, in August 2020 (Appendix A).
A. islandica was observed as a dominant bivalve mollusc within this area and found close to wild *Porphyra.* Based on these observations, it was concluded that *A. islandica* may be a suitable substrate for *Porphyra* conchocelis cultivation. After collection, the shells were transported in a plastic container to PolarAlge AS, a seaweed cultivation facility located in Skjellvik, Sandhornøy (Gildeskål municipality). Once in the cultivation facility, shells were rinsed and cleaned to eliminate any possible epiphytic organism and avoid species contamination. Rinsing and cleaning of shells were performed through exposure to hot water (~ 70°C) for a minimum of 5 minutes and manually cleaning using a sponge/brush. Finally, shells were left to dry.

Fertile *Porphyra dioica* Brodie & L. Irvine and *Porphyra purpurea* (Roth) C. Agardh gametophytes were collected from the lower intertidal zone in Løpsvika, Bodø, on the 6th Of October 2020 (Appendix A). Species were identified by morphology. Both species have previously been identified at this given location by DNA sequencing carried out by Belghit et al. (2017). Before collection of gametophytes, the presence of reproductive tissue such as patches or areas containing gametes was inspected regularly, using a stereo loupe VisiScope® ZBL350, VWR with a magnification of four-folds. The following reproductive characteristics were used to identify species and fertility:

1. In the dioecious *P. dioica*, fertile material shows male gametes as cream straw-yellow in colour (Figure 4 B) and unfertilised female gametes as cream- to pale green, appearing red to dark red once fertilised (Figure 4. D). Reproductive tissue is marginal and appears red in fertile female individuals and pale straw-yellow in male individuals (Figure 4 A, C).
2. In the monoecious *P. purpurea*, fertile material shows male pale-yellow gametes and female dark red gametes to create a vertical line segregating the male and female part

of the thallus (Figure 5 A, B). This line being distinct is an indication of fertility as it appears less evident in non-fertile individuals.

3. For both *P. dioica* and *P. purpurea*, zygotospores form red circular cells (Figure 5 C). The presence of such zygotospores on top of female individuals or female parts of the thallus indicates fertile material (Figure 5 B, C) (G. M. Guiry, In Guiry, & Guiry, 2020b, 2020c; Holmes & Brodie, 2004; Redmond et al., 2014).

Approximately 4 kg wet biomass of *Porphyra* gametophytes were collected and transported to the cultivation facility in a plastic bag. Once in the cultivation facility, thalli were rinsed several times in seawater, and visible epiphytes where manually removed. Simultaneously, each individual was identified and sorted by species. Species identification was carried out based on fertility characteristics identified by the eye, hence the marginal colour on the dioecious *P. dioica* and the presence of a sharp vertical line on the monoecious *P. purpurea* (Figure 4 A, C and Figure 5 A). Before further handling, gametophytes were kept in a 90 L bucket filled with seawater. Material stored overnight was kept moist without additional water. All material was rinsed and sorted within 30 hours after the time of collection.

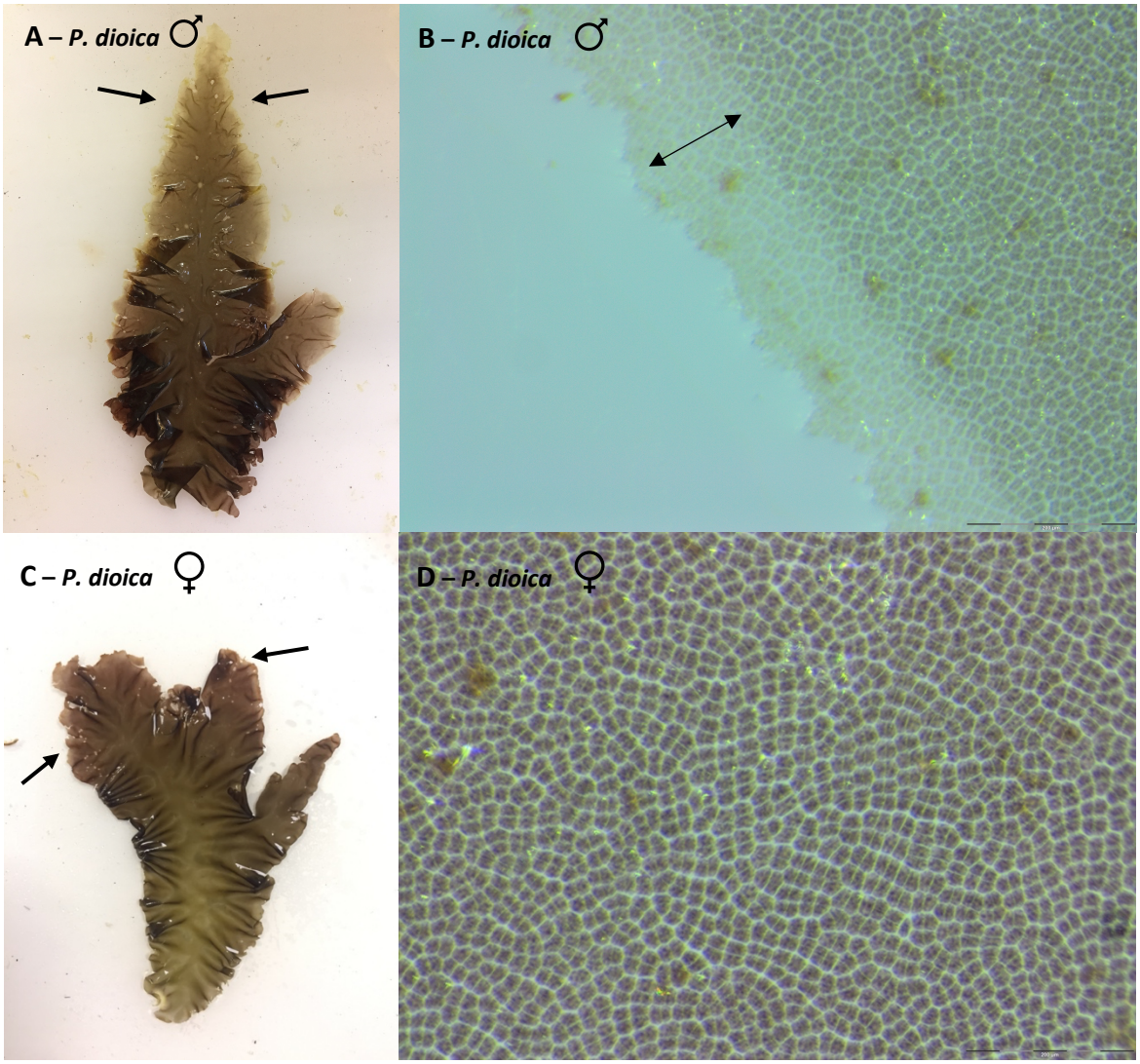

Figure 4 *Porphyra dioica*, fertility and species identification

Morphological characteristics and reproductive structures used for fertility and species identification.

(A, B) *P. dioica*, male individual. **(A)** pale-yellow margins on both sides of the tip of the blade indicating a male individual (See arrows). **(B)** Magnified image captured from the very edge of the blade showing male gametes as white or pale-yellow in colour, creating a pale-yellow or white margin along the edge of the blade (Se area in between arrows)

(C, D) *P. dioica*, female individual. **(C)** Red margins on both sides of the tip of the blade are indicating a female individual. **(D)** A Magnified image captured from the edge of the blade shows female gametes appearing red in colour.

(B, D) Images are captured using an Olympus SZX16 stereo loupe with an Olympus Soft Imaging camera, model:SC100. Scale bare shows 200 µm.

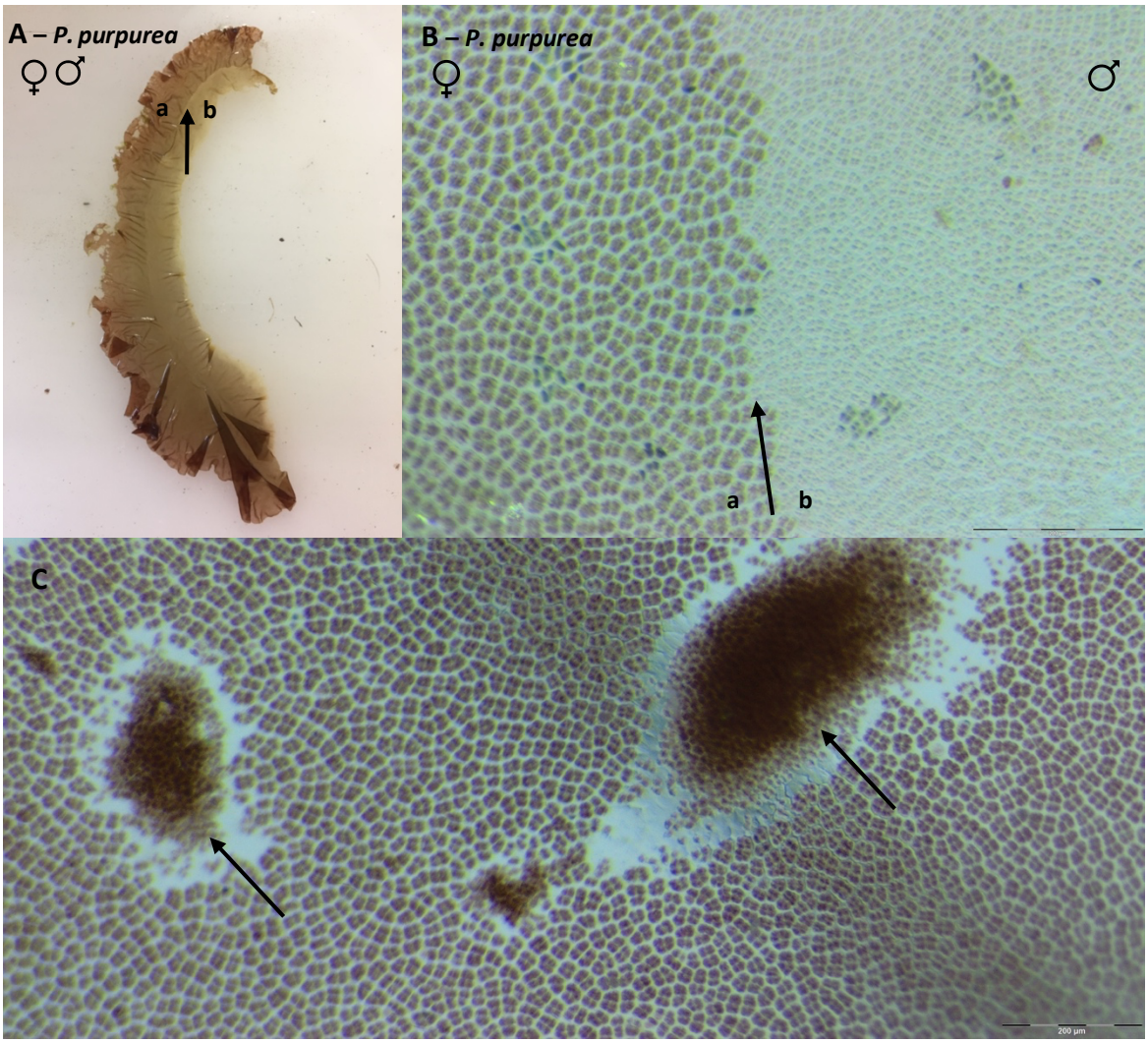

Figure 5 *Porphyra purpurea*, fertility and species identification

Morphological characteristics and reproductive structures used for fertility and species identification.

(A*)* *P. purpurea* individual, defined vertical line (indicated by arrow) segregating the female ((a) red in colour) and male ((b) pale-yellow in colour) part of the blade. **(B)** Magnified image show female (a) gametes as red in colour and noticeably larger in size than the male (b) gametes that are white or pale-yellow in colour. The vertical line is shown by an arrow.

(C) A collection of zygotospores (see arrows) in a *P. purpurea* individual. Each zygotospore is circular in shape and red in colour. The collection of zygotospores is located next to female gametes.

(B, D) Images are captured using an Olympus SZX16 stereo loupe with an Olympus Soft Imaging camera, model:SC100. Scale bare shows 200 µm.

2.2 Experimental cultivation set-up

Cultivation was carried out at a water temperature of 8–12^{o}C with a light intensity varying from 10–18 µmol photons m^{-2} s^{-1} (14 $\pm$ 4), measured from the water surface. Light–dark cycle was set to 10:14 h. Light sources used in the experiment were cool/warm white fluorescent light. The shells were organised to cover the bottom of four aquaculture tanks (each 2 x 2 x 0.5 m) in one layer to avoid overlapping. They were further arranged with the inside facing upwards since this arrangement is optimal for conchocelis growth (Figure 6 A) (Personal communication, Prof. Duan Delin, IOCAS, China). All shells were covered by seawater, and the water level was 5–10 cm above the shells (Appendix A). The seawater used in the experiment was filtered through a 5 µm filter. After the arrangement of shells, fertile gametophytes were introduced into the tanks. Zygotospores could then be released from the gametophytes and settle on available shell substrate (Figure 6 C, D). During collection of gametophytes, a large amount of *P. dioica* and a limited amount of *P. purpurea* were found. Therefore, *P. dioica* gametophytes were introduced into three of the four tanks (T1–T3: ~ 80–90 g dry weight in each tank), and *P. purpurea* gametophytes were introduced into the fourth tank (T4: 26 g dry weight). Weigh of gametophytes were measured as wet weight. The gametophytes were collected in mash bags, and water was squeezed out before weighing (Appendix B). Gametophytes were incubated in the cultivation set-up for 10–13 days. Within this duration of time, successful germination of zygotospores and formation of filamentous conchocelis was observed on most shells (Figure 7 A, B). Germination of zygotospores was verified using a stereo loupe (VisiScope® ZBL350, VWR) (Figure 6, B). After the gametophytes were removed from tanks, the water level was adjusted to ~ 5 cm above shells. The total incubation period for conchocelis was 74 days (Figure 6 E, F). During incubation, water quality was maintained through stirring of water every 2–3 days and regularly exchanging 10–40% of the water body every 1–2 weeks.

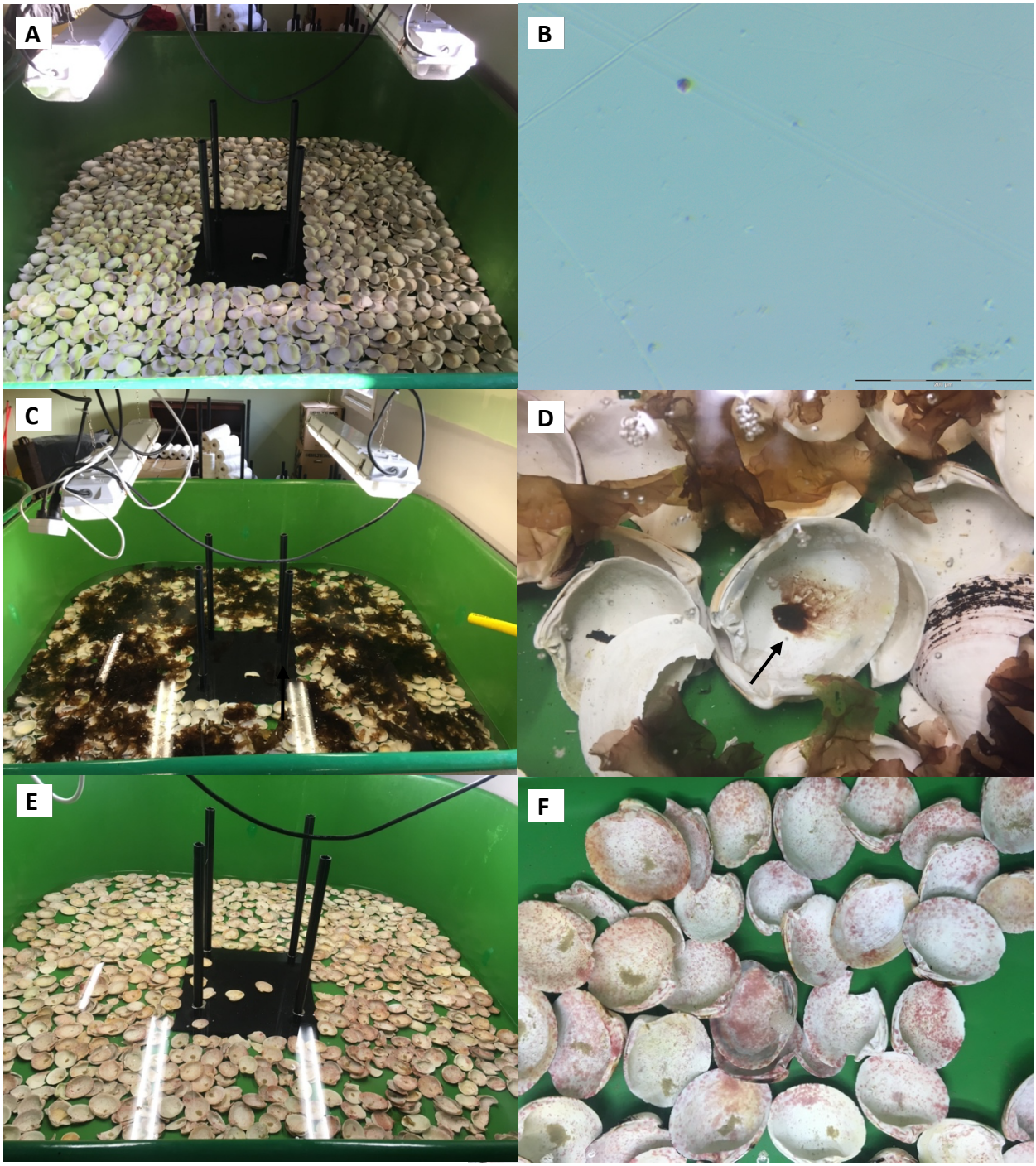

Figure 6 Experimental cultivation set-up

(A) Arrangement of shells in aquaculture tank (2 x 2 x 0.5 m). Day 0, Tank nr 1.
(B) Zygotospore, image is captured using an Olympus SZX16 stereo loupe with an Olympus Soft Imaging camera, model:SC100. Scale bare shows 200 µm.
(C) *Porphyra* gametophytes together with shells during spore release. Day 1–13, Tank 1, *P. dioica*, 17 th of October 2020.
(D) Accumulation of zygotospores on shell substratum (see arrow).
(E) Cultivation of conchocelis in tank. Day 13–74, Tank 1, *P. dioica*, 7th of December 2020.
(F) Conchocelis as pink colouration on shells. Image is captured from shells in a cultivation tank. Tank 1, *P. dioica*, 7th of December 2020.

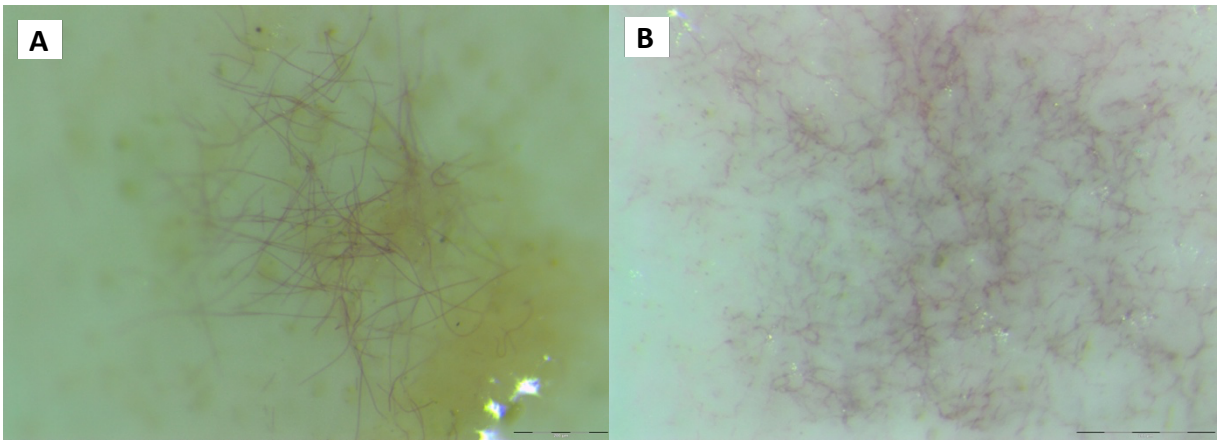

Figure 7 Conchocelis Filaments

(A) Filamentous conchocelis growing on top of a shell (*Porphyra* spp.).
(B) Filamentous conchocelis has bored into shell substratum and grows inside the shell (*Porphyra* spp.).
Images are captured using an Olympus SZX16 stereo loupe with an Olympus Soft Imaging camera, model:SC100. Scale bare shows 200 µm.

2.3 Imaging of conchocelis

The conchocelis development on shell substratum was captured through imaging. An 8-megapixel iSight camera with 1.5 µ pixels from a Phone 6 smartphone was used to take the pictures. The smartphone was placed in a tripod (Dacota platinum selfie stick tripod) and orientated in a vertical position, with the aperture at a fixed distance of 16 cm from a white background. The shell was placed in the centre of the image, orientated with the inside facing upwards and put in focus. For camera settings, the flash was turned on. A remote control connected to the smartphone camera was used to capture the images, thereby avoiding interference of unregular shade.

Two separate approaches were used in the selection of shells for imaging:

1. The first approach aimed to collect pictures of conchocelis over a given duration of time. Among all the shells used in the experimental cultivation set-up, 40 shells were selected for imaging, hence ten shells from each Tank (T1–T3: *P. dioica*, T4: *P. purpurea*). The selection of shells was performed before the incubation of *Porphyra* thalli, and shells with natural white colourisation and minimal structure were explicitly chosen. Since shells are used as substrate, these features are ideal when distinguishing colourisation made by conchocelis from natural shell colour shade. Selected shells were organised in one corner of their given tanks to facilitate

accessibility. Pictures of conchocelis on shell substratum were obtained every 2–3 days for a total of 74 days, starting from day ten. Throughout the cultivation, the presence/absence of conchocelis on shells was investigated using a stereo loupe (VisiScope® ZBL350, VWR). For some shells, the development of conchocelis seemed to be absent. If conchocelis was not observed by day 20, the shell was removed and exchanged for a shell with visible conchocelis growth. New shells were collected among the additional shells within that specific tank. Therefore, regardless of the imaging starting point, all shells within their respective tanks were exposed to the same conditions and fertile gametophyte exposure time.

2. The second approach aimed to collect pictures of different stages in the conchocelis growth development captured at one timepoint. Imaging and chlorophyll *a* assessment were performed on these shells to investigate the relationship between R, G, B and chlorophyll *a* content (See also section 2.4 "Determination of chlorophyll a content"). Fifteen shells were acquired from the experimental cultivation set-up at the end of the cultivation period, on day number 58. For each tank, shells were selected by the level of conchocelis development, ranging from low to high. Thereby, seven different levels of conchocelis coverage were captured from each tank. Seven of the shells were from *P. dioica* culture (Tank T1), seven from *P. purpurea* culture (Tank T4), and one shell that was not exposed to conchocelis. The latter was included as a reference for natural shell colour shade.

2.4 Determination of chlorophyll *a* content

The procedures of chlorophyll *a* measurements were performed on shells, one by one. First, a picture of a shell with conchocelis was taken as described in section 2.3 "Imaging of conchocelis". Immediately after the picture was taken, the shell was shattered into small pieces, placed in a 50 ml tube, then covered in Methanol (CH_3OH, anhydrous 99.8%, Sigma-Aldrich) (Johan, Jafri, Lim, & Wan Maznah, 2014). In total, approximately 0.6 L methanol was used in the experiment. After collection of samples, they were placed in a -20°C freezer for storage.

In the following step, the samples containing the methanol-chlorophyll extract were taken out of the freezer to quantify chlorophyll *a* content. Chlorophyll *a* content in the sample extracts was measured according to the 90% acetone method described by Jeffrey and Humphrey (1975). Briefly; for one sample at a time, 0.3 ml of the extract was mixed with 2.7 ml acetone (CH_3COCH_3, for HPLC, ≥ 99.9%, Sigma-Aldrich). These samples, consisting of 10% extract and 90% acetone, were then transferred to a quartz cuvette, where the absorbance was measured at wavelengths of 750, 664, 647 and 630 using a GENESYS 10S UV Vis spectrophotometer (Thermo Scientific) as described by Jeffrey and Humphrey (1975).

The wavelength 664, 647 and 630 is in the spectral peak absorbance of chlorophyll *a*. The measured value at wavelength 750 was subtracted from the these three wavelengths to correct for turbidity (Jeffrey & Humphrey, 1975; Johan et al., 2014):

$$750\ nm - 664\ nm = \text{Value of absorbance at wavelength } 664 \text{ nm} = E_{664}$$

$$750\ nm - 647\ nm = \text{Value of absorbance at wavelength } 647 \text{ nm} = E_{647}$$

$$750\ nm - 630\ nm = \text{Value of absorbance at wavelength } 630 \text{ nm} = E_{630}$$

Acetone (100%) was used as a blank/reference value and for calibration. The calculation described by Jeffrey and Humphrey (1975) was applied to measure the weight of chlorophyll *a* in a quarts cuvette:

$$Chl\ a\ (\mu g/ml) = (11.85xE_{664}) - (1.54xE_{647}) - (0.08xE_{630})$$

After successfully measuring chlorophyll *a*, the weight of each sample was determined using an analytical balance (Sartorius CP 124S). Shells were dried for one week before weighing in order for methanol to evaporate completely. Weight of chlorophyll *a* per gram shell material was quantified with the formula:

$$\mu g\ Chl\ a\ per\ gram\ shell\ = (Chl\ a\ (\mu g/ml)\ x\ V(ml) x 10)/S(g)$$

In which:

Chl *a* = Chlorophyll *a*

V = Volume of sample with methanol-chlorophyll extract in ml

S = Weight of shell fragments in gram

10 = Factor used to account for dilution of methanol-chlorophyll extract into 90% acetone sample (10% methanol chlorophyll extract and 90% acetone).

2.5 Observations on conchocelis growth

Development of conchocelis on shell substratum was visually observed as a qualitative measure of conchocelis growth throughout the experimental period. A stereo loupe was used to confirm the presence of conchocelis structures not visible by the eye. The images captured throughout the cultivation period from day ten untilled day 74 were used as a basis for visual growth measurements. For all 40 shells, pictures for the following days: 10, 20, 28, 35, 41, 48, 55 and 74, were organised by shell to get an impression of the conchocelis growth throughout the experimental period. Growth was observed as overall growth within each tank separately.

2.6 Histogram analysis and data preparation

After images were captured, they were transferred to a computer and analysed in ImageJ software. The colour histogram function in ImageJ was used to obtain mean brightness values of the primary colours R, G and B. The entire image was used when extracting R-, G- and B values, including background.

For R-, G- and B values collected over time (from pictures of 40 shells collected regularly for 74 days), the pictures from day ten were used as a reference for natural shell colour shade. R-, G-, B values for day ten was subtracted from R-, G-, B values for all days within their respective shell series. The following calculations were performed on the data for each of the 40 shell series for R values, G values and B values:

$$d(t_x) = (t_x) - (t_{10})$$

In which (shown by R value):

$d(t_x)$ = Change in R value for day x (x= 10, 13, 20… 74)

t_x = R value measured on day x (x= 10, 13, 20… 74)

t_{10} = R value measured on day 10

2.7 Statistical analysis

All statistical analyses were conducted with the software RStudio 1.2. Normality and homogeneity were ensured using the function “Fitdist”, revealing parametric data.

The R-,G-, and B features were analysed individually in all analysis, hence “Change in R”, “Chance in G”, and “Change in B”. Two-way repeated measure ANOVAs were conducted to test the effect of cultivation time (a total of 74 days) and tanks (Tank T1, T2, T3 and T4) on R-, G-, and B values from images of shells with conchocelis growth. The two-way repeated measure ANOVA is an analysis of variance, which tests for the effect of two independent variables against one dependent variable consisting of repeated measurements from the same individuals. A Likelihood ratio (LR)(Knuth, 1981) test was performed to test the significance of the factors “tank” and “time” on the growth. The LR test compares two models with and without a random effect to test wheatear there is a significant contribution to the results (Bolker et al., 2009). When cultivation time was found to affect any of the RGB features significantly, the relationship between R, G, B and cultivation time was analysed by the Pearson correlation coefficient test. When tanks were found to affect any of the RGB features significantly, a post hoc Student-Newman-Keuls (SNK)(Keuls, 1952) test was performed to compare tanks.

When investigating the relationship between the two continuous variables RGB-values (R, G and B) and chlorophyll *a*, the Pearson correlation coefficient test was performed to investigate if a correlation could be detected. The analysis was performed on the combined dataset for both species*; P. dioica* and *P. purpurea.*

3 Results

3.1 The effect of time and tank on the change in R, G and B

To test the method of measuring conchocelis growth, the effect of time and tank on RGB features was investigated for images from 40 shells with conchocelis growth captured regularly throughout their cultivation period of 74 days. The two-way repeated measure ANOVA shows a significant effect of cultivation time on all tree RGB features (Change in R: LRT = 28.2, $p < 0.001$, Change in G: LRT = 32.4, $p < 0.001$, Change in B: LRT = 38.3, $p < 0.001$). This effect is visualised in figure 8 A-F. The relationship between time and RGB features is further shown to have a positive correlation for all RGB features (Change in R: $r = 0.27$, $p < 0.001$, Change in G: $r = 0.27$, $p < 0.001$, Change in B: $r = 0.28$ $p < 0.001$). The correlation line illustrates this in figure 8 A, C and E. These results thereby shows that RGB features, presented by "changes in R", "Change in G" and "Change in B", increases during the 74 days of conchocelis cultivation. Throughout this total positive increase, smaller occurrences of increases and decreases can be visualised in Figure 8 B, D and F.

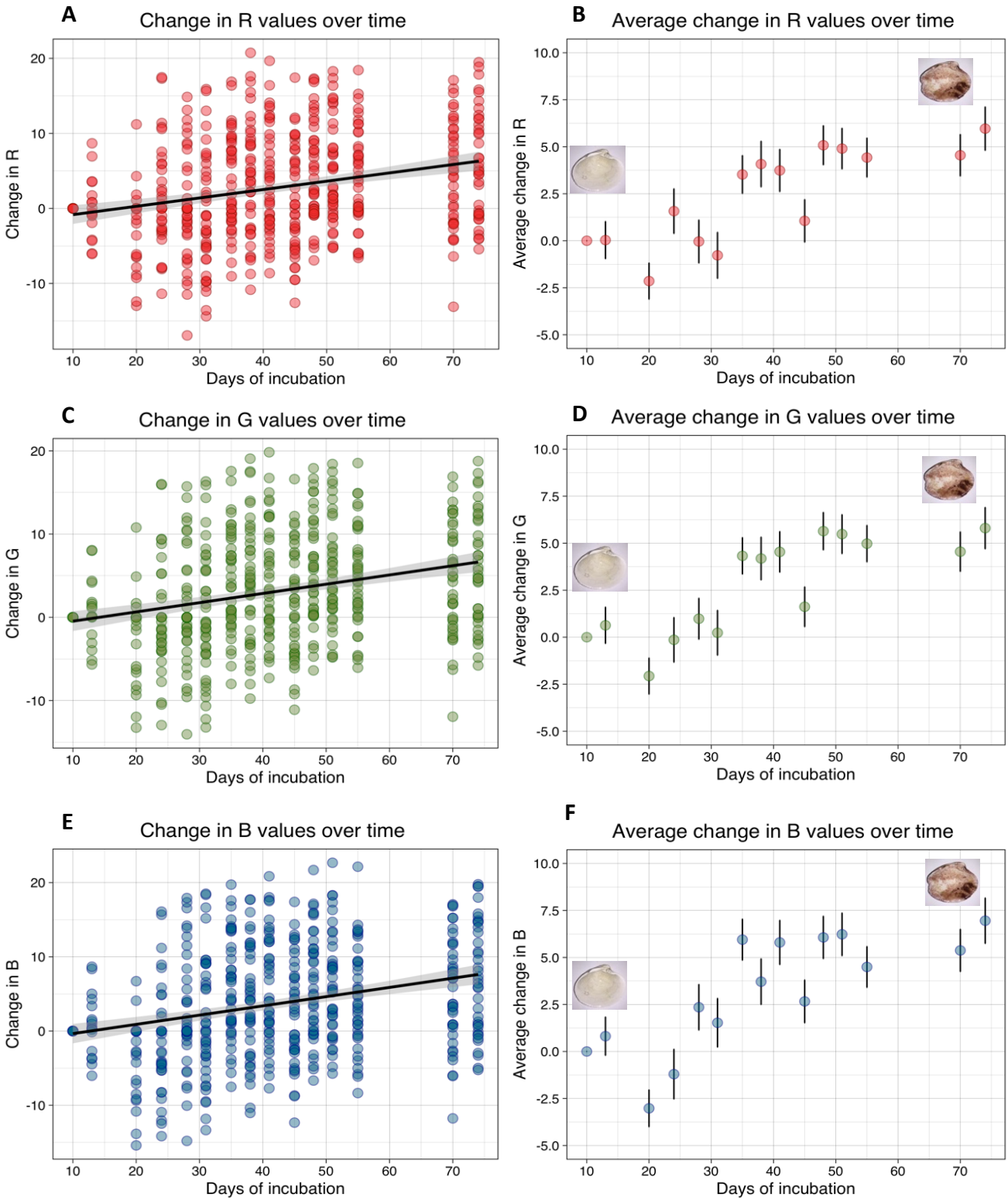

Figure 8 Change in R-, G-, and B values over time

(A, C, E) The change in R-, G-, and B values for all four tanks collectedly during the time of cultivation. The correlation line is presented using a Pearson correlation coefficient analysis, Df = 528.
(B, D, F) The average change in R-, G-, and B values for all four tanks collectedly during the time of cultivation, starting from day ten and until day 74. The standard errors are presented using error bars. Conchocelis growth is represented by images of shells collected from the experimental cultivation set up on day ten and day 74.

The 40 shells used for imaging were cultivated in four separate tanks, where tank T1–T3 contained *P. dioica* and tank T4 contained *P. Purpurea*. The main effect of tanks was therefore investigated. The two-way repeated measure ANOVA shows a significant effect of tanks for all RGB features (Change in R: LRT = 14.2, $p < 0.001$, Change in G: LRT = 8.8, $p < 0.01$, Change in B: LRT = 17.5, $p < 0.001$). These results shows that the development in RGB features, presented by "changes in R", "Change in G", and "Change in B", were different among tanks. A Student-Newman-Keuls (SNK) test further revealed which tanks significantly differed from each other. The significant difference between tanks is illustrated in figure 9 A-C, where tanks with the same letters (a–c) are not statistically different.

For all RGB features, the highest means were found in tank T4 (*P. purpurea*), closely followed by tank T1, and then tank T3 and tank T2, respectively (T1–T3: *P. dioica*) (Figure 9). The mean of tank T4 was significantly higher than that of tank T2 and tank T3 for all RGB features. For B value, tanks T1 and T4 were significantly higher than tanks T2 and T3 (Figure 9 C). For R values, the mean in tank T4 was significantly higher than the mean value in tank T1, and the mean in tank T1 was significantly higher than the mean value in tank T3 (Figure 9A).

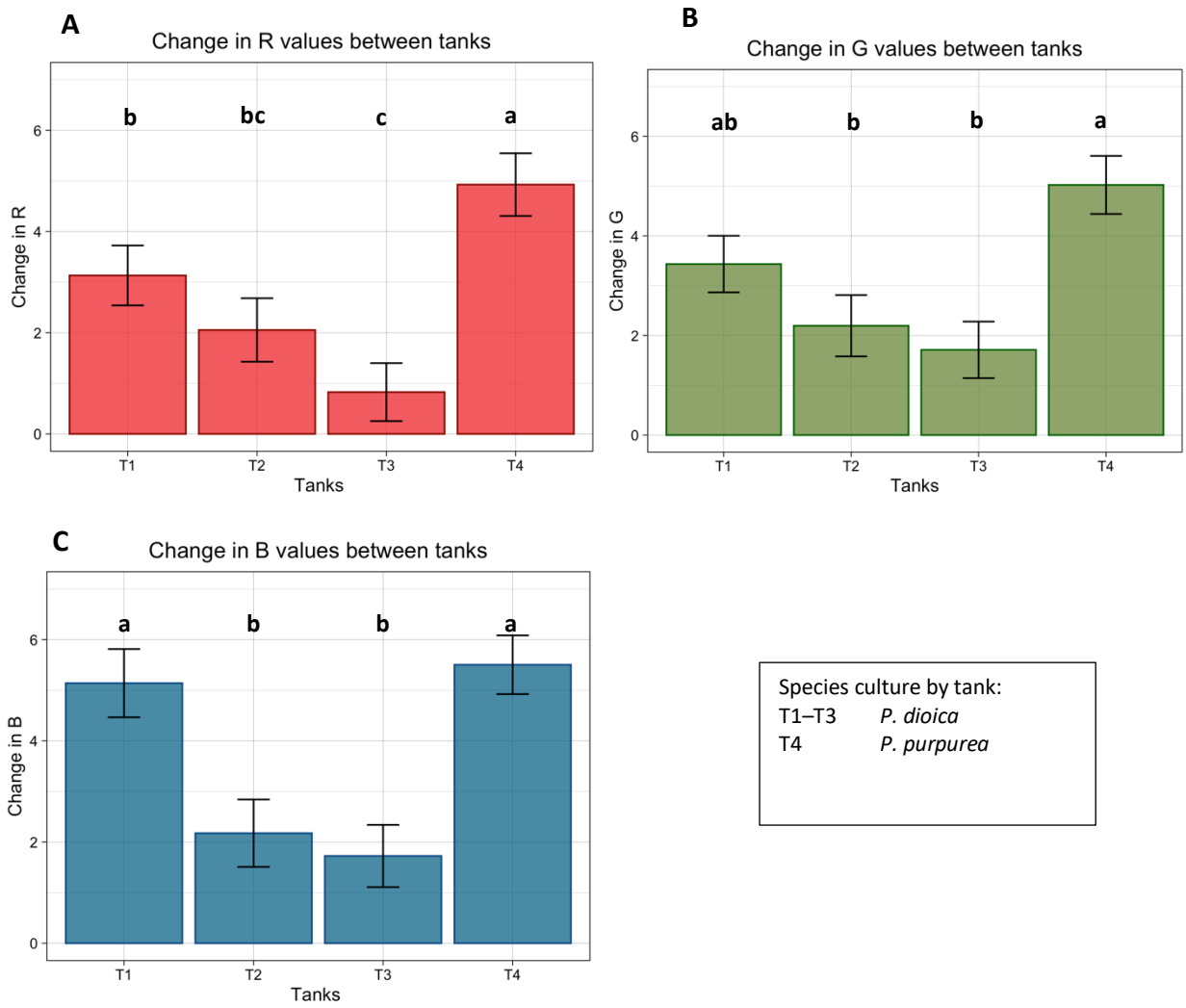

Figure 9 Change in R-, G- and B values between tanks

The difference in means between tanks for **(A)** the change in R values, **(B)** change in G values and **(C)** change in B values. The standard error of the mean is presented using error bars. Tanks labelled with the same letters (a-c) are not statistically different (Statistical test: Student-Newman-Keuls (SNK))

3.2 Observations on conchocelis growth

To validate whether R-, G-, B values are, in fact, measuring conchocelis growth, observations on conchocelis growth development were used as a control. During the experimental period, conchocelis structures were observed on all shells (Figure 10 C, D) and confirmed using a stereo loupe (Figure 7 A, B). Results showed that the degree to which conchocelis altered the colour shade of the shell substratum differed among tanks and among shells within each of the tanks. The overall growth was continuously increasing throughout the cultivation period in tank T1 (*P. dioica*) and tank T4 (*P. purpurea*). In tank T1, a sudden acceleration in growth was observed mid-way (Around day nr 48), resulting in the strongest growth being within tank T1 (Figure 10 A). In tanks T2 and T3 (T2, T3: *P. dioica*) the overall growth stagnated mid-way in the cultivation period. Stagnation in growth was observed around day 41 in tank T2 and day 35 in tank T3, with the weakest growth in tank T3 (Figure 10 B).

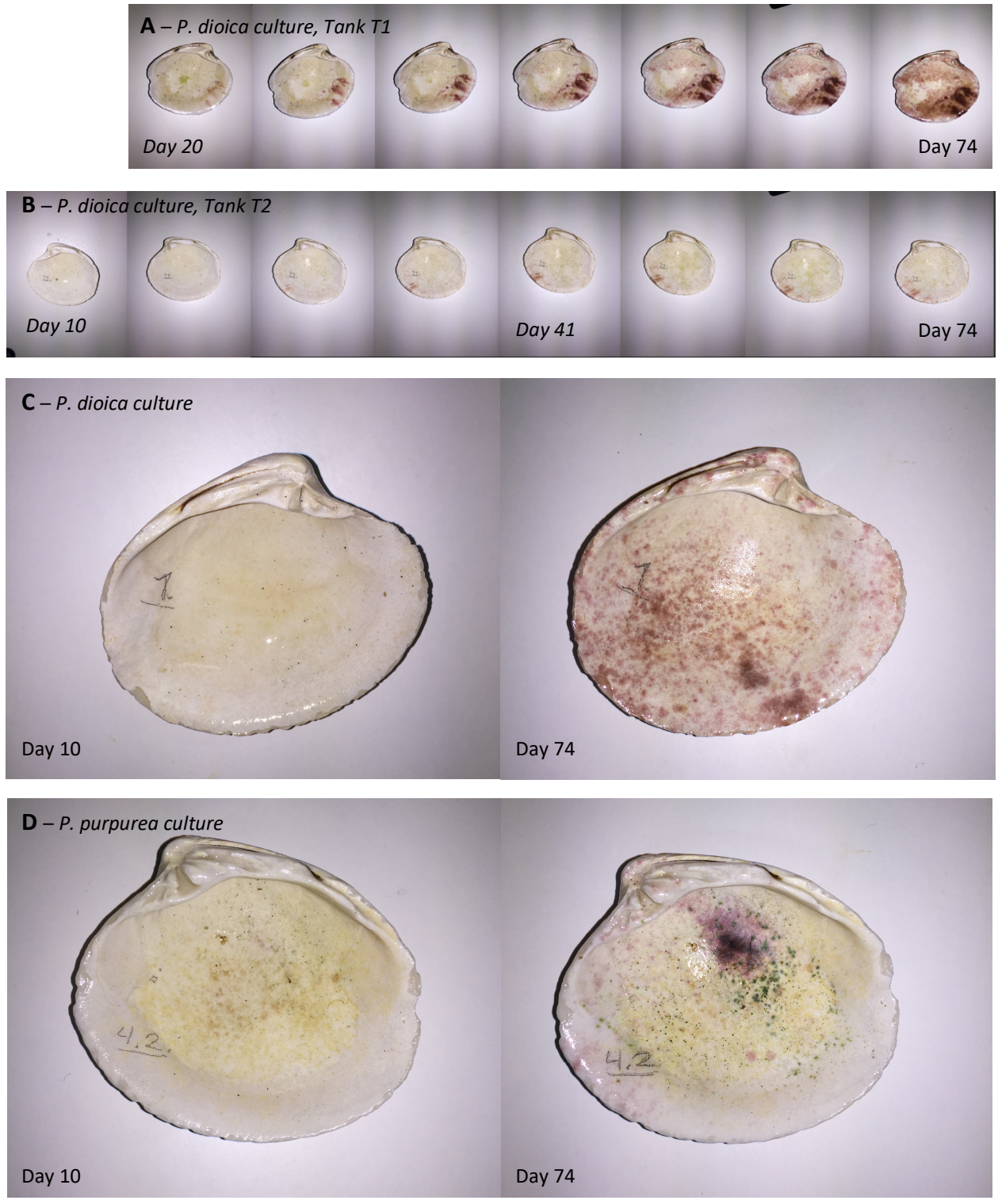

Figure 10 Development of conchocelis growth

(A) Continuous increase in conchocelis development over time, from day 20 to day 74.
(B) Stagnation in growth mid-way in the cultivation period, occurring on day 41.
(C) *P. dioica* culture on day ten and day 74 of cultivation (Shell 1 from tank T1).
(D) *P. purpurea* culture on day ten and day 74 of cultivation (Shell 4.2 from tank T4).

3.3 Relationship between R, G, B and chlorophyll *a*

R-, G-, and B values from imaging were investigated against chlorophyll *a* concentration for 15 shells with conchocelis development, collected at in the end of the experimental period. Seven shells were collected from the species *P. dioica* and seven from *P. purpurea*. No correlation between chlorophyll *a* and RGB values for any of the colours R, G or B was detected (R: r = 0.07, p = 0.8095, G: r = -0.07, p = 0.8032, B: r = -0.27, p = 0.3264*)*. Results is presented in figure

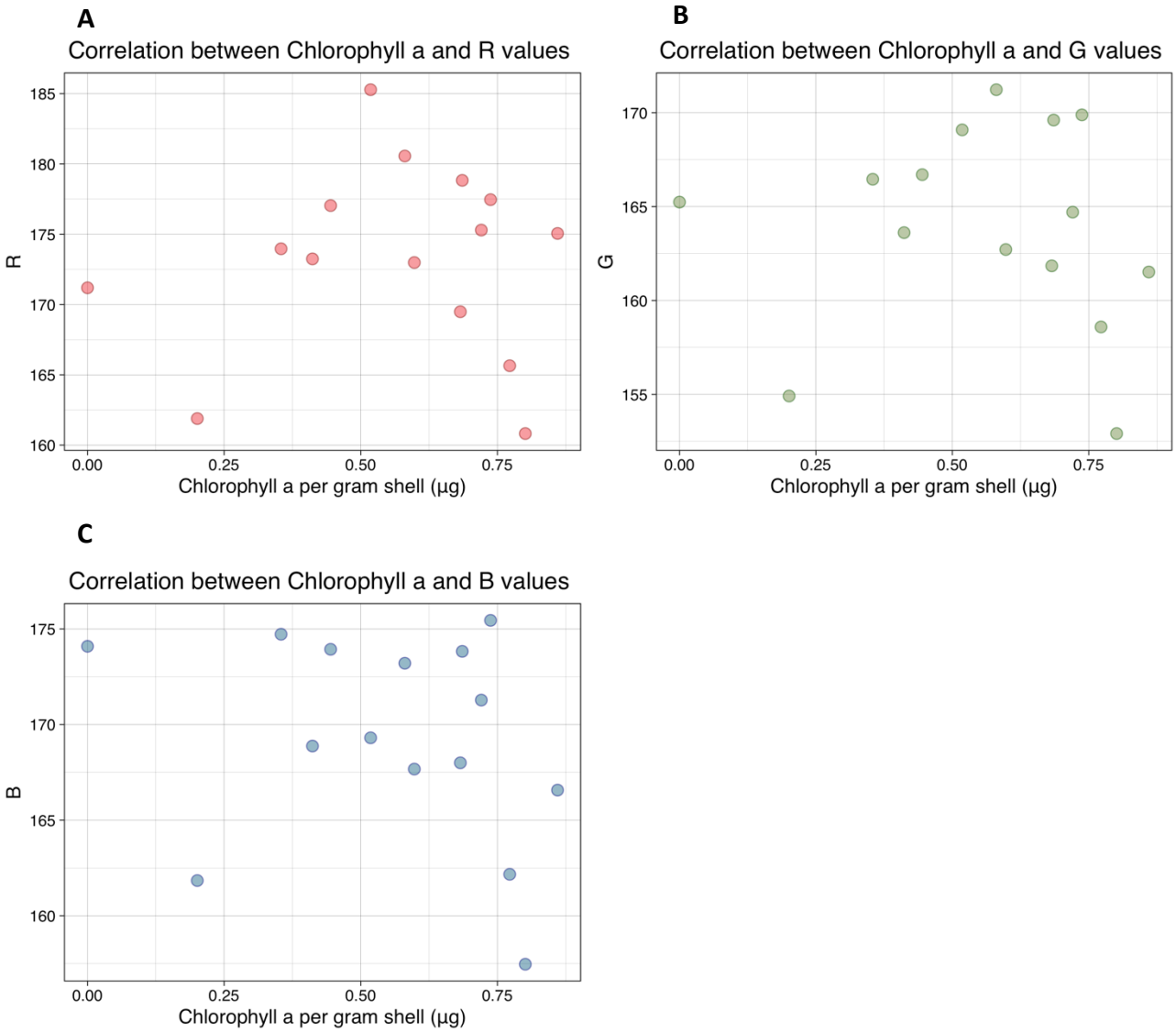

Figure 11 Relationship between R, G, B and chlorophyll *a*

The relation between **(A)** chlorophyll *a* and R values, **(B)** chlorophyll *a* and G values and **(C)** chlorophyll *a* and B values. Statistical analysis: Pearson correlation coefficient, Df = 13.

4. Discussion

In industrial *Porphyra* (or *Pyropia*) cultivation, conchocelis growth is commonly monitored by observations of growth development based on colour alteration of the shell- or substrate surface. This method may often be supported by microscopy sampling, a time-consuming and labour intensive approach. Although providing an elementary estimate of growth development, a quantitative method of growth would provide a higher level of control regarding conchocelis growth monitoring and development. The R-, G- and B- features from images of conchocelis were investigated as a potential quantitative method of measuring growth during conchocelis development, in an effort to increase efficiency within the early stage of industrial *Porphyra* cultivation.

4.1 The potential for RGB-based image analysis to assess conchocelis growth

The first objective of this thesis was to test if the alteration in R-, G-, and B values measured by image analysis using RGB features could be a suitable method for conchocelis growth assessment. To test this method, the development in R-, G- and B values from images of 40 shells with conchocelis growth was measured throughout a cultivation period of 74 days. The results show a significant increase in R, G and B over time, presumably caused by conchocelis growth. To validate whether R-, G-, B values are, in fact, measuring conchocelis growth, observations on conchocelis growth development were used as a control. The observational control found conchocelis to successively alter the colouration of most shells during cultivation, supporting the hypothesis that R, G and B are, in fact, measuring conchocelis growth. Herby, the results indicate that image analysis using RGB features can measure conchocelis growth over time and, therefore, may have the potential to serve as a suitable method for conchocelis growth assessment.

The 40 shells used in the experiment were divided into four different tanks, with ten shells in each tank. The results show a significant difference among tanks for the different R-, G-, and B values.

The reported difference among tanks is probably a result of variance in environmental conditions. Within the design of this experiment, the following biotic and abiotic factors may have caused such variations: amount of zygotospores, nutrient supply through water exchange, minor variations in light conditions and/or biological contamination *e.g.* bacteria, protozoans (Brawley et al., 2017; Wang, Zhang, Chen, Wang, & Liu, 2013), small crustaceans (observed), and other microalgae (Brawley et al., 2017; Wang et al., 2013).

Since tanks were found to differ significantly, it was investigated whether the means in R-, G- and B values (Figure 9) and the observations on growth (Figure 10) showed a similar pattern for each of the tanks, respectively. All in all, means of R, G, B and overall observations were found to show a similar pattern for each of the tanks. Higher mean R-, G-, B values were found in tanks where growth was observed to be high (Figure 9 and Figure 10 A) and lower mean R-, G-, B values were found in tanks where growth was observed to be low (Figure 9 and Figure 10 B). The strongest pattern was found between mean B values and observations. Mean B values were found to be significantly higher in tanks T1 and T4 than in tanks T2 and T3 (Figure 9 C). Simultaneously, growth was observed to be overall strong and continuously increasing in tanks T1 and T4 (Figure 10 A), and overall weak and stagnating in tanks T2 and T3 (Figure 10 B). These similar patterns found between R-, G-, and B values and observations on growth, further support the hypothesis that RGB features can measure conchocelis growth. Furthermore, the results indicated B values to have the strongest relationship to the observed growth, suggest that some RGB features may measure conchocelis growth better than others. For investigation of RGB-based image analysing method performed in a related field of studies, in higher plants, similar findings are suggested of which some of the RGB features present a better relationship than others (Do Amaral et al., 2019; Hu et al., 2013; Kawashima & Nakatani, 1998; Mohan & Gupta, 2019). To further conclude what RGB feature that best fit the measurements of conchocelis growth and how well the RGB-based image analysis can measure the growth development, a more accurate control method than observations is needed.

4.2 Relationship between R, G, B and chlorophyll *a*

The second objective of this thesis was to study the possible correlation between R-, G- and B values measured by image analysis of conchocelis and chlorophyll *a* content of conchocelis. The measurement of chlorophyll *a* content is a well-established method of measuring algae growth (Jeffrey & Humphrey, 1975). Therefore, a potential correlation between R, G, B and chlorophyll *a* would support the ability of R-, G-, and B values to measure conchocelis growth. To investigate this possible correlation, shells at different levels of conchocelis growth, thereby different levels of colouration, were analysed at one given time point. Thus, the relationship between R, G, B and chlorophyll *a* content at different growth levels could be investigated, although limited to detect only a simple relationship. However, in the results of this thesis, no correlation was found. These findings may suggest that R-, G-, and B values cannot assess chlorophyll *a* in conchocelis. Therefore, within the present study, this method could not be used to validate the ability of RGB features to measure conchocelis growth.

Investigation of the relationship between R, G, B and chlorophyll *a* is based on the hypothesis that there is a direct relation between chlorophyll *a* and the phycobiliprotein phycoerythrin, responsible for the red colouration of conchocelis. The existing literature, though, investigating the relationship between phycoerythrin and chlorophyll *a* is contradictory on whether such a relationship can be found. Mishra et al. (2012) and Hiroyuki (2002) reported a correlation in their studies. On the other hand, Sheath, Hellebust, and Sawa (1977) did not find such a correlation. Within the result of this thesis, a correlation was not found, similar to the results reported by Sheath et al. (1977). It has been showed that the phycoerythrin content in the conchocelis stage of different *Porphyra* species (Lin & Stekoll, 2011) and *P. yesoensis* (Hiroyuki, 2002) are affected by nitrogen availability. In the present experimental design, both biological contamination and water exchange may have caused nitrogen to fluctuate during cultivation. Such factors influencing the phycoerythrin content may explain why one could not use R, G, B to assess chlorophyll *a* in the present study.

4.3 Limitations, method development and future perspectives

This thesis intends to contribute in the first step to develop a quantitative method of measuring conchocelis growth, using R-, G-, and B values measured from images of conchocelis. The results did show an increase in R, G and B over time that were validated by observations of conchocelis growth, thereby indicating that RGB-based image analysis can measure conchocelis growth. The positive results shown in this study encourage further investigation of this method. Thereby, the limitations within this study, suggestions for method development for future studies and the future perspectives of this method are discussed below.

4.3.1 Improvements for chlorophyll *a* assessment

The relationship between R, G, B and chlorophyll *a* was studied. Even though a correlation could not be established using the approach within this thesis, it would be interesting to investigate this relationship further. Firstly, as previously stated, phycoerythrin concentration in conchocelis is argued to be affected by fluctuations in nitrogen (Hiroyuki, 2002; Lin & Stekoll, 2011). Therefore, if a relationship between R, G, B and chlorophyll *a* can be detected, a stable nitrogen concentration in the water would make this potential relationship easier to detect. The nitrogen concentration can be stabilised by reducing or eliminating biological contamination. This could be achieved in a more controlled lab environment. Secondly, an alternative approach for the collection of samples may ease the detectability of this potential relationship. The approach used within this thesis intended to demonstrate the possible correlation between R, G, B and chlorophyll *a* by collecting samples at a given time point from shells with different levels of conchocelis development. This approach aimed to show the potential correlation over time based on samples at different stages in the growth development captured at one timepoint. However, for future research, a random collection of samples performed throughout the cultivation period, preferably every day, creating a time series, is suggested. This approach would provide a series of samples collected over time for randomly collected shells. Although, it should be noted that the shell series will include random shells within each series, as shells need to be shattered for extraction of chlorophyll *a*.

4.3.2 Improvements for control method

When investigating the R-, G-, and B values over time, observations from the images themselves were used as a control method for conchocelis growth. However, there is a high level of uncertainties associated with visual observations alone to measure growth. In this thesis, using observations as a control method can support or contradict whether the findings are, in fact, a result of the conchocelis growth development. Thereby, this study can provide information on the interest of future investigation of this matter.

A relationship between R, G, B and chlorophyll *a* may be detected in future studies if applying the suggested improvements for experiment design, hence a stabilisation of nitrogen concentration and an alternative approach for collecting samples. If such a relationship can be found, chlorophyll *a* assessment should be used as a control instead of the observational control used in the present study. However, if such a relationship proves challenging to find, an alternative control method to the observational control can be applied. For some species of *Porphyra,* within laboratory cultivation, growth of conchocelis is measured through the diameter of conchocelis patches on shells or through the diameter of conchocelis tufts in free-living cultures (Waaland, Dickson, & Duffield, 1990). This method, measuring the diameter of conchocelis patches on shells, would provide a relatively high level of accuracy for growth monitoring. Furthermore, another method to measure conchocelis growth in laboratory cultivation, developed by Varela-Alvarez, Stengel, and Guiry (2004), includes the technique of image processing as a quantitative measure of conchocelis growth. For this method, the growth of conchocelis is determined using a stereo loupe with a video camera, whereby the camera is connected to an interfaced digital imaging processor, of which conchocelis is measured by area (mm^2). These two methods could potentially be used as a control method to verify RGB-based image analysis to assess growth. However, there are various standard methods for monitoring algae growth that potentially could serve as a control.

4.3.3 The procedure of conchocelis imaging

Within this study, positive results were found despite using a standard iPhone 6 camera to capture images, suggesting the use of low-cost equipment to be sufficient. Furthermore, no image- or colour alteration was performed on pictures before colour histogram analysis. Alteration of images through reduction or elimination of background noise is likely to improve the method's accuracy and provide a more accurate result. Such adjustments to the method would be of interest for further investigation. However, the fact that a significant increase in R-, G-, and B values over time were found from images with no alteration is interesting on its own.

For future research , it would be interesting to investigate the use of a matte black background to eliminate background noise in the image, in contrast to the white background used within this study. A matte black background may reduce the variation in light and shadow between pictures.

4.3.4 Future perspectives

If future research proves this method efficient, a higher level of control could be achieved during growth monitoring of conchocelis in industrial *Porphyra* production. Such control would be beneficial to increase efficiency during the production phase. The equipment used to perform this method was not explicitly developed for this purpose but generally available, making it easy to implement and affordable for potential production companies. A standard iPhone camera was used to obtain images, and the free software program ImageJ, downloaded on a standard personal computer, was used for image analysis. The cost advantage of generally available equipment makes it particularly interesting for the industry. Furthermore, the method is non-invasive to the conchocelis and provides a low labour technique. Such qualities increase the prevalence within the industry, making it accessible for extensive, along with basic cultivation facilities. Further development of an app, where images are analysed in the in-build processor of a smartphone instead of a separate software program for a personal computer would be a future goal, providing a growth monitoring method for real-time data on growth development.

5 Conclusion

The present study indicate that R-, G-, and B values acquired from conchocelis images can measure conchocelis growth. In view of these findings, RGB-based image analysis is suggested to have the potential to serve as a suitable method for conchocelis growth assessment. The study further indicate that some of the RGB features are likely to measure growth better than others. Although, a more accurate control method than observations is needed to conclude which RGB features provide the best measure and to what existent growth can be measured.

The present study demonstrates no correlation between RGB features and chlorophyll *a,* suggesting that R, G, B may not be suitable to assess chlorophyll *a* in conchocelis. However, by improving the experimental design, one might detect a relationship. The following improvements are suggested: stabilisation of nitrogen in the waterbody during cultivation and the use of a series of samples collected over time for randomly collected shells. If such a relationship is detected based on these improvements, proving R, G, B to assess chlorophyll *a*, this method may serve as a control. The chlorophyll *a* assessment would provide a more accurate control than the observational control applied in the present study. However, If such a relationship proves challenging to detect, possibly due to a complex relationship between phycoerythrin and chlorophyll *a* concentration, an alternative laboratory method for conchocelis growth assessment can be applied as a control method.
Additional suggestions for method improvements include using a matte black background during imaging to reduce the variation in light and shadow between pictures.

In light of the positive results demonstrated within this study, RGB-based image analysis is suggested to have the potential to serve as a quantitative measure of conchocelis growth to increase efficiency in the early stages of industrial *Porphyra* cultivation. Furthermore, RGB-based image analysis offers the possibility for a low-cost, non-invasive and low labour intensive method making it accessible for extensive and basic cultivation facilities. Further research to determine the extent to which this method can assess conchocelis growth are thus encouraged. Finally, the development of an app where images are analysed in the in-build processor of a smartphone would be a future goal, providing a growth monitoring method for real-time data on growth development.

6 References

Alam, T. (2019). Extraction of Natural Colors from Marine Algae. *Journal of Agricultural and Marine Sciences [JAMS], 23*, 81-91. doi:10.24200/jams.vol23iss0pp81-91

Belghit, I., Rasinger, J. D., Heesch, S., Biancarosa, I., Liland, N., Torstensen, B., . . . Bruckner, C. G. (2017). In-depth metabolic profiling of marine macroalgae confirms strong biochemical differences between brown, red and green algae. *Algal research, 26*, 240-249. doi:10.1016/j.algal.2017.08.001

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*(1), 29-37. doi:10.1016/j.tplants.2010.10.004

Bolker, B. M., Brooks, M. E., Clark, C. J., Geange, S. W., Poulsen, J. R., Stevens, M. H. H., & White, J.-S. S. (2009). Generalized linear mixed models: a practical guide for ecology and evolution. *Trends in Ecology & Evolution 24*(3), 127-135. doi:10.1016/j.tree.2008.10.008

Brawley, S. H., Blouin, N. A., Ficko-Blean, E., Wheeler, G. L., Lohr, M., Goodson, H. V., . . . Prochnik, S. E. (2017). Insights into the red algae and eukaryotic evolution from the genome of Porphyra umbilicalis (Bangiophyceae, Rhodophyta). *Proceedings of the National Academy of Sciences, 114*(31), E6361-E6370. doi:10.1073/pnas.1703088114

Broom, J. E., Nelson, W. A., Yarish, C., Jones, W. A., Aguilar Rosas, R., & Aguilar Rosas, L. E. (2002). A reassessment of the taxonomic status of Porphyra suborbiculata, Porphyra carolinensis and Porphyra lilliputiana (Bangiales, Rhodophyta) based on molecular and morphological data. *European Journal of Phycology, 37*(2), 227-235. doi:10.1017/S0967026202003566

Chen, Z., Wang, F., Zhang, P., Ke, C., Zhu, Y., Cao, W., & Jiang, H. (2020). Skewed distribution of leaf color RGB model and application of skewed parameters in leaf color description model. *Plant Methods, 16*(1), 23-23. doi:10.1186/s13007-020-0561-2

Cho, T. J., & Rhee, M. S. (2019). Health Functionality and Quality Control of Laver (Porphyra , Pyropia): Current Issues and Future Perspectives as an Edible Seaweed. *Marine Drugs, 18*(1), 14. doi:10.3390/md18010014

Değirmenci, A. (2017). *Computer Based Grading of Bladder Carcinoma* (Master's thesis). Ankara Yıldırım Beyazıt University, Ankara. Retrieved from http://acikerisim.ybu.edu.tr:8080/xmlui/bitstream/handle/123456789/1995/457650.pdf?sequence=1&isAllowed=y

Do Amaral, E. S., Vieira Silva, D., Dos Anjos, L., Schilling, A. C., Dalmolin, Â. C., & Mielke, M. S. (2019). Relationships between reflectance and absorbance chlorophyll indices with RGB (Red, Green, Blue) image components in seedlings of tropical tree species at nursery stage. *New forests, 50*(3), 377-388. doi:10.1007/s11056-018-9662-4

Drew, K. M. (1949). Conchocelis-Phase in the Life-History of Porphyra umbilicalis (L.) Kütz. *Nature 164*(4174), 748-749. doi:10.1038/164748a0

Dumay, J., & Morançais, M. (2016). Chapter 9 - Proteins and Pigments. In *Seaweed in Health and Disease Prevention* (pp. 275-318). doi:10.1016/B978-0-12-802772-1.00009-9

FAO. (2009). Porphyra spp. In Cultured aquatic species fact sheets. . *Text by Chen, J. & Pu Xu. Edited and compiled by Valerio Crespi and Michael New*. Retrieved from http://www.fao.org/tempref/FI/CDrom/aquaculture/I1129m/file/en/en_nori.htm

FAO. (2020). *State of World Fisheries and Aquaculture. Sustainability in action*. (SOFIA 2020). Retrieved from https://doi.org/10.4060/ca9229en

Graham, L. E., Graham, J. M., & Wilcox, L. W. (2009). *Algae* (2nd ed.). San Francisco: Benjamin Cummings.

Guiry, G. M., In Guiry, M. D., & Guiry, G. M. (2020a). Porphyra C.Agardh, 1824 *AlgaeBase, World-wide electronic publication, National University of Ireland, Galway.* Retrieved from http://www.algaebase.org/search/genus/detail/?genus_id=84 Accessed 28th of September 2020

Guiry, G. M., In Guiry, M. D., & Guiry, G. M. (2020b). Porphyra dioica J.Brodie & L.M.Irvine 1997. *AlgaeBase, World-wide electronic publication, National University of Ireland, Galway.* Retrieved from http://www.algaebase.org/search/species/detail/?species_id=18864 Accessed 28th of September 2020

Guiry, G. M., In Guiry, M. D., & Guiry, G. M. (2020c). Porphyra purpurea (Roth) C.Agardh 1824. *AlgaeBase, World-wide electronic publication, National University of Ireland, Galway.* Retrieved from http://www.algaebase.org/search/species/detail/?species_id=101 Accessed 28th of September 2020

Guiry, G. M., In Guiry, M. D., & Guiry, G. M. (2020d). Pyropia J.Agardh, 1899. *AlgaeBase, World-wide electronic publication, National University of Ireland, Galway.* Retrieved from http://www.algaebase.org/search/genus/detail/?genus_id=50308 Accessed 28th of September 2020

Guiry, M. (1990). Sporangia and spors. In K. M. Cole & R. G. Sheath (Eds.), *Biology of the red algae* (pp. 347-372): Cambridge University Press.

Hiroyuki, M. (2002). Relationship between phycoerythrin and nitrogen content in Gloiopeltis furcata and Porphyra yezoensis. *Algae, 17*(2), 89-93. Retrieved from https://www.koreascience.or.kr/article/JAKO200218138125059.pdf

Holmes, M. J., & Brodie, J. (2004). Morphology, seasonal phenology and observations on some aspects of the life history in culture of Porphyra dioica (Bangiales, Rhodophyta) from Devon, UK. *Phycologia, 43*(2), 176-188. doi:10.2216/i0031-8884-43-2-176.1

Hu, H., Zhang, J., Sun, X., & Zhang, X. (2013). Estimation of leaf chlorophyll content of rice using image color analysis. *Canadian journal of remote sensing, 39*(2), 185-190. doi:10.5589/m13-026

Jeffrey, S. W., & Humphrey, G. F. (1975). New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. *Biochemie und physiologie der pflanzen, 167*(2), 191-194. Retrieved from https://doi.org/10.1016/S0015-3796(17)30778-3

Johan, F., Jafri, M. Z., Lim, H. S., & Wan Maznah, W. O. (2014). Laboratory measurement: Chlorophyll-a concentration measurement with acetone method using spectrophotometer. *Journal of Physics. Conference Series*, 744-748,. doi:10.1109/IEEM.2014.7058737

Kawashima, S., & Nakatani, M. (1998). An Algorithm for Estimating Chlorophyll Content in Leaves Using a Video Camera. *Annals of botany, 81*(1), 49-54. doi:10.1006/anbo.1997.0544

Keuls, M. (1952). The use of the „studentized range" in connection with an analysis of variance. *Euphytica, 1*(2), 112-122. doi:10.1007/BF01908269

Knoop, J., Griffin, J. N., & Barrento, S. (2019). Cultivation of early life history stages of Porphyra dioica from the British Isles. *Journal of Applied Phycology, 32*(1), 459-471. doi:10.1007/s10811-019-01930-6

Knuth, D. E. (1981). *The art of computer programming : 2 : Seminumerical algorithms* (2nd ed. Vol. 2). Reading, Mass: Addison-Wesley.

Levine, I. A., & Sahoo, D. (2010). *Porphyra: harvesting gold from the sea*: I.K. International Publishing House Pvt. Ltd.

Lin, R., & Stekoll, M. S. (2011). Phycobilin content of the conchocelis phase of alaskan porphyra (Bangiales, Rhodophyta) species: Responses to environmental variables. *Phycological Society of America, 47*(1), 208-214. doi:10.1111/j.1529-8817.2010.00933.x

Mishra, S. K., Shrivastav, A., Maurya, R. R., Patidar, S. K., Haldar, S., & Mishra, S. (2012). Effect of light quality on the C-phycoerythrin production in marine cyanobacteria Pseudanabaena sp. isolated from Gujarat coast, India. *Protein Expression and Purification, 81*(1), 5-10. doi:10.1016/j.pep.2011.08.011

Mitman, G. G. (1991). *Meiosis, Blade Development and Sex- Determination in Porphyra umbilicalis (L.) J. Agardh from Avonport, Nova Scotia Canada.* (PhD). Dalhousie University, Canada.

Mohan, P. J., & Gupta, S. D. (2019). Intelligent image analysis for retrieval of leaf chlorophyll content of rice from digital images of smartphone under natural light. *Photosynthetica, 57*(2), 388-398. doi:10.32615/ps.2019.046

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. Retrieved from https://link.springer.com/content/pdf/10.1023/A:1008174307352.pdf

Noda, H. (1993). Health benefits and nutritional properties of nori. *Journal of Applied Phycology, 5*(2), 255-258. doi:10.1007/BF00004027

Pereira, R., Kraemer, G., Yarish, C., & Sousa-Pinto, I. (2008). Nitrogen uptake by gametophytes of Porphyra dioica (Bangiales, Rhodophyta) under controlled-culture conditions. *European Journal of Phycology, 43*(1), 107-118. doi:10.1080/09670260701763393

Redmond, S., Green, L., Yarish, C., Kim, J., & Neefus, C. (2014). *New England Seaweed Culture Handbook-Nursery Systems*. (CTSG-14-01). Connecticut Sea Grant Retrieved from http://seagrant.uconn.edu/publications/aquaculture/handbook.pdf.

Rigon, J. P. G., Capuani, S., Fernandes, D. M., & Guimarães, T. M. (2016). A novel method for the estimation of soybean chlorophyll content using a smartphone and image analysis. *Photosynthetica, 54*(4), 559-566. doi:10.1007/s11099-016-0214-x

Sahoo, D., Tang, X. R., & Yarish, C. (2002). Porphyra - the economic seaweed as a new experimental system. *Current Science, 83*(11), 1313-1316. Retrieved from https://www.jstor.org/stable/24106954

Sahoo, D., & Yarish, C. (2005). Mariculture of seaweeds. In R. A. Andersen (Ed.), *Algal Culturing Techniques* (pp. 219-237): Elsevier.

Sheath, R. G., Hellebust, J. A., & Sawa, T. (1977). Changes in plastid structure, pigmentation and photosynthesis of the conchocelis stage of Porphyra leucosticta (Rhodophyta, Bangiophyceae) in response to low light and darkness. *Phycologia, 16*(3), 265-275. Retrieved from https://doi.org/10.2216/i0031-8884-16-3-265.1

Shun Gate. (2015). Nori Blessed by the Natural Resources of the Ariake Sea. Retrieved from https://shun-gate.com/en/roots/roots_28.html

Sutherland, J. E., Lindstrom, S. C., Nelson, W. A., Brodie, J., Lynch, M. D. J., Hwang, M. S., . . . Müller, K. M. (2011). A new look at an ancient order: Generic revision of the Bangiales (Rhodophyta). *Journal of Phycology, 47*(5), 1131. doi:10.1111/j.1529-8817.2011.01052.x

Varela-Alvarez, E., Stengel, D. B., & Guiry, M. D. (2004). The use of image processing in assessing conchocelis growth and conchospore production in Porphyra linearis. *Phycologia (Oxford), 43*(3), 282-287. doi:10.2216/i0031-8884-43-3-282.1

Wang, H., Zhang, W., Chen, L., Wang, J., & Liu, T. (2013). The contamination and control of biological pollutants in mass cultivation of microalgae. *Bioresource technology, 128*, 745-750. doi:10.1016/j.biortech.2012.10.158

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*(1), 453-459. doi:10.1007/BF00040270

Xiao, X., Agusti, S., Lin, F., Li, K., Pan, Y., Yu, Y., . . . Duarte, C. M. (2017). Nutrient removal from Chinese coastal waters by large-scale seaweed aquaculture. *Scientific reports, 7*(1), 46613-46613. doi:10.1038/srep46613

Yadav, S. P., Ibaraki, Y., & Gupta, D. S. (2010). Estimation of the chlorophyll content of micropropagated potato plants using RGB based image analysis. *Plant Cell, Tissue and Organ Culture 100*(2), 183-188. doi:10.1007/s11240-009-9635-6

Yoon, H. S., Nelson, W., Lindstrom, S. C., Boo, S. M., Pueschel, C., Qiu, H., & Bhattacharya, D. (2017). Rhodophyta. In J. M. Archibald, A. G. B. Simpson, & C. H. Slamovits (Eds.), *Handbook of the Protists* (2 ed., pp. 89-133): Springer International Publishing.

Zhang, Z., Zhang, Q., Wang, J., Song, H., Zhang, H., & Niu, X. (2010). Chemical modification and influence of function groups on the in vitro-antioxidant activities of porphyran from Porphyra haitanensis. *Carbohydrate polymers, 79*(2), 290-295. doi:10.1016/j.carbpol.2009.08.004

7 Appendix

Appendix A

Locations for collected material and water intake.

	Coordinates	***Location/Origen***	***Collected material/ water intake***
	67°10'15.5"N 14°13'41.7"E	*Langsanden, Sandhornøy, Gildeskål*	*Arctica islandica*
	67°10'38.6"N 14°14'01.1"E	*Litlsanden, Sandhornøy, Gildeskål*	*Arctica islandica*
	67°12'00.2"N 14°37'59.4"E	*Åselistraumen, Gildeskål*	*Arctica islandica*
	67°24'44.5"N 14°37'36.1"E	*Mjeldevika, Bodø*	*Arctica islandica*
*	*67°19'22.7"N 14°28'42.4"E*	*Løpsvika, Bodø*	*Porphyra dioica, Porphyra purpurea*
	67°03'13.1"N 14°14'37.5"E	*Holmsundfjorden (10 m depth), Gildeskål*	*Seawater*

***** Species identification by DNA sequencing is carried out by Belghit et al. (2017) within the given coordinates. *P. dioica* and *P. purpurea* are both identified at this location.

Appendix B

Arrangement, quantity and exposure time of gametophytes in cultivation set-up

Tanks	*Genetic ID*	*Wet weight (kg)*	*Dry weight (g)**	*Incubation period (days)*
T1	*Porphyra dioica*	1.1	87.67	10
T2	*Porphyra dioica*	1.0	79.7	10
T3	*Porphyra dioica*	1.0	79.7	10
T4	*Porphyra purpurea*	0.3	25.77	13

* Dry weight calculations (Personal communication, C. Bruckner)

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