Developing systems for the commercial culture of *Ulva* species in the UK

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Abstract

The green seaweed, *Ulva*, is highly valued in terms of animal feed, food and biofuel, as well in the delivery of crucial remediation services including wastewater treatment and CO_2 removal. Accordingly, *Ulva* cultivation has gained significant research interest worldwide. Notwithstanding these research efforts, *Ulva* cultivation is still in its infancy and knowledge to underpin such developments remains limited.

A common challenge in *Ulva* cultivation is the fluctuating productivity with time due to vegetative fragmentation and/or periodic reproduction. In this study, three methods were employed to address this challenge. Firstly, culture conditions were optimised to establish a balance between growth and reproduction. Secondly, a refined culture method was developed, which more than tripled growth of *Ulva* over an 18-day cultivation as compared to a standard method. Thirdly, a sterile strain was obtained by mutating a wild strain with ultraviolet radiation. This new strain grew five times faster over an 18-day cultivation and absorbed nitrate and phosphate 40.0% and 30.9% quicker compared to the wild strain respectively. The chemical composition of the sterile strain showed a lipid content of more than double that of the wild strain, while the protein content was 26.3% lower than the wild strain.

Several tissue preservation techniques were developed to enable settlement and growth trials to be conducted on demand. The merits or otherwise of the preservation techniques were determined for gametes, germlings and thalli.

In addition to cultivation-related techniques, the co-effects of climate change factors (global warming and ocean acidification) and eutrophication on *Ulva* cultivation were investigated. These three variables interacted in a complex pattern to differentially affect life history stages, as well as altering the chemical composition and functional properties of *Ulva*. These findings make tangible contributions to the ability to successfully and commercially cultivate *Ulva* in terms of culture conditions, tissue preservation and the development of mutant strains. Further, by placing *Ulva* culture in a climate change context, this work provides valuable insight into the limits to resilience of *Ulva* to a changing climate. This will inform the future development of the *Ulva* culture industry over the coming decades.

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Table of Contents

List of Tables

List of Figures

Chapter 1. Introduction

1.1 Physiology of *Ulva*

The genus *Ulva* is one of earliest taxonomic descriptions used for algae by Linnaeus. The name *Ulva* originates from Latin and describes a marshy plant. It initially included several genera that are now called *Enteromorpha*, *Porphyra* and *Nostoc* (Linnaeus, 1753). In the 19th century tubular green seaweeds were separated from *Ulva* and moved to *Enteromorpha* while blade-like green seaweeds were still maintained in *Ulva* (Link, 1820). Later, Hayden et al. (2003) suggested that *Ulva* and *Enteromorpha* are not distinct evolutionary entities and should not be divided into separate genera. Now it is widely accepted that species in *Enteromorpha* should be included in *Ulva* since *Ulva* is the older genus.

Ulva species are membranous green algae and have two layers of cells that either unite to form a distromatic blade, entire or lobed, with flat, fluted or toothed margins, or separate, surrounding a cavity to form simple or branched tubes (Brodie et al., 2007). *Ulva* is cosmopolitan and common from tropical to polar climates, from fresh water through brackish to fully saline environments by virtue of its tolerance of a wide range of salinities and water temperatures (Carl et al., 2014a). More than 275 *Ulva* species have been reported worldwide and approximate 85 *Ulva* species have been currently recognized (Hayden et al., 2003). Most of the species in the genus are found in near-shore marine and estuarine waters, in the upper to mid-intertidal (eulittoral, mideulittoral and supralittoral zones) (Robertson-Andersson, 2003).

1.1.1 Life history of Ulva

Ulva has a basic life history of an alternation of isomorphic diploid sporophytic and haploid gametophytic phases. Diploid sporophytes produce equal numbers of zoospores of two mating types through segregation of chromosomes during meiosis. Zoospores grow into haploid gametophytes that are unisexual. Gametophytes release gametes by mitosis and then return to the sporophytic phase following copulation with gametes of the opposite mating gametophytes (Figure 1.1; Tanner, 1981).

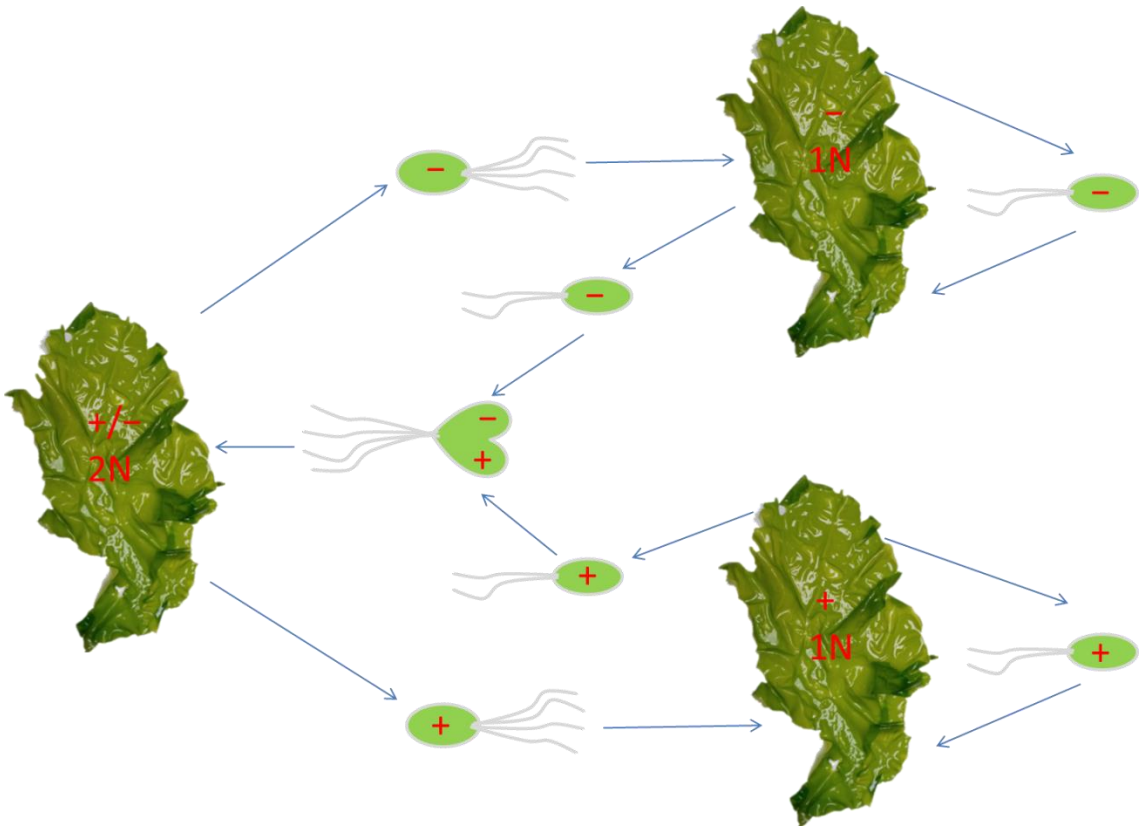

Figure 1.1 Life history of *Ulva* (adapted from Tanner (1981)).

Parthenogenetic development of gametes has also been found in *Ulva* (Bliding, 1968, Kapraun, 1970). Gametes parthenogenetically develop into two kinds of thalli: those producing biflagellate gametes and those producing quadriflagellate zoospores. Most of the parthenogenetic gametes (98–99%) in *U. mutabilis* develop into parthenosporophytes, and the remaining 1–2% become gametopytes of the same mating type (Fjeld and Lovlie, 1976).

The equilibrium between sexual and parthenogenetic reproduction could be disturbed by external factors. Low population densities and temperatures below the optimal for gamete fusion would lead to a predominance of parthenogenetic reproduction (Løvlie and Bryhni, 1978). This might be the reason why sporophytes of *U. lactuca* are quite rare on the Swedish west coast and strains of *Ulva* reproduce strictly by biflagellate neutral swarmers (Bliding, 1968, Kapraun, 1970).

Ulva species can also reproduce asexually by resumption of totipotency in blade cells (Chihara, 1968) or propagation by fragmentation (Gao et al., 2010). Additionally, it has been reported that *U. spinulosa* can reproduce asexually solely by quadriflagellate swarmers, which are produced without meiosis (Hiraoka et al., 2003).

1.1.2 Settlement of Ulva *swarmers*

There are generally three kinds of swarmers in *Ulva*, which are zoospores, male gametes and female gametes. The size of spores is commonly larger than that of gametes (Okuda, 1975, Lindstrom et al., 2006). With regard to gametes, most *Ulva* species are anisogamous, which means male and female gametes are different in size; female gametes are generally larger than male ones. For example, female gametes (length × width) of *U. lobata*, *U. angusta*, and *U. stenophylla* are 2.9–3.8 × 6.0–8.8 μm, 3.4–4.1 × 6.8–9.0 μm, and 2.6–3.2 × 5.7–6.4 μm respectively while their male gametes are 1.8–3.0 × 5.3–7.2, 1.9–3.2 × 5.2–5.8, and 1.5–2.0 × 5.7–6.4 μm respectively (Smith, 1947). It has been documented that cells of *U. lobata* sporophytes usually produce eight or 16 zoospores, while 16 or 32 female gametes and 64 or 128 male gametes are produced by cells of male and female gametophytes respectively (Smith, 1947); this could partially explain the size order of zoospores, female and male gametes. On the other hand, two gametes in a fusing pair of *U. lactuca* and *U. linza* were reported to have the same size (Føyn, 1934, Moewus, 1938).

Ulva zoospores can remain motile for up to eight days and zygotes are still viable for over six weeks, which increases the chances of an enlarged area of colonization (Maggs and Callow, 2003). Following their release and subsequent dispersal, zoospores and zygotes need to locate and adhere to a surface in order to complete their life history, although kelp spores can germinate in the water column without adhering to the substratum (Reed et al., 1992). *Ulva* gametes are usually positively phototactic. The phototaxis of the resultant zygote is reversed upon gamete fusion (Melkonian, 1980, Callow et al., 1997). *Ulva* zoospores display negative phototaxis (Melkonian, 1979, Callow et al., 1997). The positive phototaxis of gametes enhances the possibility of encounter and copulation (Togashi et al., 1999), whereas the negative phototaxis in zygotes and zoospores promotes a high rate of settlement (Fletcher, 1992). Settlement is the stage whereby zoospores detect a suitable surface, followed by the loss of the flagella, the secretion of an adhesive and the production of a new cell wall.

Settlement of *Ulva* is regulated by the perception of a series of chemotactic signals as well as physical and chemical interactions with the surface (Callow and Callow, 2011). *Ulva* zoospores preferentially settle on top of bacteria and the number of zoospores attached to a surface increases with the number of bacteria, indicating a direct interaction between the bacteria and zoospores (Joint et al., 2000). The possibility that zoospores may respond to a chemical signal produced by bacteria has been suggested (Joint et al., 2000). Further, Joint et al. (2002) established that zoospores were attracted to bacterial biofilms by N-acylhomoserine lactones (AHLs), which could be produced by diverse Gram-negative bacteria as quorum

sensing signal molecules. In addition, a number of gram-positive bacteria that did not produce AHLs also stimulated the settlement of zoospores. This was accomplished by signal peptides (Williams et al., 2007). It was found that the swimming speed of zoospores decreased in proximity to point sources of AHLs (Wheeler et al., 2006). Joint et al. (2007) proposed that calcium ion flux was related to the mechanism that zoospores respond to the AHLs. The presence of AHLs leads to an influx of calcium into the cytosol hence modulating zoospore swimming speed, which results in increased zoospore settlement on AHL-producing biofilms (Wheeler et al., 2006, Joint et al., 2007). This hypothesis was supported by manganese quenching experiments in which zoospores loaded with a calcium indicator dye displayed a decrease in fluorescence when they were exposed to AHLs, which indicated the opening of calcium channels and the corresponding calcium influx (Joint et al., 2007). The ecological benefits for *Ulva* zoospores sensing AHLs may be that bacteria can produce essential substances for normal morphology and development of *Ulva* after settlement (Wichard, 2015).

The physical properties of the substrata including surface microtopography and surface chemistry also play an important role in the selection of settlement sites for zoospores. Field observations have shown that zoospores prefer to settle on rough surfaces (Maggs and Callow, 2003). Synthetic surfaces with micro-valleys and ridges enhanced the settlement of zoospores compared with a flat surface. Settlement on roughened substrata provides a greater surface area for attachment and protects the *Ulva* zoospores from wave action, desiccation, and predation (Callow and Callow, 2002). The wettability of a surface also affects *Ulva* zoospore settlement. The number of zoospores settled on the surface increased when a surface became less wettable, which suggests the hydrophobic surfaces were more favourable for settlement (Callow et al., 2000).

1.1.3 Factors affecting germination

Light is necessary for *Ulva* swarmers to germinate. A study undertaken by Kolwlker et al. (2007) suggests that spores of *U. flexuosa* do not germinate in darkness and their survival rate decreases as the length of time in the dark increases. There was a linear decrease in germination rate with the increase of dark incubation after *Ulva* spores were exposed to light. Long term dark treatment also delayed germination since it took 21 days to germinate for spores incubated in darkness for 51 days while germination of spores at the beginning of the incubation occurred within a week. Furthermore, Finlay et al.'s (2008) study demonstrates that a threshold in reflected light of 12 photons m^{-2} s^{-1} is required to ensure that all the spores germinate in seven days. In addition, Imchen (2012) has showed the mean growth rate (30 days) of zoospores from *U. flexuosa* grown in 15 μmol L^{-1} phosphate medium increased up to

20% from 4% when light intensity varied from 20 μmol photons m^{-2} s^{-1} to 80 μmol photons m^{-2} s^{-1}.

Environmental temperature is one of most important environmental variables controlling reproduction and germination of *Ulva*. Water temperature has been suggested to be a main trigger for green tides (Keesing et al., 2011, Liu et al., 2013a). The optimal temperature for *Ulva* to germinate varies with location, which may be related to the background temperature in the location where *Ulva* were collected. The optimal temperature for germination of *Ulva* spp. from the outer Schlei (54.41 °N, 10.00 °E) in the western Baltic Sea is 10–15 ℃ (Lotze et al., 1999). The optimal temperature for germling growth of *U. intestinalis* collected from the eastern coast of Korea (35.29 °N, 29.27 °E) is 15 ℃ (Kim and Lee, 1996) while *U. fasciata* from Jaleshwar reef, Veraval (20.55 °N, 70.20 °E) North west coast of India obtained the highest germination rate (78.53 ± 10.05%) at 25 ℃ (Mantri et al., 2011). Furthermore, Woodhead and Moss (1975) found the effect of temperature on germination can be diluted by time. For example, the initial germination rate (first one to two days) in *U. intestinalis* at 20 ℃ was more than double the rate at 10 ℃ and the gap between these two temperatures shrank with time. Finally, the germination rate at 10 ℃ caught up with that at 20 ℃ by day eight.

Ulva spores have a higher sensitivity to the variation of external nutrient concentrations than adult plants (Sousa et al., 2007, Imchen, 2012). *Ulva* spore biomass shows a significant increase at high concentration of PO_4-P (6.4 μmol L^{-1}), compared to that at intermediate (2 μmol L^{-1}) or low conditions (0.8 μmol L^{-1}) (Sousa et al., 2007). Imchen (2012) suggested that the stimulating effect of nitrate on the growth of *U. flexuosa* zoopores was more significant than phosphate and both nutrients show a positive interactive effect when crossed with salinity. On the other hand, the biomass of *Ulva* spores tended to decrease with increasing NH_4-N concentrations ranging from 12.8 to 102.4 μmol L^{-1} of NH_4-N (Sousa et al., 2007). In addition, excess nitrate (80 μmol L^{-1}) or phosphate (15 μmol L^{-1}) also inhibited the growth of zoospores from *U. flexuosa* at 25 psu salinity (Imchen, 2012). These findings indicate *Ulva* spores may have a higher sensitivity to the variation of external nutrient concentrations than adult plants (Sousa et al., 2007).

Salinity is another environmental factor that affects the germination of *Ulva* spores. The biomass of *Ulva* spore was strongly affected by salinity. Higher salinity levels (20 and 35 psu) clearly increased growth of the spores compared with the low salinity of 5 psu (Sousa et al., 2007). Likewise, *U. fasciata* displays a linear relationship between germination and salinity (from 15 to 30 psu) with the highest germination rate of 78.53 ± 10.05% found at 30

psu (Mantri et al., 2011). In addition, the positive interactions between salinity and nitrate/phosphate were demonstrated in biomass of *U. flexuosa* spores. In the salinity and nitrate experiment, the highest growth rate of spores was achieved by a combination of 25 psu and 150 μmol L^{-1} nitrate and 25 psu and 8 μmol L^{-1} phosphate resulted in highest growth rate of spores in the salinity and phosphate experiment (Imchen, 2012).

Biotic factors that can affect germination mainly include extracellular compounds from bacteria or other organisms (such as plant hormones which are necessary to obtain normal germination and growth for *Ulva* spores (Provasoli, 1958)) and animal grazing. Contrary to the positive effects of bacteria on settlement of *Ulva*, bacteria usually inhibit the germination of *Ulva*. A collection of 56 marine bacteria were examined for their effects on the germination of *U. lactuca* by Egan et al. (2001). Of these bacteria, 43 displayed non-inhibitory effects on the germination rate (70–100%), 10 were assessed as slightly inhibitory (30–70% germination rate), and the remaining three bacteria including *Pseudoalteromonas tunicata* demonstrated strongly inhibitory effects (0–30% germination rate). It was also found that *P. tunicata* can inhibit germination by secreting extracellular components that are heat-sensitive, polar and in a molecular size of 3–10 kDa (Egan et al., 2001). Furthermore, Silva-Aciares and Riquelme (2008) found that the extracellular products secreted by *Alteromonas* sp (clone Ni1-LEM), *P. tunicata* and *Halomonas marina* (ATCC 25374) that inhibited germination of *U. lactuca* were proteins or peptides, thermostable, hydrophilic, and equal to or greater than 3.5 kDa in size. Meanwhile, Twigg et al. (2014) demonstrated the negative effects of *Sulfitobacter* spp. and *Shewanella* spp. on germination and early growth of *U. linza* were from N-acylhomoserine lactones. Apart from the inhibition from bacteria, marine fauna can also affect the germination of *Ulva*. It was reported that a water-borne substance exuded by the reef anthozoan *Condylactis gigantean* imposed negative effects on the germination of *Ulva* sp. (Bak and Borsboom 1984). In addition, the distinct effects of *Idotea chelipes* and *Gammarus locusta* were found on spore germination of *Ulva* spp. In a laboratory experiment, the presence of *Idotea chelipes* or *Gammarus locusta*, led to only 4.4% and 6.9% of germination rate respectively, which suggests grazers, apart from ecophysiological constraints, may play a decisive role in the early life stages of *Ulva* and following green tides (Lotze et al., 1999).

Antibiotics commonly reduce the germination rate of spores, even during pre-treatment, as they can prevent protein synthesis which is necessary for germination (Agrawal and Sarma, 1980, Srivastava and Sarma, 1980). However, the addition of low doses of ampicillin dramatically increased the viability and germination of *U. fasciata* spores. No

germination was found when *U. fasciata* was kept at 4°C for 18 days. On the contrary, more than 90% of the *U. fasciata* spores germinated with the addition of the ampicillin (Bhattarai et al., 2007). The underlying reason is that ampicillin inhibits the growth of bacteria that could degrade spores while the low concentration did not negatively affect spore germination (Bhattarai et al., 2007).

1.1.4 Factors affecting growth and reproduction

Photosynthesis rates of 12 seaweed species examined under different levels of desiccation indicated that differences were not related to intertidal levels at which those seaweeds inhabited but the abilities of each species to prevent water loss (Ji and Tanaka, 2002). Sven and Eshel (1983) showed that photosynthesis of *Ulva* sp. remained consistent when relative water content was above 70%, then declined with a decrease of relative water content and became zero at a water content of 35%. This indicates that *Ulva* growing in the lower mid-littoral zone would not experience a reduction in photosynthetic rates due to little exposure time to air. *Ulva* at higher levels of the intertidal zone might not experience net positive photosynthesis for a large part of a day and thus suffer an overall carbon loss. Similar results were reported for *U. pertusa*. Gao et al. (2011) found the electron transport rates of PSII reached zero under severe desiccation conditions whereas the electron transport of PSI still functioned, suggesting that PSI-driven cyclic electron flow may increase *Ulva* tolerance to desiccation.

Environmental factors, such as light, temperature, nutrients, and CO_2 also impose important effects on the growth and reproduction of *Ulva*. They were introduced in sections 2.1.1–2.1.3, 3.1.2, and 6.1.4.

1.2 Cryopreservation of algae

Pioneering works on the freezing of algal cells were reported in the early 1960s. A range of microalgae were successfully cryopreserved by Holm-Hansen (1963), Leibo and Jones (1963) and Hwang and Horneland (1965). The successful freezing of *U. intestinalis*, was also documented during this period (Terumoto, 1961). Subsequently, work on algal cryopreservation has focused predominantly on microalgae.

1.2.1 Freezing methodology

In general, there are two methods of cryopreserving algae related to differing cooling rates: rapid cooling and two-step cooling. Algal specimens, suspended in culture medium, are plunged into liquid nitrogen at −196 ℃ in the rapid cooling method. Success of this uncontrolled, fast-freezing method has been reported in blue-green and green algae (Holm-

Hansen, 1963, Ben-Amotz and Gilboa, 1980a, b). Although algae were stored at –196 ℃ for relatively short periods (no more than one day) in these studies, it may be feasible that this technique can be used for long term preservation. For instance, Day et al. (1997) showed that some microalgae can successfully be cryopreserved for time periods of at least one year.

The rapid cooling technique appears to be the simplest method implemented to date. However, it can lead to supercooling of the internal water, increasing the possibility of damage by intracellular ice formation (Meryman, 1966, Karlsson and Toner, 1996). To avoid this a two-step cooling method was developed, which was initially described by Luyet and Keane (1955) and subsequently applied to a number of cell-types (Farrant et al., 1977). Materials are cooled from room temperature to an intermediate temperature (usually at or above the temperature of homogeneous ice nucleation, –35 to –40 ℃) at a slow cooling rate (generally ca. –1 ℃ $minute^{-1}$), then plunged into liquid nitrogen to complete the freezing process. This method is considered to enhance viability since the slow cooling rate allows sufficient time for intracellular water to exit the cell, which has an important cryoprotective effect as it reduces the amount of water available to form ice (Taylor and Fletcher, 1999a). At sufficiently low temperatures (approximately –40 ℃), the cell membrane effectively becomes impermeable (Karlsson et al., 1994). Thus, no osmotic equilibrium would occur between intracellular and extracellular solutions below that temperature. Generally, two-step cooling promotes greater viability of algae compared to rapid cooling. Hwang and Horneland (1965) found the number of intact cells, the number of cells retaining their viability, and the mitotic activity of the viable cells in 16 strains of microalgae were unanimously higher in samples frozen by slow cooling (from room temperature to –30 ℃ at a rate of –1 ℃ per minute, then transferred directly to –79 ℃) than those frozen by rapid cooling (plunged directly to –79 ℃) for short term storage (one to two hours). Day et al. (1997) reported great success of two-step cooling in some microalgae for long term storage (up to 22 years), in which post-thaw viability levels could reach over 95%. With respect to seaweeds, Van der Meer and Simpson (1984) reported that all seven species they studied survived with little or no evidence of freezing damage for one hour storage in a two-step procedure. The survival of *Porphyra yezoensis* was approximately 60% for at least 300 days storage using two-step cooling (Kuwano et al., 1993).

In addition to the conventional freezing methods mentioned above, the technique termed vitrification is worthy to state. The phenomenon of vitrification was first investigated and described by the founder of cryobiology, Luyet, in early 20^{th} century. The potential of achieving an ice-free, structurally arrested state for cryopreservation was recognised in

Luyet's study (Luyet, 1937). Vitrification refers to the physical process by which a highly concentrated cryoprotective solution supercools to very low temperatures and finally solidifies into a metastable glass, without undergoing crystallization at a practical cooling rate (Fahy et al., 1984). Compared to conventional freeze-thaw methods vitrification attempts to prevent ice formation in both intracellular and extracellular environments rather than minimize the probability of intracellular ice formation. The glass fills the space in a cell so that it may prevent additional cell collapse and pH alteration during dehydration. In addition, glass is exceedingly viscous and stops all chemical reactions that require molecular diffusion, its formation results in dormancy and stability over time (Burke, 1986). Thus, vitrification is an effective freeze-avoidance technique. The procedure of vitrification basically involves preconditioning the specimen with an appropriate cryoprotectant and osmoprotectant, loading the cells in an extremely concentrated vitrification solution for dehydration, rapid cooling to –196 ℃ for storage in liquid N_2. Rapid rewarming is necessary to avoid potential damage of devitrification and recrystallization events during thawing and it is advised that dilution of the suspending medium and washing of samples should be conducted as rapidly as possible in order to minimise the toxicity to cells (Sakai and Engelmann, 2007).

Vitrification as a simple and reliable method for cryopreservation has been applied in the storage of cultured cells, meristems, and somatic embryos (Sakai, 1995, Sakai, 1997, Engelmann and Takagi, 2000, Sakai and Engelmann, 2007). However, little is reported about its use with algae—perhaps due to the high toxicity of the solutions to algae (Day et al., 2000). The only literature relates to the cryopreservation of protoplasts of *Porphyra yezoensis* for which a high viability (66.5%) was achieved (Liu et al., 2004). Apart from the potential damage of vitrification solutions to algae, it is also difficult to simultaneously treat a large number of samples with this technique, as the duration of the successive vitrification steps is often very short. These steps require very precise timing and it is fairly inconvenient to manipulate small sized materials. Consequently, a new technique termed encapsulation dehydration was developed for cryopreservation of pear and potato shoot-tips (Fabre and Dereuddre, 1990). This technique involves encapsulation of explants in calcium alginate beads, preculture in a liquid medium with a high sucrose concentration, and dehydration of the encapsulated explants by sterile air drying followed by immersion and storage in liquid N_2. The method has the advantage that no toxic cryoprotectants are required; hence it has successfully been employed in cryopreservation of both microalgae (Hirata et al., 1996) and seaweed (Vigneron et al., 1997). However, no studies on *Ulva* have been reported yet.

1.3 Values of seaweed

1.3.1 Food

Humanity has a long history of using seaweeds as food, dating back to the late Pleistocene (14, 220 and 13, 980 years ago) at Monte Verde in southern Chile. Four species (*Durvillaea antarctica*, *Macrocystis pyrifera*, *Porphyra columbina*, and *Sarcothalia crispata*) among nine recovered from hearths at Monte Verde II are edible (Dillehay et al., 2008). These findings support the archaeological interpretation of the site and suggest that the inhabitants used seaweed from distant beaches and estuarine environments as food and medicine (Dillehay et al., 2008). In the Far East and Pacific, there has been a long tradition of consuming seaweeds as sea vegetables and a total of 145 species of red (79), brown (38) and green (28) seaweeds are used as food today (Pereira, 2011). Seaweed has formed part of the daily diet in China, Japan and Korea for centuries and demand far outstrips supply whereas in western cuisine seaweed is vastly underexploited (Pereira, 2011). The principal uses of seaweeds in the West have been as sources of phycocolloids, thickening agents and gelling agents for various industrial applications (Pereira, 2011). Despite a long history of use in coastal areas of the British Isles with a well-established role in folklore (such as laverbread in Wales) and traditional medicine, seaweed is not an ingredient currently found in many British kitchen cupboards. Britain has a coastline that is almost as big as the coastline of Japan, which is the greatest seaweed-eating culture in the world, and has a variety of seaweeds (630 species), yet in Britain seaweeds remain an essentially untapped food resource (Steele et al., 2010).

There is renewed interest in seaweed in western countries due to scientific interest into their unique biochemical compounds (Balasubramaniam et al., 2013, Wilcox et al., 2014). Food and nutrition science has moved from identifying and correcting nutritional deficiencies to designing foods that promote optimal health and reduce the risk of disease. Functional foods, which refer to natural or processed foods that contain known biologically-active chemicals that provide clinically proven health benefits, have been proposed with this movement. Functional foods are important in the prevention, management and treatment of chronic diseases of the modern age. Seaweeds contain a large number of bioactive substances, such as dietary fiber, polysaccharides, polyphenols with antibacterial and antiviral properties in addition to various minerals and vitamins (Arasaki and Arasaki, 1983, Chandini et al., 2008, Mariya and Ravindran, 2013). Thus, seaweeds could be potential functional foods with multiple benefits.

The term “dietary fibre” first appeared in 1953 in reference to the non-digestible residue in foods, to replace “crude fibre” (Potty, 1996). In 1972, dietary fibre was defined as “the remnants of edible plant cell polysaccharides, lignin and associated substances which escape hydrolytic enzymatic digestion in the upper gastrointestinal tract” (Trowell, 1972). Cumming and Englyst (1991) proposed that dietary fibre should be defined as the non-starch polysaccharides (NSP) in plant foods for analytical purposes. However, NSP excludes resistant starches that are formed during processing, as well as lignin. Thus, no definition has been universally accepted to date.

Dietary seaweeds contain 33–62% total dietary fibre on a dry mass basis (58% for *Undaria*, 50% for *Fucus*, 30% for *Porphyra* and 29% for *Saccharina*), which is higher than levels found in higher plants (Lahaye, 1991, Murata and Nakazoe, 2001, Dawczynski et al., 2007). The dietary fibres included in seaweeds can be divided into two types: soluble dietary fibres such as agars, alginic acid, furonan, laminaran and porphyran; and insoluble fibres such as cellulose, mannans and xylan. *Undaria pinnatifida* (wakame), *Chondrus* and *Porphyra* have the highest content of soluble dietary fibres (15–22%) while *Fucus* has the highest content of insoluble dietary fibres (40%) (Fleury and Lahaye, 1991). Dietary fibres from seaweed contain valuable nutrients and bioactive substances, which make them potential sources of functional food and pharmaceuticals. There now follows a brief consideration of two representative seaweed dietary fibres: alginate and ulvan.

Alginates are unbranched saccharides that are isolated from the cell walls of several brown seaweeds. Alginates were first discovered by a British pharmacist E. C. C. Stanford in the 1880s and industrial production began in California in 1929 (Holdt and Kraan, 2011). Commercially available alginates currently only come from algae, typically from *Laminaria hyperborea*, *L. digitata*, *L. japonica*, *Ascophyllum nodosum*, and *Macrocystis pyrifera*, although certain bacteria including *Pseudomonas aeruginosa* can also produce alginates as an extracellular matrix (Lee and Mooney, 2012). The alginate contents are 22–30% for *A. nodosum* and 25–44% for *L. digitata* on a dry mass basis (Qin, 2008). Alginate is comprised of sequences of M (M-blocks) and G (G-blocks) residues interspersed with MG sequences (MG-blocks) (Figure 1.2). G blocks are believed to be important for alginate structure as they participate in intermolecular cross-linking with divalent cations (e.g. Ca^{2+}) to form hydrogels. MG blocks allow polysaccharide chain flexibility. Therefore, MG areas will reduce alginate solution viscosity (Smidsrød and Draget, 1996). The ratios of M/G sequences, G-block length, and molecular weight are thus critical factors determining the biophysical properties of alginates (George and Abraham, 2006).

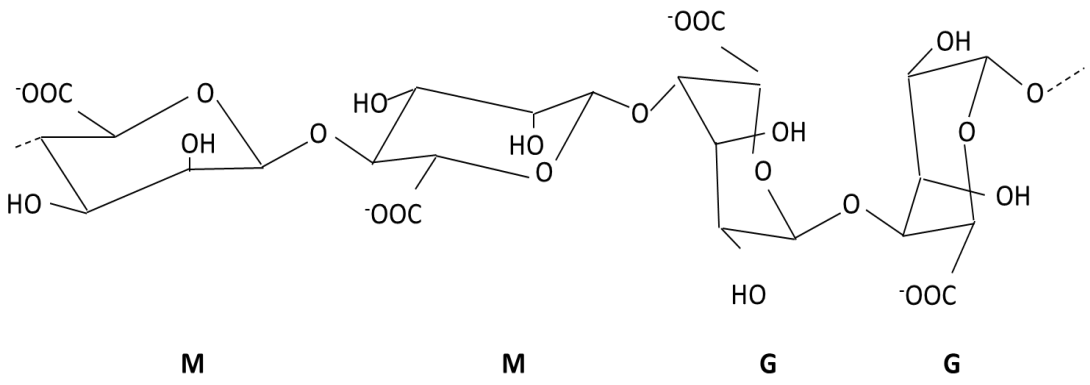

Figure 1.2 Structure of M-block and G-block in alginate.

Alginates are expected to reduce intestinal uptake and thus glycaemic response and/or cardiovascular disease risk in humans (Simpson et al., 1981, Jenkins et al., 2002). This is because once the alginate comes into contact with acid in the stomach, it will become a gel. The viscosity of the gel could cause a considerably slower rate of intestinal absorption. This property makes alginates clinically useful in reducing blood cholesterol and postprandial glycaemia, decreasing risks of obesity and Type II diabetes (Salmerón et al., 1997, Ludwig et al., 1999, Willett et al., 2002). Energy intake in non-dieting overweight and obese women was significantly reduced when they consumed a novel calcium-gelled, alginate-pectin beverage twice daily for seven days (Pelkman et al., 2007). Similar findings were reported by Paxman et al. (2008), in which a strong-gelling alginate formulation restricted energy intake of free-living adults compared to a commercially available control formulation. This reduced 7% (134.8 kcal) energy intake was underwritten by significant reductions in mean daily carbohydrate, sugar, fat, saturated fat and protein intakes. Such effects may be explained by the potential of alginate to enhance satiety (Dettmar et al., 2011). Use of foods designed to enhance satiety could be an effective adjunctive therapy for obesity. Additionally, alginates have also been demonstrated to bind up potential toxins, mutagens, or carcinogens that may occur in the colon (Ferguson and Harris, 1996, Harris et al., 1998, Kavas, 1999). All of these physiological effects of alginates result in a reduction in colonic mucosal and the rest of the body exposure to the wide range of potentially damaging agents.

The term ulvan stems from ulvin and ulvacin introduced by Kylin defining different fractions of *U. lactuca* water-soluble sulphated polysaccharides (Lahaye and Robic, 2007). It is now being used to refer to polysaccharides from Ulvales. Ulvans are highly charged sulphated polyelectrolytes composed mainly of rhamnose, uronic acid and xylose and containing a common constituting disaccharide: the aldobiuronic acid, [→4)-D-glucuronic

acid-(1→4)-L-rhamnose3-sulphate-(1→] as shown in Figure 1.3. The average molecular weight of ulvans ranges from 189 to 8, 200 kDa. The ulvan yield varies from 8% to 29% of dry seaweed matter, depending on the extraction and purification procedures (Lahaye and Robic, 2007).

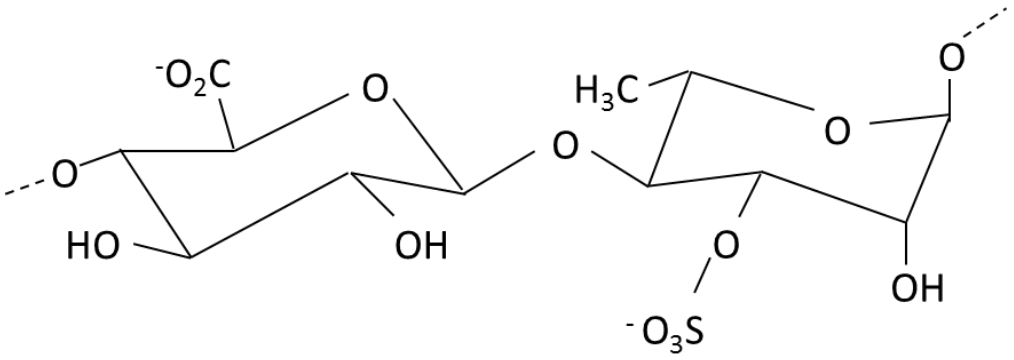

Figure 1.3 Structure of the main repeating disaccharide in ulvan.

On the basis of the characteristic chemical composition of ulvans, they can be used as sources of sugars to synthesise fine chemicals. Particularly, they are sources of iduronic acid, the only occurrence of which up to now in plants (Lahaye and Ray, 1996). Iduronic acid is usually utilised in the synthesis of heparin fragment analogues that serve as anti-thrombotic medications. To date, iduronic acid is synthesized in several complicated steps so it would be more cost-effective if they could be obtained from natural sources (Lahaye, 1998). Ulvans are also among the few polysaccharides found in nature containing high amounts of rhamnose. Rhamnose is a rare sugar that is a precursor in the synthesis of aroma compounds. The production of rhamnose from desulfated ulvan-like polysaccharides of *Monostroma*, a Japanese species of *Codiales*, has been patented (Lahaye and Robic, 2007).

Other potential applications are based on their biological properties. Ulvan cannot be digested by human endogenous enzymes and favour the water retention capacity of the fibre (Bobin-Dubigeon et al., 1997), which is a typical characteristic of dietary fibre acting as bulking agents and reducing the probability of pathologies related to intestinal transit dysfunctions (Freeman, 2001). Ulvan may also modulate lipid metabolism. Low serum levels of high-density lipoprotein cholesterol (HDL-cholesterol) and high levels of low-density lipoprotein cholesterol (LDL-cholesterol) and triglycerides are considered to be risk factors associated with atherosclerosis (Asztalos and Schaefer, 2003). Ulvan could significantly decrease the level of LDL-cholesterol and triglycerides and increase the levels of serum HDL-cholesterol. Therefore, ulvans can decrease the risk of cardiovascular diseases (Pengzhan et

al., 2003a, b). Additionally, Kaeffer et al. (1999) found ulvans were able to modify the adhesion and proliferation of human colonic tumour cells. Ahd El-Baky et al. (2009) reported that sulphated polysaccharides from *U. lactuca* could significantly inhibit cell proliferation of two cultured human cancer cell lines and showed high levels of antiviral activity against herpes simplex virus-1, which indicated that *U. lactuca* could be a potential functional food preventing cancers.

1.3.2 Animal feed

Seaweed could be a potentially valuable source of animal feed in the future (Makkar et al., 2016). It is generally accepted that seaweeds have higher amounts of minerals, vitamins and non-digestible polysaccharides, similar or higher contents of proteins, and lower lipid contents compared with terrestrial vegetables (Darcy-Vrillon, 1993, Norziah and Ching, 2000, Wong and Cheung, 2000). The feasibility of seaweed as feed for both terrestrial animals and marine animals has been extensively studied. In terms of *Ulva* species, *U. prolifera* enriched with microelements [Cu (II), Zn (II), Co (II), Mn (II), Cr (III)] could be potentially used as mineral feed additives in laying hens (Michalak et al., 2011). *U. lactuca* with high protein content can be considered as a medium quality forage for goats (Ventura and Castañón, 1998). Substituting 3.0% of corn with *U. lactuca* has been recommended as a diet for broiler chickens (Abudabos et al., 2013). *U. lactuca* has been categorized as a low-energy high-nitrogen foodstuff for ruminants (Arieli et al., 1993). However, Ventura et al. (1994) demonstrated that due to the high content of indigestible polysaccharides, *U. rigida* might not be a suitable ingredient for poultry diets, at least at inclusion rates of 100 g kg^{-1} or higher, which indicates that the inclusion rate should be considered when using *Ulva* as animal feed.

In regard to marine animals, the advantages of using seaweeds as feed, particularly for fish, have often been reported (Satoh et al., 1987, Mustafa et al., 1995, Gabrielsen and Austreng, 1998, Balasubramaniam et al., 2013). For instance, the body weight gain and feed efficiency of red sea bream *Pagrus major* was enhanced by the addition of 5% algae meal (Mustafa et al., 1995). Algae-fed groups were higher in liver glycogen, triglycerides and protein accumulation in muscle, thus reducing the nitrogen output to the environment (Mustafa et al., 1995). Furthermore, seaweed feed can improve the stress response and disease resistance of marine animals. The level of lysozyme (3.28 ± 0.49 g mL^{-1}) in Atlantic salmon, *Salmo salar* fed *Ascophyllum nodosum* was significantly higher than controls (2.99 ± 0.65 g mL^{-1}), implying an immunostimulating effect of alginate (Gabrielsen and Austreng, 1998). A 5% *Ulva* meal increased phagocytosis of the granulocytes against *Pasteurella piscicida*, bactericidal activity against *Escherichia coli* and *P. piscicida* in red sea bream *Pagrus major*,

thus enhancing disease resistance of the fish without impairing growth (Satoh et al., 1987). In addition to fish, the beneficial effects of using seaweed in the feeding of abalones (Naidoo et al., 2006) and sea urchins (Cyrus et al., 2015) were also reported. Abalone fed on a mixed diet of *U. lactuca*, *Gracilaria gracilis*, and kelp grew at a rate of 0.066 mm day^{-1} shell length and 0.074 g day^{-1} body weight while abalone fed on dried kelp grew at only 0.029 mm day^{-1} shell length and of 0.021 g day^{-1} body weight (Naidoo et al., 2006). The likely reason for the success of the mixed diet is that the *U. lactuca* and *Gracilaria gracilis* were grown in animal aquaculture effluent, with increased protein content (Naidoo et al., 2006). Furthermore, the effects of diets supplemented with varying amounts of *Ulva* (0, 50, 150, and 200 g kg^{-1} designated 0U, 5U, 15U, and 20U) on feed consumption rate and digestible protein intake of *Tripneustes gratilla* were also investigated (Cyrus et al., 2015). Mean dry feed consumption rates were higher in urchins fed the 20U diet (1.96 g $urchin^{-1}$ day^{-1}) compared with urchins fed diets with 5U (1.68 $urchin^{-1}$ day^{-1}) and 0U (1.64g $urchin^{-1}$ day^{-1}). Daily digestible protein intake was also significantly enhanced by 20U diet (360 mg $urchin^{-1}$ day^{-1}) compared with 0U diet (305 mg $urchin^{-1}$ day^{-1}) (Cyrus et al., 2015). The use of seaweeds in the diet of marine animals appears to be a promising prospect, especially given their function in improvement of the natural defences of fish.

1.3.3 Bioremediation

With growing demand for aquatic products and declining catches in the marine fishery industry, aquaculture has expanded and become one of the fastest-growing segments of the food economy (Chen and Qiu, 2014, Ferreira et al., 2014). It was estimated by the Food and Agricultural Organization that the total aquaculture production in 2011 was 63.6 million metric tons, accounting for nearly 48.6% of all the fish consumed by humans (Mathiesen, 2012). Among aquaculture methods, intensive monoculture is the predominant activity in bays or coastal lagoons (Diana et al., 2013, Troell et al., 2003). This approach also gives rise to severe environmental problems. Modern intensive monoculture requires high inputs of feeds and fertilisers which inevitably lead to considerable wastes. Unused feeds and faeces are degraded into inorganic pollutants consisting mainly of nitrate and phosphate, which results in eutrophication (Hu et al., 2013, Farmaki et al., 2014). The high nutritional status changes the characteristics of the ecosystem and causes a series of ecological events, including red tides, green tides and other disasters (Nagasoe et al., 2010, Smetacek and Zingone, 2013).

The use of live seaweed to address excess dissolved nutrients in aquaculture effluents—seaweed bioremediation—is widely mooted as an efficient and cost effective wastewater treatment method (Ridler et al., 2007, Barrington et al., 2009, Chopin et al., 2012).

Purified seawater facilitates the growth of aquatic animals and dissolved nutrients absorbed by seaweed can also enhance the biomass of seaweed (Ridler et al., 2006). This is particularly helpful for land-based aquaculture systems, where production can be limited by insufficient feed inputs due to strict environmental regulations of water quality discharged by aquaculture. The application of seaweed bioremediation reduces nutrient concentration in effluents prior to discharge, thus providing an opportunity to increase feed inputs and consequently aquaculture productivity (De Paula Silva et al., 2008, Nobre et al., 2010, Abreu et al., 2011). Among these seaweeds, the integrated culture of *Ulva* with abalones, shrimps, fishes, etc. has been suggested as an effective method to counteract the release of dissolved nutrients because of the high nutrient uptake capability of *Ulva* (Bolton et al., 2009, Cruz-Suárez et al., 2010, Al-Hafedh et al., 2014).

Seaweeds are also promising heavy metal biosorbents. Compared with the conventional treatment, the advantages of seaweed biosorbents lie in their ready availability in seas and oceans and high sorption capacity (Volesky, 1990, Rincon et al., 2005). Seaweeds possess a high metal binding capacity due to their large surface area exposed to the environment and the presence of various metal binding functional groups such as carboxyl, hydroxyl, sulphate, and amino groups (Holan and Volesky, 1994, Yun et al., 2001). For instance, brown seaweeds are widely used as bioremediation substrates due to their alginates and fucoidans acting as metal chelators (Singh and Sinha, 2013).

1.4 *Ulva* cultivation: history and methods

Due to its potential value as food, animal feed, biofuel and bioremediation, *Ulva* species have been grown in many parts of the world in pilot commercial systems (Table 1.1). The earliest trials of commercial *Ulva* culture were in America where *Ulva* was biomass for biomethane conversion along with the initiation of US Ocean Food and Energy Farm project in 1973 (Fannin, 1983). Due to the limit of developing offshore techniques, *Ulva* cultivation for biofuels was suspended. Meanwhile, there is a tradition in Japan to consume *Ulva* as food. Due to the decline of wild stocks, *Ulva* is farmed to meet market demand (Dan et al., 2002, Hiraoka and Oka, 2008). In addition, *Ulva* has also been cultivated as a biofilter in integrated multi-trophic systems where *Ulva* culture is combined with aquaculture of marine animals in Israel (Neori et al., 2000) and Saudi Arabia (Al-Hafedh et al., 2014). The *Ulva* harvested from the integrated system was also used as animal feed, which has been intensively studied in South Africa (Robertson-Andersson et al., 2008, Bolton et al., 2009). With growing demands in energy and decreasing stocks of fossil fuels, seaweeds have been reconsidered as sources of renewable energy. Therefore, *Ulva* was cultivated in outdoor tanks

to investigate its biomass production potential for bioenergy in Denmark (Bruhn et al., 2011). In a bid to enhance the market value of *Ulva*, land-based *Ulva* cultivation has recently been carried out in Australia to explore its potential in nutraceuticals and cosmeceuticals (Mata et al., 2016). Despite these trials, *Ulva* is not yet grown commercially outside Asia where it is grown for human food (Bolton et al., 2009).

Species	Systems	Timescale	Countries	Aims	References
U. lactuca	700 L tank	Eight months	America	Feed or biofuel	(DeBusk et al., 1986)
U. lactuca	3.3 m^2 integrated cultivation with fish and abalone	One year	Israel	Biofilter	(Neori et al., 2000)
U. prolifera	500 L tank	Nearly one year	Japan	Food	(Hiraoka and Oka, 2008)
U. lactuca	3000 L tank integrated cultivation with abalone	18 months	South Africa	Feed	(Robertson-Andersson et al., 2008)
U. lactuca	0.6 m^3 tank	Four months	Denmark	Biofuel	(Bruhn et al., 2011)
U. lactuca	1 m^3 integrated cultivation with fish	10 days	Saudi Arabia	Biofilter	(Al-Hafedh et al., 2014)
U. ohnoi	10,000 L tank	Over six months	Australia	Biorefinery	(Mata et al., 2016)

Table 1.1 *Ulva* cultivation in different countries with varying aims.

In terms of cultivation methods, they can be divided into different groups according to different criteria. Based on manipulating the life cycle of seaweeds, there are two categories: (1) starting from microscopic spores, the whole life cycle of the seaweed is under full control during cultivation; (2) starting from macroscopic seaweed fragments, only part of the life cycle is under control during cultivation. Based on the number of cultured species, the cultivation systems can be divided into monoculture and polyculture (or integrated culture). Open water cultivation and land-based cultivation can be defined according to cultivation

sites. Generally, both whole life cycle and partial life cycle methods, both monoculture and polyculture can be employed in open water cultivation and land-based cultivation.

1.4.1 Open water cultivation

Open water cultivation is the oldest form of aquaculture, which could date from 2000 BC in Japan and 100 BC in Italy (Iversen, 1968). In this cultivation system, seaweeds are grown in natural water; therefore the seaweeds have to be fixed in place. Generally, seaweeds are anchored to some artificial substrate (rafts, ropes, nets and cages) where they are buoyed at varying depths in the water column. According to the distance from the shore, open water cultivation can be divided into inshore cultivation and offshore cultivation. Currently there is no universally recognised definition for 'the distance' due to differing environmental conditions in every location, instead it is generally accepted that offshore cultivation is the movement of farm installations from nearshore sheltered environments to more exposed environments (Troell, 2009).

Inshore systems have several advantages compared with land-based systems. Firstly, they do not occupy land and thus do not compete with terrestrial plants in use of land. Secondly, it saves the cost of pumping in seawater and adding nutrients as the organisms are cultivated in the sea and take advantage of the natural nutrients in the coastal waters. Thirdly, this system requires less management and less time is spent in monitoring and maintenance. Therefore, most large scale commercial cultivation, such as *Laminaria*, *Porphyra*, and *Gracilaria* use this system (Titlyanov and Titlyanova, 2010). A fixed, off-bottom monoline method is widely used in inshore cultivation systems due to its low installation and maintenance costs. In this system, stakes are driven into the sea bed, spaced at 10 m intervals and 1 m apart in rows. One end of the nylon monofilament line is tied to one stake, stretched and the other end is tied to another stake in the opposite row. The monolines are constructed to form plots of a standard size (Sahoo and Yarish, 2005).

On the other hand, intensive inshore cultivation of seaweeds affects the natural community structure and also hampers the development of local tourism. Accordingly, offshore cultivation has been promoted to overcome such drawbacks and meet increasing world seafood demand (Troell et al., 2009). Pilot offshore net and rope systems have been conducted in America, Japan, and Germany. A production level of 20 tonnes of dry mass seaweeds per ha can be foreseen in a scale of 5, 000 m of lines per ha (Buck and Buchholz, 2004). Apparently, offshore cultivation encounters many operational problems, such as drifting of the anchoring systems and loss of the attached seaweed due to heavy waves and currents (van den Burg et al., 2013).

1.4.2 Land based cultivation

Although there are some strengths for open water cultivation, the fluctuation in environmental conditions results in variability in growth performance and the quality of the product. In addition, extreme weather can lead to catastrophic consequences for open water cultivation. Thus, land-based systems (which include indoor and outdoor systems) have been developed to address these limitations. Seawater is pumped into the system from the sea. These systems reduce the risks posed by grazers, poaching, and weather conditions. Due to the controllability of the culture conditions, such as nutrients, light intensity and temperature, the productivity of seaweed cultivated in land based systems is commonly high and the product quality is consistent. Seaweeds can be grown in tanks, ponds or raceways. In terms of *Ulva*, the outdoor tank systems are widely employed (DeBusk et al., 1986, Hiraoka and Oka, 2008, Bruhn et al., 2011, Mata et al., 2016). The whole thalli, fragments of thalli, germlings, and swarmers may serve as planting stock in a tank system. For thalli and germlings, they can be grown unattached, and be kept suspended in the water column in most cases by means of vigorous aeration, while ropes or nets have to be used as attachment substrates if *Ulva* cultivation starts from swarmers. For instance, the whole thalli were used in tank cultivation of *U. lactuca* in Florida. Aeration was supplied by a perforated pipe placed along the bottom of each tank to suspend *Ulva* thalli (DeBusk et al., 1986). The germlings of *U. prolifera* were grown in 500 L tanks and deep seawater pumped up from over 300 m depth was used to supply abundant nutrients. The average daily growth rate throughout a year reached 37% in this system (Hiraoka and Oka, 2008). Meanwhile, the swarmers of a tropical species of filamentous *Ulva* served as seeds in outdoor tank cultivation in Australia. Ropes were seeded at a density of 621,000 swarmers m^{-1} rope, maintained under nursery conditions for five days, then transferred to aerated flow-through outdoor tanks. The specific growth rate was more than 65% day^{-1} between seven and 14 days of outdoor cultivation and the maximum biomass yield of 23.0 $\pm$8.8 g dry mass m^{-1} was achieved after 13 days of cultivation (Carl et al., 2014a).

1.4.3 Integrated Multi-Trophic Aquaculture

In spite of its advantages in producing high yields of seaweed, land based systems have distinct weaknesses, which include the high cost of seawater pumping, filtering, aeration, infrastructure, labour, etc. Integrated Multi-Trophic Aquaculture (IMTA) has been proposed as a solution for mitigating aquaculture waste release. Seaweeds can ‘purify’ the farm effluents by acting as biofilters. Within this context the seaweeds are often referred to as extractive organisms. Further, IMTA can also increase farm revenues by providing additional

commercial crops (principally seaweeds) and by reducing the discharge of effluents. Thus, IMTA is considered as an environmentally friendly (or perhaps more appropriately less environmentally damaging) and cost effective aquaculture system. Seaweeds can be grown using three methods in integrated cultivation: (1) seaweeds are grown in ponds or tanks separately and receive aquaculture wastes from culture systems of animals; (2) seaweeds are grown in ponds or tanks together with animals; (3) seaweeds are grown in the open water near cages with animals. For instance, the integrated culture of *U. lactuca* and abalone was carried out in South West coast of South Africa. In this system, abalones and *U. lactuca* were grown in 12,000 L and 3,000 L tanks respectively. After the water passed through two seaweed tanks from abalone tanks, 25% (1, 500 L $hour^{-1}$) was pumped back to the abalone tanks (Robertson-Andersson et al., 2008). In another integrated system, shrimp and *U. clathrata* were co-cultured in 2000 L tanks and 1.5 kg *U. clathrata* was suspended on a horizontal polyethylene 2.5 × 2.5 cm mesh floating on the water surface (Cruz-Suárez et al., 2010). In addition, round fragments of *U. ohnoi* were cultured in transparent chambers beside a fish cage in the open sea. The specific growth rate of *Ulva* was 12.8–23.6%, significantly higher than those at a control station (without fish cages). The nitrogen uptake rate was 2–13.9 mg N g DW^{-1} day^{-1}, which was among the highest levels of seaweed biofilters reported (Yokoyama and Ishihi, 2010).

1.4.4 Advantages and disadvantages of Ulva *cultivation*

To better understand the future directions of *Ulva* cultivation, the strengths and weaknesses of *Ulva* cultivation are summarised in Table 1.2. Compared with other commercially cultivated seaweeds, a noteworthy advantage of *Ulva* is its fast growth. The daily growth rate of *U. intestinalis* reached 43% when cultured on a 16-hour daily photoperiod for 14 days (Kim and Lee, 1996) while the growth rate of *Porphyra* (Pereira et al., 2006, Monotilla and Notoya, 2010) and *Laminaria* (Bolton and Lüning, 1982, Roleda et al., 2004, Omoregie et al., 2010) are usually below 30% and 20% respectively. Additionally, *Ulva* species have extraordinary tolerance of high nitrogen concentrations. For instance, the optimal nitrogen level for growth of *U. prolifera* occurs at 50 μM but it also can grow well when DIN reaches 500 μM (Li et al., 2010). The strong capacity for nutrient assimilation, especially ammonium, qualifies this species for bioremediation purposes and integrated multi-trophic systems (Neori et al., 2003, da Silva Copertino et al., 2009, Cahill et al., 2010). Furthermore, *Ulva* is a cosmopolitan green seaweed and common from tropical to polar climates, from fresh water through brackish to fully saline environments by virtue of its tolerance of a wide

range salinity and water temperature (Carl et al., 2014a), which makes it easy to obtain for cultivation.

In spite of the advantages, challenges for *Ulva* cultivation have been defined. The morphology of *Ulva* species can be substantially changed by environmental factors, particularly by epiphytic bacteria (Matsuo et al., 2005, Wichard et al., 2015), leading to difficulties in identification. Consequently, many species names have been misapplied (Silva et al., 1996). For instance, the records of *U. lactuca*, shows this species to have a wide distribution. *U. lactuca* accounts for approximately 30% of the peer-reviewed international literature on *Ulva* (1499 of 4854 studies searched in Scopus) (Kirkendale et al., 2013). However, the validity of such a broad application of the name has been doubted by several studies. Heesch et al. (2009) only found *U. lactuca* infrequently in New Zealand from disturbed habitats and Kraft et al. (2010) did not recover *U. lactuca* from extensive survey work in southern Australia. As Stegenga et al. (1997) suggested, most records might be incorrect. This creates challenges for *Ulva* cultivation since morphologically similar or closely related species may have unique ecophysiological and chemical characteristics (Eswaran et al., 2002, Michael, 2009, Paulert et al., 2010). Another weakness of *Ulva* cultivation is the instability of productivity. Vegetative fragmentation and/or the formation of reproductive cells effectively terminate *Ulva* growth and lead to disintegration of part or all of the thalli (Oza and Sreenivasa Rao, 1977). In addition, the market for *Ulva* needs further exploration. Until now, *Ulva* is mainly consumed by the Japanese as food (Bolton et al., 2009). The value of *Ulva* as animal feed is relatively low.

Advantages	Disadvantages
Fast growth	Difficulty in identity of *Ulva* species
Grow well in eutrophic waters and high ammonium uptake rate	Instability of long-term growth
Cosmopolitan distribution	Commercial benefit not obvious
High stocking density	

Table 1.2 Advantages and disadvantages of commercial *Ulva* cultivation.

1.5 Effects of ocean acidification and warming on seaweeds

1.5.1 Effects of ocean acidification on seaweeds

The atmospheric concentration of carbon dioxide increased by 40% to 391 ppm between 1750 and 2011 because of human activity; a rate of increase that is unprecedented within at least the last 800,000 years (IPCC, 2013). When CO_2 dissolves in seawater it forms carbonic acid and as more CO_2 is taken up by the ocean's surface, the pH decreases, moving towards a less alkaline and therefore more acidic state, termed ocean acidification.

Studies on the ecological and physiological impacts of elevated CO_2 concentrations on seaweeds dated from the early 1990s (Gao et al., 1991, Gao et al., 1993). Seaweeds have developed multiple strategies in inorganic carbon (Ci) acquisition, with different carboxylation efficiencies associated with different photosynthetic affinities for Ci (Giordano et al., 2005, Zou and Gao, 2010). Most seaweeds can take up HCO_3^- and/or CO_2 by active transport (termed carbon concentrating mechanisms, CCMs), while a few red and green seaweeds acquire Ci solely by diffusion of dissolved CO_2 (Raven et al., 1995, Hurd et al., 2009). Therefore, the influence of ocean acidification and a changed seawater carbonate system on seaweeds may be species-dependent. Species that can actively use bicarbonate seem unlikely to show stimulation of photosynthesis or growth with an increase in atmospheric CO_2 levels since increased atmospheric CO_2 will result in a small proportional change in HCO_3^- compared to CO_2 and CO_3^{2-} concentrations in seawater (Beardall et al., 1998). On the other hand, species relying on CO_2 uptake by passive diffusion might benefit more than those that can take up CO_2 actively with an active CCM. For instance, increasing atmospheric CO_2 concentrations have been demonstrated to enhance the growth of the red algae *Porphyra yezoensis*, *Gracilaria* spp., and *Lomentaria articulata* (Gao et al., 1991, Gao et al., 1993, Kübler et al., 1999). Increased CO_2 also promoted meiospore germination of brown macroalga *Macrocystis pyrifera* (Roleda et al., 2012). However, reduced growth rate under high CO_2 concentrations was observed in *P. linearis*, which was attributed to promoted dark respiration rate under low pH consuming photosynthate accumulated during light period (Mercado et al., 1999).

Compared to non-calcareous seaweeds, the effects of ocean acidification on calcareous seaweeds are relatively straightforward. Decreased pH and carbonate concentration under high CO_2 levels are known to negatively affect calcareous seaweeds, reducing calcification of the red coralline algae (Gao et al., 1993, Gao and Zheng, 2010), brown *Padina* spp. (Johnston and Raven, 1990), and green *Halimeda* spp. (Sinutok et al., 2011). Therefore, the fundamental changes in the seawater carbonate system due to increasing atmospheric CO_2 levels may

enhance the competitive advantage of non-calcifying versus calcifying species (Kuffner et al., 2008, Hepburn et al., 2011).

Responses of seaweed may be determined by their morphology and life history, apart from carbon acquisition and calcification processes. For example, filamentous turf algae with shorter generation times may be able to acclimatise and adapt to the changing environment faster than large canopy-forming kelp species with a longer generation time (Roleda and Hurd, 2012).

1.5.2 Effects of ocean warming on seaweeds

The globally averaged combined land and ocean surface temperature has risen by 0.85 ℃ (0.65 to 1.06 ℃), over the period 1880 to 2012. The oceans dominate the increase in energy stored in the climate system, absorbing more than 90% of the energy accumulated between 1971 and 2010. Over 60% of the net energy increase in the climate system is stored in the upper ocean (0–700 m). On a global scale, the largest ocean warming is near the surface, and the upper 75 m has warmed by 0.11 ℃ (0.09 to 0.13 ℃) per decade over the period 1971 to 2010 (IPCC, 2013).

It is extremely likely that more than half of the observed increase in global average surface temperature from 1951 to 2010 was caused by the anthropogenic increase in greenhouse gas concentrations and other anthropogenic forcings. The global ocean will continue to warm during the 21st century (IPCC, 2013). Best estimates of ocean warming in the top 100 m are approximately 0.6 ℃ (Representative Concentration Pathway 2.6) to 2.0 ℃ (Representative Concentration Pathway 8.5), and approximately 0.3 ℃ (Representative Concentration Pathway 2.6) to 0.6 ℃ (Representative Concentration Pathway 8.5) at a depth of about 1000 m by the end of the 21st century (IPCC, 2013). The larger land area in the northern hemisphere results in a larger increase of temperature under the same radiative forcing compared to that in the southern hemisphere. Moreover, the decrease of sea-ice cover in summer magnifies the summer warming. In contrast, minimum warming is predicted in the Southern ocean due to strong ocean heat uptake in this region (Bartsch et al., 2012).

Temperature can affect seaweeds from community to cellular levels. Temperature change plays a crucial role in the geographical distribution of seaweeds since temperature is the chief abiotic factor directly controlling geographic boundaries of seaweed species (Hoek, 1982, Lüning, 1990) and also has recently been identified as the most important environmental predictor in terms of structuring cross-taxon marine biodiversity (Tittensor et al., 2010). The effect of temperature on the physiological traits of plants, such as growth and photosynthesis, is typically demonstrated by temperature-response curves. Both growth and

photosynthetic rates of seaweeds generally show the same trend, rising with temperature, reaching a peak at an optimal level, and then decreasing until ceasing (Figure 1.4). Manifold useful parameters (e.g. the minimum, maximum, optimum temperature, and the performance breadth) can be drawn from temperature response curves. For instance, the performance breadth (the 80% or 20% temperature ranges) can be used to define eurythermy and stenothermy. Eurythermal seaweeds have a wide "performance breadth" and can grow in environments with large fluctuations in seawater temperature. By contrast, stenothermal species have a narrow performance breadth and are only found in regions with small temperature fluctuations. Generally, responses of seaweeds to temperature change can be summarised as three types based on different timescales: (1) physiological regulation (seconds to minutes) (2) phenotypic acclimation (hours to days) (3) genetic adaptation (up to years) (Eggert, 2012).

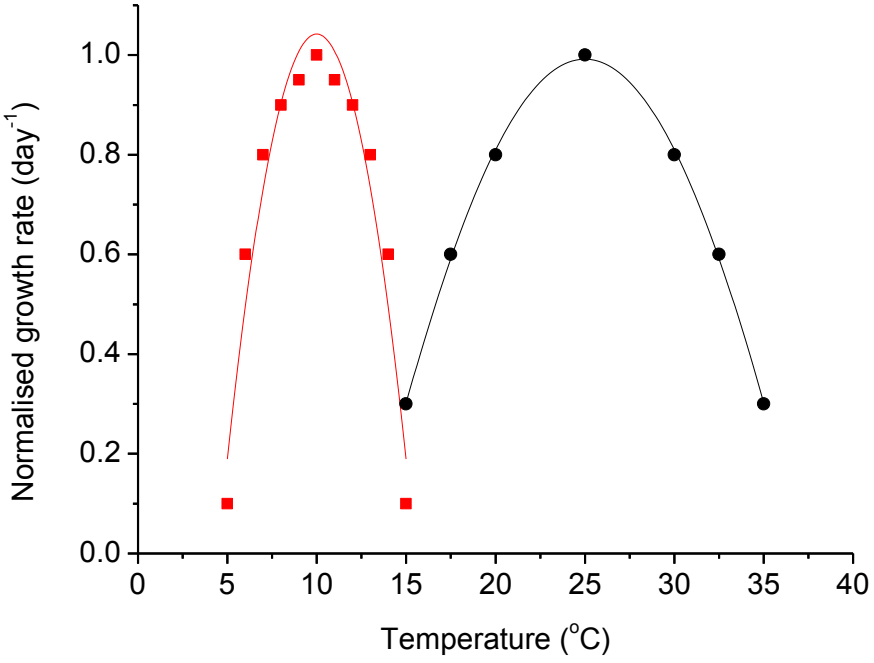

Figure 1.4 Typical normalized growth temperature-response curves of the stenothermal temperate seaweed (red squares) and eurythermal tropical seaweed (black circles). This plot was redrawn from Eggert (2012).

Change of temperature can be perceived very quickly by temperature sensors (membrane fluidity, protein conformation, cytoskeleton depolymerisation) and transduced into the interior of cells (Horváth et al., 1998, Los and Murata, 2004), by which physiological regulation of organisms can be initiated rapidly. For instance, the difference of photochemical efficiency in *U. rotundata* under different temperatures could be detected within 15 minutes

in response to a change of temperature (Franklin, 1994). Enzyme and protein adjustments occur constantly to compensate for temperature alteration. These responses can occur in both acclimation and adaption depending on different timescales (Clarke, 2003). These cellular level adjustments can involve two or more strategies: quantitative (changing the concentrations of enzymes or proteins) or qualitative (protein variant/isozyme with different thermal characteristics) strategy (Eggert, 2012). For instance, Antarctic *Chloromonas* species counteracted the effect of low temperature via increasing enzyme concentration, i.e. quantitative adjustments (Devos et al., 1998) while Antarctic diatoms showed a qualitative strategy: they could allow RuBisCo to bind CO_2 effectively at low temperature by an isozyme with modified kinetic properties (Descolas-Gros and de Billy, 1987). Molecular tools such as cDNA and oligonucleotide DNA microarrays ("gene chips") are also used to detect temperature induced changes in the transcriptome. The gene encoding ice-binding proteins have been found in Antarctic sea ice diatoms as a very specific adaptation to freezing temperatures in this extreme habitat. The extracellular proteins have been shown to interfere with the growth of ice and to have the ability to act as cryoprotectants to prevent freezing injury (Janech et al., 2006). However, studies on temperature adaptation of thermal related proteins or genes are lacking for seaweeds.

1.5.3 Combined effects of ocean acidification and warming

Ocean acidification and warming are not proceeding in isolation, and instead they are developing simultaneously. Most studies of the interactive effects of ocean acidification and warming focus on invertebrate animals (Pörtner, 2008, Pandolfi et al., 2011, Rodolfo-Metalpa et al., 2011, Kroeker et al., 2013), with very few on calcifying seaweeds (Diaz-Pulido et al., 2012), and none on non-calcifying seaweeds species. The interactive effects of multiple factors may be completely different or be of a larger magnitude, compared to effects that either single stressor imposes. For example, either increased CO_2 or temperature did not exert an influence in calcification of the crustose coralline alga (CCA) *Lithophyllum cabiochae*. However, the combination of increased CO_2 and temperature led to a 50% decline in calcification and an accelerated and extensive tissue necrosis (Martin and Gattuso, 2009). The amplified negative effects of high CO_2 under higher warming conditions was found in the CCA *Hydrolithon onkodes* as well. The rates of advanced partial mortality of CCA increased from 9% to 15% under high CO_2 when the temperature rose from 26 ℃ to 29 ℃. Furthermore, dissolution of *H. onkodes* which only occurred in the high CO_2 treatment was dramatically enhanced by the increased temperature (Diaz-Pulido et al., 2012).

1.6 Research aims and objectives

The development and deployment of culture systems for seaweeds is a growing focus for European aquaculture. Algae are increasingly viewed as central to resolving Europe's demand for biomass as a raw material for food, animal feed, fuel and the delivery of vital ecosystem services such as wastewater bioremediation. Seaweed farming will become increasingly important for north-western European nations. There are at least 13 kinds of *Ulva* species in the UK and the North Sea climate is favourable for production of seaweed. Considering that seaweed cultivation is at its infant stage in Europe, this research will assist in developing systems for the commercial culture of *Ulva* species in the UK and will therefore position Britain at the very heart of the European drive to deliver a viable and vibrant seaweed aquaculture industry with associated supply and value chains. Meanwhile, climate changes, such as ocean acidification and warming will inevitably impact the physiological features of *Ulva* and thus *Ulva* cultivation in the future. Accordingly, the research aims and objectives to be addressed in this thesis are as follow:

1. To optimise conditions of *Ulva* growth by growing *Ulva* under varying levels of light, nutrient, temperature, aeration time and period (Chapter 2) and define techniques for long-term *Ulva* cultivation by multiple harvests and strain improvement (Chapters 2 and 4);
2. To refine methods for out-of-season maturation and reproduction with the goal of providing spore release on demand by investigating the combined effects of internal and external factors on reproduction of *Ulva* (Chapter 3);
3. To develop methods for preserving *Ulva* thereby enabling growth trials to be conducted on demand by comparing viability of *Ulva* thalli, germlings, and gametes after storing at different temperatures (Chapter 5);
4. To investigate the effects of ocean acidification, warming, and eutrophication on life cycle and food quality of *Ulva* by measuring settlement, germination, growth, reproduction, chemical composition, and functional properties of *Ulva* grown under ambient, future ocean conditions, and varying nitrate levels (Chapter 6).

Chapter 2. Growth conditioning of *Ulva rigida*

2.1 Introduction

The green seaweed, *Ulva*, has been gaining significant interest due to its potential value in animal feed (Cyrus et al., 2015, Ahmed et al., 2015), human food (Ortiz et al., 2006, Yaich et al., 2015), biofuel (Bruhn et al., 2011, Neveux et al., 2014) as well as the delivery of crucial ecosystem services such as wastewater bioremediation (Chung et al., 2002, Al-Hafedh et al., 2014) and CO_2 removal (Kaladharan et al., 2009, Chung et al., 2011). Therefore, *Ulva* species have been cultivated in many areas of the world in pilot commercial systems including Japan (Hiraoka and Oka, 2008), America (DeBusk et al., 1986), Israel (Neori et al., 2000), and South Africa (Bolton et al., 2009). The first and foremost aim for seaweed cultivation is to obtain a fast growth rate. Several factors influence optimal growth of *Ulva* species, including nitrate, light, temperature and aeration. These factors are interdependent, and their effects differ between species.

2.1.1 Nitrate

Nitrate is one of most important factors affecting *Ulva* growth. Research by Steffensen (1976) demonstrated that the addition of nitrate stimulated growth of *U. lactuca* with optimum levels being 43 μmol L^{-1}. The specific growth rate of *U. rigida* is also significantly positively related to dissolved inorganic nitrogen (DIN) in the water column when DIN varied from 3–75 μmol L^{-1} (Viaroli et al., 1996). However, it is important to note that if nitrate levels are too high, they can reduce the growth of *Ulva*. With levels of nitrate above 43 μmol L^{-1}, decreased growth of *U. lactuca* was reported (Steffensen, 1976). High nitrate load level (38.32 g N m^{-2} day^{-1}) was also shown to reduce the growth of *U. lactuca* (Msuya and Neori, 2008). In addition, effects of nitrate on growth were affected by other factors. For example, nitrate addition did not enhance the growth of *U. fasciata* Delile at low light conditions of 109 μmol photons m^{-2} s^{-1} but growth increased with nitrate at high light conditions of 305 μmol photons m^{-2} s^{-1} (Lapointe and Tenore, 1981).

2.1.2 Light

Light is a prerequisite in terms of growth of seaweeds due to its role in driving photosynthesis. The relationship between irradiance and photosynthesis abides by classic photosynthesis-irradiance curves in which photosynthesis rises with irradiance, reaches a peak at some point (termed saturation irradiance) and keeps maximum photosynthesis rate or declines (photoinhibition) over saturation irradiance (Jassby and Platt, 1976, Behrenfeld and

Falkowski, 1997). The relationship between growth and irradiance is similar to that of irradiance and photosynthesis since growth depends on photosynthesis to a large extent.

The saturation irradiance for *Ulva* growth or photosynthesis varies within a large range between 10–689 μmol photons m^{-2} s^{-1}, which is species and location dependent (Arnold and Murray, 1980, Fortes and Lüning, 1980, Beach et al., 1995, Han et al., 2003, Choo et al., 2005, Kim et al., 2011). The lowest saturation irradiance reported is 10 μmol photons m^{-2} s^{-1} for *U. pertusa* collected from the eastern coast of Korea (Han et al., 2003). *U. lactuca* from the North Sea also has a relatively low saturation irradiance of 70 μmol photons m^{-2} s^{-1} (Fortes and Lüning, 1980). The highest saturation irradiance of 689 μmol photons m^{-2} s^{-1} was found in *U. linza* from the eastern Yellow Sea (Kim et al., 2011). Above saturation irradiance, a minority of *Ulva* sp. show photoinhibition, which indicates that higher light intensity could inhibit growth (Arnold and Murray, 1980). For instance, the light intensities of 1956, 1405, 1950 μmol photons m^{-2} s^{-1} reduced photosynthesis of *U. intestinalis*, *U. lobata*, and *U. rigida* by 39%, 57%, and 32% respectively, compared with maximum photosynthesis (Arnold and Murray, 1980).

2.1.3 Temperature

In addition to light and nitrate, temperature is an essential factor affecting growth of *Ulva*. Generally, the growth rate of *Ulva* increases with temperature but begins to decrease as temperature levels become damaging. For instance, the growth rate of *U. fasciata* increased as the temperature rose from 15 ℃ to 25 ℃, while fragmentation of the fronds and bleaching was observed when the temperature was above 30 ℃ and all fronds died when it reached 35 ℃ (Mohsen et al., 1973). The optimal temperature for *Ulva* growth is species and location dependent. The relative growth rate of *U. curvata* increased with temperature from 5 ℃ to 20 ℃ and decreased when the temperature passed 20 ℃ (Duke et al., 1989). For *U. fenestrata*, water temperature of 10 ℃ is the optimum condition for growth (Kalita and Tytlianov, 2003). The growth response of *U. lactuca* from two localities with differing water temperature was investigated by Steffensen (1976). The plants from the locality with the higher mean monthly temperature showed the higher optimal temperature for growth.

2.1.4 Aeration

In modern seaweed cultivation ponds, bottom aeration is extensively employed to stir the suspended seaweed (Msuya and Neori, 2008). This approach to seaweed cultivation was developed by the group led by Ryther in the 1970s and concisely described by Huguenin (1976), DeBusk et al. (1986), and Bird (1989). Vertical movement produced by bottom aeration allows each seaweed frond to be exposed to an optimal light condition and pass

nutrient-rich water over them. The productivity of *U. lactuca* cultivated over eight months under aerated condition was nearly three times more than that under non-aerated condition (DeBusk et al., 1986). However, aeration did not stimulate growth of *U. lactuca* when enough nutrient were supplied, indicating aeration is not essential for growth when nutrient concentrations are high enough (Msuya and Neori, 2008). On the other hand, aeration has accounted for much of the operating cost in pond cultivation of seaweed since the early 1970s (Huguenin, 1976, Ben-Ari et al., 2014). In order to reduce cost, intermittent aeration was used during the 1980s and 1990s but has been almost ignored in recent years (DeBusk et al., 1986, Friedlander and Ben-Amotz, 1991, Ugarte and Santelices, 1992, Caines et al., 2014). As daily aeration decreased from 12 to four hours, productivity of *Gracilaria* sp. decreased from an average of 21 to 12 g m^{-2} day^{-1} while no difference was detected between a daily aeration period of 12 and 24 hours (Guerin and Bird, 1987). A similar result was reported in *U. lactuca* (Vandermeulen and Gordin, 1990). The production of *G. chilensis* decreased dramatically (from 103 $\pm$8 to 63 $\pm$4 g m^{-2} day^{-1}) when time of air bubbling was reduced from 10 hours to five hours per day. However, a further 50% reduction in air bubbling time only caused 13% decrease in production (Ugarte and Santelices, 1992). Short intermittent aeration cycles of one minute to two hours have been successfully used in seaweed cultivation (DeBusk et al., 1986, Friedlander and Ben-Amotz, 1991) but extra electricity consumption during frequent starting of air compressors would offset the cost savings with these intermittent regimes (Caines et al., 2014).

2.1.5 Aims and objectives

It would appear that little information on optimal growth conditions of *Ulva* from the North Sea is available. This chapter aimed to obtain optimal conditions for growth of *U. rigida*. To achieve that, growth of *U. rigida* cultured under varying light intensities, nutrient levels, temperatures, and aeration rates and periods was measured. In addition, a multiple harvest method was developed in an effort to maintain a high growth rate of *U. rigida*.

2.2 Materials and methods

2.2.1 Seaweed collection and identification

Adult vegetative *U. rigida* of 50–60 mm in length were collected from the low intertidal of Cullercoats beach, UK (55.03^{o} N, 1.43^{o} W) after a spring tide. The fronds were placed in a plastic bag and transported to the laboratory in Newcastle University within one hour. The fronds were then rinsed gently in sterile seawater (one micron filtered) to remove any sediment, epiphytes or small grazers. *U. rigida* thalli were placed in 500 ml conical flasks (one thallus per flask) and aerated continuously in all experiments, except experiments where

aeration was a variable. The light and darkness cycle was 16L: 8D. All experiments were in triplicate. All flasks used in this thesis were conical flasks. All media in this thesis were made with natural seawater, which was collected from the Blue Reef Aquarium®, Tynemouth, Tyne and Wear, UK (55.03^{o} N, 1.43^{o} W), very close to the *U. rigida* collection site.

Species was identified by the techniques of DNA barcoding in the Institute of Oceanology, Chinese Academy of Sciences. Total DNA was extracted with the CTAB method described by Cuvelier et al. (2010) after the seaweed specimen was washed with sterile seawater, and approximately 100 μg of thalli (fresh mass) was carefully ground in a 1.5 ml centrifuge tube with the addition of liquid nitrogen and silica beads. The extracted DNA concentration was quantified with NanoDrop 2000c (Thermo Fisher Scientific Inc.) and preserved at –20 ℃ until PCR amplification.

The ribosomal ITS region was amplified with a pair of universal primers designed for eukaryotic organisms, ITS-F(a) (5'-TCGTAACAAGGTTTCCGTAGG-3') and ITS-R(d) (5'-TTCCTTCCGCTTATTGATATGC-3') (Leskinen and Pamilo, 1997). The volume of PCR reactions was 20 μl, containing 12.68 μl ddH_2O, 2 μl 10 × Taq Buffer (Mg^{2+}), 1.6 μl dNTP Mix (2.5 mM each NTP), 2 μl of DNA template (20 ng DNA in each PCR reaction), 0.8 μl of each PCR primer (10 pmol μl^{-1}), and 0.12 μl rTaq DNA polymerase (5 U μl^{-1}, Takara). PCR reactions were performed with an initial denaturation at 94 ℃ for five minutes, 36 cycles at 94 ℃ for 50 s, 54 ℃ for 50 s, and 72 ℃ for one minute, and a final elongation step of 10 minutes at 72 ℃.

The PCR product was confirmed by electrophoresis in 1% agarose gel. The DNA sequences of ITS regions (approximately 600 bp) were purified using Agarose Gel DNA Purification Kit (Takara). The purified DNA sequence was sequenced from both ends by Sanggon Biothen Co. LTD. and the sequences were assembled by ContigExpress in Vector NTI 11 (Invitrogen). The sequences were searched for similarity using the BLAST algorithm at web servers of NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). It was found that the sequence excluding the primers at both ends absolutely matched *U. rigida* SSBO0102 isolated from Skara Brae, Orkney, Scotland.

2.2.2 Seaweed culture under different light and nutrient levels

Nitrate was used as the source of nitrogen as it is the primary form of inorganic nitrogen in seawater (Lobban, 1994). Two levels of light (40 (low light, LL) and 80 (high light, HL) μmol photons m^{-2} s^{-1}) were fully crossed with three levels of nitrate and phosphate (6 μM N with 0 μM P (low nutrient, LN), 150 μM N with 7.5 μM P (moderate nutrient, MN), and 750 μM N and 37.5 μM P (high nutrient, HN)) in the first experiment for 12 days. Media

were made from natural seawater with the addition of $NaNO_3$ and KH_2PO_4. The growth measurements were recorded on days six, nine, and 12. In order to ensure optimal light and nitrate conditions, two levels of light (80 (low light, LL) and 140 (high light, HL) μmol photons m^{-2} s^{-1}) and two levels of nitrate (150 μM N with 7.5 μM P (low nutrient, LN) and 300 μM N with 15 μM P (high nutrient, HN) were deployed in the second experiment for 12 days. The set of light and nutrient levels was based on previous studies on *Ulva* sp. (Fortes and Lüning, 1980, Viaroli et al., 1996). The light was supplied by lamps (SYLVANIA, F58W/865). The growth measurements were recorded on days four, eight, and 12. Both experiments were in triplicate with one thallus placed in each 500 ml flask.

2.2.3 Seaweed culture under different temperatures

U. rigida thalli were cultured at three temperatures (12°C (LT), 15°C (MT), 18°C (HL)) for 16 days to investigate effects of temperature on growth. Temperature selection was based on two previous studies of *Ulva* (Mohsen et al., 1973, Kalita and Tytlianov, 2003). The experiments were in triplicate with one thallus placed in each 500 ml flask. To explore reproduction at different temperature levels, three flasks, each containing 24 *Ulva* disks of 7 mm in diameter were cultured at each temperature simultaneously. The growth and reproduction measurements were recorded on days four, eight, 12, and 16.

2.2.4 Seaweed culture under different aeration conditions

U. rigida thalli were cultured under four different aeration rates (0, 50, 100, and 200 L $minute^{-1}$) for 12 days in the first aeration experiment. Aeration was continuously conducted under the conditions of 50, 100, and 200 L $minute^{-1}$. In the second aeration experiment (12 days), five daily levels of intermittent aeration were used, in which the on and off periods were (in hours) 4: 20, 8: 16, 12: 12, 16: 8, and 24: 0. The aeration rate of 100 L $minute^{-1}$ was employed in these five levels of intermittent aeration as this rate was optimal for *Ulva* growth based on the first aeration experiment. The set of aeration rates and on and off periods were chosen according to previous studies (Guerin and Bird, 1987, Vandermeulen and Gordin, 1990, Caines et al., 2014). The nutrient level was 150 μM N with 7.5 μM P, and the light intensity was 80 μmol photons m^{-2} s^{-1} with a 16: 8 of light: dark period. One thallus was placed in a 500 ml flask and three flasks were used per treatment. The growth measurements were recorded on days four, eight, and 12.

2.2.5 Multiple harvests method

In order to maintain high growth rate over a longer term, a multiple harvest method was employed as it could remove the low productivity part of *U. rigida* thalli. *U. rigida* were grown under a light level of 80 μmol photons m^{-2} s^{-1} at 18°C for 21 days. The initial biomass

was 0.34 ± 0.01 g and part of the thalli was cut off on days nine and 18 to return to the initial biomass mass. Culturing under the same conditions without cutting was set as the one-off harvest method. One thallus was placed in a 500 ml flask, with five flasks per treatment. The growth measurements were recorded on days three, six, nine, 12, 15, 18, and 21.

2.2.6 Nitrogen and phosphorus uptake determination

Nitrate concentration was measured by a rapid spectrophotometer method (Collos et al., 1999). Nitrate standards were produced by serial dilution of a sodium nitrate stock. Standards of 0.8 ml were transferred to a quartz cuvette (1-cm light path). Absorbance intensity at 220 nm was recorded in a UV/Vis spectrophotometer (Cary 100 Bio, Australia). A standard curve (Figure A1.1) was obtained through regression analysis in Microsoft Excel 2013. Samples of 2 ml were taken from every flask in section 2.2.2 to 2.2.5, centrifuged at 1000 *g* for five minutes to remove precipitates. Afterwards, 0.8 ml of supernatant was transferred to the quartz cuvette (1-cm light path). Absorbance intensity at 220 nm was used to calculate concentration of nitrate by the standard curve.

Phosphate was determined by the phosphomolybdenum blue colorimetry method (Murphy and Riley, 1962). Mixed reagent was made by mixing thoroughly 125 ml of 0.8 M sulphuric acid, 37.5 ml of 0.2 M ammonium molybdate, 75 ml of 0.1 M ascorbic acid solution, and 12.5 ml of 0.004 M potassium antimonyl tartrate solution. This reagent was prepared as required as it cannot keep for more than 24 hours. Phosphate standards were produced by serial dilution of sodium potassium dihydrogen phosphate stock. One ml of phosphate standards were added to 5 ml mixed reagent, then placed at room temperature for 15 minutes. A mixed solution of 0.8 ml was transferred to a quartz cuvette (1-cm light path). Absorbance intensity at 882 nm was recorded in a UV/Vis spectrophotometer (Cary 100 Bio, Australia). A standard curve (Figure A1.2) was obtained through regression analysis in Microsoft Excel 2013. Samples of 2 ml were taken from every flask (section 2.2.2 to 2.2.5), centrifuged at 1000 *g* for five minutes to remove precipitates. Afterwards, 1 ml of supernatants were mixed with 5 ml mixed reagent, placed at room temperature for 15 minutes before transferring to the quartz cuvette (1-cm light path). Absorbance intensity at 882 nm was used to calculate the concentration of phosphate by the standard curve.

2.2.7 Growth measurement

Growth of *U. rigida* was determined by weighing fresh thalli. *Ulva* thalli were blotted gently with tissue paper to remove water on the surface of the thalli before weighing. Specific growth rate (DGR) was calculated by the formula: $DGR\ (\%) = [\ln (M_2/M_1)]/t \times 100$, where M_2 is the final mass, M_1 is the initial mass, and t is the number of culture days.

2.2.8 Reproduction measurement

The reproductive *U. rigida* disks were recognized by a colour change. Formation of reproductive cells in *Ulva* is accompanied by a change in thalli colour from green (vegetative state) to yellowish (reproductive state) and then to white (after release of swarmers). This was verified via microscopic observation at 400 × magnification, by which *Ulva* spores could be found within the sporangia. Partial sporulation formation in more than half the disk area was considered equivalent to complete sporulation formation. Reproduction rate was expressed as the ratio of reproductive disks to all disks in a flask.

2.2.9 Statistical analysis

Results were expressed as means of replicates ± standard deviation. Data were analyzed using the software SPSS v.21. A confidence interval of 95% was set for all tests.

Effects of light and nitrate on growth

In the first light and nitrate experiment, the specific growth rates of *U. rigida* under every treatment on days six, nine, and 12 conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the six samples on days six, nine, and 12 could be considered equal (Levene's test, $F < 2.41$, $P > 0.05$). Three two-way ANOVAs were conducted to assess the effects of light and nitrate on growth of *U. rigida* on days six, nine, and 12, respectively. Tukey HSD was conducted for *post hoc* investigation. In the second light and nitrate experiment, the specific growth rates of *U. rigida* under every treatment on days four, eight, and 12 conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the six samples on days four, eight, and 12 could be considered equal (Levene's test, $F < 3.48$, $P > 0.05$). Three two-way ANOVAs were conducted to assess the effects of light and nitrate on growth of *U. rigida* on days four, eight, and 12, respectively. *Post hoc* tests were not conducted because there were no significant main effects of light or nitrate.

Effects of temperature on growth

The specific growth rates of *U. rigida* under every temperature treatment on days four, eight, 12, and 16 conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the three samples on days four, eight, 12, and 16 could be considered equal (Levene's test, $F < 2.29$, $P > 0.05$). Four one-way ANOVAs were conducted to assess the effects of temperature on growth of *U. rigida* on days four, eight, 12, and 16, respectively. Tukey HSD was conducted for *post hoc* investigation. The reproduction rates of *U. rigida* under every temperature treatment on days eight, 12, and 16 conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the three samples on days eight, 12,

and 16 could be considered equal (Levene's test, $F < 2.90$, $P > 0.05$). Three one-way ANOVAs were conducted to assess the effects of temperature on the reproduction rates of *U. rigida* on days eight, 12, and 16, respectively. Tukey HSD was conducted for *post hoc* investigation.

Effects of aeration on growth

The specific growth rates of *U. rigida* at every aeration rate on days four, eight, and 12 conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the four samples on days four, eight, and 12 could be considered equal (Levene's test, $F < 1.33$, $P > 0.05$). Three one-way ANOVAs were conducted to assess the effects of aeration rate on growth of *U. rigida* on days four, eight, and 12, respectively. Tukey HSD was conducted for *post hoc* investigation. The specific growth rates of *U. rigida* at every aeration time on days four, eight, and 12 conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the five samples on days four, eight, and 12 could be considered equal (Levene's test, $F < 1.50$, $P > 0.05$). Three one-way ANOVAs were conducted to assess the effects of aeration rate on growth of *U. rigida* on days four, eight, and 12, respectively. Tukey HSD was conducted for *post hoc* investigation.

Effects of harvest method on growth

The specific growth rates of *U. rigida* in both harvest methods on days three, six, nine, 12, 15, 18, and 21 conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the two samples on days three, six, nine, 12, 15, 18, and 21could be considered equal (Levene's test, $F < 4.89$, $P > 0.05$). Seven independent samples t-tests assuming equal variances were conducted to assess the effects of harvest method on the growth of *U. rigida* on days three, six, nine, 12, 15, 18, and 21, respectively. The mean specific growth rate data over the 21-day culture in both harvest methods conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$). The variances of the two samples could be considered equal (Levene's test, $F = 4.918$, $P > 0.05$). A t-test assuming equal variances was conducted to assess the effects of harvest method on the mean specific growth rate of *U. rigida.*

2.3 Results

The key questions that this chapter aimed to answer are: (1) what are the optimal conditions for growth of *U. rigida* in terms of light, nutrient, temperature, and aeration? (2) how a high growth rate can be maintained in the long term?

2.3.1 Effects of light and nutrients

The specific growth rates of *U. rigida* under various light and nitrate treatments over 12-day culture were observed (Figure 2.1). Nutrient and light had significant interactive effects on the specific growth rates on day six (Table 2.1) since moderate nutrient (MN) and high light (HL) increased the specific growth rates by 206.74% and 206.68% separately while a 602.42% increase was reported when these two factors were combined (Figure 2.1). Whilst continuing to give higher growth rates, the trend became weaker on day nine (Table 2.2) as moderate nutrient and high light conditions separately resulted in 177.89% and 133.95% increase of the specific growth rates respectively, these factors combined resulted in increased specific growth rate of 401.78% (Figure 2.1). These interactive effects of nutrient and light were no longer apparent on day 12 while nutrient had a main effect on the specific growth rates (Table 2.3). *Post hoc* Tukey HSD comparison ($P = 0.05$) showed that the highest specific growth rate occurred at the moderate nutrient (MN) level (4.79 ± 0.48% at LL and 6.12 ± 0.57% at HL) and the high nutrient decreased the specific growth rates by 24.37% at LL and at 35.93 at HL compared to the moderate nutrient.

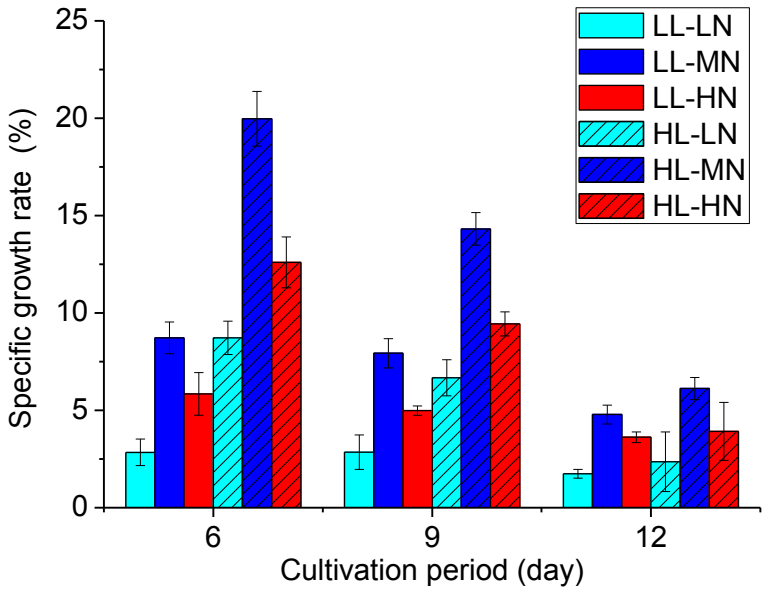

Figure 2.1 Specific growth rate of *U. rigida* cultured at low nutrient (LN, 6 µM N and 0 µM P), moderate nutrient (MN, 150 µM N and 7.5 µM P) and high nutrient (HN, 750 µM N and 37.5 µM P) conditions when exposed to low light (LL, 40 µmol photons m^{-2} s^{-1}) and high light levels (HL, 80 µmol photons m^{-2} s^{-1}). The error bars indicate the standard deviations (n = 3).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Light	285.000	1	285.000	253.392	<0.001
Nutrient	222.924	2	111.462	99.100	<0.001
Light* Nutrient	24.952	2	12.476	11.092	0.002
Error	13.497	12	1.125		

Table 2.1 Two-way analysis of variance of the effects of light and nutrient on the specific growth rate of *U. rigida* on day six (experiment 1). The confidence interval was 95%.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Light	107.513	1	107.513	192.761	<0.001
Nutrient	123.445	2	61.722	110.662	<0.001
Light* Nutrient	5.364	2	2.682	4.809	0.029
Error	6.693	12	0.558		

Table 2.2 Two-way analysis of variance of the effects of light and nutrient on the specific growth rate of *U. rigida* on day nine (experiment 1). The confidence interval was 95%.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Light	2.517	1	2.517	2.875	0.116
Nutrient	34.662	2	17.331	19.796	<0.001
Light* Nutrient	0.839	2	0.419	0.479	0.631
Error	10.505	12	0.875		

Table 2.3 Two-way analysis of variance of the effects of light and nutrient on the specific growth rate of *U. rigida* on day 12 (experiment 1). The confidence interval was 95%.

In order to investigate whether a further increase in light levels would increase the specific growth rate and whether the moderate nutrient level in the experiment is the optimal

condition, a subsequent experiment was carried out. Light levels were set as 80 (low light, LL) and 140 μmol photons m^{-2} s^{-1} (high light, LL), and a high nutrient level was set (300 μM N with 15 μM P (high nutrient, HN)) at double that of the moderate level (150 μM N with 7.5 μM P (low nutrient, LN)) in the previous experiment (Figure 2.2). Neither the increase in light nor nutrient enhanced the specific growth rate during the 12-day cultivation (Table 2.4–2.6), which indicated 80 μmol photons m^{-2} s^{-1} and 150 μM nitrate is an effective combination for rapid growth of *Ulva*.

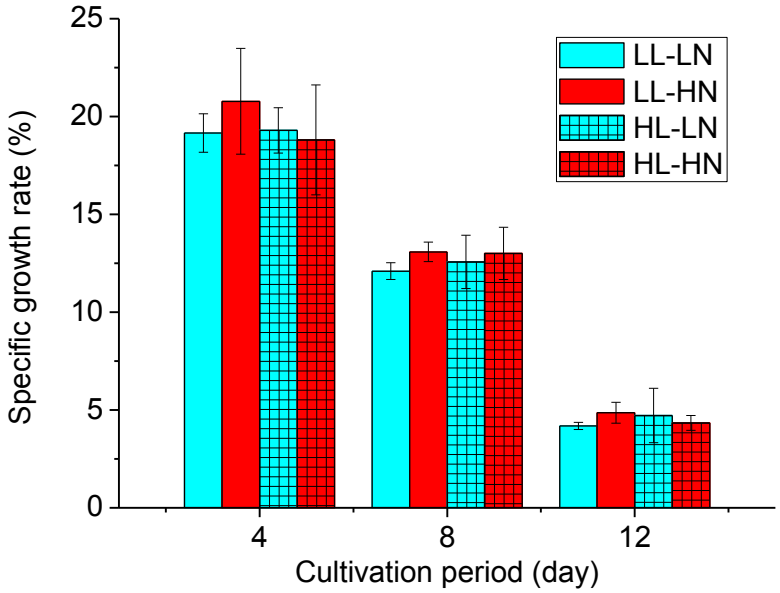

Figure 2.2 Specific growth rate of *U. rigida* under various light and nutrient conditions during the 12-day cultivation. LL-LN, low light and low nutrient (80 μmol photons m^{-2} s^{-1}, 150 μM N with 7.5 μM P); LL-HN, low light and high nutrient (80 μmol photons m^{-2} s^{-1}, 300 μM N with 15 μM P); HL-LN, high light and low nutrient (140 μmol photons m^{-2} s^{-1}, 150 μM N with 7.5 μM P); HL-HN, high light and high nutrient (140 μmol photons m^{-2} s^{-1}, 300 μM N with 15 μM P). The error bars indicate the standard deviations (n = 3).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Light	2.546	1	2.546	0.583	0.467
Nutrient	0.956	1	0.956	0.219	0.652
Light* Nutrient	3.322	1	3.322	0.760	0.409
Error	34.948	8	4.368		

Table 2.4 Two-way analysis of variance of the effects of light and nutrient on the specific growth rate of *U. rigida* on day four (experiment 2). The confidence interval was 95%.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Light	0.115	1	0.115	0.114	0.745
Nutrient	1.526	1	1.526	1.503	0.255
Light* Nutrient	0.225	1	0.225	0.221	0.651
Error	8.124	8	1.016		

Table 2.5 Two-way analysis of variance of the effects of light and nutrient on the specific growth rate of *U. rigida* on day eight (experiment 2). The confidence interval was 95%.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Light	<0.001	1	<0.001	<0.001	0.992
Nutrient	0.068	1	0.068	0.113	0.745
Light* Nutrient	0.822	1	0.822	1.372	0.275
Error	4.790	8	0.599		

Table 2.6 Two-way analysis of variance of the effects of light and nutrient on the specific growth rate of *U. rigida* on day 12 (experiment 2). The confidence interval was 95%.

2.3.2 Effects of temperature

The effects of temperature on the specific growth rates of *U. rigida* were investigated (Figure 2.3a). During the first four days of cultivation, there was a statistically significant

difference in the specific growth rate of *U. rigida* between temperatures (ANOVA, F = 19.938, df = 2, 6, P = 0.002). *Post hoc* Tukey HSD comparison (P = 0.05) showed that the specific growth rate increased with temperature (9.18 ± 1.85% at LT, 14.18 ± 1.30% at MT, 18.24 ± 2.04% at HT, Figure 2.3 a). Moderate temperature increased the specific growth rate by 54.49% compared with low temperature and a further rise in temperature to 18^{o}C resulted in a 28.58% increase relative to the moderate temperature. When cultivation time was extended to eight days, the specific growth rate of *U. rigida* between temperatures were significantly different as well (ANOVA, F = 3.481, df = 2, 6, P < 0.001). *Post hoc* Tukey HSD comparison (P = 0.05) showed moderate and high temperatures increased the specific growth rate by 42.34% and 48.66% respectively compared to low temperature and there was no significant difference in the specific growth rate between the moderate and high temperature. The effects of temperature on the specific growth rate of *U. rigida* disappeared on day 12 (ANOVA, F = 18.283, df = 2, 6, P = 0.099). At the end of the 16-day culture, the significant effect of temperature on the specific growth rate of *U. rigida* appeared again (ANOVA, F = 17.093, df = 2, 6, P = 0.003). *Post hoc* Tukey HSD comparison (P = 0.05) showed that the high temperature substantially reduced the specific growth rate, which was 18.48% lower than the moderate temperature and 21.04% lower than the low temperature.

Reproduction rates of *U. rigida* were also investigated during a 16-day culture period (Figure 2.3 b). No reproduction was observed under all three temperature treatments by day four. There were statistically significant differences in reproduction rates of *U. rigida* between temperatures (ANOVA, F > 10.857, df = 2, 6, P < 0.010) on days eight, 12, and 16. *Post hoc* Tukey HSD comparison (P = 0.05) showed that the reproduction rates of *Ulva* under high temperature were 125.00% higher than that (11.11 ± 2.41%) under low temperature and 50.00% higher than that under moderate temperature by day eight. There was no significant difference between moderate and low temperature. On day 12, the reproduction rate under low temperature was 16.67 ± 4.17%. *Post hoc* Tukey HSD comparison (P = 0.05) showed that moderate temperature did not enhance reproduction rate while high temperature increased reproduction rate by 175.00% compared with low temperature and by 73.68% compared with moderate temperature. Toward the end of the 16-day culture, reproduction rate under low temperature was 22.22 ± 2.41%. *Post hoc* Tukey HSD comparison (P = 0.05) showed that moderate temperature enhanced reproduction rate by 81.3% and there were more than two times (218.75%) the levels of reproduction rate under high temperature compared with low temperature.

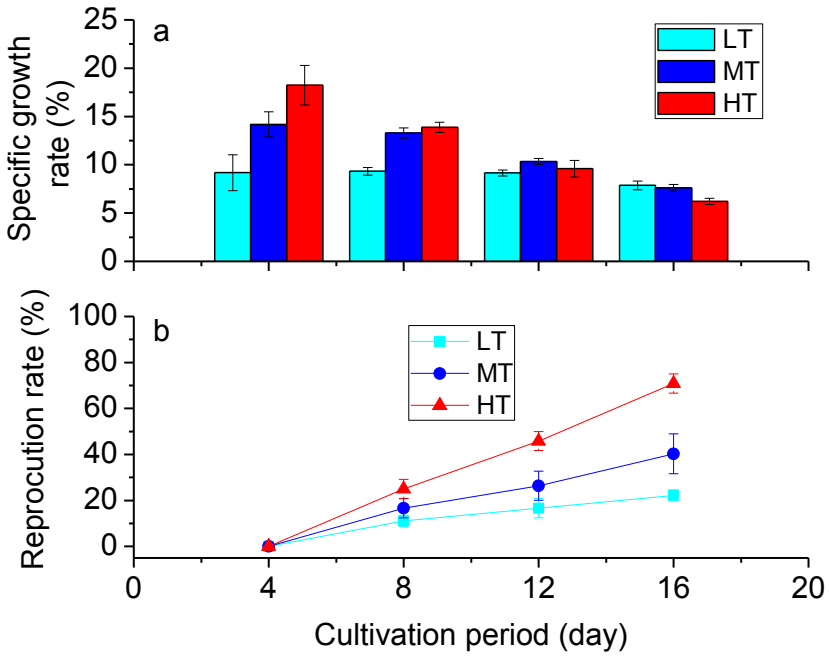

Figure 2.3 Specific growth rate (a) and reproduction rate (b) of *U. rigida* grown at different temperatures. LT, low temperature (12°C); MT, moderate temperature (15°C) and HT, high temperature (18°C). The error bars indicate the standard deviations (n = 3).

2.3.3 Effects of aeration

The effects of aeration rates on the specific growth rate of *U. rigida* over 12-day culture were investigated (Figure 2.4 a). During the first four days of cultivation, there was a statistically significant difference in the specific growth rate of *U. rigida* between aeration rates (ANOVA, $F = 43.191$, df = 3, 8, $P < 0.001$). *Post hoc* Tukey HSD comparison ($P = 0.05$) showed *Ulva* growth increased with aeration rate, and reached a peak (19.63 ±2.39%) at 100 L minute^{-1} and did not change when aeration rate rose to 200 L minute^{-1} (Figure 2.4 a). A similar pattern was found on day eight. When the culture time was extended to 12 days, significant differences in growth rate between aeration rates (ANOVA, $F = 21.253$, df = 3, 8, $P < 0.001$) were also detected. But *post hoc* Tukey HSD comparison ($P = 0.05$) showed there was no significant difference between static and 50 L minute^{-1} aeration rate and the specific growth rate increased by 180.3% at 100 L minute^{-1} compared with the static condition and by 83.6% relative to the 50 L minute^{-1} treatment. A further increase to 200 L minute^{-1} did not enhance the specific growth rate compared to 100 L minute^{-1}.

With regard to effects of aeration time on the specific growth rate of *U. rigida* (Figure 2.4 b), there were statistically significant differences in the specific growth rate of *U. rigida* between aeration rates on days four, eight, and 12 (ANOVA, $F > 8.255$, df = 4, 10, $P < 0.05$). During first four days, *post hoc* Tukey HSD comparison ($P = 0.05$) showed that the specific

growth rate of *Ulva* increased with aeration time. *Ulva* grew 36.71% quicker under eight hours aeration than four hours aeration while there was no significant difference between eight hours and 12 hours aeration. Aeration of 16 hours increased growth rate by 22.03% compared with 12 hours aeration while 24 hours aeration did not stimulate growth further. On day eight, *post hoc* Tukey HSD comparison ($P = 0.05$) showed that the specific growth rate of *Ulva* was 34.54% higher under eight hours aeration than four hours aeration and the difference between eight hours and 12 hours was still statistically non-significant. Aeration of 16 hours or 24 hours did not increase the specific growth rate of *U. rigida* compared with 12 hours but increased the specific growth rate by 42.85% and 48.37% compared with eight hours. On day 12, *post hoc* Tukey HSD comparison ($P = 0.05$) showed that there were no significant differences between any two adjacent aeration times. The positive effects of 16 hours and 24 hours aeration on the specific growth rate became insignificant compared with eight hours.

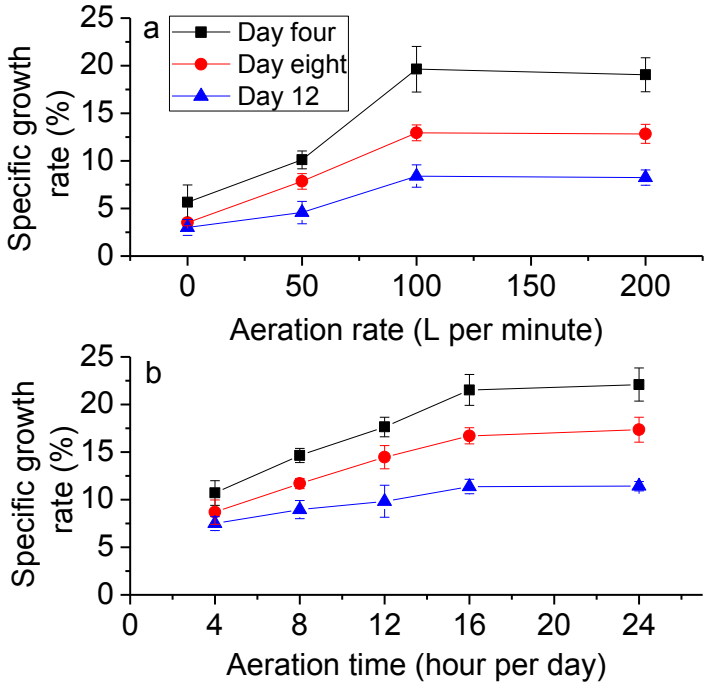

Figure 2.4 Specific growth rate of *U. rigida* cultured at different aeration conditions. The error bars indicate the standard deviations (n = 3).

2.3.4 Multiple harvests

The specific growth rates of *U. rigida* treated by two harvest methods were compared (Figure 2.5 a). There were no significant differences between the harvest methods on days three, six, and nine (Independent samples t-test, $t > -1.383$, $df = 8$, $P > 0.05$) but the specific growth rates in the multiple harvests method were higher than one-off method on days 12 (14.78 ± 3.03% versus 0.52 ± 1.03%, 15 (10.78 ± 2.32% versus –3.93 ± 1.47%), 18 (9.38 ±

2.74% versus –15.57 ± 2.37%, and 21(10.53 ± 2.60 versus –6.87 ± 1.43%) (Independent samples t-test, t < –9.963, df = 8, $P < 0.001$). The average specific growth rate over 21-day cultivation was 12.37 ± 0.63% which was more than five times higher than that without cutting (Figure 2.5 b, Independent samples t-test, t = –34.171, df = 8, $P < 0.001$).

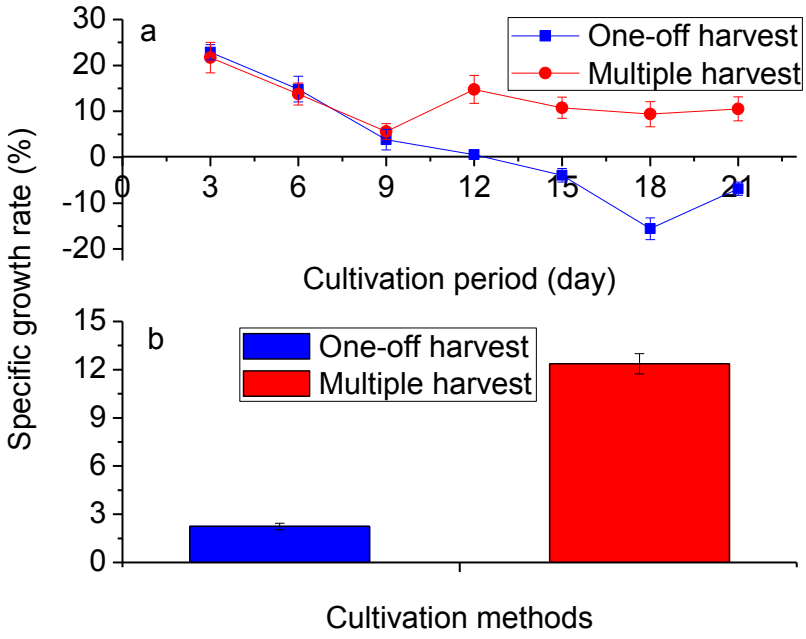

Figure 2.5 Specific growth rate (a) and mean specific growth rate (b) of *U. rigida* cultivated by one-off harvest method and multiple harvests method during the 21-day cultivation. The error bars indicate the standard deviations (n = 5).

2.4 Discussion

2.4.1 Nutrient and light

Nitrogen and phosphorus, two key nutrient components for algal growth, are generally thought to be limiting in marine systems (Howarth, 1988, Fang et al., 1993, Elser et al., 2007, Müller and Mitrovic, 2015). Therefore, adding extra nitrogen and phosphorus to natural seawater can stimulate growth of algae. In the present study, moderate nutrient levels dramatically increased growth irrespective of light intensity. Similarly, biomass yield of *U. lactuca* rose from 147 ± 20 to 365 ± 119 g m^{-2} day^{-1} without aeration and from 209 ± 61 to 376 ± 86 g m^{-2} day^{-1} with aeration when nutrient load changed from very low levels (1.4 g N m^{-2} day^{-1}) to high level (7.31 g N m^{-2} day^{-1}) (Msuya and Neori, 2008). Furthermore, a strong positive relationship between specific growth rates of *U. rigida* and DIN in the water column has been stated (Viaroli et al., 1996). Meanwhile, a photosynthesis-irradiance like curve of growth versus nitrogen concentration was plotted in *U. prolifera* and *U. linza*, in which

growth increased with nitrogen concentration and reached the plateau when the nitrogen was approximately 100 μM (Luo et al., 2012). Conversely, in this present study the high nutrient level led to a decline in growth, indicating a possible negative feedback loop governing *Ulva* biomass productivity. This phenomenon was also found in *U. lactuca*, where very high nutrient load levels (38 g N m^{-2} day^{-1}) decreased the biomass yield from 365 ± 119 to 279 ± 148 g m^{-2} day^{-1} without aeration and from 376 ± 86 to 296 ± 158 g m^{-2} day^{-1} under tidal simulation conditions in comparison to high nutrient load level (7 g N m^{-2} day^{-1}) (Msuya and Neori, 2008). The reason for decreased growth under high nutrient levels was attributed to toxicity of high N (Steffensen, 1976). However, the decreased growth may be due to more reproduction being induced by high nutrient levels, as in the findings in temperature experiments in this study.

In this study, the irradiance of 80 μmol photons m^{-2} s^{-1} increased growth significantly compared with low irradiance (40 μmol photons m^{-2} s^{-1}) during the first nine days while higher irradiance (140 μmol photons m^{-2} s^{-1}) did not stimulate further growth regardless of nitrate levels. The saturation irradiance for *Ulva* species growth or photosynthesis varies in a large range from 10 to 689 μmol m^{-2} s^{-1}, which is species and location dependent (Arnold and Murray, 1980, Fortes and Lüning, 1980, Beach et al., 1995, Han et al., 2003, Choo et al., 2005, Kim et al., 2011). The low light requirements in this study might be vital to maintain rapid growth of *U. rigida* located on the coast of the North Sea since there is not enough solar light available particularly in winter (Dring et al., 2001). The daily maxima of irradiance at Helgoland from November to January in 1994–1999 were in the range of 17.76–58.36 μmol photons m^{-2} s^{-1} (Dring et al., 2001). When tested as combined variables, nutrient and light had a strong impact on the growth of *Ulva* compared to when tested separately. Interactive effects between nitrogen and light on growth were also found in *U. fasciata* (Lapointe and Tenore, 1981). Nitrogen enrichment resulted in more growth at high light intensity as high light resulted in increased demand for nitrogen. In addition, more chlorophyll content was synthesised under high nitrogen levels which could capture more light for photosynthesis under high light conditions (Lapointe and Tenore, 1981). The interactive effect between light and phosphorus on growth was also reported in *U. flexuosa* (Imchen, 2012) and *Ulva* sp. (Sousa et al., 2007). The findings in the present research when combined with previous studies indicate that interactions between light and nutrients on growth may widely exist in *Ulva* species.

2.4.2 Temperature

In this study, growth increased with temperature during the first four days. The positive effect decreased with time and at the end of the 16 days cultivation period, high temperature reduced growth compared with moderate or low temperature. However, the negative effect was not due to the fatal damage of high temperature but increased reproduction under high temperature, which may be described as a form of inverse somatic growth or a somatic to reproductive phase transition. More reproduction induced by high temperature was also observed in *U. fenestrata* (Kalita and Tytlianov, 2003) and *U. fasciata* (Mantri et al., 2011). The sporification surface area of the total area of the disk in *U. fenestrata* were 6.1 ±3.6%, 71.3 ±31.8%, and 37.2 ±6.8% at 10°C, 15°C, and 20°C, which suggested the optimal temperature of reproduction in *U. fenestrata* was 15°C and the degree of zoospore induction in *U. fasciata* increased when temperature changed from 15°C to 25°C (Mantri et al., 2011). The varying optimal temperature for *Ulva* reproduction might be due to interspecies differences or particular adaptations to local biogeographic conditions, i.e. ecotype responses.

2.4.3 Aeration

The hydrodynamic environment is a key determinant for the productivity of seaweed, influencing directly or indirectly physiological performance and community structure. This can affect abiotic and biotic factors which control net seaweed production, such as photon flux density, CO_2 and nutrient availability, temperature, inter- and intraspecific competition for space and resources, and rates of herbivory (Hurd, 2000). In this study, increased aeration rate noticeably enhanced the growth of *U. rigida* compared with static conditions and it reached the saturated point at 100 L minute^{-1}. The increased growth could be attributed to increased photosynthesis and nutrient uptake under fast water motion (Wheeler, 1980, Gerard, 1982, Gao, 1991, Gao et al., 1992a, Gonen et al., 1993, Gonen et al., 1995, Hurd et al., 1996). Furthermore, high water velocity reduces the diffusion boundary layer around the seaweed surface and thus enhances mass transfer of inorganic carbon and nutrients (Hurd, 2000). Additionally, seaweed release metabolic products such as photosynthetic products (OH^- and O_2) into seawater. Within the diffusion boundary layer, the accumulation influences the seawater chemistry at the thallus surface and then photosynthesis of seaweed. For instance, the photosynthetic rate of *Gracilaria conferta* was reduced due to the accumulation of OH^- at the thallus surface which might be caused by the changed pH within the diffusion boundary layer (Gonen et al., 1993). In addition, decreased O_2 due to water motion could lead to an elevated ratio of CO_2/O_2 and depress photorespiratory losses as seen in the red alga *Porphyra*

yezoensis (Gao et al., 1992a, b). Therefore, the increased growth of *Ulva* under high aeration rate could be a combined consequence of the enhanced supply of inorganic carbon across the diffusion boundary layer and the decreased flux of photosynthetic products (OH^- and O_2) on the surface of thallus. However, it should be noted that water motion could also impose negative effects on seaweed. The drag and acceleration forces associated with breaking waves can dislodge entire kelp beds during storms (Graham, 1997). One study has shown that fast water motion does not always increase productivity. For instance, Gerard and Mann (1979) demonstrated that *Laminaria longicruris* grew more rapidly in a sheltered site during eight months of the year (50 cm s^{-1}) than in an exposed site (100 cm s^{-1}). *L. longicruris* had to change its morphology in a bid to adapt to the disturbance caused by high intensity water movement, which overweighed the benefits in diffusion rates of nutrient and other dissolved materials. Furthermore, flowing seawater could also stimulate the leakiness of the HCO_3^- pump and the flux of CO_2 out of the cell (Hurd, 2000). In the present study, growth at the high aeration rate did not decrease which indicates the rate of 200 L $minute^{-1}$ did not bring about obvious negative effects.

In addition to aeration rate, aeration period is another essential parameter for cultivation as it affects both productivity and cost (Ben-Ari et al., 2014). In this study, growth rate generally increased with aeration time until 16 hours of aeration per day, although the increased growth induced by prolonged aeration time shrank with days of cultivation, which indicated that growth under 16 hours of aeration per day was quickest. However, Guerin and Bird (1987) found 12 hours aeration was optimal for growth of *Gracilaria* sp. Vandermeulen and Gordin (1990) also reported that 12 hours aeration increased growth of *U. lactuca* while continuous aeration did not stimulate growth further. Optimal aeration time may depend on stock density and nutrient concentration in seawater. High nutrient availability in seawater could remit the stimulating effect of aeration on growth of *Ulva* species (Msuya and Neori, 2008). Therefore, less aeration time could satisfy growth when nutrients were abundant. Although the stock density in the present study was comparable to that in other's work, the nitrate concentration in this study was 150 μM, which was much lower than that used in Vandermeulen and Gordin's (1990) study (2.0 mM N, 0.15 mM P). In addition, different optimal aeration times per day might be due to species difference as 16 hours aeration increased growth of Basin Head *Chondrus crispus* but did not affect Charlesville *Chondrus* or *Palmaria palmata* (Caines et al., 2014). Long aeration time increases growth but at a cost. Therefore, a balance between growth and cost needs to be considered in practice while continuous aeration would seem unnecessary.

The optimal range of *Ulva* growth conditions (light, nutrients, temperature, or aeration), as discussed above and mentioned in the introduction section, varies in space and time, even within the same species. This is because *Ulva* species selected from differing ecotypes will likely have differing requirements for light and nutrients, ect. An ecotype is a genetic 'type' or species that is precisely adapted to the prevailing conditions within a given habitat (Hufford and Mazer, 2003). If *Ulva* is exposed to condtions outside of its genetically predefined ranges then growth and plant health may be compromised. It is possible to predict optimal ranges, and even select for optimised traits within species/cultivars using quantitative trait loci (QTL) analysis; however, for QTL analysis to be effective there needs to be robust genomic resources that an investigator can call upon (termed QTL mapping) (Collard et al., 2005). *Ulva* species have been shown very challenging when it comes to genome sequencing (J. Callow, personal communication). Indeed, it would appear that QTL mapping has rarely been applied to macroalgae (Shan et al., 2015) despite it being commonplace for aquacultured animals (Liu and Cordes, 2004).

2.4.4 Harvest methods

Based on the knowledge that the remaining basal portions of the *Ulva* thalli can grow to harvestable size again, a multiple harvest method was used in this study. The reason that multiple harvests maintained a relatively high growth is that it removed low productive parts, including reproductive and decaying parts. A multiple harvest method clearly enhanced growth of *U. rigida* over 21-day cultivation while a disadvantage may be increased labour cost in commercial cultivation. In addition, the changes in quality of *Ulva* with harvest times remains unknown since morphology and amino acids in *Porphyra yezoensis* changed with the progression of the number of harvests, which could affect texture and taste of dried nori (Niwa et al., 2008). The impact of a multiple harvest regime on *Ulva* quality therefore remains an open question.

2.5 Conclusions

The optimal conditions of nutrient (150 μM N and 7.5 μM P), light (80 μmol photons m^{-2} s^{-1}), temperature (18^{o}C), and aeration rate (100 L $minute^{-1}$) and time (16 hours day^{-1}) were obtained for a high growth for *U. rigida*. This should facilitate culturing *Ulva* species in the UK. Intermittent aeration was not used in nitrate, light or temperature experiment as those experiments had been carried out before the aeration experiment. Future work needs to be done to ascertain whether effects of nitrate, light or temperature would change if *U. rigida* is grown under intermittent aeration. A multiple harvest method, while it may incur significant labour costs, did significantly enhance growth rate by 404.45% compared with the one-off

harvest method. A more effective method needs to be developed in order to maintain high growth in long term culture with lower costs.

Chapter 3. Reproduction regulation of *Ulva rigida* by internal and external factors

3.1 Introduction

Ulva is a cosmopolitan genus of green seaweed that can be found from tropical to polar climates and from fresh water to fully saline environments. *Ulva* species are particularly rich in a rare cell-wall polysaccharide, termed ulvan, which is an important source of human dietary fibre (Lahaye, 1991, Lahaye and Robic, 2007). *Ulva* is also a potential source of vitamin A, B2, B12, C and tocopherol (Ortiz et al., 2006, Taboada et al., 2010). In addition, compounds extracted from *Ulva* have shown antibacterial and antiviral properties in *in vitro* assays (Ivanova et al., 1994, Awad, 2000). Therefore, interest in Ulvales as a functional food or nutritional supplement is expanding in both Asian and western countries (Peña-Rodríguez et al., 2011, Balasubramaniam et al., 2013, Wilcox et al., 2014). Apart from commercial exploitation in the food industry, *Ulva* is also considered to be an ideal biofilter due to its strong affinity for nitrogen and phosphorus uptake (Hernández et al., 2002, Yokoyama and Ishihi, 2010). Recently, production of biofuels from *Ulva* has been gaining increasing interest given its rapid growth rate and high carbohydrate content (Bruhn et al., 2011, Schultz-Jensen et al., 2013). Consequently, cultivation of *Ulva* species is been conducted in many countries including Japan, the US and South Africa to meet this increasing demand.

To gain an understanding of the reproductive biology and ecology of *Ulva* is key for cultivation as it not only directly affects productivity but can also be used to supply swarmers for seedlings and should eventually lead to full domestication. *Ulva* species tend to have complicated life cycles. The general life cycle involves an alternation of isomorphic diploid sporophytic and haploid gametophytic phases. In addition, parthenogenetic development of gametes has also been found in *Ulva* (Kapraun, 1970). In all cases, reproductive cells are transformed from vegetative cells directly (Hiraoka and Enomoto, 1998).

3.1.1 Internal factors regulating reproduction of Ulva

Internal regulatory factors are considered vital for this transformation. Föyn (1959) found that renewal of medium induced transition of *U. mutabilis* from vegetative cells to reproductive cells, and that the release of zoospores or gametes occurred two to three days after a change of medium. Thiadens and Zeuthen (1966) reported that the degree of sporulation of *U. mutabilis* increased with rising proportions of fresh medium (up to 40% renewal). It was inferred from this that some substances in the fresh medium induced the sporulation rather than factors in the old medium suppressed the transition from commitment

to mitosis to commitment to meiosis followed by sporulation. However, Nilsen and Nordby (1975) demonstrated that the formation of zoospores or gametes in *U. mutabilis* was blocked by some substances, which were extracted from living thallus fragments; these substances were identified as high molecular carbohydrates. Further, Stratmann et al. (1996) found that there were two kinds of regulatory factors maintaining the vegetative state of *U. mutabilis*. One was a glycoprotein, termed sporulation inhibitor-1 (SI-1); the other was not proteinaceous and was termed sporulation inhibitor-2 (SI-2). The SI-1 is a cell wall component and the excretion decreases with maturation of thallus while SI-2 exists in the inner space between two blade cell layers and the overall concentration of SI-2 in the thallus remains constant throughout the life cycle. Either of them can inhibit the gametogenesis and the following *U. mutabilis* gamete release.

3.1.2 External factors affecting reproduction of Ulva

On the other hand, the reproduction of *Ulva* species is also driven by external factors. For instance, temperature is an important factor affecting reproduction of *Ulva*. The reproduction of *U. fenestrata* (Kalita and Tytlianov, 2003) and *U. pertusa* (Han and Choi, 2005) was prevented when cultured at low temperatures (5 ℃). Reproductive *U. lactuca* in the field was found only in warmer months in Groton, USA (Niesenbaum, 1988). *U. pseudocurvata* in the North Sea was reported to have biweekly peaks of gametophytic reproduction during the colder seasons and approximately weekly peaks during the summer (Lüning et al., 2008). The effect of temperature on rhythms of reproduction was further tested by laboratory experiments. Kalita and Titlyanov (2011) showed that the reproductive period of *U. fenestrata* decreased from 30 to five days when the temperature increased from 10 to 20 ℃. Furthermore, rapid changes in temperature have been found to be an effective tool to induce swarmer release. The mean discharge (34.4 ± 4.6%) of swarmers in an unidentified/new *Ulva* species (listed by the authors as 'sp. 3') experiencing temperature shock was nearly double than that of controls (18.5 ± 3.9%) two days after treatment (Carl et al., 2014b). Moreover, all thalli of *U. lactuca* became reproductive 18 hours after a 2^{o}C wash (Niesenbaum, 1988).

Dehydration can also induce the release of *Ulva* swarmers. Smith (1947) found that most *Ulva* blades discharged swarmers five to 10 minutes after one hour of dehydration and then rehydration. Furthermore, Corradi et al. (2006) demonstrated that *Ulva* sp. could liberate gametes by experiencing less time (10 or 20 minutes) dehydration. On the other hand, dehydration had no effects on the sporulation of *Ulva* sp. 3, a tropical filamentous species (Carl et al., 2014b).

Fragmentation has been considered as a powerful factor inducing reproduction of *Ulva* for many years (Nordby and Hoxmark, 1972, Norby 1974, Nordby, 1977, Hiraoka and Enomoto, 1998, Dan et al., 2002, Gao et al., 2010). Fragmentation dramatically improved the sporulation rate of *U. mutabilis* from 15.8 to 80.0% (Nordby, 1977). Hiraoka and Enomoto (1998) reported that zooid formation of *U. pertusa* was induced by fragmenting the thalli two or three days after treatment and the degree of zooid formation increased when disk diameter decreased from 10 mm to 0.9 mm. Gao et al. (2010) demonstrated that nearly whole disks of *U. prolifera* became sporangia when the diameter of disks was 0.5 mm and the sporangia were formed only at the marginal and submarginal cells in larger disks. Fragmentation of *Ulva* thalli commonly occurs in the sea due to the action of grazers, waves, and propellers, which is suggested as one of the most important factors hastening the occurrence of green tides by inducing a large number of spores.

In terms of nutrients, the only literature reporting nutrient effects on *Ulva* reproduction is from Mohsen et al. (1974). Their research demonstrated that nitrogen enrichment induced rapid sporogenesis and sporulation whereas depleted nitrogen led to zygospore formation.

3.1.3 Differentiation and age

Although the *Ulva* thallus is simply organized with little functional differentiation within the thallus, it does consist of at least two cell types: rhizoidal cells in the basal parts and blade cells in the marginal parts of the thallus. Different degrees of reproduction have been shown between these regions of the *Ulva* thallus. More than 90% of disks from the upper parts of the thallus in *U. pertusa* sporulated while almost all disks from the basal parts did not mature three days after excision (Hiraoka and Enomoto, 1998). A similar trend was also found in *U. pseudocurvata*, in which the degree of fertility increased from the basal to apical part of thallus (Lüning et al., 2008). Different reproductive performances across the *Ulva* thallus might be due to the uneven distribution of sporulation inhibitors within the thallus, with the highest concentration near the holdfast (Stratmann et al., 1996). The studies aforementioned used the mature *Ulva* and little is known about the effects of internal and external factors on reproduction of the blade and basal parts of *Ulva* at varying age.

3.1.4 Aims and objectives

Although numerous studies have been carried out on the reproduction of *Ulva* species, the reproductive mechanism of *U. rigida* is still unclear. For instance, what is the interplay between internal and external factors? Do certain factors dominate this process? Do the effects of internal or external factors on different parts of thalli change with age of the plant?

This chapter aimed to test four hypotheses: (1) external factors induce reproduction as environmental stresses; (2) external factors stimulate the maturity of *Ulva* as nutrients; (3) external factors synergistically enhance the form of reproductive cells combined with internal factors; and (4) differential reproductive performance between blade and basal parts of the thallus is due to the their varying excretion of an inhibitor(s) with the plant ages. The corresponding objectives were: (1) to examine reproduction of *Ulva* experiencing temperature shock and dehydration; (2) to assess the time of reaching maturity from gametes grown under different temperature and nitrate conditions; (3) to examine reproduction of *Ulva* experiencing combined treatments of temperature shock, dehydration and fragmentation; (4) to examine the reproduction of blade parts and basal parts with plant age and the reproduction of blade disks mixed with varying proportions of basal disks from adult *U. rigida*.

3.2 Materials and methods

3.2.1 Seaweed collection

Vegetative *U. rigida* plants of 12–15 cm in length were collected from the low intertidal of Cullercoats beach, UK (55.03^{o} N, 1.43^{o} W) after a spring tide in October 2014. The fronds were placed in a plastic bag and transported to the laboratory at Newcastle University within one hour. The fronds were then gently rinsed in sterile seawater (1 micron filtered) to remove any sediment, epiphytes or small grazers.

3.2.2 Temperature shock

Ulva disks with a diameter of 20 mm were excised from the upper regions of vegetative thalli with a length of 12–15 cm using a stainless steel punch. The disks were rinsed with autoclaved seawater and transferred to 500 ml flasks with 300 ml seawater at 4^{o}C. After experiencing 0, 10, 20, 30, 60, 120, 180, or 360 minutes, the disks were returned to 18^{o}C. The chosen sets of temperature and time treatments were based on a previous study on *Ulva* (Carl et al., 2014b). Afterwards, they were cultured at 18^{o}C with a 16: 8 (L: D) cycle and light intensity of 80 μmol photons m^{-2} s^{-1} for five days. The reproduction rates on days three, four and five were recorded. The aim of this experiment was to define a method that can effectively induce reproduction of *U. rigida*, therefore the experiment was ended when the reproduction rate under a treatment was over 90% and the results were recorded on the days near the end. Reproductive rate was determined as the ratio of reproductive disks to all disks within a treatment. To further understand the temperature shock, another experiment was conducted. *Ulva* disks were treated in five conditions. One group was kept at 18^{o}C for two weeks without experiencing temperature shock (Zero); another group was transferred from 18^{o}C to 4^{o}C, kept for two hours at 4^{o}C, then returned to 18^{o}C, and grown at 18^{o}C for two

weeks; the remaining three groups were transferred from 18°C to 4°C and then kept at 4°C for one, two, and three weeks respectively. In both experiments, 25 disks were placed in each flask, with three flasks per treatment.

3.2.3 Dehydration

In order to investigate the effect of dehydration on reproduction, *Ulva* blade disks with a diameter of 20 mm were placed in a 50 × 30 cm plastic tray, air-dried at room temperature (20°C) for 0, 15, 30, 60, 120 and 180 minutes, and were then rehydrated in seawater and grown under the same conditions as mentioned above for two weeks. The reproduction rates on days 10, 12, and 14 were recorded. The set of dehydration times was based on a previous study on *Ulva* (Carl et al., 2014b). The aim of this experiment was to define a method that can effectively induce reproduction of *U. rigida*, therefore the experiment was ended when the reproduction rate under some treatment was over 90% and the results were recorded on the days near the end. Twenty five disks were placed in each flask, with three flasks per treatment.

3.2.4 Maturation of U. rigida

To investigate the effects of external factors on maturation of *U. rigida*, *Ulva* gametes were culture under two temperatures (14, 18°C) and nitrate concentrations (6, 100 μmol L^{-1}), in which the two temperatures and two nitrate levels were fully crossed. Therefore, there were four treatments in total and they were low temperature and low nitrate (LTLN), low temperature and high nitrate (LTHN), high temperature and low nitrate (HTLN), and high temperature and high nitrate (HTHN). Temperature and nitrate were selected as external factors due to their possible stimulative effects on maturation of *Ulva* (Mohsen et al., 1974, Kalita and Titlyanov, 2011). Discharged gametes from reproductive cells produced from the experiments described above were attracted to a point-source light using a fibre optic illuminator, collected with a pipette, and transferred to sterile seawater. Afterwards, 20 ml gametes suspensions with a concentration of 1.0×10^5 ml^{-1} were placed in Petri dishes and kept in darkness for 24 hours to ensure the attachment of gametes to the bottom of the Petri dishes. Settled gametes were grown in the conditions mentioned above. When germlings reached 1 mm, they were detached from the Petri dishes and ten individuals under every treatment were randomly selected for further culture. Each individual was grown in a 500 ml conical flask. The time taken for germlings to reach a length of 1.5 cm was recorded. The length was measured every three or four days.

3.2.5 Effect of fragment size

To investigate the role of fragment size, the upper regions of *U. rigida* thalli were punched into disks with diameters of 2.5, 4, 6, 8, and 10 mm. The set of disk sizes was based on previous studies on *Ulva* (Hiraoka and Enomoto, 1998, Gao et al., 2010). To explore the potential mechanism behind fragmentation, one portion of these disks was cultured in media without renewal while the other portion was cultured in media that was renewed daily for seven days. The reproduction rates on days five, six, and seven were recorded. The aim of this experiment was to define a method that can effectively induce reproduction of *U. rigida*, therefore the experiment was ended when the reproduction rate under some treatment was over 90% and the results were recorded on the days near the end. The total areas of disks at different sizes were made identical to guarantee equal densities across treatments. Twenty five disks were placed in each flask, with three flasks per treatment.

3.2.6 Combined effect of temperature shock, dehydration, and fragment size

Disks with diameters of 2.5, 4, and 6 mm were punched from the upper regions of 12–15 cm *Ulva* thalli (FR). Then they were treated with one hour of dehydration (FR+ DE), or with six hours of temperature shock (FR+TS), or with six hours of temperature shock and one hour of dehydration with (FR+TS+DE) in a bid to probe the combined effects of internal and external factors. The disk size, temperature shock, and dehydration time were selected due to their effects on induction of reproduction based on the experiments in sections 3.2.2, 3.2.3, and 3.2.5. Twenty five disks were placed in each flask, with three flasks per treatment.

3.2.7 Reproduction of blade and basal parts of Ulva *thalli with age*

Ulva disks with diameter of 2.5 mm excised from the upper and basal regions of thalli at different ages (20, 30, 40, 50, 60 days) were used to investigate the effects of internal and external factors on reproductive capability of *U. rigida* over development. Twenty five disks were placed in each flask, with three flasks per treatment. Every flask experienced six hours of temperature shock before being cultured for 14 days. The reproduction rates on days seven, 10, and 14 were recorded. The period was selected because it was enough for the reproduction of the *blade parts* based on the experience of normal laboratory culture. Different ages of *Ulva* thalli were obtained from the development of gametes. The discharged gametes from reproductive cells produced from the experiments described above were attracted to a point-source light using a fibre optic illuminator, collected with a pipette, and transferred to sterile seawater. Afterwards, 20 ml gametes suspension with a concentration of 1.0×10^5 ml^{-1} were placed in Petri dishes and kept in darkness for 24 hours to ensure the attachment of gametes to the bottom of the Petri dishes. Settled gametes were cultured at 18^oC, 100 mM nitrate, and 80

μmol photons m^{-2} s^{-1} at a 16: 8 of light: dark period. *Ulva* thalli grown for 20, 30, 40, 50, 60 days were used.

3.2.8 Inhibitory effects of blade cells

Disks from upper and basal parts of the thalli with a length of 12–15 cm were used to study the potential mechanisms that maintain vegetative status across the whole life history in basal cells. Four ratios of blade to basal disks were set up, which were blade: basal (1:0), blade: basal (1:1), blade: basal (1:2) and blade: basal (1:3). The set of these ratios was based on a preliminary experiment showing the ratio of 1:3 completely inhibited the reproduction of blade disks. The period was selected because it was enough for the reproduction of blade parts based on the experience of normal laboratory culture. The numbers of disks in these four ratios were 20 blade disks (B), 20 blade + 20 basal disks (B+20B), 20 blade + 40 basal disks (B+40B), 20 blade + 60 basal disks (B+60B), respectively. The experiment was conducted for 15 days and in triplicate.

3.2.9 Determination of reproduction

The reproductive *Ulva* disks were recognized by a colour change. Formation of reproductive cells is accompanied by a change in thallus colour from green (vegetative state) to yellowish (reproductive state) and then to white (after release of swarmers). This was verified via microscope observation (see Figure 3.1). Sporulation formation in more than half the disk area was considered as equivalent to complete sporulation formation. Reproductive rate was expressed as the ratio of reproductive disks to all disks within a treatment.

3.2.10 Statistical analysis

Results were expressed as means of replicates ± standard deviation. Statistical analysis was carried out with SPSS v.21. A confidence interval of 95% was set for all tests.

Effects of temperature shock on reproduction rate

The reproduction rates of *U. rigida* experiencing all temperature shock times on days three, four and five conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the eight samples on days three, four and five could be considered equal (Levene's test, $F < 1.714$, $P > 0.05$). Three one-way ANOVAs were conducted to assess the effects of temperature shock on reproduction of *U. rigida* on days three, four and five, respectively. Tukey HSD was conducted for *post hoc* investigation.

Effects of dehydration on reproduction rate

The reproduction rates of *U. rigida* experiencing all dehydration times on days 10, 12, and 14 conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the six samples on days 10, 12, and 14 could be considered equal (Levene's test, $F < 1.257$, $P > 0.05$). Three one-way ANOVAs were conducted to assess the effects of dehydration on reproduction of *U. rigida* on days 10, 12, and 14, respectively. Tukey HSD was conducted for *post hoc* investigation.

Effects of temperature and nitrate on maturity

The times to reach maturity of *U. rigida* grown under varying temperature and nitrate treatments conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the four samples could be considered equal (Levene's test, $F = 0.271$, $P > 0.05$). A two-way ANOVA was conducted to assess the effects of temperature and nitrate on the time to reach maturity. *Post hoc* tests were not conducted because each factor had only two levels.

Effects of fragmentation and media on reproduction rate

The reproduction rates of *U. rigida* experiencing all fragmentation and media treatments on days five, six and seven conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the 10 samples could be considered equal (Levene's test, $F < 1.296$, $P > 0.05$). Three two-way ANOVAs were conducted to assess the effects of fragmentation and media on reproduction of *U. rigida* on days five, six and seven, respectively. Tukey HSD was conducted for *post hoc* investigation.

Effects of temperature shock, dehydration, and fragmentation on reproduction rate

The reproduction rates of *U. rigida* under all temperature shock, dehydration, and fragmentation treatments on days two, three, and four conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the 12 samples on days two, three, and four could be considered equal (Levene's test, $F < 1.603$, $P > 0.05$). Three three-way ANOVAs were conducted to assess the effects of temperature shock, dehydration, and fragmentation on reproduction of *U. rigida* on days two, three and four. Tukey HSD was conducted for *post hoc* investigation.

Effects of age on reproduction rate

The reproduction rates of blade disks at different ages on days seven, 10, and 14 conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the five samples could be considered equal (Levene's test, $F < 3.080$, $P > 0.05$). Three one-way

ANOVAs was conducted to assess the effects of age on reproduction of blade disks on days seven, 10, and 14, respectively. Tukey HSD was conducted for *post hoc* investigation.

Effects of basal cells on blade cells

The reproduction rates of *U. rigida* in four ratios of blade to basal disks on days 13, 14, and 15 conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the four samples on days 13, 14, and 15 could be considered equal (Levene's test, $F < 1.895$, $P > 0.05$). Three one-way ANOVAs were conducted to assess the effects of basal disks on reproduction of blade disks on days 13, 14, and 15, respectively. Tukey HSD was conducted for *post hoc* investigation.

3.3 Results

The key questions that this chapter aimed to answer were: (1) how do internal and external factors affect reproduction of *U. rigida*? (2) do the effects of internal and external factors on different parts of thalli change with age of the plant?

3.3.1 Transformation from vegatative to reproductive cells

The cells in the surface area of the disk were green in colour, with several granular chloroplasts (Figure 3.1 a). When thalli were about to become fertile they became pale green. Microscopical examination showed that this change in colour was due to a shift in the position of the chloroplasts so that they lay towards one side instead of towards the free face of the cells along with the swelling vacuoles (Figure 3.1 b). Approximately 24 hours later, pyriform gametes were formed within the sporangia (Figure 3.1 c) leading to the thalli developing a yellowish appearance and, subsequently the gametes were liberated from the sporangia (Figure 3.1 d). The cells become empty after discharging all gametes (Figure 3.1 c).

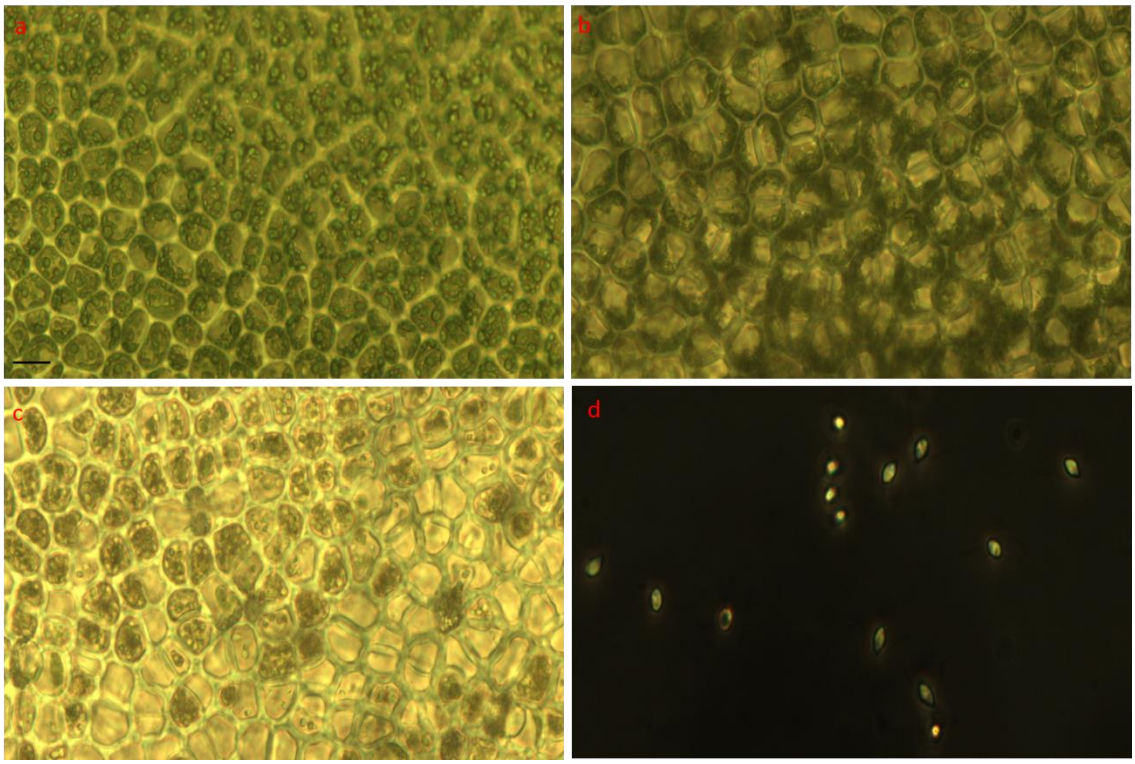

Figure 3.1 Light micrographs of *U. rigida* in the process of reproduction. (a) vegetative cells, (b) reproductive cells before discharging gametes, (c) reproductive cells discharging gametes, and (d) discharged swarmers. The scale bar represents 20 μm.

3.3.2 Temperature shock

The effects of temperature shock on the reproduction rates of *U. rigida* were investigated (Figure 3.2). There were statistically significant differences in reproduction rates of *U. rigida* between temperature shock times on days three, four, and five (ANOVA, $F > 439.929$, df = 7, 16, $P < 0.001$). On day three, *Ulva* disks experiencing less than 20 minutes temperature shock did not show any reproduction. The reproduction rate of 24.00 ± 4.00% was found in *Ulva* disks experiencing 30 minutes temperature shock and it increased to 36.00 ± 4.00% when temperature shock time extended to 60 minutes (Tukey HSD, $P < 0.05$). Afterwards, the increase of reproduction rate was not statistically significant (Tukey HSD, $P > 0.05$) until 360 minutes temperature shock where reproduction rate reached the maximal 48.00 ± 4.00% (Tukey HSD, $P < 0.05$). On day four, *Ulva* disks experiencing 20 minutes temperature shock demonstrated a 28.00 ± 4.00% reproduction rate and it increased to 76.00 ± 4.00% when temperature shock time was 30 minutes (Tukey HSD, $P < 0.05$). Afterward, the increase of reproduction rate was not statistically significant with culture time (Tukey HSD, $P > 0.05$). On day five, *Ulva* disks experiencing 10 minutes temperature shock became reproductive (36.00 ± 4.00%). The reproduction rate reached up to 94.67 ± 2.31% in disks treated with a 20-minute temperature shock. Extended exposure periods to 4°C did not lead to

a significant increase in reproduction (Tukey HSD, $P > 0.05$). An interesting finding was that *Ulva* disks did not form any sporulation if they experienced low temperature (4°C) for one, two, and three weeks without returing to high temperature (18°C) (Figure 3.3).

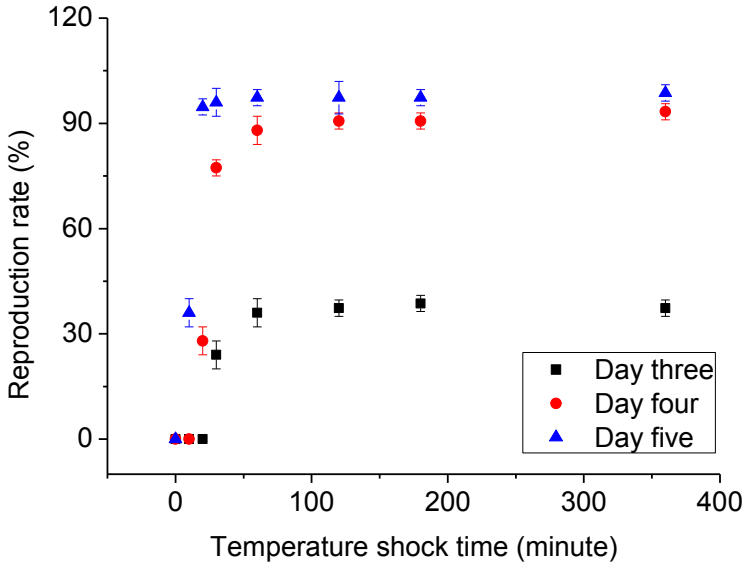

Figure 3.2 The reproduction rates of *U. rigida* treated by increasing temperature shock periods (in minutes). The reproduction rates of the disks were assessed on three, four, and five days post temperature shock treatment. Data are the means ± SD (n = 3) with each replicate including 25 disks.

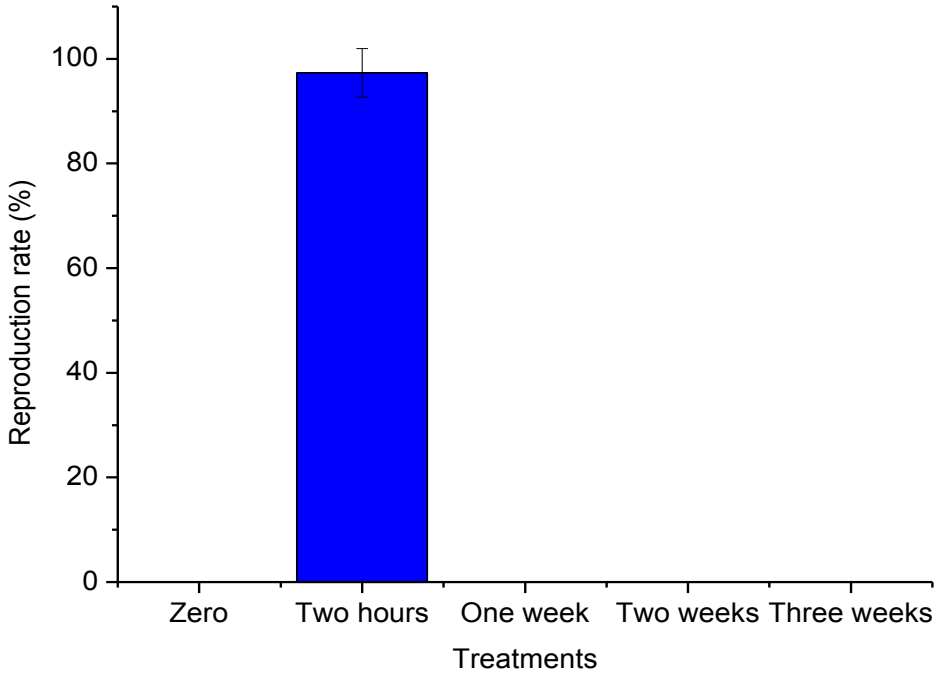

Figure 3.3 Effects of two-step temperature shock on the reproduction rates of *U. rigida*. The results were obtained on day five for the group of two hours. Zero represents disks kept at 18°C for two weeks without experiencing temperature shock, and two hours represents disks experiencing two hours 4°C treatment and returning to 18°C. One week, two weeks and three weeks indicate disks transferred from 18°C to 4°C and kept at 4°C for one week, two weeks, and three weeks respectively. Data are the means ± SD (n = 3) with each replicate including 25 disks.

3.3.3 Dehydration

The reproduction rates of *U. rigida* under various dehydration times were observed (Figure 3.4). There were statistically significant differences in reproduction rates of *U. rigida* between dehydration times on days 10, 12, and 14 (ANOVA, $F > 439.929$, df = 7, 16, $P < 0.001$). After rehydration and growing at 18°C for 10 days, 41.33 ± 2.31% of disks experiencing 15 minutes drying in the air showed zooid formation. As dehydration time increased, a conspicuous decrease in reproduction rate was observed (Figure 3.4; Tukey HSD, $P < 0.05$). No reproduction occurred when disks were dehydrated for three hours and all disks had bleached. The trends on days 12 and 14 were similar to day 10.

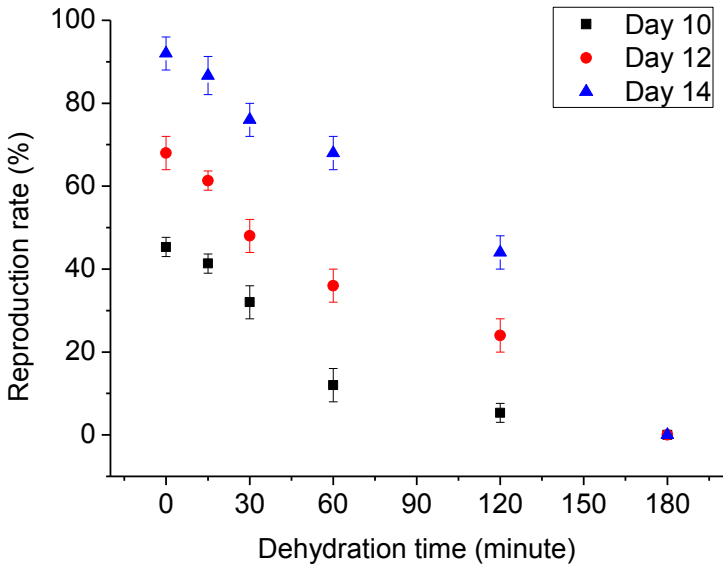

Figure 3.4 The reproduction rates of *U. rigida* treated by increasing dehydration periods (in minutes). The reproduction rates of the disks were assessed on 10, 12, and 14 days post dehydration treatment. Data are the means ± SD (n = 3) with each replicate including 25 disks.

3.3.4 The development of gametes

The development of gametes released from the excised disks into new individuals is shown in Figure 3.5. The pyriform spores became round after attaching to the Petri dishes (Figure 3.5 a). Subsequently, the settled gametes started to divide (Figure 3.5 b). Two days later, the basal cells increased in length and the apical cells underwent further division (Figure 3.5 c). With the number of cells increasing, the rhizoid and the linear thalli were formed (Figure 3.5 d and e). When germlings reached around 1.5 cm, the linear thalli developed transversely and the blade cells became mature (Figure 3.5 f). However, it did not form sporulation unless fragmentation happened. When *Ulva* became reproductive, the colour of the blade part of the thallus changed from green to yellow (Figure 3.5 g) and to white after gametes were released (Figure 3.5 h).

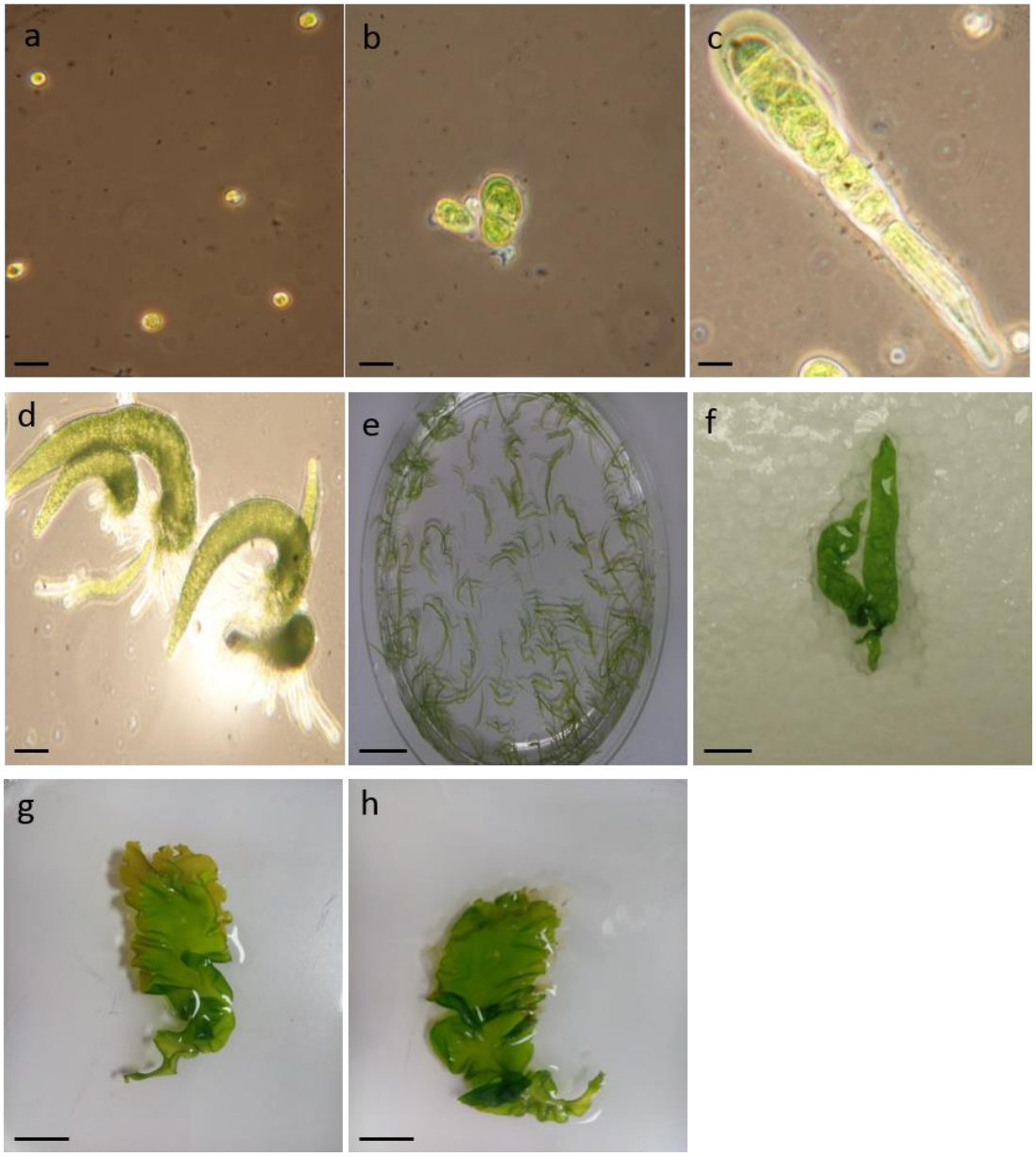

Figure 3.5 The phases of development in *U. rigida*. The scale bars represent 10 μm in panel a–c, 20 μm in panel d, 1 cm in panel e, g and h and 400 μm in panel f.

3.3.5 Effects of temperature and nitrate on maturity of U. rigida

The time taken to reach 1.5 cm length in *Ulva* thalli grown under different conditions was investigated (Figure 3.6). Temperature and nitrate had a significant interaction on maturity time of *U. rigida* and either of them had a main effect (Table 3.1). It took 62.10 ± 2.76 days to reach 1.5 cm length when plants were grown under conditions of low temperature and low nitrate (LTLN). High temperature (HTLN) and high nitrate (LTHN) shortened the time to 40.30 ± 2.21 and 41.50 ± 2.50 days, respectively. Furthermore, the high temperature and high nitrate conditions (HTHN) synergistically reduced the time to 28.40 ± 2.01 days.

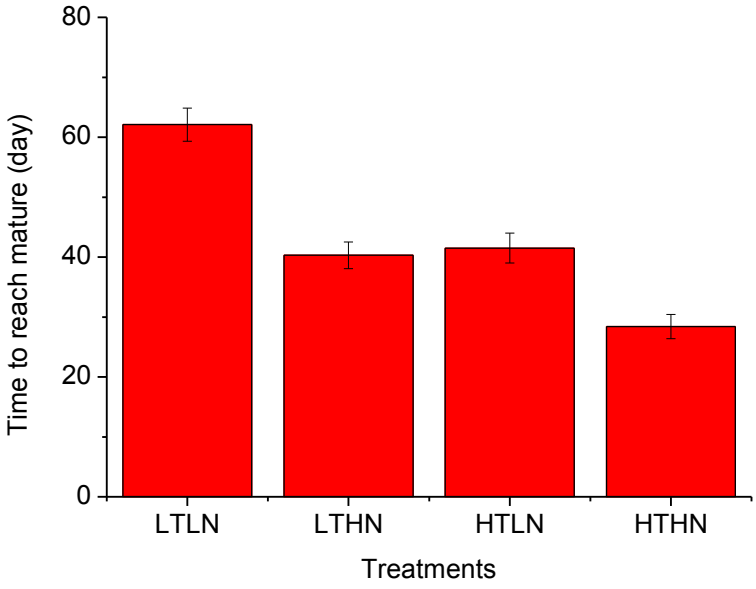

Figure 3.6 Effects of temperature and nitrate on maturation of *U. rigida*. LTLN means low temperature and low nitrate, LTHN means low temperature and high nitrate, HTLN means high temperature and low nitrate and HTHN means high temperature and high nitrate. Data are the means ± SD (n = 10).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Temperature	2640.625	1	2640.625	461.693	<0.001
Nitrate	3045.025	1	3045.025	532.399	<0.001
Temperature * Nitrate	189.225	1	189.225	33.085	<0.001
Error (Time)	205.900	36	5.719		

Table 3.1 Two-way analysis of variance of the effects of temperature and nitrate on maturity time of *U. rigida*. The confidence interval was 95%.

3.3.6 Fragmentation

The effects of fragmentation and media on the reproduction rates of *U. rigida* over seven days of culture were investigated (Figure 3.7). On day five, there was a significant interaction between fragmentation and media (Table 3.2). The gap between two kinds of media was reduced with the increase of disk size (Figure 3.7). For instance, the reproduction rates of 2.5 mm disks were 75.52 ± 2.39% (renewed media) and 39.06 ± 1.56% (unrenewed media), 24.27 ± 2.01% (renewed media) and 18.13 ± 2.01% (unrenewed media) in 4 mm

disks, and zero (both renewed and unrenewed media) in 10 mm disks. Fragmentation size affected the reproduction of *U. rigida* (Table 3.2). *Post hoc* Tukey HSD comparison (P = 0.05) showed that there were significant differences in reproduction of *Ulva* disk between sizes. The maximum were 75.52 ±2.39% (renewed media) and 39.06 ±1.56% (unrenewed media) in 2.5 mm disks. Then the reproduction rates decreased with the increase of disk size and reached the minimal of zero in 10 mm disks. The pattern on day six was similar to day five (Table 3.3 and Figure 3.7). On day seven, there was no significant interaction between fragmentation and media but they affected reproduction independently (Table 3.4). *Post hoc* Tukey HSD comparison (P = 0.05) showed that the reproduction rate decreased with the increase of disk size with a maximum of 92.19 ±5.63% (renewed media) and 64.69 ±2.67% (unrenewed media) in 2.5 mm disks and a minimum of 20.00 ±5.00% (renewed media) and 5.00 ±5.00% (unrenewed media) in 10 mm disks. The reproduction rate of *U. rigida* grown in renewed media (56.16 ±25.96%) was higher than that in unrerewed media (38.96 ±22.15%).

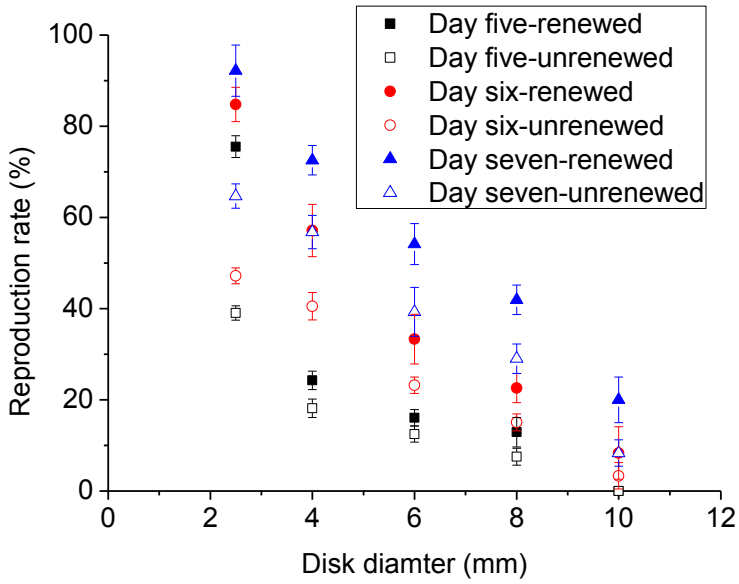

Figure 3.7 Effects of fragment size and media on the reproduction rates of *U. rigida* on days five, six, and seven. Closed squares represent unrenewed media treatment and open squares represent renewed media treatement. Data are the means ±SD (n = 3). Each replicate in 2.5 mm diameter includes 320 disks, each replicate in 4 mm diameter includes 125 disks, each replicate in 6 mm diameter includes 56 disks, each replicate in 8 mm diameter includes 31 disks, and each replicate in 10 mm diameter includes 20 disks.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Fragmentation	11580.672	4	2895.168	666.569	<0.001
Media	830.492	1	830.492	191.208	<0.001
Fragmentation * Media	1301.317	4	325.329	74.902	<0.001
Error (Time)	86.868	20	4.343		

Table 3.2 Two-way analysis of variance of the effects of fragmentation and media on the reproduction rates of *U. rigida* on day five. The confidence interval was 95%.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Fragmentation	11580.672	4	2895.168	666.569	<0.001
Media	830.492	1	830.492	191.208	<0.001
Fragmentation * Media	1301.317	4	325.329	74.902	<0.001
Error (Time)	86.868	20	4.343		

Table 3.3 Two-way analysis of variance of the effects of fragmentation and media on the reproduction rates of *U. rigida* on day six. The confidence interval was 95%.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Fragmentation	15730.738	4	3932.685	215.529	<0.001
Media	2219.704	1	2219.704	121.650	<0.001
Fragmentation * Media	205.382	4	51.345	2.814	0.053
Error (Time)	364.933	20	18.247		

Table 3.4 Two-way analysis of variance of the effects of fragmentation and media on the reproduction rates of *U. rigida* on day seven. The confidence interval was 95%.

3.3.7 Combination of temperature shock, dehydration, and fragmentation

The combined effects of temperature shock, dehydration, and fragmentation on the reproduction rates of *U. rigida* over four days of culture were investigated (Figure 3.8). On day two, temperature shock had interactive effects with fragmentation or dehydration on reproduction of *U. rigida* (Table 3.5). The reproduction rates of three sizes of disks with temperature shock (FR+TS) were 64.00 ±4.00% (small disks), 48.00 ±4.00% (medium disks), and 34.67 ±2.31% (large disks) respectively while none of them showed reproduction if they did not experience temperature shock (FR). Similarly, disks experiencing dehydration (FR+DE) did not show reproduction while the reproduction rates were 44.00 ±4.00% (small disks), 24.00 ±4.00% (medium disks), and 6.67 ±6.11% (large disks) respectively when temperature shock was added (FR+DE+TS). On day three, three factors had an interactive effect and any two of them had an interactive effect on the reproduction rates of *Ulva* (Table 3.6). Disks in all sizes without temperature shock did not reproduce while disks of varying sizes with temperature shock showed different reproduction rates. Furthermore, small disks demonstrated the biggest decline in reproduction rate when dehydration was added (Figure 3.8). All three factors had a main effect on reproduction (Table 3.7). *Post hoc* Tukey HSD comparison ($P = 0.05$) showed that the highest reproduction (97.33 ±2.31%) was in the smallest disks in combination with temperature shock. The pattern on day four was similar to day three.

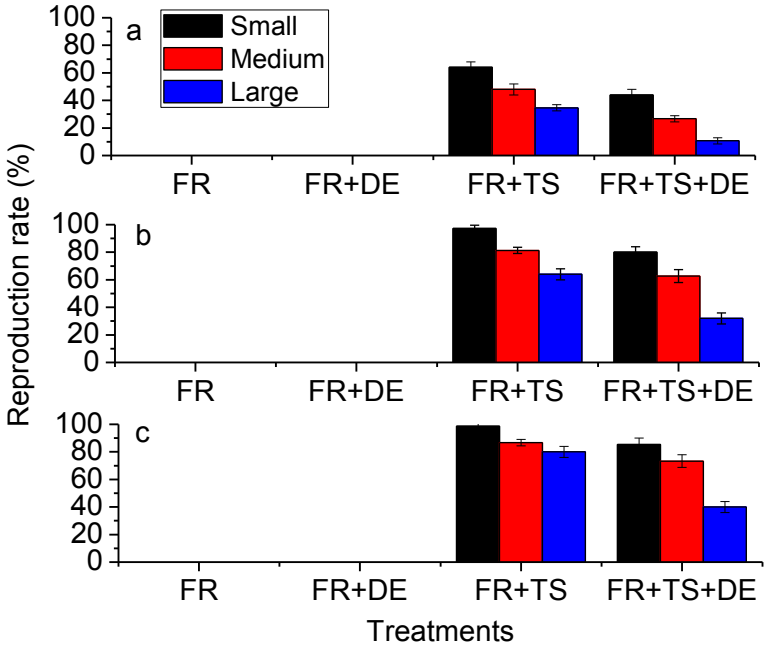

Figure 3.8 Combined effects of temperature shock, dehydration and fragment on reproduction of *U. rigida* on days two (a), three (b), and four (c). Small, Medium, and Large mean disks with a diameter of 2.5 mm, 4 mm, and 6 mm respectively. FR, FR+DE, FR+TS, and FR+TS+DE represent three sizes of disks without temperature shock or dehydration treatment, three sizes of disks with one hour-dehydration treatment, three sizes of disks with six hour-temperature shock treatment, and three sizes of disks with six hour-temperature shock and one hour-dehydartion treatment, respectively. Data are the means ± SD (n = 3) and every single repetition includes 25 disks.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
FR	1670.222	2	835.111	93.950	<0.001
TS	12247.111	1	12247.111	1377.800	<0.001
DE	1296.000	1	1296.000	145.800	<0.001
FR*TS	1670.222	2	835.111	93.950	<0.001
FR*DE	24.000	2	12.000	1.350	0.278
TS*DE	1296.000	1	1296.000	145.800	<0.001
FR*TS*DE	24.000	2	12.000	1.350	0.278
Error (Time)	213.333	24	8.889		

Table 3.5 Three-way analysis of variance of the effects of temperature shock (TS), dehydration (DE) and fragmentation (FR) on the reproduction rates of *U. rigida* on day two. The confidence interval was 95%.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
FR	2518.222	2	1259.111	202.357	<0.001
TS	43820.444	1	43820.444	7042.571	<0.001
DE	1111.111	1	1111.111	178.571	<0.001
FR*TS	2518.222	2	1259.111	202.357	<0.001
FR*DE	107.556	2	53.778	8.643	0.001
TS*DE	1111.111	1	1111.111	178.571	<0.001
FR*TS*DE	107.556	2	53.778	8.643	0.001
Error (Time)	149.333	24	6.222		

Table 3.6 Three-way analysis of variance of the effects of temperature shock (TS), dehydration (DE) and fragmentation (FR) on the reproduction rates of *U. rigida* on day three. The confidence interval was 95%.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
FR	1347.556	2	673.778	68.909	<0.001
TS	52593.778	1	52593.778	5378.909	<0.001
DE	1111.111	1	1111.111	113.636	<0.001
FR*TS	1347.556	2	673.778	68.909	<0.001
FR*DE	366.222	2	183.111	18.727	<0.001
TS*DE	1111.111	1	1111.111	113.636	<0.001
FR*TS*DE	366.222	2	183.111	18.727	<0.001
Error (Time)	234.667	24	9.778		

Table 3.7 Three-way analysis of variance of the effects of temperature shock (TS), dehydration (DE), and fragmentation (FR) on the reproduction rates of *U. rigida* on day four. The confidence interval was 95%.

3.3.8 Reproduction of U. rigida *at different ages*

The reproduction rates of *U. rigida* treated by a combination of internal and external factors at different ages were recorded (Figure 3.9). There were statistically significant differences in reproduction rates of blade disks between ages on days seven, 10, and 14 (ANOVA, $F > 115.578$, df = 4, 10, $P < 0.001$). On day seven, 20 days disks showed little reproduction ($1.33 \pm 2.31\%$) and the reproduction rate of 30 days disks was $16.00 \pm 4.00\%$.

Post hoc Tukey HSD comparison ($P = 0.05$) suggested that reproduction rate increased with disk age (from 30 to 60 days) and reached the maximal of 84.00 ± 4.00% in 60 days disks. On day 10, the reproduction rate increased from 1.33 ± 2.31% to 84.00 ± 4.00% when disk age changed from 20 to 40 days (Tukey HSD, $P < 0.05$). The different in reproduction rate between 40 days and 50 days disks or 50 days and 60 days were not statistically significant (Tukey HSD, $P > 0.05$). But 60 days disks had a higher reproduction rate (97.33 ± 2.31%) compared with 40 days disks. The pattern on day 14 was same as day 10. Contrary to the changes of blade disks at different ages with culture time, no reproduction was found in basal disks regardless of age and culture time (Figure 3.9).

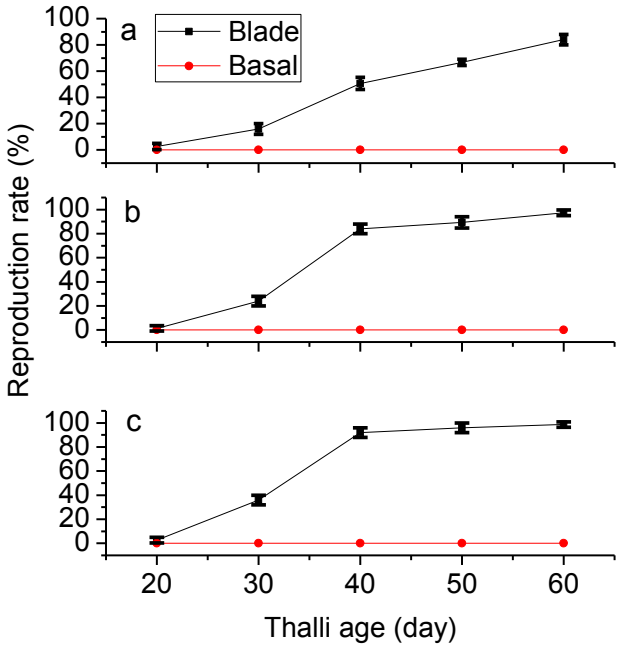

Figure 3.9 Reproduction rates of blade cells and basal cells in *U. rigida* at different ages observed on seven (a), 10 (b), and 14 (c) days post fragmentation and temperature shock treatments. Data are the means ± SD (n = 3).

3.3.9 Inhibitory effects of basal cells

The inhibitory effects of basal cells on blade cells over 15 days of culture were investigated (Figure 3.10). There were statistically significant differences in reproduction rates of blade disks with varying addition of basal disks on days 13, 14, and 15 (ANOVA, $F > 122.769$, df = 3, 8, $P < 0.001$). On day 13, blade disks alone (B) had 60.00 ± 5.00% reproduction rate (Figure 3.10). The reproduction rate of blade disks declined to 25.00 ± 5.00% when 20 basal disks were added (B+20B) and further to 8.33 ± 2.89% when 40 basal disks (B+40B) were added (Tukey HSD, $P < 0.05$). No reproduction was found in blade disks mixed with 60 basal disks (B+60B). Similar pattern were detected on days 14 and 15 except

that blade disks with 60 badal disks showed little reproduction rates (3.33 ± 2.89% on day 14 and 8.33 ± 2.89% on day 15).

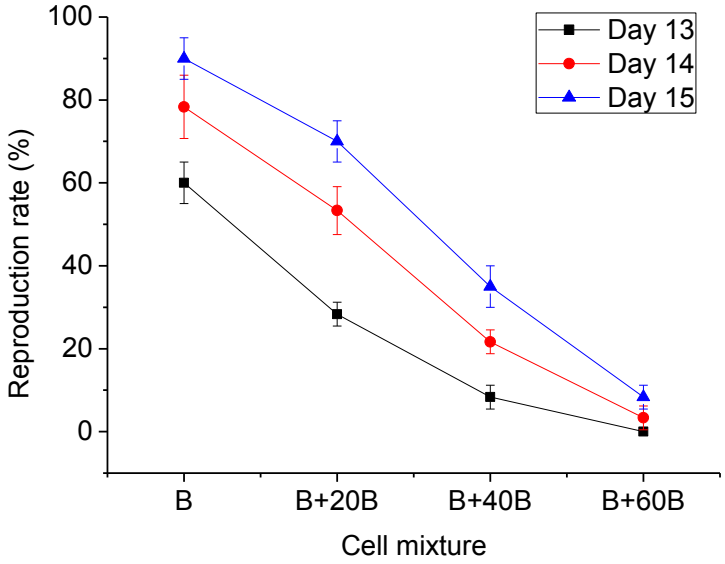

Figure 3.10 Inhibitory effects of basal disks on reproduction rates of blade disks on days 13, 14, and 15. B represents 20 blade disks, B+20 B represents 20 blade disks with 20 basal disks, B+40 B represents 20 blade disks with 40 basal disks and B+60 B represents 20 blade disks with 60 basal disks. Data are the means ± SD (n = 3).

3.4 Discussion

3.4.1 Effects of temperature shock

Temperature shock of 10 minutes led to a crucial change in reproduction (36.00 ± 4.00%) after culturing for five days since no reproduction was observed in the condition without temperature shock. Carl et al. (2014b) reported that 10 minutes was the saturation time for reproduction induction and further exposure to 4°C did not enhance zooid formation, but the data presented here show reproduction did not reach a maximum until 20 minutes in the low temperature treatment. This might be attributed to species differences considering the *Ulva* species investigated in their research was a tropical filamentous one. *Ulva* grown at a higher seawater temperature in a tropical area may be more sensitive to cold temperature stimulation. In addition, the *Ulva* disks were switched between 4°C and 25°C in their study therefore experiencing an exaggerated temperature difference compared with the *U. rigida* used in the present study (4°C and 18°C). This would equate to a stronger temperature shock signal in the tropical species versus the temperate species used in this thesis. If this logic is correct, then by extrapolation it would be expected that *Ulva* from more polar environments would experience even lower reproductive rates as a drop to 4°C for a polar species would be

the weakest temperate shock signal relative to its ecological norm. As far as the author is aware, this latitudinal relationship between temperature shock and reproduction in *Ulva* has not been investigated.

In terms of release period, it took five days to reach 94.7 ± 2.3% reproductive rate of *U. rigida* after temperture shock treatment in this study. However, 43.36 ± 11.9% sporulaton in *Ulva* sp. 3 under 4°C treatment was detected two days after the initiation of experiments (Carl et al., 2014b). Furthermore, all thalli of *U. lactuca* became reproductive 18 hours after a 2°C wash (Niesenbaum, 1988). The shorter time required for fertility of *Ulva* species experiencing temperature shock in Carl et al. (2014b) and Niesenbaum (1988) studies should be down to the pre-fertile status of *Ulva* species because swarmers discharge was also found in the control group. The temperature shock only accelerated the process of swarmers discharge. It is suggested that a minimun of two days is needed for the transition from vegatative status to reproductive status (Pettett and Dip, 2009, Wichard and Oertel, 2010). The enhanced reproduction under temperature shock could be a survival strategy under unfavourable conditions (Li and Brawley, 2004). Whether temperature shock stopped the excretion of sporulation inhibitors or triggered another pathway for reproduction of *Ulva* remains to be determined. The process appears to involve a two-step mechanism. The first step is from high temperature to low temperature and the second is returning to a high temperature. Neither is dispensable because continuous low temperature induction for three weeks without returning to high temperature did not lead to any reproduction (Figure 3.3).

3.4.2 Effects of dehydration

The effects of dehydration on reproduction were also investigated in this study. The dehydration time varied from 15 minutes to 180 minutes, however dehydration did not stimulate reproduction of *U. rigida* during any time exposure. On the contrary, reproduction decreased as dehydration time extended. This was inconsistent with the result described by Corradi et al. (2006), in which thalli subjected to 10 or 20 minutes dehydration released gametes within three days. Furthermore, Smith (1947) reported that *Ulva* blades kept away from seawater for one hour liberated swarmers five to 10 minutes after reimmersion. It is possible that *Ulva* thalli already became productive and dehydration just stimulated the release of swarmers in that case. The reason that a longer dehydration time (up to 180 minutes) was used in the present study compared to previous studies (no more than 60 minutes) was that short term dehydration (no more than 60 minutes) did not induce any reproduction. However, an extended dehydration time did not induce reproduction, either. The reason that dehydration did not serve as an environmental stimulus for reproduction of *Ulva* like

temperature shock can be attributed to *Ulva* getting used to periodic dehydration and rehydration in the field. The negligible effect of dehydration on sporulation was also found in a tropical species of *Ulva* (Carl et al., 2014b).

3.4.3 Effects of external factors on maturation

The induction of reproduction by short time exposure to temperature shock and dehydration are likely the responses of *Ulva* to environmental stress (Hypothesis 1). Apart from that, external factors affect reproduction by accelerating the maturation of *Ulva* (Hypothesis 2). For example, high temperature or nitrate shortened the time of *Ulva* reaching maturity and made them reproduce earlier in this study. Moderately high temperatures can accelerate the process of growth and reproduction by increasing the metabolic activity of enzymes and increased nitrogen availability will increase the synthesis of nucleotides and proteins (Iken, 2012). In an important study, Heinrich et al. (2012) determined that up to 32% of genes in *Saccharina latissima* had altered expression profiles in respose to changes of temperature and light, with the highest transcriptionrates at the high temperature and light treatments. In terms of maturation period, thalli of *U. mutabilis* became fertile at an age between 18 and 24 (±2) days (Stratmann et al., 1996), which was earlier than the *U. rigida* (28 ±2 days) even under optimal conditions in this study. This can mainly be ascribed to species difference since the *U. mutabilis* used was a fast-growing mutant.

3.4.4 Effects of fragmentation

Smaller disks induced more reproduction in the present study, which was also reported before (Hiraoka and Enomoto, 1998, Gao et al., 2010). The disks of 0.9, 1.5, 2.1, 3.0, 6.0, 9.0, 12.0, and 15.0 mm diameter were employed in Hiraoka and Enomoto's study (1998). The highest degree of zooid formation (93%) of *U. pertusa* was found in the disks of 0.9 mm diameter and it delined as disk size increased, to a minimum of 12% in the disks of 9.0 mm diameter. Gao et al. (2010) also found nearly 100% disks of 0.5 and 0.75 mm diameter formed sporangia while it decreased to ~4% in disks of 4.0 mm diameter. Due to the limitations in the size of the disk puncher, the minimal disk diameter in the present study was 2.5 mm and a smaller size should induce more reproduction based on the literature (Hiraoka and Enomoto, 1998, Gao et al., 2010). In addition, it took six days for *U. rigida* to form reproductive cells in this study which was longer than that detailed in the literature. Zooid formation was found three days after excision of various-sized disks from thalli of *U. pertusa* (Hiraoka and Enomoto, 1998). Peak sporulation was also dectected in *U. intestinalis*, *U. lactuca* and *U. prolifera* 72 hours after fragmentation treatment. The different time periods needed for sporulation formation could be attributed to species differences as the degrees of

sporulation in *U. intestinalis* and *U. lactuca* were 48% and 21% respectively 48 hours after treatment whereas there was negligible sporulation in *U. prolifera* (Pettett and Dip, 2009).

There are two hypothesises on fragmentation inducing reproduction. The first is that wounding can trigger the same set of gene expression as sex-inducing pheromones in green multicellular alga *Volvox* (Amon et al., 1998), suggesting a molecular link between environmental stress and sexual reproduction. Smaller disks have a higher ratio of wounded cells to total cells which indicates stronger stimulation of reproduction. This could now be tested in *U. linza* given that a baseline set of transcripts is available (Zhang et al., 2012), but as yet the molecular tools are unavailable for *U. rigida*. The second is that blade cells of *Ulva* can produce two kinds of regulatory factors. One is excreted into cell walls and the other is located in the inner space between the two cell layers (Stratmann et al., 1996). The mechanical cutting breaks the structure of the cells walls and extracelluar matrix and it is therefore easier for small disks to leak out inhibitors so that they can get away from reproduction inhibition and form more zooids (Hiraoka and Enomoto, 1998). This hypothesis was given some support by differences in reproduction between *Ulva* grown in old media and in renewed media. More wounding sites resulted in increased release of inhibitors into the media. However, if they are not removed from the media they can still inhibit reproduction. When the seawater was renewed daily, the inhibitors in the media were maintained at a low level indicating out of control of vegetative state. This properly explained that *Ulva* grown in renewed media showed more reproduction. To fully verify this hypothesis would require a detail metabolomics study, such as those advocated by Simpson et al. (2011), Goulitquer et al. (2012) and Gupta et al. (2014). To the authors best knowledge, such a focused metabolomics study has not yet been done on *Ulva* in relation to growth and reproduction inhibitors.

3.4.5 Combined effects of external and internal factors

A combination of fragment and temperature shock remarkably shortened the time of zooid formation and discharge compared to each single factor. This indicates the regulation of reproduction of *Ulva* is a comprehensive and interactive process of internal and external factors (Hypothesis 3). Fragmentation led to the leak of sporulation inhibitor, which initiated the transformation from vegetative to reproductive status of *Ulva* cells while the temperature shock accelerated the process, thereby serving as an environmental stress. This promoting effect of fragmentation and temperture was alleviated by dehyration, which suggests dehyration imposes adverse effects rather than stimulating fertility.

3.4.6 Reproduction of blade and basal cells with age

The reproduction rate of blade cells increased with age of *U. rigida* under the same fragmentation treatment. This could perhaps indicate that the blade cells of older *Ulva* are more sensitive to envrionmental stress. This changing sensitivity might be due to the different excretion capabilities of regulatory factors in blade cells with age. Stratmann et al. (1996) reported that the excretion of sporulation inhibitor (SI-1) in blade cells decreased with maturation of *U. mutabilis*. That is why young *Ulva* seldom become reproductive and release swarmers. On the other hand, no reproduction was found during 14 days of culture after basal cells from either young or mature *Ulva* thalli that experienced temperature shock and fragment treatment, which suggests the basal cells can maintain vegetative status over the whole life span. This could be due to their lifetime excretion of sporulation inhibitors since when they were mixed up with blade cells, the reproduction of blade cells was significantly inhibited (Hypothesis 4). The sporulation inhibitor excreted by basal cells can be excreted into the medium when they are massively produced inside the cells (Stratmann et al., 1996). Furthermore, the excretion of sporulation inhibitors in basal cells was not affected by environmental stresses, such as temperature shock and fragmentation. It could possibly be that the different internal factors controlling excretion in basal cells and blade cells with age has led to the functional differentiation between them (Hypothesis 4). Blade cells are more sensitive to environmental change. High productivity and quick growth of the blade part of *Ulva* permit it to rapidly invade primary substrata as an opportunistic species when conditions are favouble. Meanwhile, when conditions become unfavouble, the blade part can quickly transform it's state from vegetative to reproductive, discharge swarmers and finally die off. While the basal part persists from which new thalli arise vegetatively during each subsequent growing season.

3.5 Conclusions

In this chapter the interaction of external and internal factors regulating reproduction of *U. rigida* with age were investigated for the first time. Temperature shock was a powerful external factor while dehydration did not induce any reproduction. The degree of zooid formation in blade cells increased as the size of the blade fragments decreased. Renewing seawater further promoted reproduction within fragments at all sizes. A combination of temperature shock and fragmentation induced reproduction on day two while fragmentation alone did not induce reproduction by day four. Blade disks excised from *U. rigida* at different ages showed different degrees of reproduction while basal cells did not switch to reproduction at any age. When blade cells were cultured with basal cells, the reproduction of blade cells

decreased. Conditions of high temperature and high nitrate shortened the period to maturity from 62.1 ± 2.8 days to 28.4 ± 2.0 days. These findings indicate that external factors like temperature shock can induce reproduction as environmental stresses and also stimulate maturity. Furthermore, internal factors such as sporulation inhibitors dominate reproductive regulation of *U. rigida* which can negate external factors in some cases. The differentiation of *Ulva* cells with time lead to different reproductive performance in different regions of the plant which not only supports the rapid growth of *Ulva* when environmental conditions are favourable but also aids survival during unfavourable conditions. The findings provide tangible support for the supply of *U. rigida* gametes out of season and improvements in *Ulva* cultivation.

Chapter 4. Growth, nutrient uptake, chemical composition and functional properties of a sterile strain of *Ulva rigida*

4.1 Introduction

4.1.1 The main challenge of Ulva *cultivation*

Ulva species should, in theory, be ideal cultivation candidates given their cosmopolitan distribution (Kirkendale et al., 2013), very high growth rates (Bruhn et al., 2011), and wide environmental tolerances (Luo and Liu, 2011). However, previous studies (Oza and Sreenivasa Rao, 1977, Ale et al., 2011, Bruhn et al., 2011, Castelar et al., 2014) have experienced marked difficulty in maintaining a vegetative state for most *Ulva* species in culture. Vegetative fragmentation and/or the formation of reproductive cells effectively terminates *Ulva* growth and leads to a disintegration of part or all of the thalli, which dramatically reduces *Ulva* productivity (Oza and Sreenivasa Rao, 1977). Ale et al.'s (2011) study showed that the specific growth rate of *U. lactuca* reached the maximum of 16.4 ± 0.18% on day five when NH_4^- was used as the nitrogen source and the maximum of 9.4 ± 0.72% on day six when NO_3^- was used as the nitrogen source during 10 days of laboratory culture. After that, growth began to decrease and was nearly zero for both conditions at the end of culture. Outdoor cultivation also demonstrated the noticable fluctuation of *Ulva* growth over time. For example, the daily growth rate of *U. flexuosa* grown at sea after 15 days of culture was 17.9 ± 4.3% but it decreased to 6.6 ± 1.1% after 30 days of culture (Castelar et al., 2014). The growth rate of *U. lactuca* grown in outdoor tanks also showed periodic fluctuations of between 0.3 ± 0.5% and 9.2 ± 2.1% (Bruhn et al., 2011). Apart from the change of environmental factors (light, temperature, etc.), periodic reproduction was the main reason behind the growth fluctuations (Bruhn et al., 2011). A longer term study (from 1981 to1983) that attempted year round cultivation of *U. lactuca* was conducted in outdoor tanks in Florida. However, the cultivation could not be maintained in summer (June and July) due to fragmentation of the *U. thalli* although they grew well in the winter and spring (DeBusk et al., 1986).

4.1.2 Methods to obtain robust strains

Therefore, a robust strain of *Ulva* with either reduced or absent reproduction as a trait is in great need for commercial cultivation. There are several methods currently available to achieve that goal. The simplest approach is the selection of superior strains that preferentially express somatic growth over gamete production from the existing wild populations (van der Meer, 1983). For example, fast growing clones *Chondrus crispus* (Neish and Fox, 1971),

Eucheuma (Doty and Alvarez, 1975), and *Gigartina exasperata* (Waaland, 1978) have been discovered and through various nation state-funded programmes, have been developed into cultivars and distributed to seaweed farmers. In terms of *Ulva* sp., a sterile mutant of *U. pertusa* was found at Omura Bay, Kyushu province in Japan in 1973, which only developed vegetatively without sexual reproduction and could be maintained with ease under axenic laboratory conditions (Migita 1985, Murase et al. 1993, Kakinuma et al., 2006). Although it is straightforward to look for robust strains from the wild species, the process can be time, labour intensive and requiring a certain amount of luck, as it would require prolonged oberservation of a wide range of ecotypes. Instead, mutagenesis may create all types of variation in theory and produce the expected strain after screening. Therefore, it can shorten the breeding period and enhance the efficiency (Toker et al., 2007, Fu et al., 2014). Mutagenesis has been employed for both microalgae and seaweeds (Zhang and Lee, 1997, Wang et al., 1998, Ong et al., 2010, Niwa et al., 2011). Physical mutagens, such as ultraviolet light (UV), have been widely used in higher plants and algae due to its convenience, safety, and high mutation rate (Anderson, 1995, Huang et al., 2006, Zhang et al., 2007). High productivity of algal strains can be produced by UV mutagenesis. *Spirulina platensis* was induced by UV and two good mutants (M1-3 and M5-1) were obtained; M5-1 was bigger and M1-3 was thinner and longer compared with the parent strain. Both strains had higher photosynthesis and growth rate (Li et al., 2001). Likewise, three strains of *Chlorella vulgaris* mutants, named M51, M59 and M73, were screened from 120 clones. Compared to the parent strain, the growth rates of the mutants M51, M59, and M73 were 6.23, 3.80, and 5.92% higher respectively and their protein contents were 2.5, 3.1, and 1.9% higher respectively (Zhang et al., 2007).

With development of molecular biotechnology, genetic modification has also been used to change the traits of algae by knocking out genes or adding new genes. Most studies on genetic modification focus on microalgae due to their relatively simple genomes and preferable values in biofuels and bioproducts (Qin et al., 2012, Jijakli et al., 2015). For instance, the eukaryotic freshwater green alga *Chlamydomonas reinhardtii* is one of the most successful algal genetic transformation systems to date (Jijakli et al., 2015). All three genomes of *C. reinhardtii* have been fully sequenced and modifications of nucleus and chloroplast have reached promising commercial relevance (Merchant et al., 2007, Neupert et al., 2009). In terms of seaweeds, work has been done on genetic transformation of economically important seaweeds such as *Laminaria*, *Porphyra*, *Gracilaria*, and *Undaria* (Qin et al., 2004). A model transformation system of *Laminaria* has been set up, which

involves a series of methods of gene insertion, vector construction, transformants' regeneration and screening (Qin et al., 2004). By utilising this model, strain improvement serving particular purposes can be achieved. Genetic modification is efficient for algal strain improvement whilst it needs information on genomes of organisms and is technically challenging.

These studies demonstrate that developing sterile, or at least low level reproductive strains is technically achievable and would be beneficial for long term cultivation. To date, sterile strains of *U. rigida* have not been reported.

4.1.3 Aims and objectives

This chapter aimed to obtain a sterile stain of *U. rigida* that could demonstrate high growth in long term culture. To achieve that, varying doses of UV radiation were employed to mutate a wild strain of *U. rigida*. In addition, patterns in growth, nutrient uptake, and chemical composition were examined to shed light on whether the sterile strain can be used for bioremediation and food purposes.

4.2 Materials and methods

4.2.1 Culture of U. rigida

Vegetative *U. rigida* fronds of 50–60 mm in length were collected from the low intertidal of Cullercoats Bay, UK (55.03^{o} N, 1.43^{o} W) after a spring tide. The fronds were placed in a plastic bag and transported to the laboratory at Newcastle University within one hour. They were then rinsed gently in sterile seawater in sterilised natural seawater (1 micron filtered) to remove any sediment, epiphytes or small grazers. This wild type strain and the sterile mutants described below were cultured at 18 $\pm$ 1^{o}C with a photoperiod of 16L: 8 D. Light intensity was 80 μmol photons m^{-2} s^{-1}.

4.2.2 Mutation of Ulva *gametes and isolation of sterile mutants*

Gametes were obtained by inducing reproduction of *U. rigida* disks. Thalli of *U. rigida* were punched into 2.5 mm disks with a metal puncher. One hundred disks were rinsed with autoclaved seawater and transferred to a flask containing 200 mL of autoclaved seawater. The flask was placed at 4^{o}C for six hours and then transferred to an 18^{o}C incubator with 80 μmol photons m^{-2} s^{-1} light intensity and a 16: 8 of L: D cycle. Gametes were released three days later and collected by pipette using their phototactic response. Gametes were concentrated by centrifuging (1000 *g*, five minutes) and then placed into Petri dishes (85 mm in diameter) with the final number of 2×10^{7} per Petri dish. Penicillin G (final concentration 1000 mg L^{-1}) and streptomycin sulfate (final concentration 250 mg L^{-1}) were added to the

media to minimise bacterial growth. Six Petri dishes were exposed to UV lamps (40 W, 254 nm) for 18 s, 36 s, 54 s, 72 s, 90 s and 108 s (one dish for each treatment), to experience different UV doses. These UV doses were chosen because no gametes survived when they were exposed to UV lamps for more than 120 s in a preliminary experiment. Afterwards, Petri dishes were placed in the dark for 24 hours to allow the gametes to settle. Then Petri dishes were incubated in conditions as described above. Germlings (112, 486 individuals) from six Petri dishes were detached from the dishes and transferred to six tanks (13 L) when they reached the length of 1 cm. Thalli which showed reproductive feature were removed and those that maintained the vegetative state were kept and grown further. This screening process continued for four months. Thalli that failed to demonstrate any reproductive tendencies were identified as sterile.

4.2.3 Growth measurement

Five wild thalli (one thallus per 500 ml conical flask) and three sterile thalli (one thallus per 500 ml conical flask) were grown in conditions as described in 4.2.1. The lower quantity in the sterile thalli was due to the limited availability of the sterile thallus. Fresh masses of wild and sterile *U. rigida* were recorded every three days for a period of 18 or 27 days. The 27-day culture only applied to the sterile strain as the wild strain tissue was lost by day 18 due to decay. The fresh mass was determined after gently blotting the thalli with tissue paper. Specific growth rates (SGR) of wild and sterile *Ulva* were calculated by the formula: DGR (%) = $[\ln (M_2/M_1)]/t \times 100$, where M_2 is the final mass, M_1 is the initial mass and t is the number of culture days.

4.2.4 Reproduction assessment

Disks of 7 mm diameter from wild or sterile strains were placed in 500 ml of flasks (three flasks for each strain, each flask containing 25 disks) and cultured at the same conditions as per the growth trial. The reproductive *Ulva* disks were recognized by an associated colour change. Formation of reproductive cells in *Ulva* is accompanied by a change in thalli colour from green (vegetative state) to yellowish (reproductive state) and then to white (after release of swarmers). This was verified via microscope observation. Partial sporulation formation in more than half the disk area was considered equivalent to complete sporulation formation. Reproduction rate was expressed as the ratio of reproductive disks to all disks in a flask.

4.2.5 Nutrient measurement

Assessment of nitrate and phosphate was the same as described in section 2.2.6. Triplicates were set for both strains.

4.2.6 Dry mass

Three fresh *Ulva* fronds of the wild and sterile strains were cultivated in 500 ml flasks (one frond in one flask) at 18^{o}C and 80 µmol photons m^{-2} s^{-1} for 12 days then oven dried at 50 ℃ until consistent mass (for 24 hours). The mass was recorded as dry mass. They were ground to a powder, sieved and placed into tubes, which were kept in a desiccator pending analyses of chemical composition and functional properties.

4.2.7 Protein content

Total protein was determined by the Kjeldahl method. The crude protein was calculated using nitrogen content multiplied by 5.45 based on the mean value of three species of *Ulva* (Shuuluka et al., 2013), and total nitrogen was measured by Elementar Vario Macro Cube (Elementar, Hanau Germany). Data were expressed as percentage of dry mass. Triplicates were set for both strains.

4.2.8 Amino acids analysis

Amino acids were analysed using a high-performance liquid chromatography (Ultimate 3000, USA)-tandem mass spectrometry (API 3200 Q-TRAP, USA) (HPLC-MS/MS) system (Shimbo et al., 2010). *Ulva* powder (100 mg) was transferred to an ampoule and hydrolysed with 4 ml of 6 N hydrochloric acid at 110^{o}C for 24 hours. Fifty µl of this solution was dried under nitrogen and then re-dissolved in 200 µl of deionized water. Eighty µl of borate buffer (0.1 M, pH 8.8) was added to 40 µl of sample solution and mixed. The solution was derivatised with 40 µl of diethyl ethoxymethylene-malonate at 55^{o}C for 15 minutes. Samples were transferred to insert vials and 20 µl were injected into the HPLC. The HLPC system consisted of a SRD-3600 Solvent Rack with analytical 6-channel vacuum degasser, a DGP-3600A pump, WPS-3000TSL analytical autosampler, and a tcc-3200 column compartment. Chromatographic separations were performed on a MSLab HP-C18 column (150 × 4.6 mm, 5 µm). The mobile phase consisted of water (A) and acetonitrile (B). The solvent was delivered to the column at a flow rate of 0.8 ml $minute^{-1}$ as follow: 0–0.1 minute from A-B (95:5) to A-B (95:5); 1–8 minutes from A-B (95:5) to A-B (40:60); 8–8.1 minutes from A-B (40:60) to A-B (0:100); 8.1–10 minutes from A-B (0:100) to A-B (0:100); 10-10.1 minutes from A-B (0:100) to A-B (95:5); 10.1–15 minutes from A-B (95:5) to A-B (95:5).

The conditions for MS-MS detection were optimized to obtain the highest signal intensity and were as follows: mode: positive-ion mode; ion spray voltage: 5500 V; nebulizer gas pressure: 55 psi; curtain gas pressure: 20 psi; collision gas pressure: medium; turbo gas temperature: 500^{o}C; entrance potential: 10 V; collision cell exit potential: 2 V. Nitrogen gas was used as the collision gas in the multiple Reaction monitoring (SRM) mode. The data were

obtained using Analyst software version 1.5.1 (Applied Biosystems). Amino acid standard solution (Aladdin, China) was injected for the system's calibration and amino acid quantification. Triplicates were set for both strains

4.2.9 Lipid extraction

Lipid was extracted according to the Folch method (Folch et al., 1957) with some modifications. Homogenized samples (3 g) were extracted with 60 ml chloroform: methanol (2:1) solution. After vortexing for 20 minutes at room temperature, NaCl (0.88%) of 12 ml was added to the aqueous phase to aid stratification. Then the samples were centrifuged for five minutes at 1000 *g* and the upper phase was removed. Sixty ml methanol: water (1:1) was used to rinse the tubes. After centrifuging and removing the upper phase once more, the bottom phase was dried under a steady stream of nitrogen. When the chloroform was completely evaporated the crude lipid was weighted. Results were expressed as percentage of dry mass. Triplicates were set for both strains

4.2.10 Fatty acid analysis

Fatty acid methyl ester (FAME) preparation was carried out according to Joseph and Ackman (1992). A mixture of 100 mg of fat matter, 1 ml internal standard (25 mg C23:0 in 25ml isooctane) and 1.5 ml 0.5 N methanolic NaOH (2.0 g NaOH in 100 ml methanol) was heated at 100^{o}C for five minutes. After reaching room temperature, 2 ml boron trifluoride 12% in methanol was added to the solution, which was then heated at 100^{o}C for 30 minutes. After cooling for five minutes at room temperature, 1.0 ml isooctane and 5 ml saturated NaCl solution were added. The solution was centrifuged for five minutes at 859 *g* and the upper layer was transferred to a new tube. One ml isooctane was added to the first solution and the procedure was repeated. The two layers of isooctane were dried under a steady stream of nitrogen and the residue was re-dissolved in 1.0 ml of hexane.

Analysis of FAME was carried out with a gas chromatograph (Shimadzu, GC-2014, Kyoto, Japan) using an Agilent CP-SIL 88 fused silica capillary column (100 m X 0.25 mm ID X 0.2 μm film thickness). Purified helium was used as a carrier gas with a head pressure of 210 kPa and a column flow of 1 ml minute^{-1}. A split injection system was used with an auto injector (Shimadzu, AOC-20i) with a split ratio of 50.0 and an injector temperature of 255 ℃. FAME peaks were detected by flame ionisation detection at 260 ℃. Samples of 1 μl were injected at an initial column temperature of 70 ℃, which was held for one minute. The temperature was then raised at 5 ℃ minute^{-1} to 100 ℃, where it was held for two minutes, and then increased at 10 ℃ minute^{-1} to 160 ℃, where it was held for 61 minutes. Finally, the temperature was increased to 240 ℃ at a rate of 5 ℃ minute^{-1}, where it was held for 21

minutes, thus giving a final gradient of 113 minutes total runtime. Peaks were identified using a commercial 52 FAME standard (GLC463, Nu-Chek Prep Inc., Elysian, MN, USA). Literature resources which present peak separation in chromatograms in detail were used for the identification of peaks for which a standard was not available, such as isomers of C18:1 and non-conjugated isomers of C18:2 (Stergiadis et al., 2015). Quantification of FA was based on peak areas of individual identified and non-identified FA, expressed as a percentage of the total peak areas for quantified FA. Triplicates were set for both strains.

4.2.11 Ash content

Ash was determined by incinerating samples in a muffle furnace at 550^{o}C for 24 hours. The total ash content was expressed as percentage of dry mass. Triplicates were set for both strains.

4.2.12 Carbohydrate

Carbohydrate was estimated by subtracting content of protein, lipid, and ash from total content. Triplicates were set for both strains.

4.2.13 Swelling capacity (SWC)

SWC of *U. rigida* was estimated by a slightly modified method of bed volume after equilibrating in excess solvent (Yaich et al., 2011). *Ulva* powders (0.2 g) were placed into a 10 ml of measuring cylinder. Ten ml of distilled water was added and immediately vigorously mixed. The measuring cylinder was left to stand for 18 hours at 37^{o}C. The swelling volume was recorded and SWC was expressed as ml of swollen sample per gram of sample. Triplicates were set for both strains.

4.2.14 Water holding capacity (WHC)

WHC of *U. rigida* was analysed by the centrifugation method (Yaich et al., 2011). *Ulva* powder (0.2 g) was placed into a pre-weighed centrifuge tube. Five ml of distilled water was added and the mixture was stirred vigorously. The dispersion was centrifuged for 25 minutes at 3000 *g* after standing at 37^{o}C for one hour. The supernatant was removed and the residue was oven-dried at 50^{o}C for 25 minutes. The water holding capacity was expressed as grams of water bound per gram of the sample. Triplicates were set for both strains.

4.2.15 Oil holding capacity (OHC)

OHC of *U. rigida* was measured by the method described as Yaich et al. (2011). *Ulva* powder (0.5 g) was dispersed in 5 ml of corn oil and then placed in a centrifuge tube. The mixture was stirred and left at 37^{o}C for one hour, followed by centrifugation at 3000 *g* for 25 minutes. The oil supernatant was then transferred to a 10 ml measuring cylinder and measured.

The OHC of *U. rigida* was expressed as grams of oil held by 1 g of sample (DW). The density of the corn oil was 0.92 g ml^{-1}. Triplicates were set for both strains.

4.2.16 Statistical analysis

Results were expressed as means of replicates ± standard deviation. Data were analysed using the software of SPSS v.21.

Effects of culture time on specific growth rate and reproduction rate of two strains

The repeated measures ANOVA or Friedman test could not be conducted to assess the effects of culture time on the specific growth rates and the reproduction rates and the possible reason is that the sample size was less than the number of time points. Instead, one-way ANOVAs were conducted and a confidence interval of 99% was set to deal with the error in the statistical test caused by pseudo-replication. The specific growth rates of the wild strain at all culture times conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the six samples could be considered equal (Levene's test, $F = 0.743$, $P > 0.05$). A one-way ANOVA was conducted to assess the effects of culture time on growth of the wild strain. Tukey HSD was conducted for *post hoc* investigation. The specific growth rates of the sterile strain at all culture times conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the nine samples could be considered equal (Levene's test, $F = 2.257$, $P > 0.05$). A one-way ANOVA was conducted to assess the effects of culture time on the specific growth rate of the sterile strain. Tukey HSD was conducted for *post hoc* investigation. The mean specific growth rate of the wild and sterile strains over the first 18-day culture conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$). The variances of the two samples could be considered equal (Levene's test, $F = 2.208$, $P > 0.05$). Therefore a t-test assuming equal variances was conducted to compare the mean specific growth rate data of wild and sterile strains over the first 18-day culture.

The reproduction rates of the wild strain at all culture times conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the six samples could be considered equal (Levene's test, $F = 3.300$, $P > 0.05$). A one-way ANOVA was conducted to assess the effects of culture time on reproduction rate of the wild strain. Tukey HSD was conducted for *post hoc* investigation.

Comparisons of nutrient uptake, chemical composition and functional properties between strains

Nutrient uptake, protein, lipid, ash, carbohydrate, and functional properties data of the wild and sterile strains conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$). The

variances of the two samples could be considered equal (Levene's test, $F < 6.750$, $P > 0.05$) therefore t-tests assuming equal variances were conducted to compare these parameters between the wild and sterile strains. A confidence interval of 95% was set for all tests.

Comparisons of amino acids and fatty acids between strains

All amino acids in the wild strain and total amino acids in sterile strain conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) except for total essential amino acids (Shapiro-Wilk, $P = 0.010$). The variances of the two samples in all amino acids could be considered equal (Levene's test, $F < 6.750$, $P > 0.05$) except for arginine (Levene's test, $F = 11.144$, $P = 0.029$) and aspartic acid (Levene's test, $F = 11.046$, $P = 0.029$). A one-way multivariate ANOVA (MANOVA) was used to compare the differences of amino acids content between the two strains considering that MANOVA is reasonably robust to violations of normal distribution and homogeneity of variance particularly when the group sizes are equal (Pallant, 2010). Bonferroni adjustment was used to reduce the chance of Type I error. A new alpha level of 0.0024 (0.05 divided by 21) was set since there were 21 dependent variables. All fatty acids in the wild strain and PUFA and n6-fatty acids in sterile strain conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) except for C18:2_{n6} (Shapiro-Wilk, $P = 0.011$) and C18:2_{n3} (Shapiro-Wilk, $P = 0.040$). The variances of the two samples in all fatty acids could be considered equal (Levene's test, $F < 6.750$, $P > 0.05$) except for C20 (Levene's test, $F = 13.398$, $P = 0.022$) and C20:4_{n6} (Levene's test, $F = 13.408$, $P = 0.022$). A one-way MANOVA was used to compare the differences of fatty acids content between the two strains considering that MANOVA is reasonably robust to violations of normal distribution and homogeneity of variance particularly when the group sizes are equal (Pallant, 2010). Bonferroni adjustment was used to reduce the chance of Type I error. A new alpha level of 0.0014 (0.05 divided by 37) was set since there were 37 dependent variables.

4.3 Results

The key questions that this chapter aimed to answer were whether there were differences in growth rate, reproductive capacity, protein, amino acid, lipid, fatty acid, ash, carbohydrate, SWC, WHC, and OHC between the sterile and wild strains.

4.3.1 Growth and reproduction

The specific growth rates of the wild and sterile strains were recorded every three days (Figure 4.1) The ANOVA showed that specific growth rates of the wild strain were significantly different between culture times ($F = 229.310$, df = 5, 24, $P < 0.001$). During the first three days, the specific growth rate reached 22.8 ± 1.7% but declined close to zero on day

12 (Tukey HSD, $P < 0.001$; Figure 4.1 a). From day 12 onwards, mass loss overtook growth, and the specific growth rate dropped to –15.6 ±2.4% during the last three days (Figure 4.1 a). The ANOVA showed that specific growth rates of the sterile strain were significantly different between culture times ($F = 11.009$, df = 8, 18, $P < 0.001$). In contrast to the wild strain, the sterile strain maintained a positive specific growth rate over the course of the 27-day culture, albeit at a reduced rate with time (Tukey HSD, $P < 0.01$), with the specific growth rate on the last three days still relatively high (11.4 ±2.3%; Figure 4.1 b). There were significant differences in mean specific growth rate between wild and sterile strains over the first 18-day culture (Figure 4.1 c). The mean specific growth rate of the sterile strain was five times faster than the wild strain (Independent samples t-test, $t = –48.173$, df = 6, $P < 0.001$).

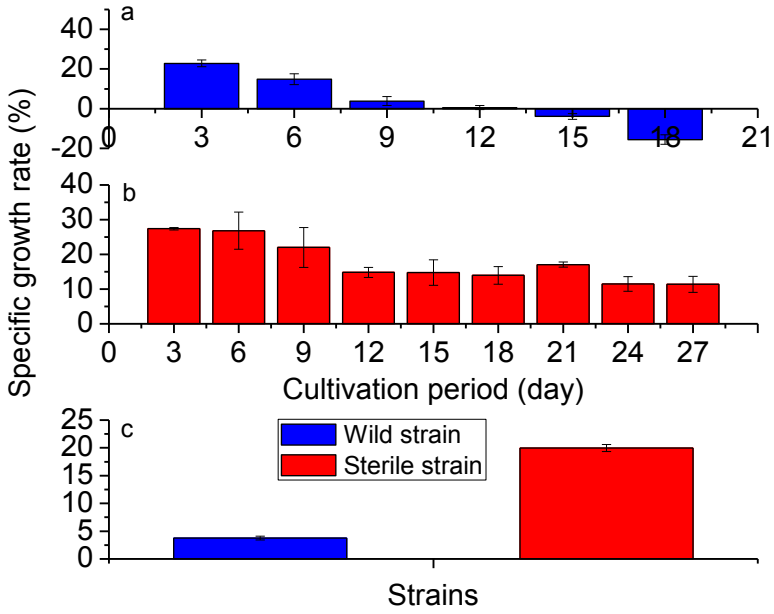

Figure 4.1 The specific growth rates of wild and sterile strains of *U. rigida*. a) Specific growth rate of the wild strain, b) specific growth rate of the sterile strain, and c) mean specific growth rates of wild and sterile strains over an 18-day cultivation. The error bars indicate the standard deviations (n = 3 or 5, 3 for sterile strain and 5 for wild strain).

The ANOVA showed that the reproduction rates of the wild strain were significantly different between culture times ($F = 251.289$, df = 5, 12, $P < 0.001$). The wild strain did not reproduce during the first six days and 18.7 ±2.3% of thalli became fertile on day nine (Figure 4.2). Then the reproduction rate increased with time (Tukey HSD, $P < 0.001$) and reached up to 82.7 ±6.1% on day 18. In contrast, all sterile disks remained in the vegetative status during the 27-day cultivation. As the sterile thalli disks did not experience biomass loss

from swarmer release, the resulting fronds were able to reach a considerable size, more than 50 cm long (Figure 4.3), which could not be found in the wild strain during laboratory culture.

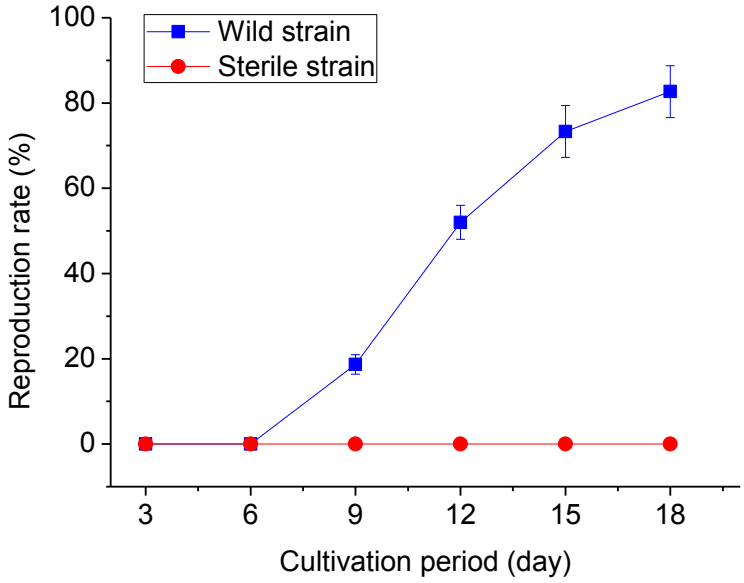

Figure 4.2 Reproduction rates of the wild and sterile strains of *U. rigida* over an 18-day cultivation. The error bars indicate the standard deviations (n = 3).

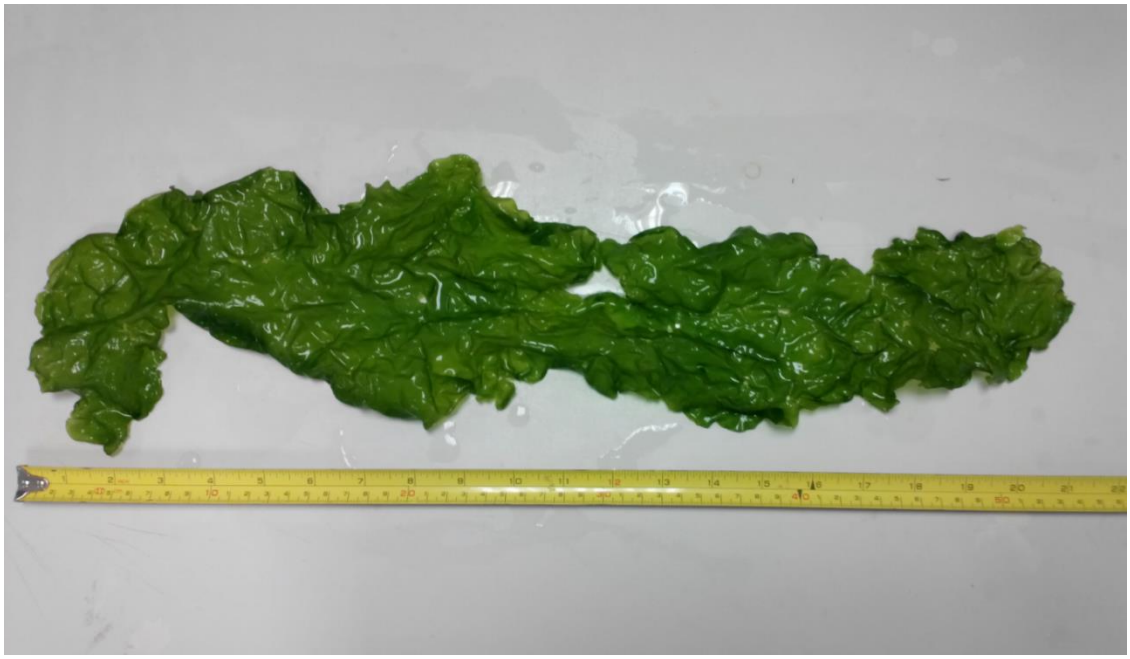

Figure 4.3 The sterile strain of *U. rigida* obtained by mutating wild strain.

4.3.2 Nutrient uptake

Nutrient uptake of wild and sterile strains was measured during the first three days (Figure 4.4). The nitrate uptake of the sterile strain was 171.6 ± 12.1 μM g FW^{-1} day^{-1}, which

was 40.0% higher than that of the wild strain (Figure 4.4 a) (Independent samples t-test, t = –5.337, df = 4, *P* = 0.006). The sterile strain absorbed 5.7 ± 0.4 μM phosphorus g FW^{-1} day^{-1}, which was significantly higher than that of (4.4 ± 0.5 μM g FW^{-1} day^{-1}) the wild strain (Independent samples t-test, t = –3.506, df = 4, *P* = 0.025, Figure 4.4 b).

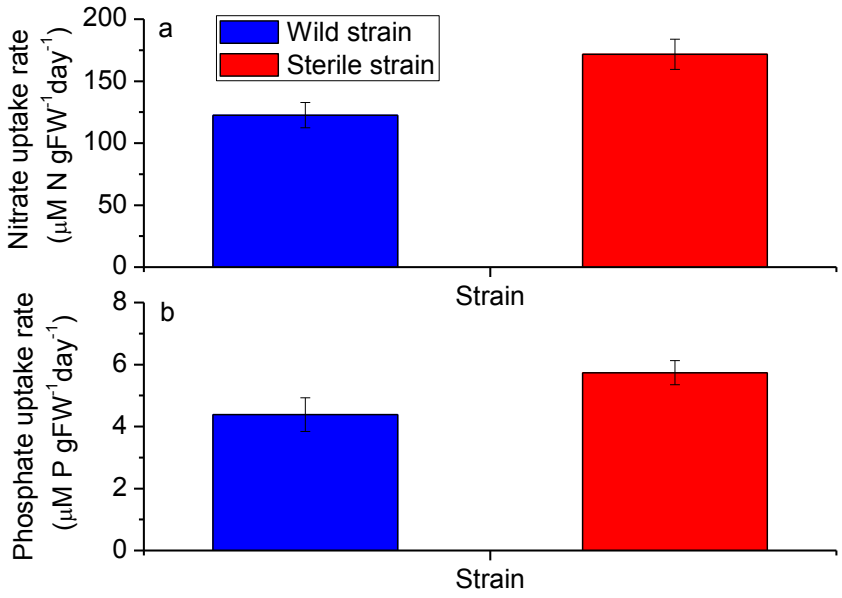

Figure 4.4 Nutrient uptake of the wild and sterile strains of *U. rigida* in nitrate (a) and phosphate (b). The error bars indicate the standard deviations (n = 3).

4.3.3 Chemical composition and functional properties

As shown in Figure 4.5, the protein content of the wild strain accounted for 24.1 ± 0.8% of *Ulva* dry mass, which was 58.8% higher than the sterile strain (Independent samples t-test, t = 8.224, df = 4, *P* = 0.001). On the other hand, the lipid content of the sterile strain was more than twice as high as the wild strain with the latter only 5.4 ± 0.4% of dry mass (Independent samples t-test, t = –19.052, df = 4, *P* < 0.001). Ash content of the sterile strain (34.0 ± 1.1%) was also slightly higher than the wild strain (31.4 ± 1.2%) (Independent samples t-test, t = –2.812, df = 4, *P* = 0.048). No significant differences between strains in carbohydrate content were found (Independent samples t-test, t = –1.007, df = 4, *P* = 0.371).

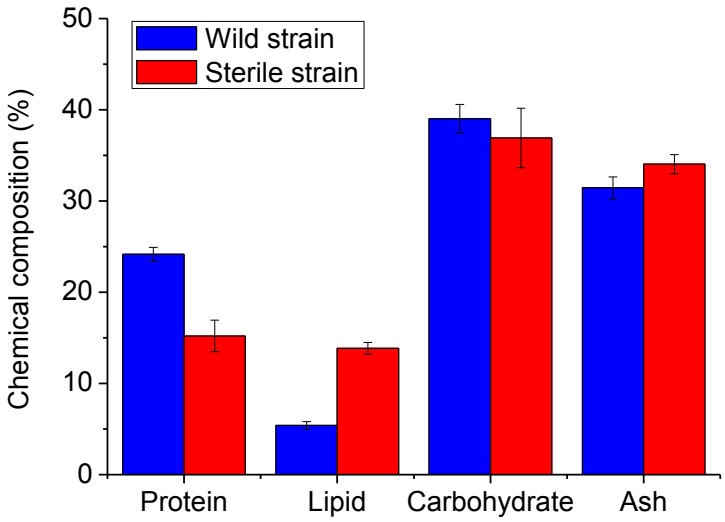

Figure 4.5 Chemical compositions of the wild and sterile strains of *U. rigida*. The error bars indicate the standard deviations (n = 3).

Functional properties of the *Ulva* strains were also assessed (Figure 4.6 a). The SWC of the sterile strain was 8.1 ± 0.3 ml g^{-1} DW, which was 27.7% lower than the wild strain (Independent samples t-test, t (4) = 30.946, $P < 0.001$). A similar trend was found in WHC and OHC. These two properties of the sterile strain were 25.3% (Independent samples t-test, t = 15.399, df = 4, $P < 0.001$) and 11.5% lower than the wild strain respectively (Independent samples t-test, t = 4.461, df = 4, $P = 0.011$).

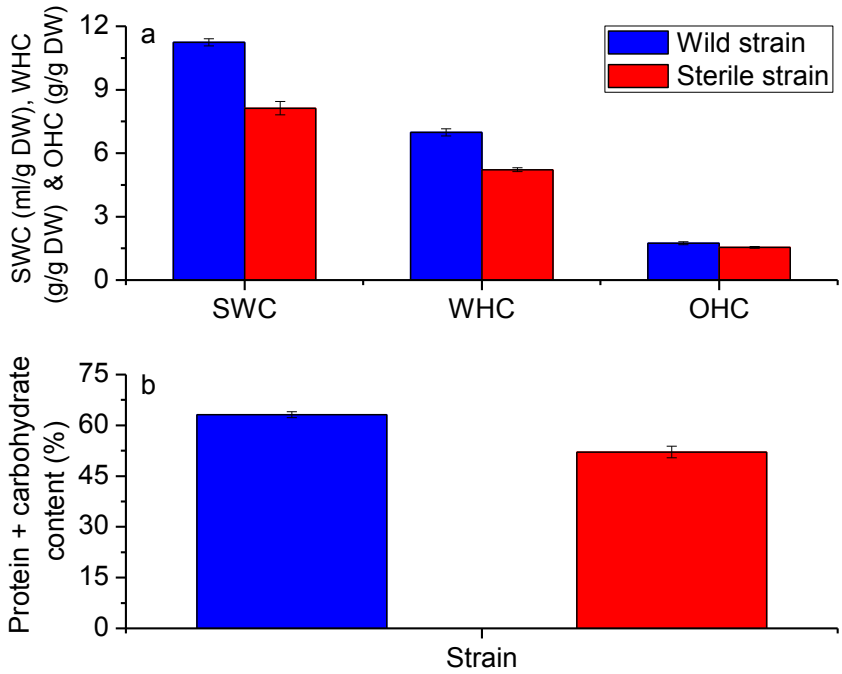

Figure 4.6 Functional properties (a) and content of protein plus carbohydrate (b) in wild and sterile strains. The error bars indicate the standard deviations (n = 3).

The amino acid composition of *U. rigida* wild and sterile strains is shown in Table 4.1. Glutamic (Glu) and alanine acid (Ala) were the most abundant amino acids (AA) in both strains. Aspartic acid (Asp) and arginine (Arg) were second highest AA group for the wild strain while they were valine (Val) and leucine (Leu) for the sterile strain. There were more histidine (His), serine (Ser), glutamic acid (Glu), isoleucine (Ile), total AA, and NEAA in the wild strain compared to the sterile strain (Table 4.1).

Amino acids	Wild strain	Sterile strain	DF	F	*P* value
His	0.36 ± 0.01	0.22 ± 0.02	1	83.248	0.001
Ser	1.27 ± 0.08	0.85 ± 0.04	1	64.233	0.001
Arg	1.57 ± 0.20	0.93 ± 0.03	1	29.797	0.005
Gly	0.83 ± 0.10	0.77 ± 0.06	1	0.918	0.392
Asp	1.94 ± 0.24	1.01 ± 0.04	1	42.525	0.003
Ala	1.98 ± 0.11	1.47 ± 0.11	1	30.452	0.005
Glu	2.21 ± 0.05	1.41 ± 0.11	1	120.476	<0.001
Thr	1.15 ± 0.15	0.89 ± 0.09	1	6.305	0.066
Pro	0.96 ± 0.10	0.66 ± 0.04	1	24.223	0.008
Lys	1.24 ± 0.11	0.79 ± 0.09	1	28.358	0.006
Tyr	0.58 ± 0.05	0.46 ± 0.03	1	12.000	0.026
Met	0.42 ± 0.04	0.38 ± 0.04	1	1.194	0.336
Val	1.39 ± 0.05	1.12 ± 0.10	1	18.229	0.013
Ile	0.81 ± 0.02	0.65 ± 0.03	1	52.285	0.002
Phe	1.17 ± 0.13	1.09 ± 0.09	1	0.726	0.442
Cys	0.18 ± 0.02	0.15 ± 0.01	1	6.487	0.064
Leu	1.12 ± 0.06	1.10 ± 0.09	1	0.135	0.732
Total	19.18 ± 0.54	13.93 ± 0.55	1	3790.801	<0.001
EAA[a]	9.22 ± 0.49	7.17 ± 0.49	1	18.732	0.007
NEAA[b]	9.95 ± 0.17	6.76 ± 0.05	1	869.035	<0.001
EAA/NEAA	0.93 ± 0.05	1.06 ± 0.06	1	8.176	0.046

Table 4.1 Amino acids profile (g 100 g^{-1} DW) in wild and sterile strains of *U. rigida*. Data are the means ± SD (n = 3). DF, F, and *P* value were from the MANOVA. Bonferroni adjustment was used to reduce the chance of Type I error. A new alpha level of 0.0024 (0.05 divided by 21) was set since there were 21 dependent variables. [a]EAA, essential amino acids: His, Arg, Thr, Lys, Met, Val, Ile, Phe, and Leu. [b]NEAA, non-essential amino acids: Ser, Gly, Asp, Ala, Glu, Pro, and Tyr.

Table 4.2 displays the fatty acids profiles of the wild and sterile strains. The most abundant fatty acid was C16:0 for both strains. In addition to C16:0, this species of *Ulva* has a high C18 unsaturated fatty acids content, such as $C18:3_{n3}$, c11 C18:1, etc. The sterile strain had more C10:0, C18:0, c11 C18:1, $C18:2_{n6}$, C20:0, $C18:3_{n6}$, $C18:3_{n3}$, $C20:2_{n6}$, $C20:3_{n6}$, $C20:4_{n6}$, and $C22:4_{n6}$ than the wild strain, which led to more MUFA, PUFA, n6-FA, and n3-FA.

Fatty acid	Wild strain	Sterile strain	DF	F	*P* value
C10:0	0.01 ± 0.00	0.03 ± 0.00	1	194.427	<0.001
C12:0	0.02 ± 0.00	0.01 ± 0.00	1	33.346	0.004
C14:0	0.47 ± 0.02	0.39 ± 0.01	1	37.970	0.004
c9 C14:1	0.03 ± 0.03	0.08 ± 0.02	1	4.617	0.098
C15:0	0.04 ± 0.00	0.06 ± 0.01	1	30.515	0.005
C16:0	11.31 ± 0.85	22.06 ± 2.36	1	55.293	0.002
t9 C16:1	0.01 ± 0.00	0.02 ± 0.01	1	7.598	0.051
c9 C16:1	0.65 ± 0.07	0.93 ± 0.13	1	10.143	0.033
C17:0	0.02 ± 0.00	0.03 ± 0.00	1	3.041	0.156
c9 C17:1	0.07 ± 0.01	0.07 ± 0.00	1	1.184	0.338
C18:0	0.17 ± 0.00	0.26 ± 0.01	1	322.978	<0.001
c9 C18:1	0.24 ± 0.04	0.46 ± 0.08	1	19.048	0.012
c11 C18:1	5.50 ± 0.23	11.33 ± 0.41	1	466.497	<0.001
c12 C18:1	0.02 ± 0.01	0.03 ± 0.00	1	0.702	0.449
$C18:2_{n6}$	0.88 ± 0.07	13.54 ± 0.71	1	956.101	<0.001
$C18:2_{n3}$	0.00 ± 0.00	0.03 ± 0.01	1	40.500	0.003
C20:0	0.03 ± 0.00	0.06 ± 0.00	1	111.518	<0.001
$C18:3_{n6}$	0.14 ± 0.01	1.61 ± 0.17	1	240.637	<0.001
$C18:3_{n3}$	6.69 ± 0.09	23.19 ± 1.06	1	714.828	<0.001
$C20:2_{n6}$	0.01 ± 0.00	0.06 ± 0.01	1	98.097	0.001
C22:0	0.57 ± 0.16	0.65 ± 0.07	1	0.806	0.420
$C20:3_{n6}$	0.07 ± 0.01	0.71 ± 0.08	1	200.229	<0.001
c13 C22:1	0.05 ± 0.00	0.09 ± 0.02	1	17.225	0.014
$C20:4_{n6}$	0.13 ± 0.00	2.90 ± 0.23	1	417.043	<0.001
$C22:2_{n6}$	0.01 ± 0.00	0.01 ± 0.00	1	1.850	0.245
$C20:5_{n3}$	0.56 ± 0.01	1.46 ± 0.07	1	548.508	<0.001
C24:0	0.05 ± 0.02	0.03 ± 0.00	1	5.271	0.083
c15C24:1	0.01 ± 0.00	0.01 ± 0.00	1	0.015	0.908
$C22:4_{n6}$	0.03 ± 0.00	0.61 ± 0.03	1	871.742	<0.001
$C22:5_{n3}$	1.38 ± 0.08	1.19 ± 0.15	1	3.843	0.121
$C22:6_{n3}$	0.00 ± 0.00	0.01 ± 0.01	1	4.517	0.101
SFA[a]	12.69 ± 0.95	23.60 ± 2.44	1	52.071	0.002
MUFA[b]	6.58 ± 0.35	13.01 ± 0.26	1	647.129	<0.001
PUFA[c]	9.93 ± 0.19	45.40 ± 0.30	1	29093.241	<0.001
n6-FA[d]	1.28 ± 0.08	19.49 ± 0.83	1	1424.768	<0.001
n3-FA[e]	8.64 ± 0.19	25.87 ± 1.14	1	668.657	<0.001
n6/n3	0.15 ± 0.01	0.76 ± 0.06	1	261.740	<0.001

Table 4.2 Fatty acid profiles (mg g^{-1} DW) of wild and sterile strains of *U. rigida*. Data are the means ± SD (n = 3). DF, F, and *P* value were from the MANOVA. Bonferroni adjustment was used to reduce the chance of Type I error. A new alpha level of 0.0014 (0.05 divided by 37) was set since there were 37 dependent variables. [a]SFA, saturated fatty acids. [b]MUFA,

monounsaturated fatty acids. [c]PUFA, polyunsaturated fatty acids. [d]n6-FA, total Omega-6 fatty acids. [e]n3-FA, total Omega-3 fatty acids.

4.4 Discussion

4.4.1 Growth, reproduction and nutrient uptake

In the present study, the growth of the wild strain decreased with time, which was consistent with other published research, both in indoor and outdoor culture. For instance, the specific growth rate of *U. lactuca* declined from 16.4% on day five to nearly zero on day 10 in a lab experiment (Ale et al., 2011) and the biomass yield of *U. lactuca* dropped from 70 g total solids m^{-2} day^{-1} to nearly zero in outdoor tank cultivation (Bruhn et al., 2011). The reason for the decrease in growth could be due to a switch to reproduction, which imposes negative effects on growth. Reproduction effectively terminates the growth of *Ulva* and leads to a pronounced disintegration of part, or all of the thalli. The reproduction rate of the wild strain increased with time in the present study, which could explain the decrease of growth with time. The dip of biomass yield in *U. lactuca* was also the result of sporulation events in outdoor cultivation (Bruhn et al., 2011).

High growth rate in the long term is commonly desired in aquaculture. The specific growth rate of sterile *Ulva* over 18 days of cultivation was 20.0 ± 0.6%, which was five times faster than the wild strain. The growth rate over 27 days of cultivation was 17.4 ± 0.3% while it was less than 10% in 10-day lab culture of *U. lactuca* (Ale et al., 2011) and 10.5% in 42-day outdoor culture of *U. lactuca* (Bruhn et al., 2011). Apart from the beneficial effects of sterility, this quick growth could also be related to high nutrient uptake capacity. The uptake rates of nitrate and phosphate were 171.6 ± 12.1 and 5.7 ± 0.4 μmol g FW^{-1} day^{-1}, which were 40.0% and 30.9% higher than that of the wild strain. Nitrogen and phosphorus are essential for growth as they are components of proteins, nucleotides, and enzymes. Rapid cell proliferation will require a large amount of nitrogen and phosphorus, and this was demonstrated with a high uptake capacity of nitrate and phosphate in the sterile strain. When compared with other species, 133.7 μmol g FW^{-1} day^{-1} in *U. fenestrata*, 109.7 μmol g FW^{-1} day^{-1} in *U. intestinalis* (Björnsäter and Wheeler, 1990), and ~70 μmol g DW^{-1} day^{-1} in *U. lactuca* (Ale et al., 2011), the nitrate uptake capacity of the sterile strain is still preferable, which makes it an efficient biofilter for bioremediation. The phosphate uptake rate of the sterile strain (5.7 ± 0.4 μmol g FW^{-1} day^{-1}) was higher than 0.637 ± 0.266 μmol g FW^{-1} day^{-1} in *U. rotundata* (Hernández et al., 2005), 0.15 μmol g FW^{-1} day^{-1} in *U. pertusa* (Seob et al., 2010), 7–8 μmol g DW^{-1} day^{-1} in *U. intestinalis* (Martínez-Aragón et al., 2002), 1.01–5.04 μmol g FW^{-1} day^{-1} in *U. fenestrata* (Björnsäter and Wheeler, 1990) and comparable to 3.42–

6.54 μmol g FW^{-1} day^{-1} in *U. intestinalis* (Björnsäter and Wheeler, 1990). The N: P uptake ratio (29.9) in the sterile strain was higher than 3.7–4.4 in *U. linza*, 4.5–5.1 in *U. prolifera* (Luo et al., 2012), 9.4 in *U. rotundata* (Hernández et al., 2005), and 16.6–16.8 in *U. intestinalis* (Björnsäter and Wheeler, 1990) but comparable with 11.8–36.0 in *U. fenestrata* (Björnsäter and Wheeler, 1990).

4.4.2 Chemical composition and functional properties

Compared with the wild strain, there was less protein, more lipid and ash in the sterile strain. The reasons for this remain unknown although less protein content in a fast-growing mutant of *U. mutabilis* was reported (Løvlie, 1969). One explanation for this may be that the faster growing sterile strain required more cell membrane (possibly both external cell membrane and membranes surrounding the organelles) for cell division. Cell membranes have a high PUFA content to maintain membrane fluidity (Heldt and Piechulla, 2005). If this was the case then this would explain why the sterile strain had both a higher overall lipid content (relative to protein) and the increased concentration of PUFA, as well as the higher metabolic demand for phosphorous. In addition, more energy was needed in the sterile strain to maintain a higher rate of cell division and then growth, which required a higher photosynthetic efficiency to convert solar energy to chemical energy that could be ultilised by the cell. However, there was no statistical difference in carbohydrate content, a crude proxy for photosynthetic efficiency, between strains. The reason could be that the ATP and NADPH produced by photosynthesis were not used to synthesised carbohydrate or protein but lipid. Why was that? Apart from the requirement from fast cell division, nitrogen limitation could also result in higher lipid content (Brennan and Owende, 2010). Although nitrogen was not deliberately limited in the media, the rapid growth of the sterile strain could lead to inadvertently limiting of nitrogen near the surface of *Ulva* thalli or in vivo where nitrogen demand outstripped supply. When nitrogen is limited, the fixed carbons are flowed to the synthesis of either lipid or carbohydrate rather than protein (Roessler, 1990, Rodolfi et al., 2009). Because lipid particularly saturated and monounsaturated fatty acids can generate more energy than carbohydrates upon oxidation compared to carbohydrate, they are given the priority of synthesis (Roessler, 1990, Rodolfi et al., 2009). The present study verified this hypothesis: more lipid and monounsaturated fatty acids while less protein was found in the sterile strain.

Although there was less protein in the sterile strain, there was a relatively high ratio of EAA to NEAA in the sterile strain (1.06 ± 0.06), which makes the sterile strain preferable in terms of EAA sources as the ratio of EAA to UEAA in other *Ulva* species is usually below 1,

such as *U. clathrata* (Peña-Rodríguez et al., 2011) and *U. rigida* (Taboada et al., 2010). The lipid content in the sterile strain was extremely high compared with the wild strain. The 13.8% lipid content was also higher than other *Ulva* species. For instance, lipid accounted for 2.5–3.5% in *U. clathrata* (Peña-Rodríguez et al., 2011) and 7.9% in *U. lactuca* (Yaich et al., 2011). In addition, there were more PUFA in the sterile strain, such as $C18:2_{n6}$, $C18:2_{n3}$, $C18:3_{n6}$, $C18:3_{n3}$, $C20:2_{n6}$, $C20:3_{n6}$, $C20:4_{n6}$, and $C22:4_{n6}$, which would have potentially greater benefits for health and nutrition applications. The ratio of n6-FA to n3-FA was higher in the sterile strain than the wild strain but the value was still far less than 10. The recommended value of the World Health Organisation (WHO) of this ratio in the diet is currently no higher than 10. Therefore, the sterile strain is an efficient food resource to reduce n6-FA/n3-FA.

The SWC of the wild strain was slightly lower than that in *U. lactuca* (13.0 ± 0.70 ml g^{-1} DW) reported by Wong and Cheung (2000). The sterile strain had a lower SWC compared with the wild strain but Yaich et al. (2011) demonstrated further lower SWC (0.3 ml g^{-1} DW) in *U. lactuca*. SWC mainly depends on the amount of protein and fibre and physical properties, such as particle size, density, pH, temperature, ionic strength, and the types of ions in solution (Robertson and Eastwood, 1981, Fleury and Lahaye, 1991). The WHC of the wild strain (6.98 ± 0.17 g g^{-1} DW) in this study was noticeably lower than that (9.71 ± 0.11 g g^{-1} DW) reported by Wong and Cheung (2000) but comparable to Yaich et al. (2011)'s finding (6.66 to 7.00 g g^{-1} DW). The sterile strain had a lower WHC (5.2 ± 0.10 g g^{-1} DW) compared with the values above. According to Robertson and Eastwood (1981), water can be associated with fibre either as bound water or trapped water. The amount of bound water depends on the chemical composition whereas trapped water refers to the structure of the fibre. WHC, determined by the centrifugation method used in this study, represented both types of water to exit the fibre. Apart from fibre, protein may also play a role in water holding that involves protein conformations and the nature of the water binding sites in the protein molecules (Chou and Morr, 1979). Therefore, the hydration properties might be mainly determined by these two chemical components. In this study, the total content of protein and carbohydrate in the sterile strain (52.1 ± 1.7%) was 17.5% lower than in the wild strain (Figure 4.6 b). This might lead to lower SWC and WHC in the sterile strain. The OHC of the wild strain (1.75 ± 0.07 g g^{-1} DW) and the sterile strain (1.54 ± 0.04 g g^{-1} DW) was much higher than the value (0.65 ± 0.03 g g^{-1} DW) reported by Wong and Cheung (2000). This may be due to different treatment temperatures. The OHC was determined at 37°C in the present study while it was 25°C in Wong and Cheung's study. The effects of temperature on OHC were investigated by Yaich et al. (2011). Our results were comparable to their findings (around 1.60 g g^{-1} DW at 40°C). In

general, proteins play a major role in fat absorption by hydrophobic bonding. Furthermore, the OHC of seaweeds are also related to the particle size, overall charge density and hydrophilic nature of fibres (Kinsella and Melachouris, 1976, Voutsinas and Nakai, 1983).

4.5 Conclusion

The results clearly demonstrate that this new sterile strain is a positive step towards increasing *Ulva* cultivation productivity in the long-term to serve multiple market applications. The sterile strain grew five times faster than the wild strain over an 18-day cultivation. In addition, it also had noticeable bioremediation advantages in nitrate (171.6 ± 12.1 μM g FW^{-1} day^{-1}) and phosphate uptake (5.7 ± 0.4 μM g FW^{-1} day^{-1}), which were 40.0% and 30.9% higher than in the wild strain respectively. In terms of chemical composition, the lipid content of the sterile strain was more than two times higher than the wild strain, and the protein content of the sterile strain was 26.3% lower than the wild strain. The wild strain contained more histidine (His), serine (Ser), glutamic acid (Glu), isoleucine (Ile), total AA, and NEAA compared with the wild strain. There were more monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), total omega-6 fatty acids (n6-FA), and total omega-3 fatty acids (n3-FA) in the sterile strain. The swelling, water holding, and oil holding capacities of the sterile strain were all lower than the wild strain. The findings above indicate that the production of a sterile *U. rigida* strain is technically achievable. Unfortunately, this sterile strain is unavailable due to improper preservation. Further, the random nature of the mutation(s) induced in the plant could be difficult to replicate. Ideally, a full pairwise genome comparison would have been conducted between the wild strain and the mutant. Unfortunately, the *Ulva* genome has been shown difficult to adequately sequence, despite numerous attempts at several international laboratories. Even if the genome was amenable to sequencing, such a pairwise comparison would necessitate a very long read sequencing platform such as PacBio, which remains an expensive option and far outside of the scope of this Ph.D. project.

Chapter 5. Comparative study of preservation of thalli, germlings, and gametes of *Ulva rigida*

5.1 Introduction

5.1.1 Significance of seaweed preservation

The successful and reliable preservation of algae is fundamental for commercial cultivation in order to maintain robust species/strains for long-term availability. In addition, preservation of seaweed will increase their potential value as experimental test organisms in diverse fields including marine ecotoxicology and in the development and testing of antifouling paints. Preservation will circumvent issues relating to seasonal fluctuation in plant availability and quality. Further, it will provide a means to safeguard desirable genetic strains, for example those that are adapted to grow well in particular biogeographic regions. The successful cryopreservation of a variety of microalgae has been documented over the last 50 years, and the approach is now commonly employed by culture collections for long-term storage of microalgae, such as in the Culture Centre for Algae and Protozoa (CCAP) (Day, 1998). In comparison, research into the preservation of seaweeds is less advanced and literature records relating to successful preservation are relatively few.

5.1.2 Methods of seaweed preservation

The majority of research in seaweed preservation has focused on commercially important edible seaweeds such as *Porphyra* (Migita, 1964, 1966, 1967, Kuwano et al., 1992, 1993, 1994, 1996), *Undaria* (Arbault et al., 1990, Ginsburger-Vogel et al., 1992, Renard et al., 1992), and *Laminaria* (Vigneron et al., 1997). Among these literatures (Kuwano et al., 1992, 1993, 1994, 1996), both conchocelis and thalli of *Porphyra* were successfully cryopreserved. With the combined cryoprotectants of 10% DMSO and 0.5 M sorbitol in 50% seawater and a two-step cooling method, the survival of *Porphyra* conchocelis stored in liquid nitrogen for one day could reach above 60% (Kuwano et al., 1993). The viability of sporothalli of *Porphyra* species (*P. seriata*, *P. yezoensis*, *P. tenera*, *P. pseudolinearis*, and *P. dentata*) stored in liquid nitrogen for one day ranged from 54.6–70.9% using the same cooling method and cryoprotectant (Jo et al., 2003). Meanwhile, the viability of gametophytic thalli of *P. yezoensis* (U-511) was much higher, exceeding 95% with mixed with a cryoprotectant of 5% DMSO and 5% dextran, PVP, or Ficoll. Furthermore, the viability in two clonal gametophytic thalli of *P. yezoensis* (TU-1, 2) and a clonal gametophytic thalli culture of *P. teneru* (TU-3) was more than 98% with a cryoprotective solution of 5% DMSO and 5% dextran (Kuwano et al., 1996). The research mentioned above has focused on the two-step cryopreservation of

seaweed thalli or conchocelis perhaps because they take less time to grow to a harvestable size compared with spores or gametes.

In addition to two-step cryopreservation, encapsulation-vitrification has also been used in seaweed preservation. Zhang et al. (2009) investigated the effect of the composition of the vitrification solution, the concentration of the loading solution, the duration of loading and dehydration treatments and of the washing method on viability of two diatom species. The results demonstrated that the highest viability (73.8% for *Nitzschia closterium* and 48.2% for *Chaetoceros muelleri*) was achieved when alginate beads containing the seaweed cells were loaded with 50% Plant Vitrification Solution 2 (60 minutes for *N. closterium* and 40 minutes for *C. muelleri*), dehydrated with 100% Plant Vitrification Solution 2 for 60 minutes at 0 ℃, frozen and rewarmed rapidly and washed with a 1.2 M sucrose solution for 30 minutes at 20 ℃. Further, Wang et al. (2011) reported female gametophytes had higher viability than male gametophytes of brown algae of *Undaria pinnatifida* by encapsulation-vitrification. The highest viability of female (31.2%) and male gametophytes (26.0%) was obtained by loading alginate beads with a mixture of 2 M glycerol and 0.6 M sucrose for 120 minutes (90 minutes for male gametophytes) at 25 ℃, dehydrated with PVS2 for 50 minutes (40 minutes for male gametophytes at 0 ℃ and washed with a 1.2 M sucrose solution. These studies indicate the conditions of encapsulation-vitrification may be species-dependent.

Apart from cryopreservation, a few studies on seaweed preservation at non-cryogenic temperatures (above –150^{o}C) have also been conducted. Up to 100% viabilities of *Porphyra renera* were showed when the thalli were stored at the temperature of –20 ℃ to –75 ℃ for short times (within one hour) (Migita, 1966). The viability generally decreases with stored time. For instance, the survival rate of *Porphyra yezoensis* conchocelis stored in 50% seawater with 5% DMSO + 0.5 M sorbitol solution at –80^{o}C for four hours was 1.9 ± 1.6% and it was reduced to 0.3 ± 0.2% when the time was extended to three days (Kuwano et al., 1992). When frozen at –30^{o}C, the survival rate of the conchocelis cells was 55% for one day storage and it decreased to 16% in seven days. Below 1% of the conchocelis cells survived in 55 days (Kuwano et al., 1992).

5.1.3 Ulva *preservation*

As far as *Ulva* is concerned, limited reports are confined to preservation of zoospores due to their value in pollution and antifouling tests (Taylor and Fletcher, 1999b, Bhattarai et al., 2007). The viabilities of *Ulva intestinalis* spores preserved at both cryogenic (–196 ℃) and non-cryogenic temperatures (–2 ℃ and –40 ℃) were investigated by Taylor and Fletcher (1999b) and nearly all spores could survive for five minutes storage regardless of preservation

temperatures. However, only spores stored at –2 ℃ survived (28.0 ± 4.2%–38.9 ± 3.3%) for more than three days of preservation. Similar results were reported by Bhattarai et al. (2007). The survival rates of *U. fasciata* spores stored at 4°C, –20°C and –70°C for five days were 4.4–38.6%, 0–2.5%, and 0% respectively. Only spores stored at 4°C survived (1.4–5.8%) for more than ten days of preservation. These findings indicate preservtion at higher temperate could improve the viability of *Ulva* spores. With respect to the preservation of *Ulva* thalli, Van der Meer and Simpson's (1984) study demonstrated that *U. lactuca* was successfully cryopreserved (100% survival) for one hour.

5.1.4 Aims and objectives

Understanding of *Ulva* preservation is poor and this may create a bottleneck for future expansion of the industry. This chapter aimed to define an optimal preservation method using appropriate *Ulva* material. Therefore, different methods used to preserve *Ulva* gametes, germlings and adults were compared. This study provides powerful support for both the theoretical understanding and practical application of *Ulva* preservation.

5.2 Materials and methods

5.2.1 Preparation of Ulva *material for freezing treatments*

Adult vegetative *Ulva* was collected from Cullercoats, eastern coast of England (55.03° N, 1.43° W) after a spring tide in June 2014. The *Ulva* thalli were punched into disks of 7 mm in diameter after rinsing gently in sterile seawater (1 micron filtered) to remove any sediment, epiphytes or small grazers. Gametes were obtained by the fragmentation method and then concentrated by point light source. Some (1×10^6) of these gametes were used to produce germlings. After settlement and germination, 387 germling individuals of around 2 mm length were selected for preservation trials.

5.2.2 Freezing and thawing treatment

DMSO was chosen as a cryoprotectant because it can pass through cell membranes more freely and is much more easily removed from cells after preservation than glycerol (Taylor and Fletcher, 1999a). The concentrations of 10% and 15% were used for *Ulva* thalli and germlings while 5% and 10% was employed for *Ulva* gametes given the lower tolerance of gametes to DMSO toxicity (Taylor and Fletcher, 1999b).

Ulva disks or germlings were placed into freezing ampoules (1.5 ml capacity) containing 10% or 15% DMSO. One disk or germling was placed in each ampoule, with 54 ampoules under each temperature treatment. Nine of these 54 ampoules were assessed for viability at every storage period (one, two, seven, 30, 90, and 180 days). Pretreatment with

DMSO was conducted at room temperature (20^{o}C) for 20 minutes. After the pretreatment, two batches of ampoules were transferred to –20^{o}C and –80^{o}C directly for preservation. Another batch was frozen to –20^{o}C at a cooling rate of –1^{o}C minute^{-1} and then plunged to –80^{o}C to complete the freezing process. As for the 4^{o}C preservation treatment, samples were placed directly into a 4^{o}C fridge, free of cryoprotectant and pretreatment. Nine *Ulva* disks or germlings without any preservation treatment were set as initial. The preservation of *Ulva* gametes was carried out using a similar method with the omission of the two-step cooling. Fifty four ampoules were used for every temperature treatment and nine of these 54 ampoules were assessed for viability at every storage period (one, two, seven, 30, 90, and 180 days). Each ampoule contained 2.5 $\times 10^{6}$ gametes. Nine gametes ampoules without any preservation treatment were set as initial. Cryopreservation with liquid nitrogen was not employed in this study given its cost in long-term preservation and low possibility of success based on a preliminary experiment.

After storage for one, two, seven, 30, 90, and 180 days, samples were quickly thawed by plunging the ampoules into a 37^{o}C water bath. As soon as the ice had melted, the media with DMSO were removed and samples were repeatedly washed with fresh medium. Afterwards, thalli and germling samples were cultured under the condition of 18^{o}C, 80 μmol m^{-2} s^{-1} photon light density, with a 16L: 8D photoperiod. Gametes were placed in darkness for 24 hours to ensure the settlement of gametes at the bottom of the Petri dishes before being incubated under the conditions described above.

5.2.3 Viability assay

The viability of thalli and germlings after the freeze-thaw cycle was assessed by methods of pigmentation index (PI) and regrowth. Part or all of the pigmentation of *Ulva* can be lost due to damage caused by freezing. This phenomenon was used to establish a 'pigmentation index' where fully pigmented *Ulva* was given a score of 10 whereas completely white, dead fronds received a score of 0 (Van der Meer and Simpson 1984). This method results in rapid assessment, although it is relatively subjective. Regrowth was estimated by recording specific growth rate (SGR) over a six-day culture period at 18^{o}C. SGR (%) = [ln (M_2/M_1)]/t $\times 100$, where M_2 is the final mass, W_1 is the initial mass, and t is the number of culture days. The SGR of thalli or germlings grown at 18^{o}C for six days without any preservation treatments were set as initial measurements prior to storage treatments.

After 14 days of culture at 18^{o}C, gametes were examined under a light microscope and viability was determined by the level of germination. Germination was defined as gametes that show cell division. The mean percentage germination over nine fields of view was

recorded and there were 30–40 gametes for each field of view. The germination rate of gametes grown at 18^{o}C for 14 days without preservation treatment was set as initial measurement prior storage treatments. Viability was expressed as a relative rate in order to eliminate effects of low settlement rate in *Ulva* gametes. V= Gt/Gi × 100, where V is viability of samples, Gt is germination rate of treated samples and Gi is germination rate of initial.

5.2.4 Statistical analysis

Results were expressed as means of replicates ± standard deviation for continuous data and medians of replicates ± interquartile range for categorical data. Data were analyzed using SPSS v.21. A confidence interval of 95% was set for all tests.

Effects of preservation time and temperature on PI and regrowth of Ulva *thalli*

Friedman tests were used to analyse the effects of preservation time on PI of *Ulva* thalli and Wilcoxon Signed Rank tests were conducted for the *post hoc* comparison. Kruskal-Wallis tests were used to analyse the effects of temperature on PI of *Ulva* thalli and Mann-Whitney *U*-tests were conducted for the *post hoc* comparison. The regrowth rates of *Ulva* thalli in all groups conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of all groups could be considered equal (Levene's test, $F = 1.457$, $P > 0.05$). A two-way ANOVA was used to assess the effects of preservation temperature and time on regrowth of *Ulva* thalli. Tukey HSD was conducted for *post hoc* investigation. One-way ANOVA was used to compare the differences of regrowth between *Ulva* thalli at 4^{o}C and the initial. Tukey HSD was conducted for *post hoc* investigation.

Effects of preservation time and temperature on PI and regrowth of Ulva *germlings*

A Friedman test was used to analyse the effects of preservation time on PI of *Ulva* germlings preserved at 4^{o}C and Wilcoxon Signed Rank tests were conducted for the *post hoc* comparison. The regrowth rates of *Ulva* germlings preserved at 4^{o}C in all groups conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of all groups could be considered equal (Levene's test, $F = 1.329$, $P > 0.05$). A one-way ANOVA was used to assess the effects of preservation time on regrowth of *Ulva* germlings. Another one-way ANOVA was used to compare the differences of regrowth between *Ulva* germlings at 4^{o}C and the initial. Tukey HSD was conducted for *post hoc* investigation.

Effects of preservation time and temperature on viability of Ulva *gametes*

The viabilities of *Ulva* gametes in all groups conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of all groups could be considered equal (Levene's test, $F = 1.117$, $P > 0.05$). A two-way ANOVA was used to assess the effects of preservation

temperature and time on regrowth of thalli. Tukey HSD was conducted for *post hoc* investigation.

5.3 Results

The key question that this chapter aimed to answer was which preservation method and which material was optimal for *Ulva* preservation.

5.3.1 Preservation of Ulva *thalli*

The effects of different preservation methods on pigmentation index of *U. rigida* were investigated (Table 5.1). There was a significant difference in PI of *Ulva* between preservation times but patterns at every temperature were different (Table 5.1, Friedman test, $K > 35.000$, $df = 5$, $P < 0.001$). PI of samples preserved at –80°C reduced to zero by day seven (Wilcoxon Signed Rank test, $Z < –2.700$, $n = 9$, $P < 0.05$) whereas it was more than 6.0 for samples stored under –20°C and 10 ± 0.0 under 4°C. PI of samples stored at –20°C decreased to zero (Wilcoxon Signed Rank test, $Z < –2.750$, $n = 9$, $P < 0.05$) by day 30 while samples stored at 4°C still had a high PI by day184. There was a significant difference in PI between preservation temperatures regardless of preservation times (Kruskal-Wallis test, $K > 50.557$, $df = 6$, $P < 0.001$). The highest PI was found at 4°C (Mann-Whitney *U*-test, $U = 4.500$, $Z = –3.571$, $n = 9$, $P < 0.05$), which was comparative to the initial up to 92 days preservation (Mann-Whitney *U*-test, $U = 40.500$, $Z = 0.001$, $n = 9$, $P > 0.05$). On the contrary, samples preserved at –80°C for one day showed extremely low PI. Two concentrations of DMSO were used in the present study. There were no significant differences between the PI in *Ulva* thalli between these two concentrations (Mann-Whitney *U*-test, $U > 37.000$, $Z > –0.394$, $n = 9$, $P > 0.05$) except that preserved at –20°C for one day (Mann-Whitney *U*-test, $U = 17.500$, $Z = –2.136$, $n = 9$, $P < 0.05$). Higher concentration (15%) of DMSO enhanced PI of *Ulva* thalli preserved at –20°C for one day (Table 5.1). Two-step cooling process to –80°C did not enhance PI compared with rapid cooling (Mann-Whitney *U*-test, $U > 33.500$, $Z > –0.742$, $n = 9$, $P > 0.05$).

Methods		Pigmentation index					
	DMSO	1d	2d	7d	30d	92d	184d
–80°C	10%	2.0 ±1.0	2.0 ±1.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
	15%	2.0 ±0.0	2.0 ±1.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
–20°C then to –80°C	10%	2.0 ±0.0	2.0 ±1.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
	15%	2.0 ±0.0	2.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0
–20°C	10%	8.0 ±1.0	8.0 ±1.0	7.0 ±1.0	0 ±0.0	0 ±0.0	0 ±0.0
	15%	9.0 ±0.0	9.0 ±1.0	8.0 ±1.0	0 ±0.0	0 ±0.0	0 ±0.0
4°C		10.0 ±0.0	10.0 ±0.0	10.0 ±0.0	10 ±0.0	10 ±0.0	9.0 ±1.0
Initial				10.0 ±0.0			

Table 5.1 Effects of different preservation methods on pigmentation index of *U. rigida*. Samples were stored at the end temperature for times ranging from one to 184 days. Initial represents initial measurement of the sample prior storage under treatments. Data are the medians ± interquartile range (n = 9).

The regrowth of *Ulva* thalli preserved at different temperatures was assessed (Table 5.2). Preservation temperature and time had an interactive effect of regrowth of *Ulva* thalli (Table 5.3), which indicates the differences in regrowth between times were not the same at different preservation temperatures. For instance, the regrowth of *Ulva* thalli preserved at –80°C decreased to near zero by day seven and *Ulva* thalli preserved at –20°C could regrow by day 30. In contrast, the regrowth rate of *Ulva* thalli preserved at –4°C had a high value even after 184 days storage (Table 5.2). Temperature had a main effect on regrowth (Table 5.3). *Post hoc* Tukey HSD comparison ($P = 0.05$) showed the highest regrowth rate (23.09 ± 2.23%) was at –4°C and it decreased to 2.60 ± 3.10% (10% DMSO) and 3.59 ± 3.98% (15% DMSO) for *Ulva* thalli at –20°C. There were no significant differences between two-step cooling (1.40 ± 2.17% at 10% DMSO, 1.45 ± 2.21% at 15% DMSO) and rapid cooling (1.33 ± 2.20% at 10% DMSO, 1.41 ± 2.21% at 15% DMSO) to –80°C. Time also had a main effect on regrowth (Table 5.3). *Post hoc* Tukey HSD comparison ($P = 0.05$) showed the regrowth rates on day one (7.69 ± 5.57%) and day two (7.85 ± 6.49%) were not statistically different. After that, it decreased with time and to 2.69 ± 7.41% on day 184. There was a significant difference between *Ulva* thalli under 4°C treatment and the initial (ANOVA, $F = 34.866$, df = 6, 56, $P < 0.001$). Interestingly, *Post hoc* Tukey HSD comparison ($P = 0.05$) showed *Ulva* thalli stored

at 4°C for two days, seven days, 30 days, or 92 days had a higher regrowth compared to the initial—this finding did not reflect the PI of *Ulva* thalli.

Methods		Regrowth (%)					
Temperature	DMSO	1d	2d	7d	30d	92d	184d
–80°C	10%	4.35±0.58	4.17±0.66	0.04±0.80	0.20±0.71	–0.22±0.49	–0.59±0.63
	15%	4.45±0.66	4.26±0.63	0.04±0.84	0.15±0.75	–0.11±0.67	–0.38±0.58
–20°C then to –80°C	10%	4.40±0.53	4.21±0.67	0.04±0.80	0.20±0.71	–0.16±0.63	–0.27±0.49
	15%	4.49±0.60	4.31±0.63	0.04±0.84	0.15±0.75	–0.11±0.67	–0.17±0.67
–20°C	10%	8.33±0.70	8.29±0.76	2.34±0.85	0.20±0.53	–0.06±0.56	–0.11±0.57
	15%	8.66±0.60	8.62±0.69	4.24±1.23	0.31±0.47	–0.11±0.57	–0.21±0.42
4°C		20.60±0.58	23.05±0.58	25.28±0.51	25.28±0.56	23.75±1.54	20.57±2.011
Initial		20.86±0.62					

Table 5.2 Effects of different preservation methods on regrowth of *Ulva* thalli. Samples were stored at the end temperature for times ranging from one to 184 days and then cultured under the condition of 18°C, 80 μmol m^{-2} s^{-1} photon light density, with a 16L: 8D photoperiod. Regrowth was estimated by recording specific growth rate over a six-day culture period. Initial represents initial measurement of the sample prior storage under treatments. Data are the means ± SD (n = 9).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Temperature	20895.104	6	3482.517	5575.569	<0.001
Time	1592.028	5	318.406	509.773	<0.001
Temperature* Time	830.959	30	27.699	44.346	<0.001
Error	209.867	336	0.625		
Total	32901.494	378			

Table 5.3 Two-way analysis of variance of the effects of preservation temperature and time on regrowth of *Ulva* thalli. The confidence interval was 95%.

5.3.2 Preservation of Ulva *germlings*

PI of *Ulva* germlings preserved by different methods are shown in Table 5.4. Compared with the thalli, *Ulva* germlings were more vulnerable to freezing temperature. PI was zero at –20°C or –80°C even after only one day of preservation although the PI of *Ulva*

germlings preserved at 4°C was still high until day 30. Friedman test showed that there was a statistically significant difference in median number of PI between storage times (Friedman test, K = 25.159, df = 5, $P < 0.001$). The PI began to decrease from day 92 (Wilcoxon Signed Ranks Test, Z = –2.333, n = 9, $P < 0.05$).

Regrowth of *Ulva* germlings preserved at –20°C, –20°C then –80°C, and –80°C was not measured due to the PI score of zero indicating that all germlings had perished under these conditions (Table 5.5). Referring to germlings preserved at 4°C, there was a significantly difference in regrowth between storage times (ANOVA, F =50.698, df = 5, 48, $P < 0.001$). The difference between one and two days preservation was not statistically significant (Tukey HSD, $P = 0.841$) but the regrowth increased by 18.42% under seven-day preservation (Tukey HSD, $P < 0.001$) and had a further 10.46% rise when the time was extended to 30 days (Tukey HSD, $P < 0.001$). However, it decreased to 21.8 ± 3.0% for 92-day preservation (Tukey HSD, $P < 0.001$). To compare the differences between the initial and treatments, another ANOVA was conducted. The result showed there was a significant difference between groups (ANOVA, $F = 43.598$, df = 6, 56, $P < 0.001$). There was no difference in regrowth between the initial and one-day (Tukey HSD, $P = 0.615$) or two-day (Tukey HSD, $P = 0.452$) preservation but seven-day and 30-day preservation increased regrowth by 22.34% and 43.22%, with 19.95% and 24.74% decrease on 92-day and 184-day preservation respectively compared with the initial (Tukey HSD, $P < 0.001$).

Methods		Pigmentation index					
Temperature	DMSO	1d	2d	7d	30d	92d	184d
–80°C	10%	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	15%	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
–20°C then to	10%	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
–80°C	15%	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
–20°C	10%	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	15%	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
4°C		10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0	9.0 ± 1.0	9.0 ± 1.0
Initial		10.0 ± 0.0					

Table 5.4 Effects of different preservation methods on pigmentation index of *Ulva* germlings. Samples were stored at the end temperature for times ranging from one to 184 days. Initial represents initial measurement of the sample prior storage under treatments. Data are the medians ± interquartile range (n = 9).

Methods		Regrowth (%)					
Temperature	DMS	1d	2d	7d	30d	92d	184d
–80°C	10%	–	–	–	–	–	–
	15%	–	–	–	–	–	–
–20°C then	10%	–	–	–	–	–	–
to –80°C	15%	–	–	–	–	–	–
–20°C	10%	–	–	–	–	–	–
	15%	–	–	–	–	–	–
4°C		27.91 ±2.87	28.17 ±2.56	33.36 ±1.93	36.85 ±2.03	21.83 ±3.03	20.52 ±3.3
Initial				27.27 ±2.40			

Table 5.5 Effects of different preservation methods on regrowth of *Ulva* germlings. Samples were stored at the end temperature for times ranging from one to 184 days. Data are the means ± SD (n = 9). Initial represents initial measurement of the sample prior storage under treatments. –Data not measured as all samples had died and also see the results presented in Table 5.4.

5.3.3 Preservation of Ulva *gametes*

The viability of *Ulva* gametes preserved at different temperatures was investigated (Figure 5.1). Preservation temperature and time had an interactive effect of viability of *Ulva* gametes (Table 5.6), which indicates the differences in viability between times were not the same at different preservation temperatures. For instance, the viability of gametes preserved for one day at 4°C, –20°C with 5% DMSO, and –80°C with 5% DMSO were 94.74 ± 4.30%, 56.14 ± 5.26%, and 12.48 ± 3.97%, respectively (Figure 5.1). Viability at –4°C and –20°C with 5% DMSO decreased to 26.12 ± 3.97% and 18.71 ± 4.30% but those kept at –80°C remained consistent (12.09 ± 5.30%) during 184 days preservation. Temperature had a main effect on viability (Table 5.6). *Post hoc* Tukey HSD comparison ($P = 0.05$) showed the highest viability (60.00 ± 23.32%) was at –4°C and it decreased to 34.55 ± 12.68% (5% DMSO) and 22.12 ± 11.96% (10% DMSO) for *Ulva* gametes at –20°C further to 11.64 ± 3.97% (5% DMSO) and 3.36 ± 2.40% (10% DMSO) *Ulva* gametes at –80°C. Time also had a main effect on regrowth (Table 5.6). *Post hoc* Tukey HSD comparison ($P = 0.05$) showed the viability decreased with time from 39.63 ± 31.60% on day one to 12.82 ± 8.56% on day 184.

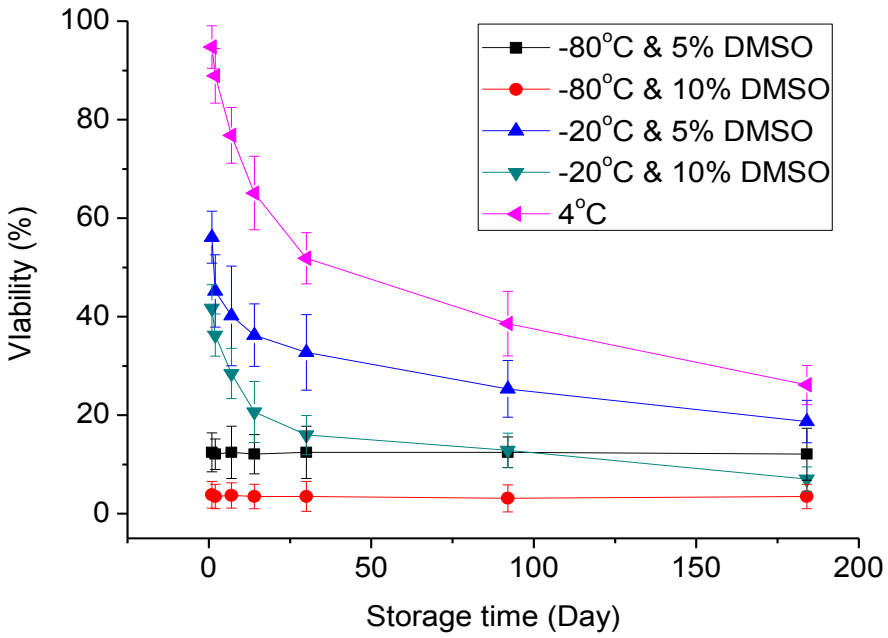

Figure 5.1 Effects of different preservation methods on viability of *Ulva* gametes. Samples were stored at the end temperature for times ranging from one to 184 days. The error bars indicate the standard deviations (n = 9).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Temperature	123632.240	4	30908.060	1361.684	<0.001
Time	24940.864	6	4156.811	183.132	<0.001
Temperature* Time	22583.563	24	940.982	44.346	<0.001
Error	6355.556	280	22.698		
Total	395947.222	315			

Table 5.6 Two-way analysis of variance of the effects of preservation temperature and time on viability of *Ulva* gametes. The confidence interval was 95%.

5.4 Discussion

5.4.1 Preservation of Ulva *thalli and germlings*

Successful preservation of seaweed thalli has been documented for relatively short term storage duration (Kuwano et al., 1993, 1996, Jo et al., 2003). For instance, in previous studies, the survival rate of *Porphyra* sp. in liquid nitrogen for one day exceeded 95% by

using a combined cryoprotectant of DMSO with dextran, polyvinylpyrrolidone (PVP) or Ficoll (Kuwano et al., 1996). The viability of *Porphyra* samples was 54.6–70.9% when a mixed suspension of 10% DMSO and 0.5 M sorbitol in 50% seawater was used as a cryoprotectant for one day (Jo et al., 2003). Long term preservation is not well documented, although sporelings and apical segments of mature thalli of several seaweeds, including *Gracilaria foliifera*, *Palmaria palmata*, *Devaleraea ramentacea*, *Chondrus crispus*, *U. lactuca* and *Gracilaria tikvahiae* were frozen and stored alive successfully in liquid nitrogen for four years. *U. lactuca* fronds survived with little damage after four years preservation in liquid nitrogen (Van der Meer and Simpson, 1984). In the present study, cryopreservation with lipid nitrogen was not employed due to its failure in a preliminary experiment (data not shown). Instead, *U. rigida* was preserved at the low temperature of 4^{o}C and freezing temperatures of –20^{o}C and –80^{o}C. The survival of *Ulva* thalli decreased with time when samples were preserved at –20^{o}C or –80^{o}C and all thalli were dead after seven days preservation at –80^{o}C and 30 days preservation at –20^{o}C. In addition, noticeable damage to *Ulva* thalli was found even after only one day of preservation. A possible reason that this approach led to lower regrowth and shorter preservation periods compared with Van der Meer and Simpson's (1984) findings is that different intermediate temperatures were used in the two-step cooling technique. The intermediate temperatures were –40^{o}C in Van der Meer and Simpson's study and –20^{o}C in the present study. A two-step cooling technique can substantially increase the survival of samples compared with a rapid cooling method because the low cooling rate from room temperature to an intermediate temperature allows sufficient time for intracellular water to move to the outside of the cell, hence reducing the formation of ice crystals (Taylor and Fletcher, 1999a). The intermediate temperature is usually set above –40^{o}C as the cell membrane becomes effectively impermeable below this temperature (Karlsson et al., 1994). It appears that –40^{o}C may be a preferable option for an intermediate temperature as the highest survival of *Porphyra* thalli was founded when samples were pre-frozen at –40^{o}C before immersion in liquid nitrogen (Kuwano et al., 1996). Due to limit of equipment, –20^{o}C rather than –40^{o}C was used as the intermediate temperature in the present study.

An interesting finding in the current study was that the regrowth rate of *Ulva* thalli or germlings was enhanced relative to the initial after 4^{o}C preservation for some times (2–92 days for thalli and 7–30 days for germlings). This might be down to the gene regulation induced by cold shock since it was found that overexpression of ERG10 enhanced survival and growth of yeast when they were frozen at –200^{o}C for five days (Rodriguez-Vargas et al.,

2002). Whereas the expression of heat shock proteins (HSP) was not determined in the current study, clones for HSP genes are available for *U. pertusa* (Tominaga et al., 2010) and *U. prolifera* (Fu et al., 2011). It would have been enlightening to evaluate HSP expression in this study; however, resources precluded this. The subsequent decrease of regrowth rate with time might be down to bacterial activity and the subsequent mortality in the *Ulva* germlings. Bacterial contamination usually occurs in low temperature preservation (Bhattarai et al., 2007). The adverse effect of bacterial degradation exceeded the promoting effect of cold shock, hence leading to the decline of regrowth rate.

5.4.2 Preservation of Ulva *gametes*

Compared with other studies (e.g. Taylor and Fletcher, 1999b, Bhattarai et al., 2007), gametes in the present study can be preserved at –20^{o}C for a longer time. For instance, spores of *U. intestinalis* were all dead after preservation at –20^{o}C with 5% DMSO for three days in Taylor and Fletcher's (1999b) study and 100% mortality of spores in *U. fasciata* was detected after preservation at –20^{o}C with 5% DMSO for five days in Bhattarai et al.'s (1999) study. Despite the species differences and conceivable differences of freezing tolerance between gametes and spores, another possible reason is down to the delayed germination of swarmers due to freezing preservation. Germination of spores after culturing for three days and seven days was employed in Taylor and Fletcher's (1999b) and Bhattarai et al.'s (2007) study respectively, to assess the viability of spores. In the present study, the culture period was extended to 14 days because gametes preserved at –20^{o}C or –80^{o}C for more than two days did not germinate until they were cultured for 10–14 days. Therefore, the present study indicates three or seven-day culture might underestimate the viability of spores and an extended period is needed to assess the viability of *Ulva* spores. Another noticeable finding was that the viability of gametes preserved at –80^{o}C was consistent while it decreased with time at –20^{o}C or 4^{o}C. This could be attributed to bacterial activity (Bhattarai et al., 2007). The low temperature such as 4^{o}C or –20^{o}C is not enough to stop bacterial activity whilst a further decrease in temperature (–80^{o}C) greatly inhibits bacterial degradation. Meanwhile, the continual loss of viability with time at –20^{o}C or 4^{o}C might result from degradative enzymatic reactions in preserved cells. The rates of most chemical reactions decrease exponentially with decreasing temperatures, including degradative enzymatic reactions. Gametes of *Ulva* frozen at –80^{o}C seemed not to be affected by these reactions in the present study although it was proposed that cell deterioration could not be completely prevented until –135^{o}C (Warren et al., 1997).

5.4.3 Differences of preservation between **Ulva** *thalli, germling and gametes*

Grout and Morris (1987) suggest that water loss is less effective during freezing in larger cells than in smaller cells since larger cells have lower surface area: volume ratios compared to smaller cells. This makes larger cells more prone to intracellular ice formation during freezing, hence suffering more freezing injury. This might partially explain why gametes could be preserved for a longer time at –20^{o}C or –80^{o}C compared with thalli or germlings. As reported, the size of vegetative cells are larger than swarmers (spores and gametes) since cells of *Ulva* sporophytes usually produce 8 or 16 zoospores with 16 or 32 female gametes and 64 or 128 male gametes generated by cells of male and female gametophytes respectively (Smith, 1947). The size of vegetative cells and gametes in this study was 19.51 $\pm$1.69 $\times$14.05 $\pm$1.81 (length $\times$width) and 5.93 $\pm$0.74 $\times$3.47 $\pm$0.40 (length $\times$width) respectively (unpublished data). In addition to this, difference between gametes and adults might be due to lipid content and composition. Coalescence of lipid droplets has been considered to play a role in reducing the formation of intracellular ice in diatoms (McLellan, 1989) and thus it could theoretically result in an improvement in viability after cryopreservation. It was reported that the existence of lipid droplets avoided or decreased modification of cell morphology and thus enhanced tolerance of freezing in gametophytes of the kelp *Undaria pinnatifida* (Ginsburger-Vogel et al., 1992). After release, swarmers need to locate and adhere to a surface following germination. Most zoospores in *Laminaria* have only one or two chloroplasts which lead to a very low photosynthetic capacity and cannot supply the whole organism with energy for movement, settlement, and germination. Therefore, there should be endogenous energy reserves to support these processes. It has been found that zoospores of the palm kelp *Pterygophora californica* consumed 43% of neutral lipid to fuel swimming over 30 hours in the dark (Reed et al., 1999). Compared to vegetative cells, there would be more lipid content to fuel these extra activities. This hypothesis was verified by Steinhoff et al. (2011) who demonstrated a decrease in lipid during development from spores to gametophytes in the brown alga *Saccharina latissima*. Furthermore, the freezing tolerance could be related to the composition of lipid as it affects permeability of the plasma membrane to water. For instance, *Saccharomyces cerevisiae* enriched in ergosterol or stigmasterol rather than campesterol or cholesterol had higher viability (Calcott and Rose, 1982). Different fatty acid profiles between zoospores and cells of gametophytes in *S. latissima* were reported, with a decrease in the fatty acid 18:1(n-9) from 45 to 30% in particular (Steinhoff et al., 2011). This decrease in unsaturated fatty acids might lead to a decline in the ability of gametophytes to endure cold temperatures compared to zoospores.

On the other hand, *Ulva* thalli or germlings performed better than gametes when they were preserved at 4^{o}C. Firstly, this may be attributed to bacterial activity. Bacterial contamination occurred in all three types of preservation as biofilms were found during storage. Bacterial degradation cannot be stopped at a temperature of 4^{o}C (Bhattarai et al., 2007), which led to the viability decrease of gametes preservation with time at 4^{o}C. Similar findings were reported in spore preservation of *U. fasciata* and *U. pertusa* and the addition of 100 μg ml^{-1} ampicillin remarkably enhanced the viability of *Ulva* spores stored at 4^{o}C (Bhattarai et al., 2007). But in the preservation of thalli or germling, bacteria biofilms in flasks were swept away with 75% ethanol cotton ball when the media were renewed monthly, avoiding or decreasing the harm from bacteria. Secondly, nutrient limitation might be another possible reason for the decrease of gametes preservation with time. Although low temperature and light level tremendously inhibited photosynthesis, metabolic activities continued at a low rate. This would keep consuming nutrient in the media. Media were not renewed for gamete preservation due to inconvenience, which might be nutrient limited for long-term preservation.

5.5 Conclusion

Pigment index is a simple but effective method to determine the viability of seaweed, which can save time and cost. *Ulva* thalli, germlings, and gametes can all be preserved at 4^{o}C for a long time (184 days) while only gametes can be stored at –80^{o}C for a long term (184 days). Germling and gamete preservation occupies less space with quicker regrowth but it requires more time to reach a harvestable size. Low temperature (4^{o}C) preservation increased the viability of *Ulva* thalli or germlings but is more labour intensive to renew the medium and clear away the bacterial biofilm. Therefore, future work should be focused in improving viability of cryopreservation of *Ulva* thalli, germlings and gametes.

Chapter 6. Interactive effects of ocean acidification and warming on physiological and biochemical properties of *Ulva rigida* grown under changing nutrient levels

6.1 Introduction

6.1.1 Ocean acidification

Due largely to burning fossil fuels and changes to land use, the level of carbon dioxide in the atmosphere has been increasing since 1750 and according to the latest data published by the National Oceanic and Atmospheric Administration, the global CO_2 partial pressure reached up to 401.24 ppm in April 2015 (NOAA, 2015). The ocean is the main sink for CO_2, which has absorbed approximately 30% of the emitted anthropogenic carbon dioxide, causing ocean acidification (IPCC, 2013) The mean surface ocean pH has already decreased by 0.1 units since the beginning of the industrial era, corresponding to a 26% increase in hydrogen ion concentration (IPCC, 2013). By 2100, concentrations of CO_2 (aq) and HCO_3^- are predicted to increase by 192% and 14%, respectively, and CO_3^{2-} to decrease by 56%, with a concomitant decline in pH to 7.65 (Raven et al., 2005). Specifically, seawater at high latitudes is expected to experience more serious acidification since more CO_2 can dissolve in cold waters compared to tropical regions (McNeil and Matear, 2008, Roleda and Hurd, 2012). Coastal waters are more susceptible to acidification than the pelagic ocean due to eutrophication (Cai et al., 2011). Therefore, impacts of acidification on coastal seaweeds are of great concern given the economic and social services provided by the coastal ecosystem that is adjacent to human living areas. Approximately two thirds of the global population lived within 60 miles of a coastline by the end of 20th century and this number is projected to reach more than 75% by 2025 (Vallega, 2013).

6.1.2 Ocean warming

The global sea surface temperature increased at a rate of 0.121 ± 0.033–0.124 ± 0.030 ℃ per decade based on *in situ* data records from the year of 1979 to 2012 (IPCC, 2013). Changes are particularly strong in polar and cold-temperate regions of the northern hemisphere (Levitus et al., 2000, Hansen et al., 2006). For example, a recent rapid regional warming (increase of 3.7 ℃ per century) was reported for the Antarctic Peninsula (Vaughan et al., 2003) and the average sea surface temperature increased by 1.13 ℃ from 1962 to 2002 in the North Sea at Helgoland (Wiltshire and Manly, 2004). The global ocean will continue to warm during the 21st century. The global mean sea surface temperatures for the months of February and August are projected to increase by 1.9 ℃ by the end of the 21st century

(Bartsch et al., 2012). The maximum warming of around 4 ℃ is predicted for high latitudes of the northern hemisphere in summer (Bartsch et al., 2012).

6.1.3 Eutrophication

Apart from ocean warming and acidification, eutrophication is another pressing environmental issue. Eutrophication can occur naturally in lakes by transferring nutrients from the sediment to the water by living or decomposing macrophytes, resuspension, diffusion, and bioturbation (Carpenter, 1981). However, anthropogenic activities have accelerated the rate and extent of eutrophication (Carpenter et al., 1998). Inevitable urbanization of a growing human population, increased use of coastal areas, and rising fertilizer use for agricultural intensification has led to accelerated nutrient inputs from land-water to coastal waters (Smith et al., 1999). These changes in nutrient availability result in eutrophication, an increasing threat for coastal ecosystems (Bricker et al., 2008). One consequence of eutrophication is that it can lead to green tide events. Green tides are now of growing concern globally due to their ecological and economic impacts. They can harm shore-based activities by preventing tourists, swimmers and small boats from accessing the sea due to their sheer physical mass. The seaweeds turn into a stinking morass, which can produce toxic hydrogen sulphide (H_2S) from their anoxic interior leading to detrimental influences on the coastal ecosystems if they are not removed quickly (Smetacek and Zingone, 2013). It cost more than US$100 million to maintain an algae-free sea area near Qingdao for the Olympic sailing competition in 2008 (Wang et al., 2009). Furthermore, the number of green tides reported from new locations all over the world increased further during the 2000s with eutrophication considered as the leading reason for the increase (Smetacek and Zingone, 2013). *Ulva* was the only genus present in the majority of green tides that have been examined (Fletcher, 1996).

6.1.4 Effects of ocean acidification, warming, and eutrophication on Ulva

The environmental changes caused by human activities would pose an effect on the physiological and biochemical traits of *Ulva*, an ecologically and economically important genus, and thus the cultivation and food quality of *Ulva*. However, little has been studied on the physiological traits, chemical composition, and functional properties of *Ulva* in the context of the effects of ocean acidification, warming and eutrophication but some indications can be obtained from the effects of higher CO_2, temperature and nitrate levels.

CO_2 plays a pivotal role in photosynthesis and growth of photoautotrophs as it is the source of carbon in the light-dependent reaction of photosynthesis and can be converted into

energy-rich organic molecules by the Calvin-Benson Cycle. The concentration of CO_2 is vital for photosynthesis since ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCo) can also catalyze ribulose-1, 5-bisphosphate with oxygen (O_2) in a process called photorespiration if the ratio of CO_2 to O_2 is low. Half saturation constants of CO_2 for algal RuBisCo carboxylase range from 20 to 70 μmol L^{-1}, varying with species (Badger et al., 1998). Although the concentration of dissolved inorganic carbon (DIC) is very high (about approximately 2 mmol L^{-1}) in seawater, its predominant form is HCO_3^-, and CO_2 accounts for less than 1% of it. In addition, CO_2 in seawater diffuses about 10,000 times slower in water than in air (Wu et al., 2008). Therefore, CO_2 concentrations in aquatic environments are usually not enough to saturate the carboxylation process. Photosynthesis and growth of seaweeds are likely to be boosted by rising CO_2 concentrations even though many seaweeds investigated so far have developed CO_2-concentrating mechanisms (CCMs) to raise intracellular CO_2 concentrations (Koch et al., 2013). Some studies have been conducted to investigate the effects of increased CO_2 level on physiological traits of *Ulva* species, such as photosynthesis and growth (Björk et al., 1993, Gordillo et al., 2001b, Xu and Gao, 2012, Rautenberger et al., 2015). High CO_2 (10, 000 ppm) more than doubled the growth rate of *U. rigida* compared to ambient air when nitrogen was sufficient (Gordillo et al., 2001b). Growth of *U. prolifera* was also significantly enhanced by high CO_2 (1000 ppm) after the plants had acclimated to the CO_2 levels for more than 50 days (Xu and Gao, 2012). On the other hand, neutral effects of elevated CO_2 on *U. rigida* were reported (Rautenberger et al., 2015). The photosynthesis and growth of *U. rigida* were not stimulated by increased CO_2 as *U. rigida* was inorganic carbon saturated (Rautenberger et al., 2015). Furthermore, negative effects of elevated CO_2 on photosynthesis have been observed in *Ulva* spp. (Björk et al., 1993). Therefore, the influence of ocean acidification and changes in the carbonate system of seawater on seaweeds may be species-dependent. Effects of high CO_2 levels on the settlement, germination and reproduction of *Ulva* have not been studied yet.

In terms of biochemical composition and functional properties, high CO_2 concentration (10,000 ppm) did not affect significantly total internal carbon, total internal nitrogen or soluble carbohydrate in *U. rigida* but reduced soluble protein compared with the normal CO_2 level (350 ppm, Gordillo et al., 2001a, b). The study on CO_2 affecting functional properties of *Ulva* has not been reported.

Higher temperatures can usually stimulate the physiological performances of *Ulva*. For instance, the number of settled zoospores in *U. intestinalis* increased with temperature with the maximum at 23^{o}C (Christie and Shaw, 1968). Likewise, the bound zoospores of *U.*

compressa increased from ~150 cells mm^{-2} to ~450 cells mm^{-2} when the temperature rose from 5°C to 25°C (Callow et al., 1997). The germination rate of *U. fasciata* from the North west coast of India was also enhanced by higher temperature, with the highest germination rate (78.53 ± 10.05%) at 25 ℃ (Mantri et al., 2011). On the other hand, the higher temperature of 15 ℃ reduced the germination of *Ulva* spp. from the western Baltic Sea compared to the low temperature of 10°C (Lotze et al., 1999). In terms of growth and reproduction, the growth rate of *U. fenestrata*, collected from 6°C seawater in Japan, was 3.349 ± 0.398% at 5°C and 40 μmol photons m^{-2} s^{-1} while it was 6.559 ± 0.312 at 10°C and 40 μmol photons m^{-2} s^{-1} (Kalita and Tytlianov, 2003). The reproduction rate of *U. fenestrata* increased from 6.1 ± 3.6% to 71.3 ± 31.8% when the temperature was increased from 10 to 15°C (Kalita and Tytlianov, 2003). A similar trend was found at tropical *Ulva* (20.55 ℃N, 70.20 °E). A 5°C increase of temperature (from 20 to 25°C) more than doubled the growth rate of *U. fasciata* when salinity was 25 psu (Mantri et al., 2011). All disks of *U. fasciata* grown at 25°C were induced to form spores and 60% of *Ulva* disks grown at 20°C became reproductive when salinity was 25 psu (Mantri et al., 2011).

Sugar contents and amino acids in *U. fasciata* increased with the rise of temperature (from 15–25 ℃), reaching their maximum around 25 ℃ and decreased at 30 ℃ even lower than at 15 ℃ (Mohsen et al., 1973). The high temperature of 25°C decreased the total lipid of *U. pertusa* from 2.7–3.6% DW to 2.6–2.7% DW compared to the low temperature of 15°C (Floreto et al., 1993). The best yield of fat in *U. fasciata* was attained at 20 ℃ followed by 15 ℃, and 25 ℃, with negligible fat at 30 ℃ (Mohsen et al., 1973). The effects of temperature on the functional properties of *Ulva* have not been documented.

The effects of high nitrate on growth and reproduction of *Ulva* have been stated in section 2.1.1–2.1.3 and 3.1.2. Higher nitrate level commonly stimulates the synthesis of amino acid and then protein content of *Ulva* (Naldi and Wheeler, 1999, Msuya and Neori, 2008, Angell et al., 2014). The nitrogen pool of protein in *U. fenestrata* increased from 43% of total nitrogen to 54% when nitrate was enriched from 10 μM to 1 mM (Naldi and Wheeler, 1999). A rise in the nitrogen concentration from 7 μM to 20 μM led to an increase in protein content of *U. lactuca* from 14.2 ± 9.6% DW to 36.6 ± 9.1% DW cultivated under condition of aeration (Msuya and Neori, 2008). Likewise, the total amino acid content of *U. ohnoi* increased linearly with internal nitrogen content ($r = 0.987$) with a range from 2.98 g 100 g^{-1} DW to 18.72 g 100 g^{-1} DW (Angell et al., 2014).

Nitrogen concentration in the culture medium can regulate the degree of cellular lipid accumulation, and nitrogen deficiency has been regarded as the most effective approach to

increase lipid content in microalgae (Brennan and Owende, 2010). Nitrogen deprivation induced a two-three-fold increase in lipid content in some green unicellular algae (Thompson, 1996). In regard to *Ulva* species, nitrogen limitation also enhanced total lipid from 64 mg g^{-1} DW to 72 mg g^{-1} DW at ambient CO_2 concentration (350 ppm) (Gordillo et al., 2001a). On the other hand, nitrogen limitation did not enhance total lipid content in *U. lactuca* compared with a nitrogen enriched condition (Kumari et al., 2014). The reason behind this remains unknown.

No reports on high nitrate levels affecting settlement, germination, and functional properties of *Ulva* have been found.

6.1.5 Aims and objectives

Neither ocean acidification nor warming is proceeding in isolation, rather there are concurrent changes in nutrient levels. The interactive effects of multiple factors may be completely different, or be of greater magnitude, compared to the effects of any single stressor. To the best of my knowledge, none of the previous studies have examined the co-effects of ocean acidification, warming and eutrophication on *U. rigida*. This chapter aimed to understand how ocean acidification, warming and eutrophication would affect *Ulva* cultivation and the food quality of *Ulva* in the future. To achieve that, the physiological (gamete settlement, germination, growth and reproduction) and biochemical (protein, lipid, carbohydrate, amino acids, fatty acids, etc.) responses of *U. rigida* to varying pCO_2, temperatures, and nutrients levels were investigated in this study.

6.2 Materials and methods

6.2.1 Sample preparation and culture conditions

Considering the potentially different responses of adult and immature *Ulva* to environmental factors, adult *U. rigida* and swarmers (zoospores or gametes) were cultured separately under the same treatments. Adult vegetative *U. rigida* of 50–60 mm in length were collected from the low intertidal of Cullercoats beach, UK (55.03^o N, 1.43^o W) after spring tide in May 2014. The fronds were placed in a zip-lock plastic bag and transported to the Ridley aquaria at Newcastle University within one hour. They were then rinsed gently in sterilised natural seawater (one micron filtered) to remove any sediment, epiphytes and small grazers. The 720 healthy individual *Ulva* plants were randomly assigned to 24 tanks of 13.5 L in volume each containing 10 L of natural seawater. In order to investigate the interactive effects of ocean acidification and warming under nitrate-limited and -sufficient conditions, the plants were incubated under two levels of pH (7.95, 7.55), temperature (14, 18^{o}C) and nitrate

(6, 150 μmol L^{-1}). The phosphate concentration was set at 50 μmol L^{-1} to obviate phosphorus limitation. Therefore, there were eight treatments in total: low nitrate, low temperature and low pCO_2 (LNLTLC); low nitrate, low temperature and high pCO_2 (LNLTHC); low nitrate, high temperature and low pCO_2 (LNHTLC); low nitrate, high temperature and high pCO_2 (LNHTHC); high nitrate, low temperature and low pCO_2 (HNLTLC); high nitrate, low temperature and high pCO_2 (HNLTHC); high nitrate, high temperature and low pCO_2 (HNHTLC); high nitrate, high temperature and high pCO_2 (HNHTHC). Three replicate tanks (30 plants per tank) were set up for each treatment. Temperature was controlled using incubators with a photoperiod of 16L: 8D. Light intensity was 80 μmol photons m^{-2} s^{-1}. The ambient pH (7.95), nitrate concentration (6 μmol L^{-1}) and summer average surface seawater temperature (14^{o}C) (Mathis et al., 2015) of seawater in the coast of North Sea were set as the current conditions. The reduced pH and elevated temperature used represent the predicted levels by the year 2100 (Baede et al., 2001). Nitrate concentration was maintained by adding the daily consumed amount after measuring this by a rapid spectrophotometer method (Collos et al., 1999). Seawater was renewed every three days. After a 12-day cultivation period, *U. rigida* fronds were collected and dried in an oven at 50 ℃ until completely dry (for 24 hours). The dried biomass was then ground to a powder, sieved and placed into tubes which were kept in a desiccator pending further analyses.

In order to obtain swarmers, fertile *U. rigida* were collected during a spring tide in June 2014 from Cullercoats (see above). The fronds were transported and rinsed as above. Swarmers were released after exposing fronds to light for one to two hours. After transferring the 300 ml swarmer suspension to a 500 ml beaker, the spores were attracted to a light source. The spores were collected with a pipette and transferred to sterile seawater. This step was repeated three times. This process excluded other unicellular organisms as well as selecting healthy spores. Swarmers released from the thalli were checked microscopically for the presence of two flagella. They were identified as gametes given their positive phototaxis. Sporophytes of *Ulva* have rarely been found in collections from the coast of North East of England during the past two years. This observation is similar to the case on the Swedish west coast, where low temperature is unfavourable for gamete fusion, leading to a predominance of parthenogenetic reproduction (Bliding, 1968, Løvlie and Bryhni, 1978). Afterwards, collected gametes were used for settlement, germination and growth experiments (60 days).

Physiological regulation (seconds to minutes) and phenotypic acclimation (hours to days) (Eggert, 2012) could be investigated by 12 days of culture for adult *Ulva* combined with

60 days culture from gametes, which covered the whole life cycle (growth, reproduction, settlement, germination, and growth) of *U. rigida.*

6.2.2 Carbonate chemistry

A custom-built, computer-controlled pH system Aqua-medic™ (Loveland, Colorado) was used to add CO_2 into an air stream via solenoid valves to maintain the pH of the cultures under low temperature and low pCO_2 (LTLC), low temperature and high pCO_2 (LTHC), high temperature and low pCO_2 (HTLC), and high temperature and high pCO_2 (HTHC). Temperature and daily salinity measurements were recorded. Total alkalinity (TA) was measured by titrations prior to water changes. Carbonate system parameters, which were not directly measured, were calculated via CO2SYS (Pierrot et al., 2006).

6.2.3 Gamete settlement

Concentrated gametes of *U. rigida* were added to Petri dishes (85 mm in diameter) to a final concentration of 1.2×10^6 ml^{-1}. Gametes were immobilized by adding 4% formaldehyde before counting using a haemocytometer. The seawater (20 ml) in the Petri dishes was adjusted to the desired levels of pH (7.95, 7.55), temperature (14, 18^oC), nitrate (6, 150 μmol L^{-1}) in advance, which were same as those for the adult *Ulva* culture. The dishes were incubated in the dark for settlement. Settled gametes were counted at four, eight, 16, 24, 32, 40 hours after washing with seawater to remove unattached spores. The variation of pH during the 40-hour dark incubation was less than 0.05 units, along with around 1% nitrate fluctuation. The number of spores within an eyepiece grid, viewed using a ×40 objective, was counted in three fields of view on each of the three replicate Petri dishes. The ratio of settled spores to all spores initially placed in the Petri dishes was defined as settlement rate.

6.2.4 Germination and growth of young **U. rigida**

After settlement, Petri dishes were placed into 5 L tanks in order to control pH in media by the Aqua-medic™ (Loveland, Colorado) pH system. The method of maintaining temperature and nutrient was as described for the adult *Ulva* culture. The germination rate was determined by counting cells that had divided vs. those that had not after two, four, six, eight days of incubation from three randomly selected microscopic fields of view in each dish. When the germlings attained 5 mm length, they were detached from the dishes and dispersed into the tanks.

6.2.5 Quantification of growth

The dry mass of individuals was measured after a 60-day incubation of young *U. rigida*. The mass of a single gamete, which served as the initial mass, was determined from

the dry mass of a known number, 2×10^8 of gametes. Changes in biomass (fresh mass) of adult *Ulva* were recorded every four days for a period of 12 days. The fresh mass was determined after removing excess water by blotting the thalli gently with tissue paper. Specific growth rates (SGR) of young and adult *Ulva* were calculated by the formula: SGR (%) = $[\ln (M_2/M_1)]/t \times 100$, where M_2 is the final mass, W_1 is the initial mass and t is the number of culture days.

6.2.6 Determination of reproduction

Reproductive thalli were recognized by their colour. The formation of reproductive cells in *Ulva* is accompanied by a change in thallus colour from green (vegetative state) to yellowish (reproductive state) and then to white (after the release of swarmers). This was verified in this study by microscope observation. The reproduction rate was expressed as the ratio of reproductive thalli to all thalli in a tank.

6.2.7 Protein content

The analysis method was the same as described in section 4.2.7. Triplicates were set for each treatment.

6.2.8 Amino acids analysis

The analysis method was the same as described in section 4.2.8. Triplicates were set for each treatment.

6.2.9 Lipid extraction

The analysis method was the same as described in section 4.2.9. Triplicates were set for each treatment.

6.2.10 Fatty acid analysis

The analysis method was the same as described in section 4.2.10. Triplicates were set for each treatment.

6.2.11 Ash content

The analysis method was the same as described in section 4.2.11. Triplicates were set for each treatment.

6.2.12 Carbohydrate

The analysis method was the same as described in section 4.2.12. Triplicates were set for each treatment.

6.2.13 Swelling capacity (SWC)

The analysis method was the same as described in section 4.2.13. Triplicates were set for each treatment.

6.2.14 Water holding capacity (WHC)

The analysis method was the same as described in section 4.2.14. Triplicates were set for each treatment.

6.2.15 Oil holding capacity (OHC)

The analysis method was the same as described in section 4.2.15. Triplicates were set for each treatment.

6.2.16 Statistical analysis

Results were expressed as means of replicates ± standard deviation. Data were analyzed using SPSS v.21. *Post hoc* tests were not conducted because each factor had only two levels in all experiments of this chapter.

The changes of parameters of the seawater carbonate system caused by decreased pH or increased temperature

pCO_2, DIC, HCO_3^-, CO_3^{2-}, CO_2, and TA at two different conditions conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$). The variances of the two samples at two different conditions could be considered equal (Levene's test, $F < 3.346$, $P > 0.05$), therefore t-tests assuming equal variances were conducted to compare the parameters at two different conditions. A confidence interval of 95% was set for all tests.

The effects of pCO_2*, temperature and nitrate on settlement rates*

The settlement rates of *Ulva* gametes under all treatments conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the eight samples at all time points could be considered equal (Levene's test, $F < 1.681$, $P > 0.05$) except that at four (Levene's test, $F = 2.776$, $P = 0.022$), eight (Levene's test, $F = 2.964$, $P = 0.010$), and 16 hours (Levene's test, $F = 2.653$, $P = 0.028$). Six three-way ANOVAs were used to assess the effects of pCO_2, temperature and nitrate on settlement rates of *Ulva* gametes at four, eight, 16, 24, 32, 40 hours considering that ANOVA is reasonably robust to violations of normal distribution and homogeneity of variance particularly when the group sizes are equal (Pallant, 2010). A confidence interval of 95% was set for all tests where data fulfilled the assumptions of normality and homogeneity and 99% for all tests where data did not fulfil the assumptions of normality or/and homogeneity.

The effects of pCO_2*, temperature and nitrate on germination rates*

The germination rates of *Ulva* gametes under all treatments conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the eight samples at all time points could be considered equal (Levene's test, $F < 1.157$, $P > 0.05$). Four three-way ANOVAs were used to assess the effects of pCO_2, temperature and nitrate on germination rates of *Ulva* gametes on days two, four, six, and eight, respectively. A confidence interval of 95% was set for all tests.

The effects of pCO_2*, temperature and nitrate on growth*

The specific growth rates of young *Ulva* under all treatment conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the eight samples could be considered equal (Levene's test, $F = 1.688$, $P > 0.05$). A three-way ANOVA was used to assess the effects of pCO_2, temperature and nitrate on the specific growth rates of young *Ulva* over 60 days of culture. The specific growth rates of adult *Ulva* under all treatments conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the eight samples at all time points could be considered equal (Levene's test, $F < 1.938$, $P > 0.05$). Three three-way ANOVAs were used to assess the effects of pCO_2, temperature and nitrate on the specific growth rates of adult *Ulva* on days four, eight, and 12, respectively. A confidence interval of 95% was set for all tests.

The effects of pCO_2*, temperature and nitrate on reproduction rates*

The reproduction rates of adult *Ulva* in all groups conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the eight samples were not equal on days eight (Levene's test, $F = 3.644$, $P = 0.015$) and 12 (Levene's test, $F = 4.755$, $P = 0.009$). Two two-way ANOVAs were used to assess the effects of pCO_2, temperature and nitrate on the reproduction rates of adult *Ulva* days eight and 12 respectively considering that ANOVA is reasonably robust to violations of normal distribution and homogeneity of variance particularly when the group sizes are equal (Pallant, 2010). A confidence interval of 99% was set as the data did not fulfil the assumptions of homogeneity.

The effects of pCO_2*, temperature and nitrate on chemical composition*

The protein contents of *U. rigida* under all treatments conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the eight samples could be considered equal (Levene's test, $F = 2.378$, $P > 0.05$). A three-way ANOVA was used to

assess the effects of pCO_2, temperature and nitrate on protein content of *U. rigida.* The lipid contents of *U. rigida* under all treatments conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the eight samples could be considered equal (Levene's test, F = 1.073, $P > 0.05$). A three-way ANOVA was used to assess the effects of pCO_2, temperature and nitrate on lipid content of *U. rigida.* The ash contents of *U. rigida* under all treatments conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the eight samples could be considered equal (Levene's test, F = 1.288, $P > 0.05$). A three-way ANOVA was used to assess the effects of pCO_2, temperature and nitrate on ash content of *U. rigida.* The carbohydrate contents of *U. rigida* under all treatments conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the eight samples could be considered equal (Levene's test, F = 2.378, $P > 0.05$). A three-way ANOVA was used to assess the effects of pCO_2, temperature and nitrate on carbohydrate content of *U. rigida.* A confidence interval of 95% was set for all tests.

The effects of pCO_2*, temperature and nitrate on amino acids and fatty acids*

The amino acid contents of *U. rigida* under all treatments conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the eight samples could be considered equal (Levene's test, F < 2.303, $P > 0.05$) except glycine (Levene's test, F = 2.786, $P = 0.042$), proline (Levene's test, F = 2.817, $P = 0.041$), and cysteine (Levene's test, F = 2.840, $P = 0.040$). A three-way MANOVA was conducted to assess the effects of pCO_2, temperature and nitrate on amino acid contents of *U. rigida* considering that ANOVA is reasonably robust to violations of a normal distribution and homogeneity of variance particularly when the group sizes are equal (Pallant, 2010). Bonferroni adjustment was used to reduce the chance of Type I error. A new alpha level of 0.0024 (0.05 divided by 21) was set since there were 21 dependent variables. The fatty acid contents of *U. rigida* under all treatments conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the eight samples in all fatty acids could be considered equal (Levene's test, F < 2.477, $P > 0.05$) except C17:0 (Levene's test, F = 2.741, $P = 0.045$), C20:0 (Levene's test, F = 3.002, $P = 0.032$), C20:4n_6 (Levene's test, F = 4.692, $P = 0.005$), and C24:0 (Levene's test, F = 3.276, $P = 0.023$). A three-way MANOVA was conducted to assess the effects of pCO_2, temperature and nitrate on fatty acid contents of *U. rigida* considering that MANOVA is reasonably robust to violations of a normal distribution and homogeneity of variance particularly when the group sizes are equal (Pallant, 2010). Bonferroni adjustment was used to reduce the chance of Type I error. A new alpha level of 0.0016 (0.05 divided by 32) was set since there were 32 dependent variables.

The effects of pCO_2*, temperature and nitrate on functional properties*

The SWC of *U. rigida* under all treatments conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the eight samples could be considered equal (Levene's test, F = 2.594, $P > 0.05$). A three-way ANOVA was used to assess the effects of pCO_2, temperature and nitrate on SWC of *U. rigida.* The WHC of *U. rigida* under all treatments conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the eight samples could be considered equal (Levene's test, F = 1.325, $P > 0.05$). A three-way ANOVA was used to assess the effects of pCO_2, temperature and nitrate on WHC of *U. rigida.* The OHC of *U. rigida* under all treatments conformed to a normal distribution (Shapiro-Wilk, $P > 0.05$) and the variances of the eight samples could be considered equal (Levene's test, F = 0.524, $P > 0.05$). A three-way ANOVA was used to assess the effects of pCO_2, temperature and nitrate on OHC of *U. rigida.* A confidence interval of 95% was set for all tests.

6.3 Results

6.3.1 Changes in seawater carbonate chemistry

The changes of the seawater carbonate system under different treatments were recorded (Table 6.1). The decrease of 0.4 units of pH in seawater lead to an increase of 187.4% and 208.9% in pCO_2, 8.2% and 14.8% in DIC, 10.4% and 17.2% in HCO_3^-, 188.1% and 173.8% in CO_2, respectively for the LT and HT treatments (Table 6.1 and 6.2). Meanwhile, the lower pH decreased the concentration of CO_3^{2-} by 57.8% at LT and 46.1% at HT. There was no significant difference in TA between the two pH levels (Table 6.1 and 6.2). Higher temperature enhanced HCO_3^- by 7.5% and 6.1%, CO_3^{2-} by 29.2 ± 13.3% and 27.8%, and TA by 8.9% and 6.9% at LC and HC respectively (Table 6.1 and 6.2).

Treatment	pH	Temperature (^{o}C)	pCO_2 (µatm)	DIC (µmol kg^{-1})	HCO_3^- (µmol kg^{-1})	CO_3^{2-} (µmol kg^{-1})	CO_2 (µmol kg^{-1})	TA (µmol kg^{-1})
LTLC	7.95 ± 0.05	14 ± 0.5	670.4 ± 81.4	2053.9 ± 128.7	1907.3 ± 78.3	95.6 ± 9.7	26.1 ± 3.4	2196.3 ± 79.0
LTHC	7.55 ± 0.05	14 ± 0.5	1926.8 ± 180.1	2221.9 ± 50.9	2106.4 ± 47.9	40.3 ± 4.1	75.2 ± 7.4	2254.8 ± 49.8
HTLC	7.95 ± 0.05	18 ± 1	753.1 ± 61.7	2194.8 ± 98.3	2049.8 ± 86.7	123.5 ± 12.7	26.0 ± 6.9	2390.7 ± 115.4
HTHC	7.55 ± 0.05	18 ± 1	2071.0 ± 248.9	2358.4 ± 52.3	2235.4 ± 48.4	51.5 ± 4.0	71.5 ± 4.8	2409.5 ± 44.6

Table 6.1 Parameters of the seawater carbonate system in different cultures. Measurements and estimation of the parameters are described in Materials and Methods. Data are the means ± SD (n = 6). LTLC, low temperature and low pCO_2; LTHC, low temperature and high pCO_2; HTLC, high temperature and low pCO_2; HTHC, high temperature and high pCO_2. DIC = dissolved inorganic carbon, TA = total alkalinity.

Comparison between treatments		pCO_2	DIC	HCO_3^-	CO_3^{2-}	CO_2	TA
LTLC&LTHC	t	–15.571	–2.974	–5.315	–12.814	–14.747	–1.532
	P	< 0.001	0.014	0.001	< 0.001	< 0.001	0.156
HTLC&HTHC	t	–12.590	–3.601	–4.581	–13.276	–13.871	–0.371
	P	< 0.001	0.005	0.001	< 0.001	< 0.001	0.719
LTLC&HTLC	t	–1.984	–2.131	–2.988	–4.278	0.063	–3.404
	P	0.075	0.059	0.014	0.002	0.951	0.007
LTHC&HTHC	t	–1.150	–4.586	–4.640	–4.811	0.850	–5.666
	P	0.277	0.001	0.001	0.001	0.415	0.001

Table 6.2 Independent samples t-test of the changes of the seawater carbonate system caused by decreased pH or increased temperature. The degree of freedom for all tests was 10. LTLC, low temperature and low pCO_2; LTHC, low temperature and high pCO_2; HTLC, high temperature and low pCO_2; HTHC, high temperature and high pCO_2. DIC = dissolved inorganic carbon, TA = total alkalinity.

6.3.2 Settlement and germination

The effects of nitrate, temperature, and pCO_2 on the settlement rates of *U. rigida* were investigated (Figure 6.1). There were no interactive effects between factors within first four hours of settlement (Table 6.3). High temperature accelerated the onset of settlement of *Ulva* gametes significantly as gametes initiated settlement (0.45 ± 0.17%) under high temperature within four hours (Table 6.3 and Figure 6.1). Nitrate, temperature, and pCO_2 had an interactive effect on settlement at eight, 16, and 24 (Table 6.4–6.6), suggesting that the effects of pCO_2 may be different at different nitrate levels and temperatures. For instance, by hour eight the gametes did not settle at LNLTLC, LNLTHC, HNLTLC, or HNLTHC, whilst the settlement rates were 1.49 ± 0.27% and 2.45 ± 0.26% at LNHTLC and LNHTHC but 1.71 ± 0.39% and 1.44 ± 0.34% at HNHTLC and HNHTHC, respectively (Figure 6.1). Temperature and nitrate had main effects on settlement rates at eight, 16, and 24 hours (Table 6.4–6.6). The settlement rates of *U. rigida* under high temperature were higher than low temperature at eight (1.77 ± 0.51% versus 0.00 ± 0.00%), 16 (2.58 ± 0.60% versus 0.65 ± 0.68%), and 24 (3.62 ± 0.74% versus 1.82 ± 0.46%) hours (Figure 6.1). On the contrary, settlement rates under high nitrate were lower than low nitrate at eight (0.79 ± 0.84%

versus 0.98 ±1.07%), 16 (1.09 ±1.15% versus 2.14 ±0.92%), and 24 (2.31 ±0.93% versus 3.13 ±1.11%) hours (Figure 6.1). The interactive effects disappeared at hour 32 and 40. Temperature still had a main effect on settlement rates (Table 6.7 and 6.8).

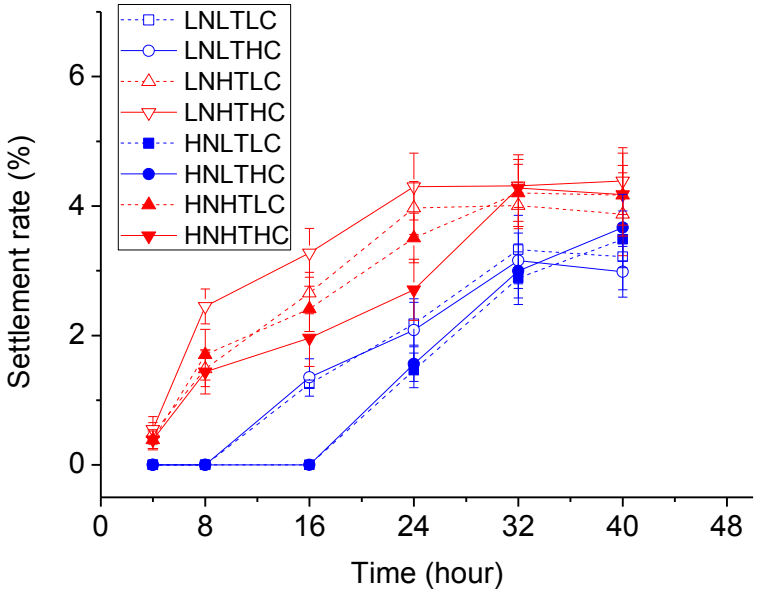

Figure 6.1 Time course of gamete settlement rates under different treatments. Each point is the mean of nine counts of view fields. Bars indicate the standard deviation. LNLTLC: low nitrate, low temperature and low pCO_2; LNLTHC: low nitrate, low temperature and high pCO_2; LNHTLC: low nitrate, high temperature and low pCO_2; LNHTHC: low nitrate, high temperature and high pCO_2; HNLTLC: high nitrate, low temperature and low pCO_2; HNLTHC: high nitrate, low temperature and high pCO_2; HNHTLC: high nitrate, high temperature and low pCO_2; HNHTHC: high nitrate, high temperature and high pCO_2.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	0.074	1	0.074	5.394	0.023
Temperature	3.707	1	3.707	271.572	<0.001
pCO_2	0.004	1	0.004	0.306	0.582
Nitrate* Temperature	0.074	1	0.074	5.394	0.023
Nitrate*pCO_2	0.004	1	0.004	0.306	0.582
Temperature* pCO_2	0.004	1	0.004	0.306	0.582
Nitrate* Temperature* pCO_2	0.004	1	0.004	0.306	0.582
Error	0.874	64	0.014		

Table 6.3 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on the settlement rates of *U. rigida* at hour four. The confidence interval was 99%.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	0.706	1	0.706	13.542	<0.001
Temperature	56.368	1	56.368	1081.926	<0.001
pCO_2	0.543	1	0.543	10.413	0.002
Nitrate* Temperature	0.706	1	0.706	13.542	<0.001
Nitrate*pCO_2	1.703	1	1.703	32.696	<0.001
Temperature* pCO_2	0.543	1	0.543	10.413	0.002
Nitrate* Temperature* pCO_2	1.703	1	1.703	32.696	<0.001
Error	3.334	64	0.052		

Table 6.4 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on the settlement rates of *U. rigida* at hour eight. The confidence interval was 99%.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	19.531	1	19.531	240.915	<0.001
Temperature	66.698	1	66.698	822.706	<0.001
pCO_2	0.081	1	0.081	0.997	0.322
Nitrate* Temperature	1.235	1	1.235	15.234	<0.001
Nitrate*pCO_2	1.539	1	1.539	18.982	<0.001
Temperature* pCO_2	0.006	1	0.006	0.074	0.786
Nitrate* Temperature* pCO_2	1.069	1	1.069	13.182	0.001
Error	5.189	64	0.081		

Table 6.5 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on the settlement rates of *U. rigida* at hour 16. The confidence interval was 99%.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	12.264	1	12.264	75.947	<0.001
Temperature	58.325	1	58.325	361.201	<0.001
pCO_2	0.254	1	0.254	1.573	0.214
Nitrate* Temperature	0.750	1	0.750	4.642	0.035
Nitrate*pCO_2	0.990	1	0.990	6.131	0.016
Temperature* pCO_2	0.254	1	0.254	1.573	0.214
Nitrate* Temperature* pCO_2	1.984	1	1.984	12.286	0.001
Error	10.334	64	0.161		

Table 6.6 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on the settlement rates of *U. rigida* at hour 24. The confidence interval was 95%.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	0.216	1	0.216	1.099	0.298
Temperature	22.125	1	22.125	112.404	<0.001
pCO_2	0.113	1	0.113	0.573	0.452
Nitrate* Temperature	0.642	1	0.642	3.261	0.076
Nitrate*pCO_2	0.003	1	0.003	0.014	0.908
Temperature* pCO_2	0.216	1	0.216	1.099	0.298
Nitrate* Temperature* pCO_2	0.295	1	0.295	1.497	0.226
Error	12.597	64	0.197		

Table 6.7 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on the settlement rates of *U. rigida* at hour 32. The confidence interval was 95%.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	1.206	1	1.206	4.447	0.039
Temperature	11.904	1	11.904	43.877	<0.001
pCO_2	0.254	1	0.254	0.936	0.337
Nitrate* Temperature	0.842	1	0.842	3.103	0.083
Nitrate*pCO_2	0.008	1	0.008	0.030	0.863
Temperature* pCO_2	0.369	1	0.369	1.360	0.248
Nitrate* Temperature* pCO_2	0.939	1	0.939	3.462	0.067
Error	17.364	64	0.271		

Table 6.8 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on the settlement rates of *U. rigida* at hour 40. The confidence interval was 95%.

The effects of nitrate, temperature, and pCO_2 on the germination rates of *U. rigida* were investigated (Figure 6.2). On day two, any single factor enhanced the germination rates, any two factors had an interactive effect and a further significant

increase was found when these three factors acted together (Table 6.9, Figure 6.2). For example, the germination rates were 6.94 ±3.04% at LNLTLC, 7.99 ±2.76% at LNLTHC, 11.11 ±3.17% at HNLTLC, 12.50 ±3.13% at HNLTHC, 19.10 ±4.82% at LNHTLC, 19.79 ±4.42% at LNHTHC, 24.65 ±6.89% at HNHTLC, and 37.50 ±11.38% at HNHTHC, which indicated that high temperature, nitrate and pCO_2 increased the germination rates by 175.22%, 60.09%, and 15.13% respectively but the combination of these three factors resulted in an increase of 440.35%. On day four, only temperature had interactive effects with pCO_2 or nitrate, although any single factor still increased the germination rates (Table 6.10). On day six, the germination-promoting effect of pCO_2 was not detected and there were no longer any interactive effects (Table 6.11). On day eight, the germination rates under all conditions were not significantly different (Table 6.12).

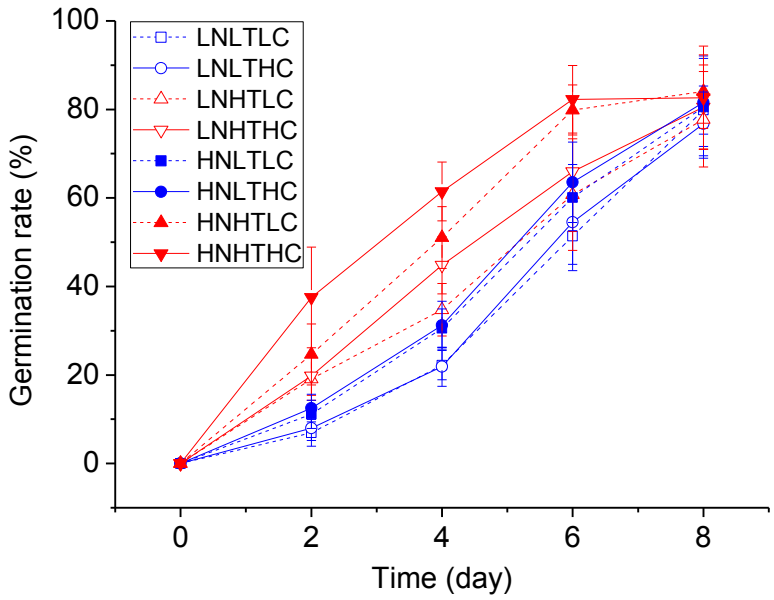

Figure 6.2 Germination rates of settled gametes with time under different treatments. Each point is the mean of nine counts of view fields. Bars indicate the standard deviation. LNLTLC: low nitrate, low temperature and low pCO_2; LNLTHC: low nitrate, low temperature and high pCO_2; LNHTLC: low nitrate, high temperature and low pCO_2; LNHTHC: low nitrate, high temperature and high pCO_2; HNLTLC: high nitrate, low temperature and low pCO_2; HNLTHC: high nitrate, low temperature and high pCO_2; HNHTLC: high nitrate, high temperature and low pCO_2; HNHTHC: high nitrate, high temperature and high pCO_2.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	1148.003	1	1148.003	35.864	<0.001
Temperature	4394.531	1	4394.531	137.288	<0.001
pCO_2	287.001	1	287.001	8.966	0.004
Nitrate* Temperature	239.258	1	239.258	7.475	0.008
Nitrate*pCO_2	175.781	1	175.781	5.492	0.022
Temperature* pCO_2	138.889	1	138.889	4.339	0.041
Nitrate* Temperature* pCO_2	156.793	1	156.793	4.898	0.030
Error	2048.611	64	32.010		

Table 6.9 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on the germination rates of *U. rigida* on day two. The confidence interval was 95%.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	2891.168	1	2891.168	93.185	<0.001
Temperature	8342.014	1	8342.014	268.870	<0.001
pCO_2	488.281	1	488.281	15.738	<0.001
Nitrate* Temperature	262.587	1	262.587	8.463	0.005
Nitrate*pCO_2	2.170	1	2.170	0.070	0.792
Temperature* pCO_2	456.272	1	456.272	14.706	<0.001
Nitrate* Temperature* pCO_2	0.543	1	0.543	0.017	0.895
Error	1985.677	64	31.026		

Table 6.10 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on the germination rates of *U. rigida* on day four. The confidence interval was 95%.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	3175.049	1	3175.049	35.070	<0.001
Temperature	3966.064	1	3966.064	43.807	<0.001
pCO_2	228.000	1	228.000	2.518	0.117
Nitrate* Temperature	352.783	1	352.783	3.897	0.053
Nitrate*pCO_2	6.646	1	6.646	0.073	0.787
Temperature* pCO_2	1.221	1	1.221	0.013	0.908
Nitrate* Temperature* pCO_2	10.986	1	10.986	0.121	0.729
Error	5794.271	64	90.535		

Table 6.11 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on the germination rates of *U. rigida* on day six. The confidence interval was 95%.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	217.014	1	217.014	2.393	0.127
Temperature	43.945	1	43.945	0.484	0.489
pCO_2	0.543	1	0.543	0.006	0.939
Nitrate* Temperature	8.681	1	8.681	0.096	0.758
Nitrate*pCO_2	<0.001	1	<0.001	<0.001	1.000
Temperature* pCO_2	13.563	1	13.563	0.150	0.700
Nitrate* Temperature* pCO_2	78.125	1	78.125	0.861	0.357
Error	5805.122	64	90.705		

Table 6.12 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on the germination rates of *U. rigida* on day eight. The confidence interval was 95%.

6.3.3 Growth of young U. rigida

The effects of nitrate, temperature, and pCO_2 on the specific growth rates of young *U. rigida* were investigated (Figure 6.3 a). There were no interactive effects of

these three factors but any two of them interacted on the specific growth rates of young *U. rigida* (Table 6.13), which indicated that high nitrate and temperature, or nitrate and pCO_2, or temperature and pCO_2 simulated the specific growth rates synergistically (Figure 6.3 a). For example, the specific growth rate at LNLTLC was 34.07 ± 0.67% and high pCO_2 (LNLTHC) and high temperature (LNHTLC) increased the specific growth rates by 0.27% and 2.14% respectively, while the combination of high pCO_2 and high temperature (LNHTHC) increased the specific growth rate by 2.81% (Figure 6.3 a). Based on the F values (Table 6.13), nitrate had the greatest effect on the specific growth rate of young *U. rigida*. High nitrate enhanced the growth rate by 14.75% compared to low nitrate. Next in importance to nitrate was high temperature (Table 6.13) with a 5.05% increase in the specific growth rate, and high pCO_2 promoted the specific growth rate by 2.42%. The positive effects of nitrate, temperature or pCO_2 on the specific growth rate seemed slight but remarkable differences in individual mass can be seen (Figure 6.3 b) since the specific growth rate was a daily mean value over a 60-day culture period. For example, the mass of individual germlings was only 0.018 ± 0.007 mg under LNLTLC while it reached up to 2.111 ± 0.366 mg under HTHNHC, so that the high temperature, nitrate, and pCO_2 increased the biomass of germlings more than 100 times over the 60-day culture period.

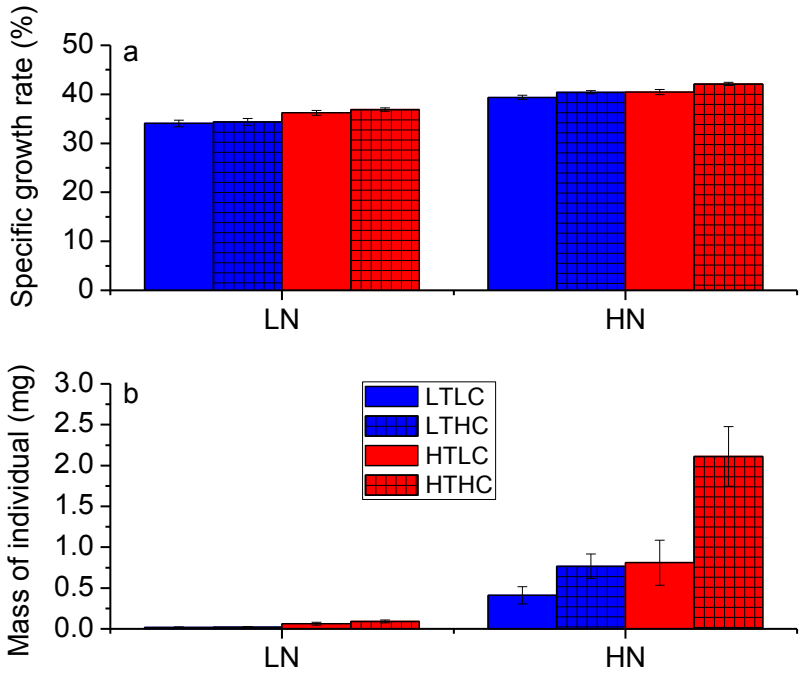

Figure 6.3 Interactive effects of nitrate, temperature, and pCO_2 on specific growth rate (a) and individual mass (b) of young *U. rigida* over a 60-day culture period. Data are the means ± SD (n = 9). LTLC: low temperature and low pCO_2; LTHC: low temperature and high pCO_2; HTLC: high temperature and low pCO_2; HTHC: high temperature and high pCO_2; LN: low nitrate; HN: high nitrate.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	490.547	1	490.547	1989.527	<0.001
Temperature	62.754	1	62.754	254.515	<0.001
pCO_2	14.983	1	14.983	60.766	<0.001
Nitrate* Temperature	3.957	1	3.957	16.047	<0.001
Nitrate*pCO_2	3.490	1	3.490	14.155	<0.001
Temperature* pCO_2	1.084	1	1.084	4.395	0.040
Nitrate* Temperature* pCO_2	0.048	1	0.048	0.196	0.659
Error	15.780	64	0.247		

Table 6.13 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on the specific growth rates of young *U. rigida*. The confidence interval was 95%.

6.3.4 Growth and reproduction of adult U. rigida

The specific growth rates of *U. rigida* over 12-day culture in different conditions of nitrate, temperature, and pCO_2 were recorded (Figure 6.4). During the first four days, temperature and pCO_2 had interactive effects on the specific growth rates (Table 6.14) since high pCO_2 did not stimulate the specific growth rates at low temperature. Temperature contributed most to growth of adult *U. rigida* compared to nitrate and pCO_2 (Table 6.14). High temperature (HT) significantly enhanced the specific growth rates of *U. rigida* by 80.78% (Figure 6.4 a). High nitrate (HN) also significantly increased the specific growth rates of *U. rigida* (45.35%, Table 6.14, Figure 6.4 a). pCO_2 was the weakest factor, increasing the specific growth rates by19.86% (Table 6.14, Figure 6.4 a). By day eight, no interactive effects were detected. Nitrate replaced temperature as the most effective factor, with an increase in the specific growth rates of 58.13% (Table 6.15 and Figure 6.4 b). The positive effect of temperature on the specific growth rates decreased to 29.74% (Table 6.15 and Figure 6.4 b). pCO_2 continued to have the smallest effect, increasing the specific growth rates by 17.53% (Table 6.15 and Figure 6.4 b). By day 12, any two factors had an interactive effect (Table 6.16). For example, high temperature decreased the specific growth rate by 8.83% at LC but it was 28.95% at HC (Figure 6.4 c), which indicates the negative effect of high temperature was more significant at HC.

No reproduction of *U. rigida* occurred during the first four days of culture regardless of conditions (Figure 6.4 d). Temperature had interactive effect with nitrate or pCO_2 (Table 6.17). High temperature (HT) alone resulted in a 6.67% increase in the reproduction rate by day eight (Table 6.17 and Figure 6.4 e), which was more significant at HC (16.67%, Table 6.17 and Figure 6.4 e) or HN (17.77%, Table 6.17 and Figure 6.4 e). This trend continued to day 12 and these three factors had an interactive effect (Table 6.18). Therefore, the highest reproduction rate ($64.44 \pm 5.09\%$) was found at high nitrate, temperature, and pCO_2 level (HNHTHC; Figure 6.4 f).

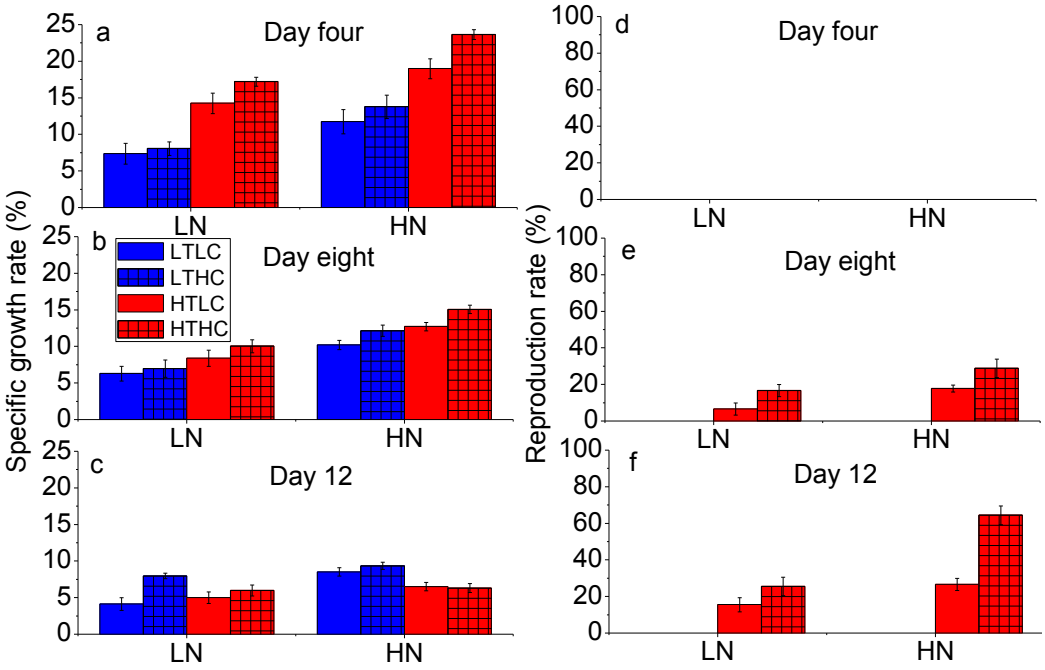

Figure 6.4 Effects of nitrate, temperature, and pCO_2 on growth (a, b, c) and reproduction (d, e, f) of adult *U. rigida* over a 12-day culture period. Data are the means ± SD (n = 3). LTLC: low temperature and low pCO_2; LTHC: low temperature and high pCO_2; HTLC: high temperature and low pCO_2; HTHC: high temperature and high pCO_2; LN: low nitrate; HN: high nitrate.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	169.536	1	169.536	105.704	<0.001
Temperature	412.221	1	412.221	257.017	<0.001
pCO_2	40.275	1	40.275	25.111	<0.001
Nitrate* Temperature	0.461	1	0.461	0.288	0.599
Nitrate*pCO_2	3.485	1	3.485	2.173	0.160
Temperature* pCO_2	8.925	1	8.925	5.565	0.031
Nitrate* Temperature* pCO_2	0.059	1	0.059	0.037	0.850
Error	25.662	16	1.604		

Table 6.14 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on the specific growth rates of adult *U. rigida* on day four. The confidence interval was 95%.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	127.656	1	127.656	169.594	<0.001
Temperature	41.964	1	41.964	55.749	<0.001
pCO_2	16.364	1	16.364	21.740	<0.001
Nitrate* Temperature	0.022	1	0.022	0.029	0.868
Nitrate*pCO_2	1.452	1	1.452	1.928	0.184
Temperature* pCO_2	0.672	1	0.672	0.893	0.359
Nitrate* Temperature* pCO_2	0.108	1	0.108	0.143	0.710
Error	12.044	16	0.753		

Table 6.15 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on the specific growth rates of adult *U. rigida* on day eight. The confidence interval was 95%.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	21.700	1	21.700	52.305	<0.001
Temperature	14.252	1	14.252	34.351	<0.001
pCO_2	11.114	1	11.114	26.789	<0.001
Nitrate* Temperature	5.768	1	5.768	13.902	0.002
Nitrate*pCO_2	6.503	1	6.503	15.674	0.001
Temperature* pCO_2	5.676	1	5.676	13.680	0.002
Nitrate* Temperature* pCO_2	1.242	1	1.242	2.992	0.103
Error	6.638	16	0.415		

Table 6.16 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on the specific growth rates of adult *U. rigida* on day 12. The confidence interval was 95%.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	204.167	1	204.167	31.500	<0.001
Temperature	1837.500	1	1837.500	283.500	<0.001
pCO_2	167.130	1	167.130	25.786	<0.001
Nitrate* Temperature	204.167	1	204.167	31.500	<0.001
Nitrate*pCO_2	0.463	1	0.463	0.071	0.793
Temperature* pCO_2	167.130	1	167.130	25.786	<0.001
Nitrate* Temperature* pCO_2	0.463	1	0.463	0.071	0.793
Error	103.704	16	6.481		

Table 6.17 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on the reproduction rates of adult *U. rigida* on day eight. The confidence interval was 99%.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	937.500	1	937.500	96.429	<0.001
Temperature	6556.019	1	6556.019	674.333	<0.001
pCO_2	856.019	1	856.019	88.048	<0.001
Nitrate* Temperature	937.500	1	937.500	96.429	<0.001
Nitrate*pCO_2	289.352	1	289.352	29.762	<0.001
Temperature* pCO_2	856.019	1	856.019	88.048	<0.001
Nitrate* Temperature* pCO_2	289.352	1	289.352	29.762	<0.001
Error	155.556	16	9.722		

Table 6.18 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on the reproduction rates of adult *U. rigida* on day 12. The confidence interval was 99%.

6.3.5 Protein

The percentage protein content had a large variation when grown under simulated climate change conditions (Figure 6.5). The lowest content of 11.17 ± 1.64% was found at LNLTLC while the highest value (24.14 ± 0.76%) was reached when *U. rigida* were grown at HNHTLC. No interactive effects between these three factors were found (Table 6.19). Temperature was the strongest factor affecting protein content (Table 6.19). The high temperature (HT) enhanced the protein content by 49.13% (Figure 6.5). Following temperature, high nitrate (HN) increased protein content by 31.06% (Table 6.19 and Figure 6.5). pCO_2 also had a main effect on the protein content and high pCO_2 (HC) decreased protein content by 6.90% (Table 6.19 and Figure 6.5).

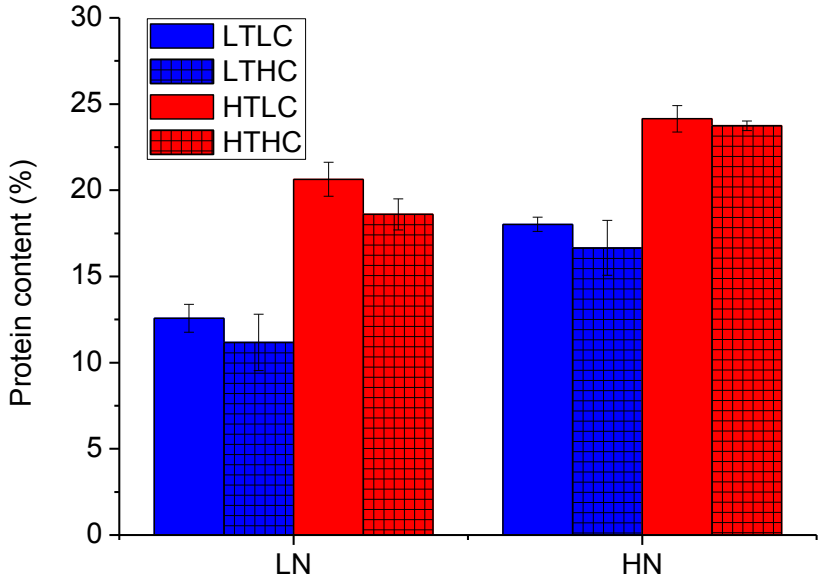

Figure 6.5 Protein content of *U. rigida* grown under different conditions. LTLC: low temperature and low pCO_2; LTHC: low temperature and high pCO_2; HTLC: high temperature and low pCO_2; HTHC: high temperature and high pCO_2; LN: low nitrate; HN: high nitrate. Data are the means ± SD (n = 3).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	189.141	1	189.141	135.916	<0.001
Temperature	406.315	1	406.315	291.977	<0.001
pCO_2	13.313	1	13.313	9.567	0.007
Nitrate* Temperature	2.584	1	2.584	1.857	0.192
Nitrate*pCO_2	1.378	1	1.378	0.990	0.335
Temperature* pCO_2	0.053	1	0.053	0.038	0.848
Nitrate* Temperature* pCO_2	1.260	1	1.260	0.906	0.355
Error	22.266	16	1.392		

Table 6.19 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on protein content of *U. rigida*. The confidence interval was 95%.

6.3.6 Lipid

The lipid content ranged from 3.84 ± 0.35% to 6.31 ± 0.32% (Figure 6.6). Temperature, nitrate, and pCO_2 had an interactive effect on lipid content (Table 6.20). These three factors alone (LNHTLC, HNLTLC, or LNLTHC) did not increase lipid content but the combination (HNHTHC) enhanced lipid content by 75.14% compared with LNLTLC (Figure 6.6). The high temperature (HT) enhanced lipid content by 28.50% (Table 6.20 and Figure 6.6). The high nitrate (HN) treatment increased lipid content by 25.18% (Table 6.20 and Figure 6.6). The high pCO_2 (HC) treatment increased the lipid content by 20.90% (Table 6.20 and Figure 6.6).

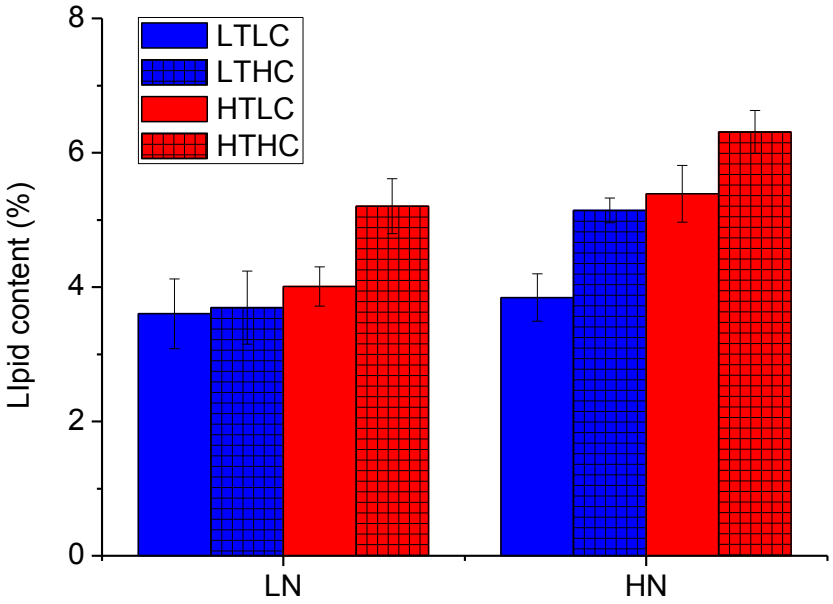

Figure 6.6 Lipid content of *U. rigida* grown under different conditions. LTLC: low temperature and low pCO_2; LTHC: low temperature and high pCO_2; HTLC: high temperature and low pCO_2; HTHC: high temperature and high pCO_2; LN: low nitrate; HN: high nitrate. Data are the means ± SD (n = 3).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	6.528	1	6.528	41.497	<0.001
Temperature	8.041	1	8.041	51.114	<0.001
pCO_2	4.606	1	4.606	29.278	<0.001
Nitrate* Temperature	0.238	1	0.238	1.515	0.236
Nitrate*pCO_2	0.324	1	0.324	2.060	0.170
Temperature* pCO_2	0.200	1	0.200	1.270	0.276
Nitrate* Temperature * pCO_2	0.821	1	0.821	5.222	0.036
Error	2.517	16	0.157		

Table 6.20 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on lipid content of *U. rigida*. The confidence interval was 95%.

6.3.7 Ash

The ash content ranged from 29.15 ± 0.46% to 36.19 ± 1.12% (Figure 6.7). The high temperature (HT) treatment reduced the ash content by 13.28% (Table 6.21 and Figure 6.7). By contrast, the high nitrate (HN) treatment increased it by 4.86% (Table 6.21 and Figure 6.7). Temperature had interactive effects with nitrate or pCO_2 (Table 6.21) since the negative effects of high temperature were more obvious under high nitrate (HN) or the pCO_2 (HC) conditions (Figure 6.7).

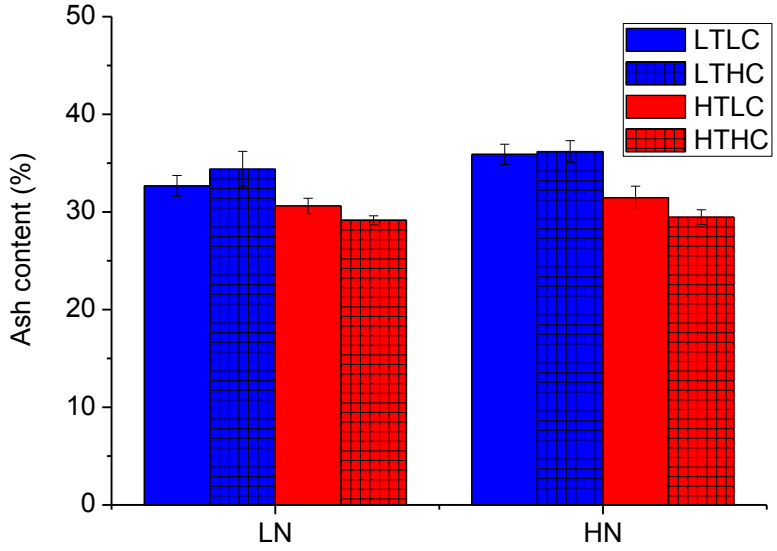

Figure 6.7 Ash content of *U. rigida* grown under different conditions. LTLC: low temperature and low pCO_2; LTHC: low temperature and high pCO_2; HTLC: high temperature and low pCO_2; HTHC: high temperature and high pCO_2; LN: low nitrate; HN: high nitrate. Data are the means ± SD (n = 3).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	14.254	1	14.254	11.883	0.003
Temperature	127.840	1	127.840	106.570	<0.001
pCO_2	0.795	1	0.795	0.663	0.427
Nitrate * Temperature	5.655	1	5.655	4.714	0.045
Nitrate * pCO_2	1.415	1	1.415	1.180	0.293
Temperature * pCO_2	11.144	1	11.144	9.290	0.008
Nitrate * Temperature * pCO_2	0.326	1	0.326	0.272	0.609
Error	19.193	16	1.200		

Table 6.21 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on ash content of *U. rigida*. The confidence interval was 95%.

6.3.8 Carbohydrate

The carbohydrate content *U. rigida* ranged from 39.02 ± 1.56% to 51.15 ± 0.24% (Figure 6.8). The high temperature (HT) treatment reduced carbohydrate by 10.75% (Table 6.22 and Figure 6.8) and high nitrate (HN) had a bigger inhibition effect of 17.77% (Table 6.22 and Figure 6.8). pCO_2 had no effects on carbohydrate content. High nitrate or pCO_2 alleviated the negative effects of high temperature (Table 6.22 and Figure 6.8). For instance, the high temperature reduced carbohydrate by 7.60% (LC) and 3.66% (HC) under high nitrate conditions while they were 12.56% (LC) and 7.29% (HC) under low nitrate conditions.

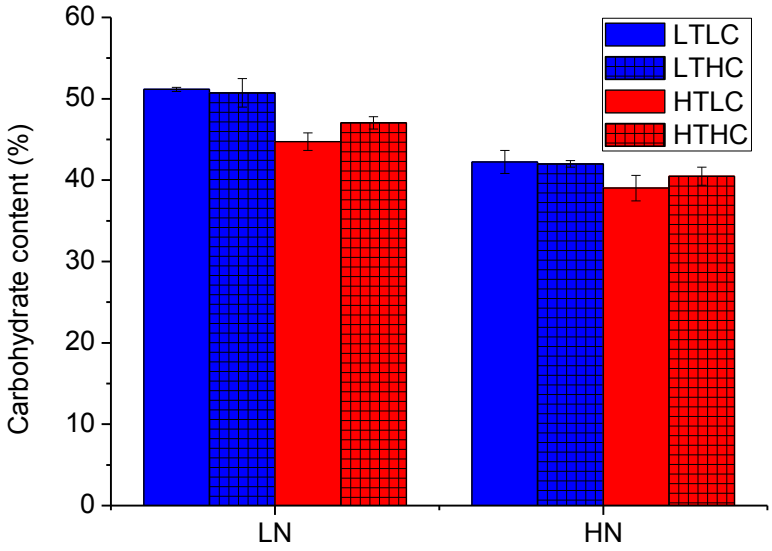

Figure 6.8 Carbohydrate content of *U. rigida* grown under different conditions. LTLC: low temperature and low pCO_2; LTHC: low temperature and high pCO_2; HTLC: high temperature and low pCO_2; HTHC: high temperature and high pCO_2; LN: low nitrate; HN: high nitrate. Data are the means ± SD (n = 3).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	403.339	1	403.339	261.589	<0.001
Temperature	136.568	1	136.568	88.572	<0.001
pCO_2	5.733	1	5.733	3.718	0.072
Nitrate* Temperature	12.231	1	12.231	7.932	0.012
Nitrate*pCO_2	0.306	1	0.306	0.198	0.662
Temperature* pCO_2	7.084	1	7.084	4.595	0.048
Nitrate* Temperature* pCO_2	0.621	1	0.621	0.402	0.535
Error	24.670	16	1.542		

Table 6.22 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on carbohydrate content of *U. rigida*. The confidence interval was 95%.

6.3.9 Amino acids

The amino acid composition *U. rigida* grown under various conditions was assessed (Table 6.23). Glutamic acid and arginine were the most abundant amino acids,

followed by aspartic acid and alanine. All samples were rich in essential amino acids. The ratios of essential to non-essential amino acids were all above 1 except under HTLC, which indicated those samples contained more essential than non-essential amino acids.

Interactive effects of temperature, pCO_2, and nitrate were found in phenylalanine (Phe) and cysteine (Cys) (Table 6.24). For instance, high temperature (HT) and nitrate (HN) increased phenylalanine (Phe) content by 26.74% and 11.63% respectively and pCO_2 did not have any effect on it. Nevertheless, high temperature (HT), pCO_2 (HC) and nitrate (HN) increased phenylalanine (Phe) content by 96.51%. Temperature and nitrate had interactive effects on arginine (Arg), glutamic acid (Glu), tyrosine (Tyr), phenylalanine (Phe), and the ratio of EAA to NEAA (Table 6.16). For instance, high temperature and high nitrate increased arginine (Arg) by 18.58% and 8.85% respectively, but the combination of the two factors increased it by 89.38% (Table 6.23 and 6.24). Temperature and pCO_2 had interactive effects on arginine (Arg), glycine (Gly), aspartic acid (Asp), glutamic acid (Glu), phenylalanine (Phe), and the ratio of EAA to NEAA (Table 6.24). For instance, temperature alone increased arginine by 18.58%, pCO_2 did not affect arginine but combination of them increased arginine by 15.04%. Similar trends were found in glycine (Gly) and phenylalanine (Phe) (Table 6.23 and 6.24). Nitrate and pCO_2 had interactive effects on arginine (Arg), methionine (Met), phenylalanine (Phe), cysteine (Cys), leucine (Leu), total AA, EAA, and NEAA (Table 6.23). For instance, high nitrate increased arginine (Arg) by 8.85% and pCO_2 did not affect threonine but high nitrate and pCO_2 increased threonine by 4.42%. Similar trends were found in methionine (Met), phenylalanine (Phe), cysteine (Cys), leucine (Leu), total AA, and EAA. Meanwhile, another pattern of interactions of nitrate and pCO_2 on fatty acids was that high nitrate increased NEAA only under high pCO_2 (Table 6.23 and 6.24). High temperature increased the content of every amino acid and thus the total amino acids. High nitrate increased the content of all amino acid except aspartic acid (Asp) and cysteine (Cys). By contrast, high pCO_2 decreased the content of aspartic acid (Asp) and glutamic acid (Glu) (Table 6.23 and 6.24).

Amino acids	LTLNLC[a]	LTLNHC[b]	LTHNLC[c]	LTHNHC[d]	HTLNLC[e]	HTLNHC[f]	HTHNLC[g]	HTHNH[h]
His	0.21±0.03	0.20±0.02	0.21±0.01	0.22±0.01	0.31±0.02	0.27±0.01	0.36±0.01	0.34±0.03
Ser	0.78±0.10	0.70±0.11	0.90±0.12	0.82±0.12	1.10±0.05	0.99±0.02	1.27±0.08	1.39±0.17
Arg	1.13±0.07	1.02±0.06	1.23±0.04	1.18±0.10	1.34±0.05	1.30±0.08	1.57±0.20	2.14±0.10
Gly	0.63±0.05	0.57±0.06	0.76±0.04	0.72±0.03	0.80±0.02	0.79±0.04	0.83±0.10	1.12±0.09
Asp	1.04±0.06	0.85±0.07	1.18±0.04	1.19±0.03	1.99±0.13	1.46±0.14	1.94±0.24	1.44±0.05
Ala	1.25±0.05	1.10±0.04	1.45±0.08	1.41±0.10	2.05±0.17	1.60±0.05	1.98±0.11	1.92±0.25
Glu	1.22±0.05	1.06±0.04	1.39±0.04	1.41±0.06	2.26±0.03	1.80±0.05	2.21±0.05	1.70±0.05
Thr	0.75±0.07	0.64±0.07	0.83±0.10	0.80±0.12	1.08±0.03	0.95±0.01	1.15±0.15	1.34±0.16
Pro	0.52±0.09	0.46±0.04	0.61±0.06	0.58±0.09	0.69±0.02	0.64±0.03	0.96±0.10	0.91±0.07
Lys	0.76±0.04	0.68±0.05	0.94±0.07	0.93±0.06	1.11±0.06	1.08±0.03	1.24±0.11	1.11±0.11
Tyr	0.39±0.05	0.35±0.06	0.41±0.03	0.39±0.07	0.50±0.03	0.49±0.04	0.58±0.05	0.80±0.05
Met	0.30±0.05	0.27±0.01	0.34±0.03	0.33±0.01	0.38±0.01	0.37±0.00	0.42±0.04	0.56±0.09
Val	0.90±0.08	0.79±0.06	1.00±0.11	0.99±0.11	1.37±0.08	1.16±0.03	1.39±0.05	1.46±0.12
Ile	0.55±0.06	0.51±0.04	0.63±0.08	0.60±0.01	0.81±0.08	0.75±0.02	0.81±0.02	0.94±0.08
Phe	0.86±0.10	0.82±0.04	0.96±0.14	0.91±0.04	1.09±0.04	1.03±0.05	1.17±0.13	1.69±0.08
Cys	0.11±0.02	0.08±0.02	0.08±0.02	0.07±0.02	0.27±0.03	0.15±0.01	0.18±0.02	0.27±0.02
Leu	0.94±0.04	0.86±0.05	1.09±0.05	1.05±0.06	1.36±0.05	1.15±0.05	1.12±0.06	1.44±0.08
Total	12.33±0.70	10.95±0.46	14.01±0.77	13.61±0.80	18.49±0.59	15.97±0.66	19.18±0.54	20.56±0.79
EAAi	6.40±0.37	5.78±0.24	7.23±0.51	7.02±0.44	8.84±0.31	8.06±0.18	9.22±0.49	11.01±0.55
NEAAj	5.94±0.30	5.17±0.24	6.78±0.26	6.59±0.36	9.65±0.36	7.91±0.09	9.95±0.17	9.55±0.32
EAA/NEAA	1.08±0.02	1.12±0.03	1.07±0.04	1.06±0.01	0.92±0.03	1.02±0.03	0.93±0.05	1.15±0.05

Table 6.23 Amino acid composition of *U. rigida* grown under different conditions (g $100g^{-1}$ DW). Values are means of three replicates ± standard deviation. [a]LTLNLC, low temperature, low nitrate and low pCO_2. [b]LTLNHC, low temperature, low nitrate and high pCO_2. [c]LTHNLC, low temperature, high nitrate and low pCO_2. [d]LTHNHC, low temperature, high nitrate and high pCO_2. [e]HTLNLC, high temperature, low nitrate and low pCO_2. [f]HTLNHC, high temperature, low nitrate and high pCO_2. [g]HTHNLC, high temperature, high nitrate and low pCO_2. [h]HTHNHC, high temperature, high nitrate and high pCO_2. [i]EAA, essential amino acids: His, Arg, Thr, Lys, Met, Val, Ile, Phe, and Leu. [j]NEAA, non-essential amino acids: Ser, Gly, Asp, Ala, Glu, Pro, and Tyr.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Temperature	His	<0.001	1	<0.001	206.571	<0.001
	Ser	<0.001	1	<0.001	81.064	<0.001
	Arg	<0.001	1	<0.001	103.194	<0.001
	Gly	<0.001	1	<0.001	76.223	<0.001
	Asp	<0.001	1	<0.001	178.610	<0.001
	Ala	<0.001	1	<0.001	129.124	<0.001
	Glu	<0.001	1	<0.001	1431.222	<0.001
	Thr	<0.001	1	<0.001	81.470	<0.001
	Pro	<0.001	1	<0.001	88.459	<0.001
	Lys	<0.001	1	<0.001	102.617	<0.001
	Tyr	<0.001	1	<0.001	110.119	<0.001
	Met	<0.001	1	<0.001	55.706	<0.001
	Val	<0.001	1	<0.001	142.512	<0.001
	Ile	<0.001	1	<0.001	119.196	<0.001
	Phe	<0.001	1	<0.001	102.223	<0.001
	Cys	<0.001	1	<0.001	243.373	<0.001
	Leu	<0.001	1	<0.001	146.595	<0.001
	Total	0.020	1	0.020	507.346	<0.001
	EAA	0.004	1	0.004	253.579	<0.001
	NEAA	0.006	1	0.006	768.449	<0.001
	Ratio	0.036	1	0.036	29.169	<0.001
Nitrate	His	<0.001	1	<0.001	23.711	<0.001
	Ser	<0.001	1	<0.001	23.060	<0.001
	Arg	<0.001	1	<0.001	55.977	<0.001
	Gly	<0.001	1	<0.001	40.117	<0.001
	Asp	<0.001	1	<0.001	4.951	0.041
	Ala	<0.001	1	<0.001	14.240	0.002
	Glu	<0.001	1	<0.001	22.872	<0.001
	Thr	<0.001	1	<0.001	18.131	0.001
	Pro	<0.001	1	<0.001	45.197	<0.001
	Lys	<0.001	1	<0.001	22.820	<0.001
	Tyr	<0.001	1	<0.001	31.750	<0.001
	Met	<0.001	1	<0.001	23.728	<0.001
	Val	<0.001	1	<0.001	19.199	<0.001

	Ile	<0.001	1	<0.001	15.609	0.001
	Phe	<0.001	1	<0.001	44.256	<0.001
	Cys	<0.001	1	<0.001	0.050	0.826
	Leu	<0.001	1	<0.001	17.520	0.001
	Total	0.003	1	0.003	86.090	<0.001
	EAA	0.001	1	0.001	64.436	<0.001
	NEAA	0.001	1	0.001	85.501	<0.001
	Ratio	0.002	1	0.002	1.877	0.190
pCO_2	His	<0.001	1	<0.001	5.329	0.035
	Ser	<0.001	1	<0.001	0.756	0.397
	Arg	<0.001	1	<0.001	4.302	0.055
	Gly	<0.001	1	<0.001	3.085	0.098
	Asp	<0.001	1	<0.001	39.676	<0.001
	Ala	<0.001	1	<0.001	11.407	0.004
	Glu	<0.001	1	<0.001	205.304	<0.001
	Thr	<0.001	1	<0.001	0.219	0.646
	Pro	<0.001	1	<0.001	3.192	0.093
	Lys	<0.001	1	<0.001	4.122	0.059
	Tyr	<0.001	1	<0.001	3.515	0.079
	Met	<0.001	1	<0.001	1.854	0.192
	Val	<0.001	1	<0.001	2.900	0.108
	Ile	<0.001	1	<0.001	0.002	0.963
	Phe	<0.001	1	<0.001	6.738	0.020
	Cys	<0.001	1	<0.001	4.571	0.048
	Leu	<0.001	1	<0.001	0.016	0.900
	Total	<0.001	1	<0.001	7.934	0.012
	EAA	<0.001	1	<0.001	0.067	0.798
	NEAA	<0.001	1	<0.001	46.223	<0.001
	Ratio	0.050	1	0.050	40.651	<0.001
Temperature*Nitrate	His	<0.001	1	<0.001	12.397	0.003
	Ser	<0.001	1	<0.001	4.054	0.061
	Arg	<0.001	1	<0.001	20.113	<0.001
	Gly	<0.001	1	<0.001	0.693	0.417
	Asp	<0.001	1	<0.001	8.348	0.011
	Ala	<0.001	1	<0.001	1.529	0.234
	Glu	<0.001	1	<0.001	75.035	<0.001

	Thr	<0.001	1	<0.001	1.750	0.204
	Pro	<0.001	1	<0.001	8.619	0.010
	Lys	<0.001	1	<0.001	5.077	0.039
	Tyr	<0.001	1	<0.001	16.739	0.001
	Met	<0.001	1	<0.001	4.148	0.059
	Val	<0.001	1	<0.001	0.024	0.878
	Ile	<0.001	1	<0.001	0.077	0.785
	Phe	<0.001	1	<0.001	14.157	0.002
	Cys	<0.001	1	<0.001	4.764	0.044
	Leu	<0.001	1	<0.001	10.132	0.006
	Total	<0.001	1	<0.001	0.834	0.375
	EAA	<0.001	1	<0.001	3.533	0.079
	NEAA	<0.001	1	<0.001	0.493	0.493
	Ratio	0.016	1	0.016	13.224	0.002
Temperature*pCO_2	His	<0.001	1	<0.001	3.573	0.077
	Ser	<0.001	1	<0.001	0.884	0.361
	Arg	<0.001	1	<0.001	14.698	0.001
	Gly	<0.001	1	<0.001	13.989	0.002
	Asp	<0.001	1	<0.001	19.538	<0.001
	Ala	<0.001	1	<0.001	2.377	0.143
	Glu	<0.001	1	<0.001	118.792	<0.001
	Thr	<0.001	1	<0.001	1.668	0.215
	Pro	<0.001	1	<0.001	0.001	0.981
	Lys	<0.001	1	<0.001	0.507	0.487
	Tyr	<0.001	1	<0.001	11.777	0.003
	Met	<0.001	1	<0.001	7.755	0.013
	Val	<0.001	1	<0.001	0.053	0.821
	Ile	<0.001	1	<0.001	2.447	0.137
	Phe	<0.001	1	<0.001	15.490	0.001
	Cys	<0.001	1	<0.001	0.022	0.883
	Leu	<0.001	1	<0.001	6.133	0.025
	Total	<0.001	1	<0.001	0.379	0.547
	EAA	<0.001	1	<0.001	7.445	0.015
	NEAA	<0.001	1	<0.001	6.950	0.018
	Ratio	0.032	1	0.032	25.508	<0.001
Nitrate*pCO_2	His	<0.001	1	<0.001	0.957	0.343

	Ser	<0.001	1	<0.001	1.802	0.198
	Arg	<0.001	1	<0.001	13.838	0.002
	Gly	<0.001	1	<0.001	10.744	0.005
	Asp	<0.001	1	<0.001	1.389	0.256
	Ala	<0.001	1	<0.001	5.762	0.029
	Glu	<0.001	1	<0.001	3.017	0.102
	Thr	<0.001	1	<0.001	5.736	0.029
	Pro	<0.001	1	<0.001	0.130	0.723
	Lys	<0.001	1	<0.001	0.076	0.786
	Tyr	<0.001	1	<0.001	9.444	0.007
	Met	<0.001	1	<0.001	6.339	0.023
	Val	<0.001	1	<0.001	7.264	0.016
	Ile	<0.001	1	<0.001	5.367	0.034
	Phe	<0.001	1	<0.001	16.100	0.001
	Cys	<0.001	1	<0.001	45.402	<0.001
	Leu	<0.001	1	<0.001	39.144	<0.001
	Total	0.001	1	0.001	22.259	<0.001
	EAA	<0.001	1	<0.001	19.401	<0.001
	NEAA	<0.001	1	<0.001	17.841	<0.001
	Ratio	0.002	1	0.002	1.919	0.185
Temperature* Nitrate*pCO_2	His	<0.001	1	<0.001	0.106	0.749
	Ser	<0.001	1	<0.001	1.769	0.202
	Arg	<0.001	1	<0.001	9.045	0.008
	Gly	<0.001	1	<0.001	7.283	0.016
	Asp	<0.001	1	<0.001	0.720	0.409
	Ala	<0.001	1	<0.001	1.929	0.184
	Glu	<0.001	1	<0.001	8.050	0.012
	Thr	<0.001	1	<0.001	2.475	0.135
	Pro	<0.001	1	<0.001	0.056	0.815
	Lys	<0.001	1	<0.001	1.942	0.183
	Tyr	<0.001	1	<0.001	6.566	0.021
	Met	<0.001	1	<0.001	3.475	0.081
	Val	<0.001	1	<0.001	1.672	0.214
	Ile	<0.001	1	<0.001	3.286	0.089
	Phe	<0.001	1	<0.001	17.441	0.001
	Cys	<0.001	1	<0.001	29.472	<0.001

	Leu	<0.001	1	<0.001	26.418	<0.001
	Total	<0.001	1	<0.001	7.958	0.012
	EAA	<0.001	1	<0.001	10.277	0.006
	NEAA	<0.001	1	<0.001	2.818	0.113
	Ratio	0.010	1	0.010	8.203	0.011
Error	His	<0.001	16	<0.001		
	Ser	<0.001	16	<0.001		
	Arg	<0.001	16	<0.001		
	Gly	<0.001	16	<0.001		
	Asp	<0.001	16	<0.001		
	Ala	<0.001	16	<0.001		
	Glu	<0.001	16	<0.001		
	Thr	<0.001	16	<0.001		
	Pro	<0.001	16	<0.001		
	Lys	<0.001	16	<0.001		
	Tyr	<0.001	16	<0.001		
	Met	<0.001	16	<0.001		
	Val	<0.001	16	<0.001		
	Ile	<0.001	16	<0.001		
	Phe	<0.001	16	<0.001		
	Cys	<0.001	16	<0.001		
	Leu	<0.001	16	<0.001		
	Total	0.001	16	0.001		
	EAA	<0.001	16	<0.001		
	NEAA	<0.001	16	<0.001		
	Ratio	0.020	16	0.001		

Table 6.24 Multivariate analysis of variance of the effects of nitrate, temperature, and pCO_2 on amino acid content of *U. rigida*. Bonferroni adjustment was used to reduce the chance of Type I error. A new alpha level of 0.0024 (0.05 divided by 21) was set since there were 21 dependent variables.

6.3.10 Fatty acids

Thirty-three fatty acids were identified of which seven accounted for less than 0.01 mg g^{-1} DW are not shown in the Table 6.25. The most abundant fatty acid was palmitic acid (C16:0). In addition, *U. rigida* had a high C18 unsaturated fatty acid content, such as C18:3_{n3} and c11 C18:1. Temperature, pCO_2, and nitrate had interactive

effects on C14:0, C18:3n_3, PUFA, and n6-FA (Table 6.26). For instance, high temperature (HT) and pCO_2 (HC) increased C14:0 by 94.44% and 5.56% respectively, along with negative effects of nitrate (–5.56%) while high temperature, pCO_2 and nitrate (HN) increased C14:0 by 211.11%. High temperature and nitrate increased C18:3n_3 by 4.97% and 5.80% respectively. High pCO_2 decreased C18:3n_3 by 4.14% while the combination of these three factors increased it by 66.46% (Table 6.25 and 6.26). Temperature and nitrate had an interactive effect on C12:0, C14:0, c9 C18, c11 C18:1, C18:2n_6, MUFA, PUFA, n3-FA, the ratio of EAA to NEAA (Table 6.26). For instance, high temperature alone increased C12:0 by 54.54%, nitrate did not have any effect, but combination of these two factors increased C12:0 by 109.09% (Table 6.25 and 6.26). Temperature and pCO_2 had an interactive effect on C12:0, C14:0, c11 C18:1, C20:0, C18:3n_6, C18:3n_3, C20:5n_3, MUFA, PUFA, and n6-FA (Table 6.26). For instance, high temperature and pCO_2 increased c11 C18:1 by 12.67% and 1.93% respectively while combination of these two factors increased it by 53.17%. Nitrate and pCO_2 had an interactive effect on C18:3n_3, PUFA, and n6-FA (Table 6.26). For instance, high nitrate increased C18:3n_3 by 5.80%, high pCO_2 decreased it by 4.14% while the combination increased it by 46.38% (Table 6.25 and 6.26). High temperature increased C12:0, C14:0, C15:0, C16:0, c9 C16:1, C18:0, c11 C18:1, c12 C18:1, C20:0, C18:3$_{n3}$, c13 C22:1, C20:5$_{n3}$, C22:5$_{n3}$, SFA, MUFA, PUFA, and n3-FA but decreased C18:2$_{n6}$, C20:3$_{n6}$, C22:4$_{n6}$, n6-FA, and n6/n3 (Table 6.25 and 6.26). High nitrate increased C14:0, C15:0, C16:0, c9 C16:1, c11 C18:1, C20:0, C18:3$_{n6}$, C18:3$_{n3}$, C22:0, C20:3$_{n6}$, c13 C22:1, C20:5$_{n3}$, C24:0, C22:4$_{n6}$, C22:5$_{n3}$, SFA, MUFA, PUFA, and n3-FA but decreased C18:2$_{n6}$, and n6/n3 (Table 6.25 and 6.26). High pCO_2 increased C12:0, C14:0, C15:0, C16:0, c9 C16:1, C18:0, c9 C18:1, c11 C18:1, C18:2$_{n6}$, C20:0, C18:3$_{n6}$, C18:3$_{n3}$, C20:5$_{n3}$, C22:5$_{n3}$, SFA, MUFA, n6-FA, n3-FA, and n6/n3 (Table 6.25 and 6.26).

Fatty acid	LTLNLC[a]	LTLNHC[b]	LTHNLC[c]	LTHNHC[d]	HTLNLC[e]	HTLNHC[f]	HTHNLC[g]	HTHNH[h]
C12:0	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.02±0.00	0.03±0.00	0.02±0.00	0.03±0.00
C14:0	0.18±0.02	0.19±0.01	0.17±0.01	0.24±0.04	0.35±0.01	0.52±0.02	0.47±0.02	0.56±0.02
c9 C14:1	0.02±0.01	0.04±0.01	0.07±0.04	0.03±0.02	0.02±0.01	0.03±0.01	0.03±0.03	0.08±0.03
C15:0	0.03±0.00	0.03±0.00	0.03±0.00	0.04±0.00	0.03±0.00	0.04±0.00	0.04±0.00	0.05±0.00
C16:0	7.30±0.61	8.15±0.67	7.05±0.15	9.84±0.52	8.83±0.56	11.70±1.03	11.31±0.85	14.24±0.38
c9 C16:1	0.31±0.02	0.36±0.01	0.33±0.01	0.46±0.08	0.47 ±0.02	0.64±0.04	0.65±0.07	0.79±0.02
C17:0	0.01±0.00	0.01±0.00	0.01±0.00	0.03±0.00	0.02±0.00	0.02±0.00	0.02±0.00	0.04±0.02
c9 C17:1	0.07±0.00	0.07±0.01	0.06±0.01	0.07±0.00	0.07±0.01	0.06±0.01	0.07±0.01	0.06±0.00
C18:0	0.14±0.02	0.15±0.04	0.11±0.00	0.15±0.01	0.16 ±0.02	0.22±0.00	0.17±0.00	0.20±0.01
c9 C18:1	0.20±0.00	0.25±0.02	0.16±0.01	0.24±0.02	0.14±0.01	0.27±0.04	0.24±0.04	0.33±0.03
c11 C18:1	3.63±0.11	3.70±0.15	3.50±0.07	4.57±0.32	4.09±0.08	5.56±0.29	5.50±0.23	6.77±0.15
c12 C18:1	0.01±0.00	0.01±0.00	0.02±0.00	0.01±0.00	0.02±0.01	0.02±0.00	0.02±0.01	0.02±0.00
$C18:2_{n6}$	1.90±0.07	2.20±0.16	1.08±0.16	1.55±0.31	0.69±0.10	1.41±0.05	0.88±0.07	1.34±0.03
$C18:2_{n3}$	0.00±0.00	0.01±0.00	0.00±0.00	0.00±0.00	0.01±0.00	0.01±0.00	0.00±0.00	0.00±0.00
C20:0	0.02±0.00	0.02±0.01	0.02±0.00	0.02±0.00	0.02±0.00	0.03±0.00	0.03±0.00	0.04±0.00
$C18:3_{n6}$	0.12±0.01	0.11±0.00	0.13±0.01	0.16±0.02	0.10 ±0.00	0.15±0.01	0.14±0.01	0.19±0.01
$C18:3_{n3}$	4.83±0.11	4.63±0.02	5.11±0.17	7.07±0.05	5.07±0.16	6.87±0.13	6.69±0.09	8.04±0.11
$C20:2_{n6}$	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.00±0.00	0.01±0.00	0.01±0.00	0.01±0.00
C22:0	0.38±0.03	0.40±0.04	0.47±0.01	0.53±0.09	0.32±0.07	0.45±0.08	0.57±0.16	0.74±0.10
$C20:3_{n6}$	0.07±0.01	0.06±0.01	0.09±0.00	0.11±0.01	0.05±0.00	0.06±0.00	0.07±0.01	0.08±0.01
c13 C22:1	0.03±0.00	0.02±0.00	0.03±0.00	0.03±0.00	0.04±0.00	0.05±0.00	0.05±0.00	0.05±0.01
$C20:4_{n6}$	0.11±0.01	0.09±0.01	0.12±0.02	0.16±0.07	0.09±0.01	0.14±0.01	0.13±0.00	0.16±0.00
$C20:5_{n3}$	0.25±0.02	0.23±0.00	0.26±0.00	0.33±0.06	0.41±0.01	0.55±0.02	0.56±0.01	0.64±0.01
C24:0	0.03±0.00	0.03±0.00	0.03±0.00	0.04±0.01	0.02±0.01	0.04±0.02	0.05±0.02	0.06±0.01
$C22:4_{n6}$	0.04±0.00	0.03±0.00	0.05±0.01	0.04±0.01	0.02±0.00	0.03±0.00	0.03±0.00	0.04±0.00
$C22:5_{n3}$	1.03±0.09	0.98±0.08	1.03±0.02	1.30±0.12	1.02±0.01	1.26±0.05	1.38±0.08	1.50±0.04
SFA[i]	8.11±0.64	9.00±0.68	7.91±0.16	10.91±0.50	9.78±0.64	13.06±0.98	12.69±0.95	15.98±0.30
MUFA[j]	4.27±0.14	4.46±0.16	4.16±0.09	5.43±0.39	4.86±0.11	6.64±0.30	6.58±0.35	8.13±0.23
PUFA[k]	8.39±0.26	8.36±0.17	7.90±0.39	10.75±0.42	7.50±0.23	10.52±0.25	9.93±0.19	12.05±0.15
n6-FA[l]	2.27±0.10	2.51±0.16	1.49±0.21	2.04±0.32	0.98±0.11	1.82±0.06	1.28±0.08	1.84±0.05
n3-FA[m]	6.11±0.17	5.84±0.11	6.40±0.18	8.71±0.12	6.51±0.17	8.68±0.19	8.64±0.19	10.20±0.12

n6/n3	0.37±0.01	0.43±0.03	0.23±0.03	0.23±0.03	0.15±0.02	0.21±0.00	0.15±0.01	0.18±0.00

Table 6.25 Fatty acids composition of *U. rigida* grown under different conditions (mg g^{-1} DW). Values are means of three replicates ± standard deviation. [a]LTLNLC, low temperature, low nitrate and low pCO_2. [b]LTLNHC, low temperature, low nitrate and high pCO_2. [c]LTHNLC, low temperature, high nitrate and low pCO_2. [d]LTHNHC, low temperature, high nitrate and high pCO_2. [e]HTLNLC, high temperature, low nitrate and low pCO_2. [f]HTLNHC, high temperature, low nitrate and high pCO_2. [g]HTHNLC, high temperature, high nitrate and low pCO_2. [h]HTHNHC, high temperature, high nitrate and high pCO_2. [i]SFA, saturated fatty acids. [j]MUFA, monounsaturated fatty acids. [k]PUFA, polyunsaturated fatty acids. [l]n6-FA, total Omega-6 fatty acids. [m]n3-FA, total Omega-3 fatty acids.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Temperature	C12:0	12.606	1	12.606	673.335	<0.001
	C14:0	4682.274	1	4682.274	1232.128	<0.001
	c9 C14:1	0.059	1	0.059	0.013	0.910
	C15:0	4.727	1	4.727	84.814	<0.001
	C16:0	707463.865	1	707463.865	168.507	<0.001
	c9 C16:1	4432.793	1	4432.793	237.985	<0.001
	C17:0	2.110	1	2.110	5.591	0.031
	c9 C17:1	0.003	1	0.003	0.009	0.925
	C18:0	144.860	1	144.860	45.631	<0.001
	c9 C18:1	66.808	1	66.808	10.255	0.006
	c11 C18:1	160110.558	1	160110.558	424.661	<0.001
	c12 C18:1	5.645	1	5.645	27.723	<0.001
	$C18:2_{n6}$	21739.577	1	21739.577	101.191	<0.001
	$C18:2_{n3}$	0.169	1	0.169	2.441	0.138
	C20:0	11.060	1	11.060	179.606	<0.001
	$C18:3_{n6}$	12.252	1	12.252	13.560	0.002
	$C18:3_{n3}$	94985.296	1	94985.296	711.925	<0.001
	$C20:2_{n6}$	0.020	1	0.020	0.888	0.360
	C22:0	324.635	1	324.635	4.743	0.045
	$C20:3_{n6}$	14.160	1	14.160	33.746	<0.001
	c13 C22:1	21.622	1	21.622	320.534	<0.001
	$C20:4_{n6}$	4.875	1	4.875	0.728	0.406
	$C20:5_{n3}$	4458.916	1	4458.916	755.019	<0.001
	C24:0	6.793	1	6.793	8.114	0.012
	$C22:4_{n6}$	4.518	1	4.518	22.337	<0.001
	$C22:5_{n3}$	2640.228	1	2640.228	52.513	<0.001
	SFA[a]	909837.320	1	909837.320	206.909	<0.001
	MUFA[b]	233819.989	1	233819.989	390.527	<0.001
	PUFA[c]	79127.840	1	79127.840	105.417	<0.001
	n6-FA[d]	21287.160	1	21287.160	81.352	<0.001
	n3-FA[e]	181648.584	1	181648.584	713.540	<0.001
	n6/n3	0.125	1	0.125	290.573	<0.001
Nitrate	C12:0	0.033	1	0.033	1.743	0.205
	C14:0	138.871	1	138.871	36.543	<0.001

	c9 C14:1	36.607	1	36.607	8.205	0.011
	C15:0	4.898	1	4.898	87.878	<0.001
	C16:0	156340.317	1	156340.317	37.238	<0.001
	c9 C16:1	799.245	1	799.245	42.909	<0.001
	C17:0	5.890	1	5.890	15.607	0.001
	c9 C17:1	0.061	1	0.061	0.168	0.687
	C18:0	5.184	1	5.184	1.633	0.220
	c9 C18:1	50.766	1	50.766	7.792	0.013
	c11 C18:1	42154.687	1	42154.687	111.807	<0.001
	c12 C18:1	0.466	1	0.466	2.288	0.150
	$C18:2_{n6}$	7038.989	1	7038.989	32.764	<0.001
	$C18:2_{n3}$	0.002	1	0.002	0.036	0.853
	C20:0	2.846	1	2.846	46.223	<0.001
	$C18:3_{n6}$	75.101	1	75.101	83.120	<0.001
	$C18:3_{n3}$	113964.836	1	113964.836	854.178	<0.001
	$C20:2_{n6}$	0.270	1	0.270	12.168	0.003
	C22:0	2135.145	1	2135.145	31.198	<0.001
	$C20:3_{n6}$	38.372	1	38.372	91.446	<0.001
	c13 C22:1	3.145	1	3.145	46.616	<0.001
	$C20:4_{n6}$	71.144	1	71.144	10.621	0.005
	$C20:5_{n3}$	500.869	1	500.869	84.811	<0.001
	C24:0	14.750	1	14.750	17.620	0.001
	$C22:4_{n6}$	6.807	1	6.807	33.655	<0.001
	$C22:5_{n3}$	3253.219	1	3253.219	64.705	<0.001
	SFA[a]	212518.051	1	212518.051	48.329	<0.001
	MUFA[b]	62071.453	1	62071.453	103.672	<0.001
	PUFA[c]	129041.422	1	129041.422	171.913	<0.001
	n6-FA[d]	3267.719	1	3267.719	12.488	0.003
	n3-FA[e]	173314.373	1	173314.373	680.802	<0.001
	n6/n3	0.050	1	0.050	115.350	<0.001
pCO_2	C12:0	1.839	1	1.839	98.208	<0.001
	C14:0	436.384	1	436.384	114.833	<0.001
	c9 C14:1	1.528	1	1.528	0.342	0.567
	C15:0	3.759	1	3.759	67.456	<0.001
	C16:0	334631.778	1	334631.778	79.704	<0.001
	c9 C16:1	852.178	1	852.178	45.751	<0.001

	C17:0	2.692	1	2.692	7.133	0.017
	c9 C17:1	0.034	1	0.034	0.094	0.764
	C18:0	72.215	1	72.215	22.748	<0.001
	c9 C18:1	504.545	1	504.545	77.446	<0.001
	c11 C18:1	56905.903	1	56905.903	150.931	<0.001
	c12 C18:1	0.095	1	0.095	0.464	0.505
	$C18:2_{n6}$	14135.281	1	14135.281	65.796	<0.001
	$C18:2_{n3}$	0.097	1	0.097	1.395	0.255
	C20:0	2.601	1	2.601	42.244	<0.001
	$C18:3_{n6}$	52.124	1	52.124	57.690	<0.001
	$C18:3_{n3}$	90645.442	1	90645.442	679.397	<0.001
	$C20:2_{n6}$	0.087	1	0.087	3.923	0.065
	C22:0	560.112	1	560.112	8.184	0.011
	$C20:3_{n6}$	1.782	1	1.782	4.247	0.056
	c13 C22:1	0.877	1	0.877	12.997	0.002
	$C20:4_{n6}$	37.280	1	37.280	5.565	0.031
	$C20:5_{n3}$	281.291	1	281.291	47.630	<0.001
	C24:0	3.084	1	3.084	3.684	0.073
	$C22:4_{n6}$	0.186	1	0.186	0.922	0.351
	$C22:5_{n3}$	1259.074	1	1259.074	25.042	<0.001
	SFA[a]	410353.842	1	410353.842	93.320	<0.001
	MUFA[b]	85327.754	1	85327.754	142.515	<0.001
	PUFA[c]	237166.055	1	237166.055	315.960	<0.001
	n6-FA[d]	17778.811	1	17778.811	67.945	<0.001
	n3-FA[e]	125041.615	1	125041.615	491.180	<0.001
	n6/n3	0.008	1	0.008	17.951	0.001
Temperature* Nitrate	C12:0	.0304	1	0.304	16.240	0.001
	C14:0	66.139	1	66.139	17.404	0.001
	c9 C14:1	2.412	1	2.412	.541	0.473
	C15:0	0.121	1	0.121	2.168	0.160
	C16:0	48120.534	1	48120.534	11.462	0.004
	c9 C16:1	173.838	1	173.838	9.333	0.008
	C17:0	1.066	1	1.066	2.824	0.112
	c9 C17:1	0.744	1	.744	2.055	0.171
	C18:0	0.745	1	.745	.235	0.635
	c9 C18:1	171.538	1	171.538	26.330	<0.001

	c11 C18:1	13233.599	1	13233.599	35.099	<0.001
	c12 C18:1	0.078	1	0.078	.384	0.544
	$C18:2_{n6}$	9425.740	1	9425.740	43.874	<0.001
	$C18:2_{n3}$	0.050	1	0.050	0.720	0.409
	C20:0	0.497	1	0.497	8.069	0.012
	$C18:3_{n6}$	3.001	1	3.001	3.321	0.087
	$C18:3_{n3}$	31.522	1	31.522	0.236	0.634
	$C20:2_{n6}$	0.206	1	0.206	9.281	0.008
	C22:0	358.931	1	358.931	5.245	0.036
	$C20:3_{n6}$	2.238	1	2.238	5.334	0.035
	c13 C22:1	0.095	1	.095	1.410	0.252
	$C20:4_{n6}$	1.314	1	1.314	0.196	0.664
	$C20:5_{n3}$	65.583	1	65.583	11.105	0.004
	C24:0	4.471	1	4.471	5.341	0.034
	$C22:4_{n6}$	0.005	1	.005	.025	0.876
	$C22:5_{n3}$	294.386	1	294.386	5.855	0.028
	SFA[a]	63704.721	1	63704.721	14.487	0.002
	MUFA[b]	20622.855	1	20622.855	34.444	<0.001
	PUFA[c]	16033.037	1	16033.037	21.360	<0.001
	n6-FA[d]	9286.160	1	9286.160	35.489	<0.001
	n3-FA[e]	947.474	1	947.474	3.722	0.072
	n6/n3	0.035	1	.035	80.398	<0.001
Temperature*pCO_2	C12:0	0.823	1	.823	43.935	<0.001
	C14:0	116.307	1	116.307	30.606	<0.001
	c9 C14:1	18.751	1	18.751	4.203	0.057
	C15:0	0.284	1	0.284	5.100	0.038
	C16:0	17554.322	1	17554.322	4.181	0.058
	c9 C16:1	72.153	1	72.153	3.874	0.067
	C17:0	0.077	1	0.077	0.205	0.657
	c9 C17:1	3.893	1	3.893	10.755	0.005
	C18:0	7.245	1	7.245	2.282	0.150
	c9 C18:1	32.054	1	32.054	4.920	0.041
	c11 C18:1	9472.590	1	9472.590	25.124	<0.001
	c12 C18:1	0.012	1	0.012	0.060	0.810
	$C18:2_{n6}$	649.161	1	649.161	3.022	0.101
	$C18:2_{n3}$	0.193	1	.193	2.775	0.115

	C20:0	1.657	1	1.657	26.904	<0.001
	$C18:3_{n6}$	29.878	1	29.878	33.069	<0.001
	$C18:3_{n3}$	7277.386	1	7277.386	54.545	<0.001
	$C20:2_{n6}$	0.065	1	0.065	2.939	0.106
	C22:0	198.811	1	198.811	2.905	0.108
	$C20:3_{n6}$	0.125	1	0.125	0.298	0.593
	c13 C22:1	0.348	1	0.348	5.152	0.037
	$C20:4_{n6}$	12.467	1	12.467	1.861	0.191
	$C20:5_{n3}$	104.139	1	104.139	17.634	0.001
	C24:0	0.815	1	0.815	0.974	0.338
	$C22:4_{n6}$	1.952	1	1.952	9.651	0.007
	$C22:5_{n3}$	82.523	1	82.523	1.641	0.218
	SFA[a]	27052.476	1	27052.476	6.152	0.025
	MUFA[b]	13160.102	1	13160.102	21.980	<0.001
	PUFA[c]	20010.034	1	20010.034	26.658	<0.001
	n6-FA[d]	1320.639	1	1320.639	5.047	0.039
	n3-FA[e]	10717.110	1	10717.110	42.098	<0.001
	n6/n3	0.001	1	0.001	1.175	0.294
Nitrate*pCO_2	C12:0	0.032	1	0.032	1.713	0.209
	C14:0	0.516	1	0.516	0.136	0.717
	c9 C14:1	0.161	1	0.161	0.036	0.852
	C15:0	0.554	1	0.554	9.940	0.006
	C16:0	14923.566	1	14923.566	3.555	0.078
	c9 C16:1	8.697	1	8.697	0.467	0.504
	C17:0	3.466	1	3.466	9.184	0.008
	c9 C17:1	0.757	1	0.757	2.092	0.167
	C18:0	0.070	1	0.070	0.022	0.884
	c9 C18:1	0.880	1	0.880	0.135	0.718
	c11 C18:1	2434.533	1	2434.533	6.457	0.022
	c12 C18:1	0.164	1	0.164	0.807	0.382
	$C18:2_{n6}$	27.549	1	27.549	0.128	0.725
	$C18:2_{n3}$	0.028	1	0.028	0.401	0.536
	C20:0	0.172	1	0.172	2.801	0.114
	$C18:3_{n6}$	6.317	1	6.317	6.992	0.018
	$C18:3_{n3}$	10967.976	1	10967.976	82.206	<0.001
	$C20:2_{n6}$	0.019	1	0.019	0.865	0.366

	C22:0	21.473	1	21.473	0.314	0.583
	$C20:3_{n6}$	1.223	1	1.223	2.914	0.107
	c13 C22:1	0.007	1	0.007	0.099	0.757
	$C20:4_{n6}$	9.918	1	9.918	1.481	0.241
	$C20:5_{n3}$	7.332	1	7.332	1.241	0.282
	C24:0	0.052	1	0.052	0.062	0.806
	$C22:4_{n6}$	0.282	1	0.282	1.392	0.255
	$C22:5_{n3}$	147.087	1	147.087	2.925	0.107
	SFA[a]	16686.473	1	16686.473	3.795	0.069
	MUFA[b]	2701.994	1	2701.994	4.513	0.050
	PUFA[c]	14810.702	1	14810.702	19.731	<0.001
	n6-FA[d]	4.898	1	4.898	0.019	0.893
	n3-FA[e]	14551.025	1	14551.025	57.158	<0.001
	n6/n3	0.003	1	0.003	6.068	0.025
Temperature* Nitrate*pCO_2	C12:0	0.245	1	0.245	13.096	0.002
	C14:0	56.279	1	56.279	14.810	0.001
	c9 C14:1	27.814	1	27.814	6.234	0.024
	C15:0	0.011	1	0.011	0.193	0.667
	C16:0	13225.498	1	13225.498	3.150	0.095
	c9 C16:1	39.770	1	39.770	2.135	0.163
	C17:0	0.001	1	0.001	0.001	0.971
	c9 C17:1	0.231	1	0.231	0.637	0.437
	C18:0	13.990	1	13.990	4.407	0.052
	c9 C18:1	16.064	1	16.064	2.466	0.136
	c11 C18:1	5460.838	1	5460.838	14.484	0.002
	c12 C18:1	0.120	1	0.120	0.590	0.453
	$C18:2_{n6}$	697.709	1	697.709	3.248	0.090
	$C18:2_{n3}$	0.123	1	0.123	1.771	0.202
	C20:0	7.499E-5	1	7.499E-5	0.001	0.973
	$C18:3_{n6}$	3.226	1	3.226	3.570	0.077
	$C18:3_{n3}$	25795.574	1	25795.574	193.341	<0.001
	$C20:2_{n6}$	0.024	1	0.024	1.102	0.309
	C22:0	0.628	1	0.628	0.009	0.925
	$C20:3_{n6}$	3.220	1	3.220	7.673	0.014
	c13 C22:1	0.770	1	0.770	11.411	0.004
	$C20:4_{n6}$	29.661	1	29.661	4.428	0.052

	$C20:5_{n3}$	83.671	1	83.671	14.168	0.002
	C24:0	0.043	1	0.043	0.051	0.824
	$C22:4_{n6}$	0.239	1	0.239	1.182	0.293
	$C22:5_{n3}$	724.813	1	724.813	14.416	0.002
	SFA[a]	16726.049	1	16726.049	3.804	0.069
	MUFA[b]	6478.125	1	6478.125	10.820	0.005
	PUFA[c]	54012.394	1	54012.394	71.957	<0.001
	n6-FA[d]	1291.146	1	1291.146	4.934	0.041
	n3-FA[e]	38384.596	1	38384.596	150.780	<0.001
	n6/n3	<0.001	1	<0.001	0.476	0.500
Error	C12:0	0.300	16	0.019		
	C14:0	60.802	16	3.800		
	c9 C14:1	71.382	16	4.461		
	C15:0	0.892	16	0.056		
	C16:0	67174.760	16	4198.422		
	c9 C16:1	298.022	16	18.626		
	C17:0	6.038	16	.377		
	c9 C17:1	5.792	16	.362		
	C18:0	50.794	16	3.175		
	c9 C18:1	104.237	16	6.515		
	c11 C18:1	6032.509	16	377.032		
	c12 C18:1	3.258	16	0.204		
	$C18:2_{n6}$	3437.383	16	214.836		
	$C18:2_{n3}$	1.111	16	0.069		
	C20:0	0.985	16	0.062		
	$C18:3_{n6}$	14.456	16	0.904		
	$C18:3_{n3}$	2134.727	16	133.420		
	$C20:2_{n6}$	0.355	16	0.022		
	C22:0	1095.017	16	68.439		
	$C20:3_{n6}$	6.714	16	0.420		
	c13 C22:1	1.079	16	0.067		
	$C20:4_{n6}$	107.179	16	6.699		
	$C20:5_{n3}$	94.491	16	5.906		
	C24:0	13.395	16	0.837		
	$C22:4_{n6}$	3.236	16	0.202		
	$C22:5_{n3}$	804.445	16	50.278		

SFA[a]	70356.610	16	4397.288
MUFA[b]	9579.660	16	598.729
PUFA[c]	12009.925	16	750.620
n6-FA[d]	4186.662	16	261.666
n3-FA[e]	4073.183	16	254.574
n6/n3	0.007	16	<0.001

Table 6.26 Multivariate analysis of variance of the effects of nitrate, temperature, and pCO_2 on fatty acid content of *U. rigida*. [a]SFA, saturated fatty acids. [b]MUFA, monounsaturated fatty acids. [c]PUFA, polyunsaturated fatty acids. [d]n6-FA, total Omega-6 fatty acids. [e]n3-FA, total Omega-3 fatty acids. Bonferroni adjustment was used to reduce the chance of Type I error. A new alpha level of 0.0016 (0.05 divided by 32) was set since there were 32 dependent variables.

6.3.11 Functional properties

The swelling capacity (SWC) trials varied from 7.59 $\pm$0.17 ml g^{-1} DW to 11.24 $\pm$0.17 ml g^{-1} DW (Figure 6.9 a). Nitrate, temperature, and pCO_2 had an interactive effect on SWC (Table 6.27). High nitrate (HNLTLC), high pCO_2 (LNLTHC) alone did not affect SWC, high (LNHTLC) temperature increased it by 25.12% but the combination of these three factors (HNHTHC) increased SWC by 15.60% compared to LNLTLC. Both temperature and nitrate had a main effect on SWC (Table 6.27). The high temperature treatment enhanced SWC by 33.17% compared to the low temperature while high nitrate reduced SWC by 5.52%. pCO_2 did not affect the SWC (Table 6.27). As far as water holding capacity (WHC) was concerned (Figure 6.9 b), the range was 5.22 $\pm$0.10 to 7.24 $\pm$0.04 g g^{-1} DW when *U. rigida* were grown under different conditions. Temperature and nitrate had interactive effects on WHC (Table 6.28) since high temperature enhanced WHC more under high nitrate (10.96%) than low nitrate (23.51%) (Figure 6.9 b). Both temperature and nitrate had a main effect on WHC (Table 6.28). The high temperature enhanced WHC by 16.97% while high nitrate reduced WHC by 8.08% (Figure 6.9 b). The pCO_2 treatments were non-significant (Table 6.28). The pattern of protein + carbohydrate (PC) was similar to SWC and WHC. Both temperature and nitrate had a main effect on PC (Table 6.29). The high temperature enhanced PC by 5.66% while high nitrate reduced it by 4.02% (Figure 6.9 c). The pCO_2 did not affect PC (Table 6.29).

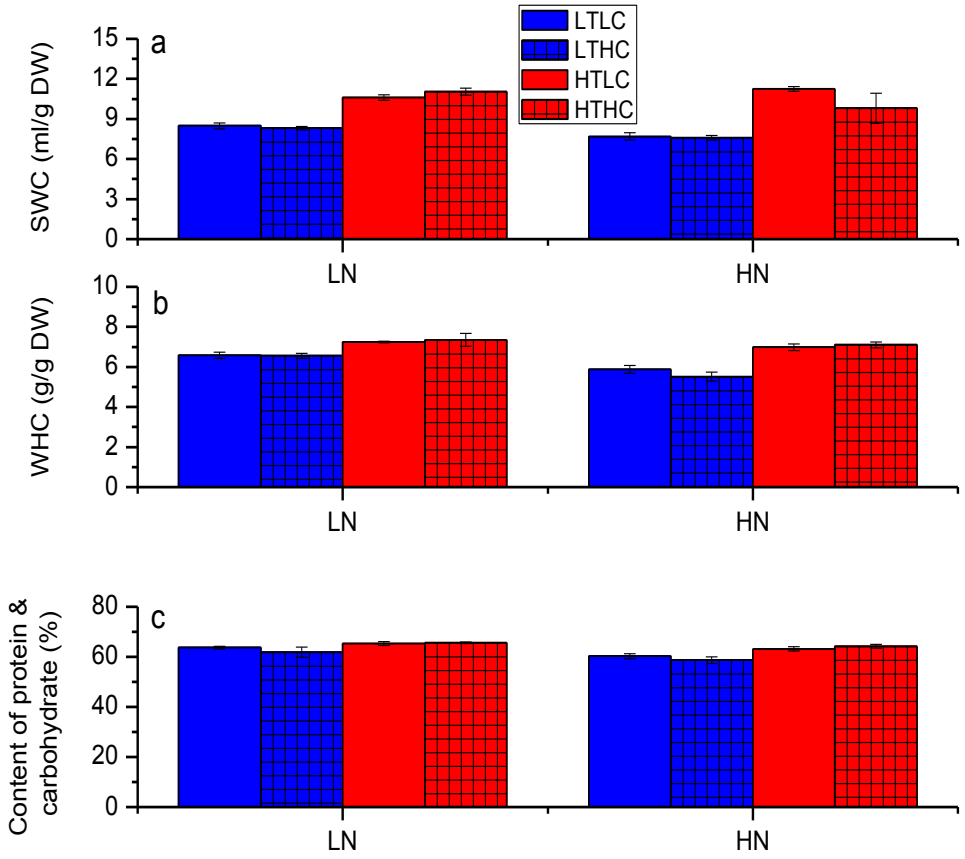

Figure 6.9 Swelling capacity (a), water holding capacity (b) and content of protein and carbohydrate (c) in *U. rigida* grown under different conditions. LTLC: low temperature and low pCO_2; LTHC: low temperature and high pCO_2; HTLC: high temperature and low pCO_2; HTHC: high temperature and high pCO_2; LN: low nitrate; HN: high nitrate. Data are the means ± SD (n = 3).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	1.712	1	1.712	8.675	0.010
Temperature	42.522	1	42.522	215.470	<0.001
pCO_2	0.607	1	0.607	3.073	0.099
Nitrate*Temperature	0.310	1	0.310	1.570	0.228
Nitrate*pCO_2	1.200	1	1.200	6.081	0.055
Temperature*pCO_2	0.202	1	0.202	1.023	0.327
Nitrate*Temperature*pCO_2	1.422	1	1.422	7.208	0.016
Error	3.158	16	0.197		

Table 6.27 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on swelling capacity of *U. rigida*. The confidence interval was 95%.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	1.896	1	1.896	53.586	<0.001
Temperature	6.432	1	6.432	181.803	<0.001
pCO_2	0.010	1	0.010	0.289	0.598
Nitrate*Temperature	0.581	1	0.581	16.425	0.001
Nitrate*pCO_2	0.038	1	0.038	1.083	0.313
Temperature*pCO_2	0.144	1	0.144	4.079	0.061
Nitrate*Temperature * pCO_2	0.045	1	0.045	1.277	0.275
Error	0.566	16	0.035		

Table 6.28 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on water holding capacity of *U. rigida*. The confidence interval was 95%.

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	40.074	1	40.074	33.762	<0.001
Temperature	71.758	1	71.758	60.454	<0.001
pCO_2	1.573	1	1.573	1.325	0.267
Nitrate*Temperature	3.571	1	3.571	3.009	0.102
Nitrate*pCO_2	0.385	1	0.385	0.324	0.577
Temperature*pCO_2	8.360	1	8.360	7.043	0.057
Nitrate*Temperature* pCO_2	0.112	1	0.112	0.095	0.762
Error	18.992	16	1.187		

Table 6.29 Three-way analysis of variance of effects of the nitrate, temperature, and pCO_2 on protein + carbohydrate content of *U. rigida*. The confidence interval was 95%.

The OHC of *U. rigida* in this study ranged from 1.46 ± 0.08–1.84 ± 0.07 g g^{-1} DW (Figure 6.10). There were on interactive effects of temperature, nitrate and pCO_2 on OHC. Temperature had a main effect on OHC (Table 6.30). Temperature had a main effect on OHC (Table 6.30). The high temperature (HT) treatment enhanced the oil holding capacity (OHC) by 18.8% (Figure 6.10) while no significant effects of nitrate or pCO_2 were found (Table 6.30).

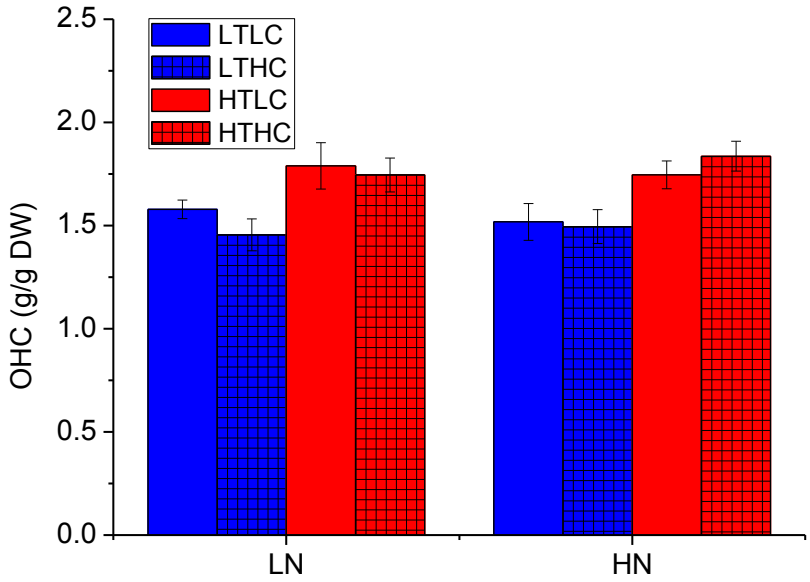

Figure 6.10 Oil holding capacity of *U. rigida* grown under different conditions. LTLC: low temperature and low pCO_2; LTHC: low temperature and high pCO_2; HTLC: high temperature and low pCO_2; HTHC: high temperature and high pCO_2; LN: low nitrate; HN: high nitrate. Data are the means ± SD (n = 3).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Nitrate	<0.001	1	<0.001	0.041	0.841
Temperature	0.429	1	0.429	66.168	<0.001
pCO_2	0.004	1	0.004	0.579	0.458
Nitrate*Temperature	0.002	1	0.002	0.269	0.611
Nitrate*pCO_2	0.021	1	0.021	3.176	0.094
Temperature*pCO_2	0.014	1	0.014	2.147	0.162
Nitrate*Temperature * pCO_2	<0.001	1	0.000	0.065	0.801
Error	0.104	16	0.006		

Table 6.30 Three-way analysis of variance of the effects of nitrate, temperature, and pCO_2 on oil holding capacity of *U. rigida*. The confidence interval was 95%.

6.4 Discussion

6.4.1 Settlement and germination

Following their release from fertile thalli and subsequent dispersal, swarmers need to locate and adhere to a surface in order to complete their life history. Settlement

is the stage whereby swarmers detect a suitable surface, lose their flagella, secrete an adhesive and produce a new cell wall, which is indispensable for the initiation of germination and subsequent growth of the germling. This process is regulated by the perception of a series of chemotactic signals as well as physical and chemical interactions with the surface (Callow and Callow, 2011). In the present study, high temperature significantly enhanced the settlement of gametes. Likewise, the number of settled zoospores in *U. intestinalis* and *U. compressa* increased with temperature (Christie and Shaw, 1968, Callow et al., 1997). However, the proportion of settled gametes in the present study was much lower, even under optimal conditions, compared to that of zoospores (Christie and Shaw, 1968, Callow et al., 1997). It was reported that spores could settle down in minutes and over 60% adhered within three hours (Callow et al., 1997). The low settlement of gametes was also found by Callow et al. (1997). One possible reason might be that gametes prefer to mate and fuse with the opposite sex rather than settle alone. In times of stress, sexual reproduction is often preferred as the exchange of genetic material can help improve the capacity for the next generation to adapt to the changing conditions (Zinn et al., 2010). In contrast, asexual reproduction does not afford any genetic recombination and therefore is less suited to adapting to stressful environmental conditions. Parthenogenetic development of gametes may occur when fusion cannot be met whereas direct settlement is the only option for spores.

Germination of *U. rigida* cultured under the low temperature treatment was lower than that under high temperature, though there was no significant difference eight days later. Temperature is considered an important factor for green tide development in relation to control of growth rate (Liu et al., 2013a). This study indicates that high temperature triggers green tide not only by stimulating growth of *Ulva* but also by shortening the time to settlement and germination. The impact of such a shortened settlement and germination time would mean that fewer gametes would be lost to predation (Xu and Yang, 2007), thereby releasing green tide algae from some of the pressures of grazer control. While it cannot be argued that this shortened settlement time is an adaptive strategy, there would seem to be inadvertent benefits for *Ulva* through the reduction of grazing pressure in the planktonic phase of the life cycle. The positive effects of temperature on germination were magnified further under high CO_2 during the first four days of culture, which suggests that future ocean warming and acidification would synergistically support green tide. *Ulva* species have been described as opportunists (Charlier et al., 2008). The results of the current experiments in relation to

key climate change parameters would tend to support this appraisal in so far as *U. rigida* appears to be sufficiently plastic in its settlement and germination patterns to allow it to take advantage of changing environmental conditions. *Ulva* will be competing with many other organisms for settlement sites, an accelerated settlement and germination process may therefore afford *Ulva* species with a vital competitive advantage in a climate change impacted ocean.

High nitrate reduced the settlement of *U. rigida* gametes, particularly within the first 24 hours. This may be attributed to high nitrogen toxicity on gametes as it has been reported that *Ulva* spores had a higher sensitivity to changes in the external nutrient concentrations than adult plants (Sousa et al., 2007). On the other hand, high nitrate increased germination of gametes. As swarmers of *Ulva* are naked (i.e. lacking cell walls), they are vulnerable to environmental change (Callow and Callow, 2006). After settlement, a new cell wall is produced, improving the cell's ability to deal with environmental stress. While it was not possible to directly assess toxicity in the present study, it would be enlightening to have looked at cellular measures of nitrogen stress such as changes in nitrate reductase—an enzyme that is widely used to process nitrogen generated free radicals (Corzo and Neill, 1991). As mentioned in earlier chapters, it would also be informative to undertake transcriptomic studies in response to nitrogen stress (as well as temperature and pH stressors). Although *Ulva* is considered as a difficult group to study at the genomic level, intense efforts from numerous laboratories across the world have succeeded in developing *Ulva* transcriomic tools (e.g. Zhang et al., 2012).

6.4.2 Different responses of young and adult **U. rigida** *in growth and reproduction*

The effects of pCO_2, temperature, and nitrate on the growth of young and adult *U. rigida* were studied for the first time. Growth of young *U. rigida* was much faster than that of adult plants irrespective of culture conditions. For instance, the specific growth rate of young *U. rigida* reached up to 42.1 ± 0.3% under HTHCHN, while it was only 6.3 ± 0.6% in adult *U. rigida*. The specific growth rate of young *Ulva* in this study was higher than the 22% over a 50-day culture period reported by Xu and Gao (2012) for *U. prolifera*. A four-hour longer day length used in the present study may account for the difference. A similar increase of 43% was reported for *U. intestinalis* when cultured on a 16-hour daily photoperiod for 14 days (Kim and Lee, 1996). The low growth of adult *Ulva* was consistent with previous studies (Ale et al., 2011, Mantri et al., 2011). The reasons for the marked differences in growth rate between young and adult

Ulva could be due to differentiation of *Ulva*. Early in the life history, all cells are initially of the same type, developing from one single cell. Cell division proceeds fast during this stage. Thereafter, cells differentiate into two types: rhizoidal cells in the basal part of the thallus and blade cells in the marginal part. Differences in cell size and photosynthetic pigments between these two cell types result in unequal growth in the thallus (Han et al., 2003, Lüning et al., 2008), which slows down the total growth of the thallus with age. This could partially explain the lower growth of adult *U. rigida*. Reproduction is another tangible reason causing a reduction in the growth of adult *Ulva* since reproduction can stop the growth of *Ulva* and the release of swarmers leads to a loss of thallus mass. In this study, the young *U. rigida* did not reproduce over 60 days of culture, while adult *U. rigida* became reproductive on day eight. It seems there is less possibility for young *Ulva* to reproduce as an extracellular sporulation inhibitor is excreted at an early stage. The excretion of a sporulation inhibitor decreases with maturation of the thalli, which allows vegetative cells to transform to reproductive cells in mature *Ulva* (Stratmann et al., 1996).

Nitrate played a very important role in the growth of young *U. rigida*. The mass of individuals grown under low nitrate was only 2.8–7.7% of that under high nitrate over a 60-day period (Figure 6.3 a). By contrast, the effect of nitrate on adult *U. rigida* changed with time. Nitrate was less important than temperature in terms of promoting growth during the first four days of culture but it replaced temperature as the most important factor after that. The different responses of young and adult *U. rigida* to nitrate and the increasing dependence of adult *Ulva* on nitrate may be due to differences in the tolerance of young and adult *U. rigida* to low nitrate. It is possible that gametes do not have enough nitrate reserves to maintain fast propagation and gamete energy reserves might be consumed during swimming and settling. Therefore, low nitrate seriously inhibits growth of germlings. In contrast, adult *Ulva* may develop strategies to deal with low nitrate. Adult *Ulva* can take advantage of internal nitrate reserves to overcome short-term nitrate limitation for example (Viaroli et al., 1996). But when nitrate reserves run out, the effect of nitrate limitation emerges.

Temperature is vital for growth of *U. rigida*. High temperature significantly enhanced the growth of both young and adult *U. rigida* during the first eight days of culture. Acceleration of growth at moderately elevated temperature is a common phenomenon among seaweeds due to increasing metabolic activity (Kim and Lee, 1996, Mantri et al., 2011). On the other hand, high temperature decreased the growth of adult

U. rigida by day 12. Positive effects of high temperature on adult *U. rigida* were completely converted into negative effects. This could be caused by more reproductive thalli at the higher experimental temperature. Meanwhile, *Ulva* thalli at the low temperature did not show any signs of reproduction during 12 days of culture. Likewise, the reproductive rhythm of *U. fenestrata* decreased from 30 to five days when temperature was increased from 10 to 20 ℃ (Kalita and Titlyanov, 2011). Clearly temperature is an important environmental cue regulating both growth and reproduction in *Ulva*.

Any of the two factors examined had interactive effects on growth of both young and adult *U. rigida* (day 12) but in different directions. All interactive effects in young *U. rigida* were positive while high temperature and pCO_2 decreased growth of adult *U. rigida* synergistically (day12), which can be attributed to more reproduction. This is the first report of high pCO_2 inducing reproduction of seaweed. The possible reason is increased photosynthesis (growth) of *Ulva* under high pCO_2 because photosynthesis produces organic matter that is required for reproduction (Lüning et al., 2008). This inductive effect was significantly greater under high nitrate or high temperature condition by day 12. Again it would be enlightening to be able to undertake detailed transcriptomic analysis throughout the *Ulva* life cycle under simulated climate change conditions. It is likely that entire gene networks are up or down regulated in response to environmental changes. Ecologically speaking, *Ulva* species would be ideal candidates for such analysis given their rapid life cycle and evident phenotypic plasticity. Whereas transcriptomic tools are slowly becoming available for *Ulva*, it was well beyond the scope of this project to engage in complex molecular investigation. Clearly, such studies will provide definitive answers and explanations for many, if not all of the trends observed in this thesis.

6.4.3 Ulva *cultivation*

High temperature shortened the time needed for gametes to settle. High temperature, pCO_2 and nitrate enhanced the germination rate; these factors also stimulated growth of young and early-stage adult *U. rigida*. This indicates that ocean warming, acidification combined with eutrophication may stimulate *Ulva* productivity and hence favour *Ulva* cultivation; therefore from an aquaculture point of view an ocean affected by climate change offers opportunities for *Ulva* farming. On the other hand, High temperature, pCO_2, and nitrate led to increased reproduction, which indicates future ocean environment (Baede et al., 2001) may pose significant challenges for *Ulva*

cultivation. This disadvantage could be eliminated by harvesting *Ulva* thalli before they become reproductive. These findings in the context of climate change can also be applied to *Ulva* cultivation in terms of deliberate manipulation of culture conditions or selecting advantageous culture sites. Higher temperatures, nitrate, and CO_2 levels are preferable when setting up culture operations. Furthermore, locating near to a power station or other such point source of CO_2 would be ideal for *Ulva* cultivation as waste heat from the power station can be utilised to increase the temperature of culture seawater and emitted CO_2 (flue gas) can aerate and enrich the seawater. This would stimulate *Ulva* growth and slow the rate of CO_2 release to atmosphere at the same time. Alternatively, effluent from aquaculture can be loaded into the cultivation system of *Ulva*, from which both *Ulva* cultivation and the water environment would benefit. From this admittedly biased practical viewpoint, *Ulva* being opportunists is a distinct advantage.

6.4.4 Green tide

While the predicted effects of ocean acidification, warming and eutrophication on settlement, germination, growth and reproduction of *U. rigida* would potentially support future *Ulva* cultivation, this may lead to adverse effects in terms of green tides as high concentrations of released swarmers are considered a prerequisite for blooms of green tide (Zhang et al., 2011). Moreover, these swarmers may be able to propagate faster in a future ocean. Nevertheless, the present study offers a clue on how to deal with green tides. That is to control nitrate levels since gametes of *Ulva* are very sensitive to low nitrate. If nitrate can be limited, it would effectively inhibit growth of germlings. When other environmental factors become unfavourable, such as temperature rising further, salinity dropping due to rainfall, green tides might fail to establish. To limit the discharge of nitrate from industry and agriculture, more strict laws need to be executed and more heavy taxes should be imposed.

6.4.5 Protein and amino acids

The protein content of *U. rigida* varied from 11.17 ± 1.64% DW to 24.14 ± 0.76% DW which was substantially higher than the value (7.06 ± 0.06%) in *U. lactuca* reported by Wong and Cheung (2000) or that (8.46 ± 0.01%) in *U. lactuca* reported by Yaich et al. (2011). However, this quantity was in the ranges for *U. lactuca* reported by Ortiz et al. (2006) (27.2 ± 1.1%) and Pádua et al. (2004) (15.23–18.35%). In the literature, the traditional conversion factor of 6.25 was used to estimate the total protein content. This approach is based on one assumption that all of the nitrogen in the sample

is present as amino acids in protein. The average nitrogen (N) content of proteins was found to be about 16% in early measurements and thus the calculation N $\times$ 6.25 was used to convert nitrogen content into protein content (Jones, 1941). However, most foods contain some proportion of nitrogen as non-protein nitrogen, which led to the inaccuracy of using 6.25 as a conversion factor. Therefore, a corrected factor is needed to account for the nitrogen found only in amino acids (Tkachuk 1969). In the present study, the conversion factor of 5.45 was based on quantitative amino acid profiles of three species of *Ulva* (Shuuluka et al., 2013), which should be more accurate to estimate the total protein content. Variations in the protein content of seaweeds can be attributed to species differences and environmental factors. Metabolic theory predicts that the metabolic rates of organisms generally rise exponentially with temperature within a certain range (Iken, 2012), leading to higher rates for most physiological processes, including nitrate assimilation. Moderate temperatures are favourable for various metabolic processes. In the present study, the high temperature (18^{o}C) significantly increased protein and amino acid content of *U. rigida* compared with the low temperature (14^{o}C). This is consistent with Mohsen et al. (1973) ‘s finding in which protein and amino acid content of *U. fasciata* increased with culture temperature (15–25^{o}C). Although optimal temperature for amino acids sysnthesis in *U. rigida* was not determined, the finding in the present study combined with the literature indicates ocean warming would enhance the amino acids and protein content of *Ulva* species in cold areas.

Nitrogen is an essential element required for amino acid and hence protein synthesis. The general pathway of nitrate assimilation in plant cells is that nitrate is reduced to nitrite in the cytosol and then nitrite is reduced further to NH^{4+} in the chloroplasts, from which amino acids are synthesised (Heldt and Piechulla, 2005). Consequently, the increase of nitrogen availability commonly enhances the amino acid and protein content. For instance, the total amino acid content of *U. ohnoi* increased linearly with internal nitrogen content (r = 0.987) (Angell et al., 2014). In the present study, the high nitrate level (150 μM) enhanced nearly all the amino acids content and thus the protein content of *U. rigida* from 15.75 $\pm$ 4.26% DW to 20.64 $\pm$ 3.57% DW compared with the low nitrate level. Due to the limit of experiment design, only two levels of nitrate (6, 150 μM) were used in the present study. More information can be known from the literature. An increase of 13 μM (from 7 μM to 20 μM) could cause a rise of protein content of *U. lactuca* from 14.2 $\pm$ 9.6% DW to 36.6 $\pm$ 9.1% DW. Naldi

and Wheeler's study (1999) demonstrated that very high nitrate concentration (up to 1000 μM) still stimulated the nitrogen pool of protein in *U. fenestrata* from 43% to 54% of the total nitrogen. The findings from the present study combined with the literature indicate eutrophication can increase the amino acid and protein content of *Ulva* regardless of the intensity of eutrophication and *Ulva* species. From a practical perspective this is advantageous as it shows that *Ulva* has the potential to be cultured as a high protein food. It also hints that *Ulva* is in effect building up a long term nitrogen store by increasing protein content within cells.

High CO_2 decreased the protein content of *U. rigida* in the present study. High CO_2 commonly leads to lower soluble protein content in higher plants (Spencer and Bowes, 1986, Van Oosten et al., 1992, Sicher et al., 1994). Van Oosten et al. (1992) suggested that the decline in soluble protein is due to the increase of soluble carbohydrates since they reduce the expression of genes related to the photosynthetic pathway. However, there was no enhanced carbohydrate production in *U. rigida* at the high CO_2 conditions in the present study. This phenomenon was also found in *U. rigida* collected from the Mediterranean Sea (Gordillo et al., 2001a, Gordillo et al., 2001b) and in *Porphyra leucosticta* (Mercado et al., 1999) which indicates that the mechanism of interaction between carbon and nitrogen assimilation in seaweeds may differ from that in higher plants. Meanwhile, CO_2 is also a key factor affecting the synthesis of amino acids as the amino acid carbon skeleton derives from CO_2 assimilation. Also, nitrate reduction, reduction of nitrite, and fixation of NH^{4+} depends largely on photosynthesis providing reducing equivalents and ATP (Heldt and Piechulla, 2005). The interaction of CO_2 and nitrate assimilation plus the effects of temperature on these processes resulted in complicated interactive effects between temperature, nitrate, and CO_2 on the synthesis of amino acids.

6.4.6 Lipid and fatty acids

Temperature is one of the most important and best-studied environmental factors affecting the lipid content and fatty acid composition of photosynthetic tissues or organisms (Guschina and Harwood, 2009 and references therein). The effect of temperature on total lipid varies with species. The total lipid content of *Isochrysis galbana* was higher when grown at 30°C than at 15°C regardless of the growth phase (Zhu et al., 1997). On the other hand, the high temperature of 25°C decreased the total lipid of *U. pertusa* from 3.6% DW to 2.6% DW at low light intensity and from 4.9% DW to 2.7% DW at high light intensity compared with the low temperature of 15°C

(Floreto et al., 1993). In the present study, the high temperature enhanced total lipid content. Apart from the difference of species, the opposite effects of temperature on total lipid aforementioned might be due to different temperatures used in different studies. The temperature range was 4^{o}C in present study while it was 10^{o}C in Floreto et al.'s (1993) study. Mohsen et al. (1973) reported that the largest lipid yield in *U. fasciata* was at 20 ℃, followed by 15 ℃, and 25 ℃. This finding was consistent with both Floreto et al.'s (1993) and the present studies since the high temperature in the present study is close to 20^{o}C and the low temperature is near to 15^{o}C. This indicates there is an optimal temperature for total lipid content and the temperatures on both sides of this optimal point would lead to the decrease of total lipid.

Synthesis of fatty acids depends on CO_2 assimilation to a large extent because photosynthesis can supply the necessary materials, such as acetyl CoA, ATP, NADPH, etc. (Gordillo et al., 2003). The total lipid content of *Scenedesmus obliquus* and *Chlorella pyrenoidosa* showed increasing trends when CO_2 rose from 0.03% to 50% concentration (Tang et al., 2011). In the present study, high CO_2 increased total lipid content except under the LTLN condition. A similar trend was also found in *Chlorella pyrenoidosa* (Pratt and Johnson, 1964). In addition, the positive effect of CO_2 on total lipid was also detected in *Dunaliella viridis*, of which the total lipid was elevated to 2.06 pg cell^{-1} under 1% CO_2 compared with 1.83 pg cell^{-1} under 0.035% CO_2 when nitrogen was limited (Gordillo et al., 1998). Meanwhile, high CO_2 (10,000 ppm) did not affect total lipid content in *U. rigida* grown under a nitrogen sufficiency condition and reduced it from 72 mg g^{-1} DW to 45 mg g^{-1} DW under a nitrogen limitation condition (Gordillo et al., 2001a). In respect to fatty acid profile, high CO_2 increased MUFA in the present study. This is consistent with Tang et al. (2011) in which high CO_2 (5%) increased unsaturated fatty acids from 65.78% to 71.39% of total fatty acids in *S. obliquus* and from 59.01% to 71.50% of total fatty acids in *C. pyrenoidosa* compared with 0.03% CO_2. On the other hand, elevated (2–5%) CO_2 concentrations resulted in a general reduction in the degree of unsaturation of fatty acids in *Chlorella vulgaris* compared with ambient (0.04%) CO_2, of which the particularly significant decrease was in C18:3, from 38.1% total fatty acids down to 17.8% total fatty acids (Tsuzuki et al., 1990). Therefore, the response of algal lipid content and fatty acid composition to CO_2 enrichment may be heterogeneous.

Nitrogen is an important factor that can affect lipid content in algae and nitrogen deficiency commonly enhances lipid accumulation in microalgae (Brennan and Owende,

2010). Generally, there are two hypothesises behind this conclusion. One is that nitrogen deficiency almost invariably causes a steady decline in cell division rate. Meanwhile, biosynthesis of fatty acids can continue under such conditions. Consequently, the synthesized fatty acids are converted to TAG lipids that are stored within existing cells thereby increasing the content per cell (Thompson, 1996, Brennan and Owende, 2010). The other hypothesis is that when nitrogen is limited, the flow of fixed carbon is diverted from protein to either lipid or carbohydrate synthesis. TAGs composed primarily of saturated and monounsaturated fatty acids can be efficiently packed into the cell and generate more energy than carbohydrates upon oxidation, thus constituting the best reserve for rebuilding the cell after the stress (Roessler, 1990, Rodolfi et al., 2009). However, high nitrate increased total lipid content in *U. rigida* in the present research. This is in inconsistent with previous hypothesis and studies as nitrogen limitation enhanced total lipid of *U. rigida* at 350 ppm CO_2 concentration in Gordillo et al.'s (2001a) study. The possible reason for this positive effect of high nitrate on total lipid content might be due to the stage of *U. rigida* when harvested. *U. rigida* was harvested at the end of 12 days of culture for biochemical analysis, when high nitrate induced more reproduction of *U. rigida*. When vegetative cells transit into reproductive cells followed by the formation of swarmers, more lipid might be required. Apart from the massive synthesis of lipid during mitosis and meiosis, swarmers may contain more lipid than vegetative cells since they are in great need of energy sources to support swimming and following settlement of swarmers. The total lipid content was 56.7% normalised to carbon in spores of *U. intestinalis* and could be 84.0% in spores of *Zonaria farlowii* (Reed et al., 1999). In addition, it has been found that the lipid content in cells decreased from 176.0 to 123.5 μg 10^{-7} per spore during 10 days development from spores to gametophytes of *Saccharina latissima* (Steinhoff et al., 2011). In Gordillo et al.'s (2001a) study, *U. rigida* was harvested after 10 days of culture when no reprocution was found. The current study indicates the chemical composition of *Ulva* would change with culture stage and it is essential to point out in which stage the samples are harvested when presenting data.

6.4.7 Carbohydrate and ash

The synthesis of carbohydrate is commonly favoured by increased temperature (Rosenberg and Ramus, 1982, Rotem et al., 1986, Marinho-Soriano et al., 2006). A positive correlation between carbohydrate and temperature was found in *Gracilaria cervicornis* ($r = 0.39$, $P < 0.05$) and *Sargassum vulgare* ($r = 0.36$, $P < 0.05$) (Marinho-

Soriano et al., 2006). However, the high temperature decreased carbohydrate content of *U. rigida* in the present study. A possible reason can be down to the stage of *U. rigida* harvested for chemical analysis, when high temperature led to dramatic reproduction of *U. rigida* compared with the low temperature. The photosynthetic capacity of reproductive cells is usually lower than vegetative cells, which indicates fewer carbohydrates are synthesised in reproductive cells. For instance, photosynthesis just offsets respiration in zoospores of *Laminaria hyperborea* even under saturating irradiance (Kain and Erin, 1964). Nitrogen limitation could increase the carbohydrate content of seaweeds (Rosenberg and Ramus, 1982, Rotem et al., 1986, Marinho-Soriano et al., 2006). This is related to the decline of protein synthesis under nitrogen limitation (Mouradi-Givernaud et al., 1993, Marinho-Soriano et al., 2006). This is consistent with the findings in the present study in which the high nitrate level decreased the carbohydrate content but increased the protein content of *U. rigida*, which indicates that eutrophication may be beneficial for protein but not for carbonhydrate content of *U. rigida*. The effect of elevated CO_2 on carbohydrate is species-dependent. High CO_2 concentration (1% in air) enhanced the carbohydrate content of *Porphyra leucosticta* from 5.3 mg g^{-1} fresh mass to 15.1 mg g^{-1} fresh mass compared with ambient CO_2 concentration (Mercado et al., 1999). On the other hand, the same concentration of high CO_2 did not increase the soluble carbohydrate of *U. rigida* (Gordillo et al., 2001a), which is similar to the finding in the present study. The various effects of CO_2 on carbohydrate may be attributed to different strengths of carbon-concentrating mechanisms (CCMs). The photosynthesis and hence the carbohydrate content of algae which hold robust CCMs are less sensitive to the change of CO_2 since photosynthesis is already saturated at ambient CO_2 level.

The high temperature decreased ash content in this study. Generally, there is an inverse correlation between ash and protein content for wild *Ulva* species (Peña-Rodríguez et al., 2011). Therefore, the reduced ash content may be related to the increased protein content of *U. rigida* at the high temperature. In addition, ash content can be associated with carbohydrate since polysaccharides have an extraordinary ability to accumulate inorganic elements present in the water (Lahaye and Jegou, 1993, Yaich et al., 2011). The high temperate treatment reduced carbohydrate content in this study, which partially explains the decreased ash content at high temperature.

6.4.8 SWC, WHC and OHC

The SWC data (ranging from 7.34 ± 0.31 to 11.24 ± 0.17 ml g^{-1} DW) was slightly lower than that reported in *U. lactuca* by Wong and Cheung (2000) (13.0 ± 0.70 ml g^{-1} DW) and Yaich et al. (2011) observed an even lower SWC (0.3 ml g^{-1} DW) in *U. lactuca*. Apart from the different water holding abilities of fibre and protein, the differences in SWC among the seaweed samples might be attributed to the different physical properties, such as particle size, density, pH, temperature, ionic strength, and types of ions in solutions (Robertson and Eastwood, 1981, Fleury and Lahaye, 1991). High temperature or low nitrate increased the SWC of *U. rigida* in this study, of which the pattern was similar to the total protein and carbohydrate content (Figure 6.9 a and c). The SWC can enhance satiety and reduce calorie intake (i.e. carbohydrate, sugar, fat, saturated fat and protein intake), and hence can be applied in an adjunctive therapy for obesity (Dettmar et al., 2011).

The WHC (ranging from 5.22 ± 0.10 to 7.24 ± 0.04 g g^{-1} DW) in this study was noticeably lower than that reported in *U. lactuca* by Wong and Cheung (2000) (9.71 ± 0.11 g g^{-1} DW) but comparative to Yaich et al. (2011) (6.66–7.00 g g^{-1} DW). Furthermore, the WHC of the *U. rigida* were also comparable to (6.60–9.00 g g^{-1} DW) some commercial dietary fibre-rich supplements (Goñi and Martin-Carrón, 1998). According to Robertson and Eastwood (1981), water can be associated with fibre either as bound water or trapped water. The amount of bound water depends on the chemical composition whereas trapped water refers to the structure of the fibre. WHC, as determined by the centrifugation method used in this study, represented both types of water-fibre association. Apart from fibre, protein may also play a role in water holding that involves the protein conformations and nature of the water binding sites in the protein molecules (Chou and Morr, 1979). In this study, there was an explicit correlation between WHC and total content of protein plus total carbohydrate in the seaweed samples, in which the high temperature and low nitrate increased both WHC and total content of protein plus total carbohydrate (Figure 6.9 b and c). Therefore, the hydration properties could be determined by both chemical components. The water-holding capacity can avoid syneresis and modify the viscosity and texture of formulated food. Furthermore, the increased viscosity due to water absorption could lead to slower rates of intestinal absorption. This property makes alginates clinically useful in reducing blood cholesterol and postprandial glycaemia, decreasing risks of obesity and Type II diabetes (Salmerón et al., 1997, Ludwig et al., 1999, Willett et al., 2002).

The OHC in this study varied from 1.46 ± 0.08–1.84 ± 0.07 g g^{-1} DW, which was much higher than the value reported in *U. lactuca* by Wong and Cheung (2000) (0.65 ± 0.03 g g^{-1} DW). This may be due to different treatment temperature. The OHC was determined at 37°C in this study while it was determined at 25°C in Wong and Cheung's study. The effect of temperature on OHC was investigated by Yaich et al. (2011). The present results were similar to their findings (around 1.60 g g^{-1} DW at 40°C). In general, proteins play a major role in fat absorption by hydrophobic bonding (Kinsella and Melachouris, 1976, Voutsinas and Nakai, 1983). Furthermore, the OHC of seaweeds is also related to the particle size, overall charge density and hydrophilic nature of fibres. Therefore, a high correlation (r = 1.00) between OHC and total amount of protein and total dietary fiber was reported (Wong and Cheung, 2000). The high temperature increased OHC of *U. rigida* in this study which can be down to the increased content of total protein and carbohydrate at the high temperature. Due to their OHC, *Ulva* species can be used as stabilizers in formulate food products. Moreover, they can reduce blood lipid level, obesity and coronary heart disease risk and thus can be a valuable functional food.

6.5 Conclusions

Little research has been done to study effects of global change variables on seaweeds. The present study is the first to investigate the effects of ocean warming, ocean acidification and eutrophication on the physiological and biochemical properties of *U. rigida*. The high temperature accelerated the onset of settlement and enhanced the number of adhered gametes. pCO_2, temperature and nitrate had interactive effects on the germination of *Ulva* during the first two days of culture. Germination rates under all treatments tended to be the same at the end of the eight-day culture period. Nitrate was the most important factor for growth of young *Ulva*, followed by temperature and pCO_2. Any combination of two factors interacted to positively affect the growth of young *Ulva*. By contrast, temperature was the dominant factor for growth of adult *Ulva* during the first four days of culture and afterwards nitrate overtook temperature as the principal stimulating factor. Based on the positive effects of high temperature, pCO_2 and/or nitrate on settlement, germination, growth and/or reproduction, future OAW may give rise to more severe green tides particularly when eutrophication cannot be controlled effectively.

High temperature and nitrate prominently enhanced protein content while pCO_2 slightly reduced it. All three factors increased lipid content. High temperature reduced

ash content and the negative effect was magnified under high pCO_2 or high nitrate. Both high temperature and high nitrate decreased carbohydrate and high nitrate or high temperature alleviated the negative effects of high pCO_2. All three factors impacted the amino and fatty acid profiles. In terms of functional properties, high temperature increased SWH while high nitrate decreased it, along with insignificant effects of pCO_2. A similar trend was found in WHC. High temperature increased OHC as well.

All findings above indicate global change variables (ocean warming, acidification, and eutrophication) will not only affect *Ulva* cultivation and evolution of green tides but also food quality of *Ulva* in the future.

Chapter 7. Conclusions and recommendations

7.1 General conclusions

In the present study research was undertaken into factors surrounding the cultivation of *U. rigida*; including optimising growth conditions, defining and manipulating reproductive mechanisms, strain improvement (in terms of promoting reproductive sterility), and low-cost preservation techniques. In addition, these practical aspects were viewed in the context of a changing climate—and by default a changing ocean (Gattuso et al., 2015)—particularly with respect to the interactive effects of ocean warming, ocean acidification, and eutrophication. Emphasis was placed on how a changing climate may impact future *Ulva* cultivation operations and also how it may alter the ecology of *Ulva*, for example by promoting the proliferation of green tide events. The physiological and biochemical properties of *Ulva* grown under a range of simulated climate change conditions were investigated. The conclusions that were drawn are broken down into two main sections: (i) innovative techniques required for *Ulva* cultivation; (ii) interactive effects of global climate change variables on future *Ulva* cultivation.

7.1.1 Innovative techniques required for Ulva *cultivation*

Ulva is an important genus ecologically and economically. As clearly highlighted in this thesis, one of the major technical obstacles limiting the extent and scope of *Ulva* cultivation is the confounding effects of periodic and destructive reproductive events. In order to combat this problem, three methods were developed. Firstly, optimal growth conditions producing low reproductive rates were investigated and determined as: a light intensity of 80 μmol photons m^{-2} s^{-1}; 150 μM N and 7.5 μM P; 12^{o}C, and an aeration rate of 100 L $minute^{-1}$ for 16 hour day^{-1}. This combination of growth parameters resulted in high and stable rates of growth (not necessarily the highest growth rates possible), however, what was pivotal was that this combination reduced reproductive rates of the plants and thereby produced a much higher biomass production over a more sustained cultivation period. Secondly, a multiple-harvest method was employed in order to maintain high growth over long-term cultivation by removing potentially reproductive thallus tissue prior to sporulation. This method achieved significantly enhanced growth (over three times improvement) over an 18-day cultivation when compared with a one-off harvest method. Nevertheless, a disadvantage of this approach will likely be increased labour costs for commercial cultivation. A

technoeconomic analysis of a multiple-harvest approach will need to be conducted before concrete recommendations can be made; however, this is not a trivial task and is deemed outside the scope of this thesis. However, it does warrant brief consideration here. Labour costs will naturally vary depending on the country, with countries with low labour costs best positioned to exploit this harvesting approach. Nonetheless, increased mechanization in seaweed farming needs to be considered in this context. It is easy enough to conceive of a harvesting machine designed along the lines of an agricultural shredder that could fragment the thalli at regular intervals with a proportion of the fragments harvested from the culture system. This could reduce the rate of reproduction within the cultured population and may even have an additional benefit in that it may stimulate/restimulate rapid somatic growth from the remaining thalli fragments. Clearly one drawback of this approach is that the size of the *Ulva* thalli that will be grown will be limited, and that may in turn restrict the market for the biomass (e.g. the wholefood sea vegetable market may have a preference for large intact thalli). However, if the target market is less sensitive to plant size (e.g. for biofuel conversion or for plant extracts), then this approach may have merit. Any prospective grower will have to factor this into their business model, obviously including the capex investment in the harvesting machinery and the sale value of their target markets (food, feed, fuel, ecosystem service, etc.).

The third strand to the anti-reproduction research involved a programme of strain improvement in the hope of developing a low reproductive, or ideally a sterile strain that would channel all or most of its resources into somatic growth rather than reproduction. A robust new *U. rigida* strain was obtained by mutating a wild strain using a combination UV radiation and chemical treatments. The new strain far exceeded expectations as it grew five times faster over an 18-day cultivation period. For the purposes of this thesis I have considered this strain to be sterile as no reproduction was observed during a three month cultivation period. Apart from markedly enhanced growth and the lack of reproductive activity, the new strain absorbed nitrate and phosphate 40.0% and 30.9% faster than the wild strain (likely linked to enhanced resource demand to fuel the accelerated somatic growth). In terms of the chemical composition and the functional properties of the mutant strain, the lipid content was more than twice that of the wild strain. This in itself is a peculiar and arguably counterintuitive result. Drawing from the extensive literature on microalgae biofuel production, enhanced lipid biosynthesis is enhanced typically in response to nitrogen

deprivation and as a result culture growth is sacrificed (Brennan and Owende, 2010, Thompson, 1996). In the case of the mutant *U. rigida* strain nitrogen availability was not deliberately limited; indeed nitrogen uptake was significantly enhanced in the mutant. How can this be explained? Perhaps the growth rate was so rapid that nitrogen availably may have inadvertently become limiting, in effect the rapid growth may have created a condition of nitrogen demand outstripping supply. If this is indeed the case then this is a potentially exciting outcome as it would suggest that there is even greater scope for growth in the mutant strain than that was demonstrated here. The protein content was less than three quarters (73.7%) that of the wild strain, which is reasonable that nitrogen limitation commonly reduces protein content in plants and algae. The ash content was slightly higher in the mutant strain whereas there was no significant difference in carbohydrate between these two strains. The swelling, water holding and oil holding capacities of the sterile strain were 27.7%, 25.3%, and 11.5% lower than the wild strain, respectively. This is likely a function of the differences in protein and carbohydrate content between the strains (see the discussion in Chapter 4). In summary, the new strain demonstrated its advantages in terms of growth, nutrient uptake and food quality (particularly the higher fatty acids content) and hence it is an attractive strain of *U. rigida* in the fields of cultivation, food, and bioremediation.

Given the importance of limiting reproduction to the success of *Ulva* cultivation further research was undertaken into some of the reproductive regulatory mechanisms of *U. rigida*. There was a clear distinction between the reproductive capacity of blade and basal cells with blade cells responsible for zooid formation. The degree of zooid formation increased as the size of tissue disks reduced. Seawater renewal further promoted reproduction of all sized fragments. This might explain the quick proliferation of *Ulva* during green tides. The formation of fragments in *Ulva* commonly occurs due to waves, propellers, and the actions of grazers. Meanwhile, the sporulation inhibitors released by *Ulva* fragments would be diluted immediately by the waves and surges. Accordingly, a large number of swarmers can be discharged from the fragments and grow up into new individuals quickly under the favourable environment (enough nutrients and optimal temperature during green tides). Combining temperature shock and fragmentation further shortened the blade cell maturation period to three days. This treatment can be employed to obtain abundant swarmers out of season for diverse fields including *Ulva* cultivation, marine ecotoxicology, and the testing of antifouling paints as natural release of *Ulva* swarmers usually occurs biweekly. An interesting result was

achieved when disks of blade cells were cultured with disks of basal cells wherein the reproduction rate of blade cells decreased in line with the number of basal disks which indicates sporulation inhibitors excreted by basal cells can inhibit the sporulation of blade cells. This case could occur in the field when *Ulva* live in a closed ditch or tide pool, or upper intertidal zone. This can partially explain why swarmer release of *Ulva* commonly occurs during spring tides when seawater can reach the highest level of the intertidal zone and substances in the intertidal zone can be diluted vigorously. Consequently, swarmers are induced and released without the control of sporulation inhibitors. Blade disks excised at different ages also showed different degrees of reproductive activity while basal disks kept vegetative status throughout the whole life cycle. Conditions of high temperature and nitrate also shortened the maturation period from 62.1 ± 2.8 days to 28.4 ± 2.0 days. These findings indicate that the internal factors such as sporulation inhibitors dominate reproduction regulation in *Ulva* while external factors can stimulate this process and accelerate maturation. The differentiation of *Ulva* cells with time leads to different reproductive performances in different regions of *Ulva* which not only supports rapid growth of *Ulva* when environmental conditions are favourable but also aids survival during unfavourable conditions. These findings will be helpful in managing the fertility of *Ulva*, particularly when trying to obtain numerous gametes out of season.

In order to expand the suit of tools available to the *Ulva* farmer a range of low cost and low-tech preservation techniques (defined as not requiring access to liquid nitrogen) were developed for *U. rigida* thalli, germlings, and gametes. *Ulva* thalli were successfully maintained at 4°C for 184 days (six months) without any detectable decline in regrowth rate. In stark contrast, viability decreased to zero after preserving at –80°C for seven days and –20°C for 30 days. *Ulva* germlings were more sensitive to these very low temperatures. All germlings died when stored at –20°C or –80°C for only one day. The regrowth rate decreased to 20.52 ± 3.32% when germlings were stored at 4°C for 184 days, which was 75.4% of initial. Therefore, despite the refrigeration approach shown very effective for thalli it was markedly less effective for germlings. Nevertheless, a regrowth rate in excess of 20% still represents a reasonable return and perhaps with further optimization the germling regrowth rate may yet be improved. In contrast, *Ulva* gametes could be preserved at –20°C or –80°C for 184 days although the viabilities were relatively low. The viability of *Ulva* gametes preserved at 4°C decreased from 94.74 ± 4.30% to 26.12 ± 3.97% when the storage time was extended from one

day to 184 days. These variations in the viabilities of *Ulva* thalli, germlings, and gametes stored at the same temperature are likely down to differing cellular morphologies and chemical composition across these three stages. The higher surface area to volume ratio of gametes results in fewer opportunities for intracellular ice formation during freezing, hence reducing freezing injury. In addition, the higher lipid content in gametes will probably play an important role in reducing intracellular ice formation and thus enhancing post thaw viability. The lower viability of gametes preserved at 4^{o}C presents something more of a mystery. Contributing factors may include nutrient limitation as the gametes will still be metabolically active at 4^{o}C albeit at a slow rate. Over the course of six months the available nutrient pool could become exhausted thereby triggering cellular stress and possible death. Further, as conditions were not axenic and as media were not renewed, bacterial action may have degraded the gametes leading to a higher failure rate. On a more practical level, the preservation of germlings and gametes occupies less physical space and generates a quicker regrowth rate but it is more time consuming to reach a decent sized plant compared with thalli preservation. *Ulva* thalli or germlings maintained high viabilities when stored at 4^{o}C but this method is quite labour intensive to renew the media and clean away any bacterial biofilms. Furthermore, the use of a pigment index was trialled and shown to be a simple but effective method to determine *Ulva* viability—at an operational level this approach will save a lot of time and cost.

7.1.2 Interactive effects of global change variables on future Ulva *cultivation*

Global change factors, such as ocean warming, acidification and eutrophication, will inevitably influence *Ulva* cultivation as most commercial seaweed cultivation is conducted outdoors, hence relying on natural environmental conditions. Previous findings are helpful in understanding how temperature, CO_2, and nitrate affect *Ulva* performance and culture, however, to the best of my knowledge, no previous research has examined the interactive effects of ocean warming, acidification and eutrophication on *Ulva* cultivation. Ocean warming and acidification are considered to occur more or less simultaneously with both being driven by our CO_2 enriched atmosphere. It does not necessarily follow that nutrient enrichment is also climatically driven, however it is anticipated that eutrophication (particularly in coastal waters) will continue to be a problem in its own right. It is therefore appropriate to consider ocean warming, acidification and eutrophication in combination. The final missing component of a changing ocean is the increase in ocean hypoxia. It is unclear how such hypoxic events

will impact seaweeds and as the events are primarily restricted to offshore waters their relevance to *Ulva* culture is questionable. As such, hypoxia was not investigated in this thesis. To begin the investigation into the effects of a changing ocean on *Ulva*, two levels of seawater temperature and pH (ambient versus that predicted for the end of this century) along with limited and enriched nitrate concentration were set up for *U. rigida* culture.

As separate factors the high temperature treatment lowered the time to settlement and increased the number of adhered gametes. In contrast, the high nitrate treatment delayed gametes settling whereas the elevated pCO_2 treatment had no impact on settlement. In combination, pCO_2, temperature, and nitrate treatments had interactive effects on *Ulva* germination during the first two days. The high pCO_2 lost its positive effect and any interactive effects had disappeared by day six. Germination rates under all treatments tended to be the same by day eight. Nitrate was the most important factor for growth of young *Ulva*, followed by temperature and pCO_2. Any combination of two factors interacted to positively affect growth of young *Ulva*. By contrast, temperature was the dominant factor for growth of adult *Ulva* during the first four days and afterwards nitrate overtook temperature as the principal factor. The positive effect of temperature on growth became negative by day 12 due to induced reproduction, which was even more prevalent at the high pCO_2 level or high nitrate and was highest when the high pCO_2 and nitrate treatments were combined.

Ocean acidification and warming shortened the time to gamete settlement, enhanced germination and also stimulated the growth of young and early-stage adult *Ulva*. These effects were further enhanced when nitrate was available at sufficient concentrations. So what are the implications of these findings for *Ulva* cultivation and ecology? On the one hand the data indicate that *Ulva* cultivation may benefit from the predicted future ocean environment as faster growth and a shorter life cycle should translate into higher profit and lower cost. Although these three factors will tend to induce more reproduction (which reduces *Ulva* productivity), a well-managed operation should be able to harvest before the negative effects of reproduction become damaging. On the other hand, the future ocean might be a harmful force in terms of the scale, frequency and intensity of green tide events as enormous releases of swarmers is considered a prerequisite for green tide blooms. What's worse, these swarmers can propagate faster in a future ocean. The stimulatory effects of high temperature and pCO_2 on settlement, germination and early-stage growth indicate peaks of green tides in the

future ocean may arise earlier. More reproduction under warmer and CO_2-richer conditions can lead to more swarmers released and thus more severe green tides. This will be exacerbated by eutrophication. Nevertheless, we can also get a clue on how to deal with green tides from the present study—the most significant measure will be to control nitrate levels as *Ulva* gametes are very sensitive to low nitrate. If nitrate availability can be limited, it would effectively inhibit the growth of germlings. When other environmental factors become unfavourable, such as temperature rising further, salinity dropping due to rainfall, green tides might fail to establish. Reducing nutrient input is a direct measure to curb eutrophication and thus green tides. However, it requires significant investment in infrastructure and agricultural practices in the catchment area and may take years to take effect. Furthermore, the implementation of this strategy is commonly hampered when it runs counter to the nation's main stream of economic development. A more short term approach to mitigate eutrophication is the direct removal of seaweed biomass from the sea as *Ulva* mats may actually contribute to the perpetuation of eutrophic conditions through rerelease of nitrate and phosphorus following decomposition. In addition, this action could reduce the magnitude of the green tides if carried out in the early stages of the blooms. The collected seaweeds can be used as fertilisers, feeds or biofuel materials.

In addition to growth and development, interactive effects of ocean warming, ocean acidification and eutrophication on chemical composition and functional properties of *U. rigida* were also investigated. Ocean warming and eutrophication prominently enhanced protein content while ocean acidification had little effect. All three factors increased lipid content. Ocean warming reduced ash content and the negative effect was magnified under ocean acidification or eutrophication. Both ocean warming and eutrophication decreased carbohydrate and eutrophication or ocean acidification alleviated the negative effects of ocean warming. In terms of functional properties, ocean warming increased SWH while eutrophication decreased it when temperature was low, along with insignificant effects of ocean acidification. A similar trend was found in WHC. Ocean warming increased OHC as well. All findings above indicate that the future ocean environment will increase the contents of protein and lipid but reduce carbohydrate and ash in *U rigida*. Due to the sum of protein and carbohydrate increases, functional properties (SWH, WHC, and OHC) would be enhanced. This suggests the value of *U. rigida* as feeds and food grown under future ocean environment may rise, because protein and lipid content are prioritised as animal

feeds and functional properties of food are highly valued by nutritionists. These data also provide some foundation of how to set cultivation conditions in order to obtain specific chemical and functional properties of *U. rigida*. For instance, higher temperature and nitrate should be used in an *Ulva* culture system if more protein content is expected.

More reproduction of *Ulva* was induced by higher temperature, pCO_2 and nitrate. On one side, these three factors may enhance reproduction by positive promoting effects. Metabolic theory predicts metabolic rates of organisms generally rise exponentially with temperature in a certain range, leading to higher rates for most physiological processes, including photosynthesis and nitrate assimilation. Higher pCO_2 and nitrate levels could also promote the rate of CO_2 fixation and nitrate assimilation since seawater is CO_2 and nitrate limited for seaweed. Both CO_2 fixation and nitrate assimilation may supply essential materials to convert vegetative cells into reproductive cells. However, it is important to note that the enhanced formation and discharge of swarmers under increased temperature, pCO_2, and nitrate might be a strategy of *Ulva* to provide more possibilities of survival under environmental stress. To meet the demand of producing numerous swarmers, more protein and lipid were synthesized along with the reduced proportion of carbohydrate and ash. Apart from enhanced reproduction, higher temperature shortened settlement time, higher temperature, CO_2 and nitrate increased germination rate, and these three factors promoted growth of young *Ulva* and early-stage growth of adult *Ulva*. Therefore, this indicates that *Ulva* may adapt to climate change by shortening its life cycle. Shorter generation time means more opportunities to adapt climate change phenotypically as well as genetically. This adaptive strategy may improve *Ulva*'s competitiveness against other seaweeds in future intertidal zones as a gradual reduction in the competency of either reproduction, recruitment, or recruit survival with increasing ocean temperature was found in kelp (Wernberg et al., 2010).

Based on the techniques developed and findings obtained in this study, two *Ulva* culture systems are proposed here. The first one is the land based system where *Ulva* is cultivated in tanks or ponds indoors. The site is preferably near a power station so that the waste heat and emitted CO_2 from the power station can be utilised to stimulate *Ulva* growth. The conditions of light, nutrient, temperature and aeration rate can be set up according to the optimal obtained in the present study. Frequent renewal of seawater is not suggested as it will dilute the concentration of sporulation inhibitors and thus

stimulate reproduction. Suspending germlings or thalli by bottom aeration rather than swarmers attached to nets should be employed as it can shorten the harvest period. This system is favourable to grow *Ulva* for high value markets, such as the pharmaceutical and cosmetic industries because stable conditions in this system can result in consistent quality of products. Meanwhile, inshore open water culture can be employed if *Ulva* is used as fertilisers or animal feeds. The product quality from this system may vary with the change of environmental factors but the cultivation cost is lower. This culture should start with swarmers which are attached to ropes since the strong adhesion can avoid detachment of material from ropes. Swarmers induction techniques in this study can be used to produce enough swarmers in demand. The multiple-harvest method can be employed in both land based and inshore open water systems to improve the productivity.

7.2 Recommendations for future research

Ulva cultivation is in its infant stages, it may develop rapidly during the next decade with growing awareness of its value and affect numerous aspects of our society, including food, culture, environment, energy, etc. The recommended research has been identified in this study.

7.2.1 Food and health

With the growing awareness of health, functional foods are gaining more interest from researchers and the food industry. However, seaweeds consumed by people in Asia, such as Chinese, Japanese, and Korean, are not accepted by Western people yet. To change a deep rooted food custom/tradition is never easy to achieve. The first thing to change this situation should be education, by which people would know the benefits of seaweeds as food. Fortunately, work is being done by some organisations, e.g. Seaweed Health Foundation and Seaweed & Co. One bold suggestion is to transform the term seaweed into sea vegetable because human beings generally would not like to eat weeds—a simple marketing strategy. In Ireland, seaweed is associated with poverty, making it a hard product to sell. One way is to incorporate it into "normal" foods. It would be acceptable for individuals to put seaweed into existing foods that they are used to, which can also enhance the flavour and nutrition. In addition, more research should be done on the benefits of consuming seaweeds. *Ulva* that have a high content of dietary fibre would be very helpful in reducing weight and thus reducing the risks of cardiovascular diseases. Furthermore, some studies have demonstrated the antibacterial and antiviral activity of sulphated polysaccharides from *Ulva*. Future work

should explore more bioactive compounds from *Ulva*, which will benefit both the food and the pharmacy industry from *Ulva*. Apart from growth, the studies on optimising the culture condition for the yield of polysaccharides, vitamins, and minerals need to be conducted in the future.

After sufficient awareness of the benefits from *Ulva*, they can be sold as a whole, like nori and kelp in Asian countries. *Ulva* may be more attractive due to its colour compared with brown or red seaweeds. Obesity is an ever-growing challenge, which is particularly severe in western countries due to excessive intake of high calorie foods. A lot of people find it difficult to stick to diet and exercise plans in order to lose weight. By using *Ulva* as a staple food, the obesity in Western countries might be tackled to some extent.

7.2.2 Bioremediation by Ulva

Nearly 70% of global population live within 60 miles of coastline by the end of 20th century and the rising trend is projected to continue (Vallega, 2013). In addition, aquaculture should expand further with increasing demand for aquatic products in the next decade. Increased urbanization, rising fertilizer use in agriculture combined with intensive aquaculture will lead to more severe environmental problems, e.g. eutrophication in the next decade. *Ulva*, as a powerful nutrients absorber, could play a more important role in bioremediation of coastal waters, particularly in some Asian countries where there are larger populations and more intensive agriculture and aquaculture. To treat the waste water from industry and agriculture, *Ulva* could be cultured in ponds or tanks near the pollution point. The effluent from the pollution point can be pumped into the ponds or tanks and then pumped out after the absorption of nutrients and heavy metals by *Ulva*. To purify the effluent from aquaculture, it would be better to conduct integrated multi-trophic aquaculture (IMTA), which has been considered as a mutually beneficial and environmentally friendly aquaculture system. Thus, the integrated cultivation of *Ulva* with marine animals, such as fishes, sea cucumbers, scallops and so on, should be developed to maximise the advantages of *Ulva* cultivation. One common challenge for IMTA is that scientists or farmers usually have expertise on either marine animals or seaweeds. More communications and cooperation between experts in marine animals and seaweeds should be made and farmers should be trained in both fields.

7.2.3 Biofuel from Ulva

With declining stocks of fossil fuels and rising concerns of environmental problems, the proportion of biofuels in energy consumption would increase in the next decade. *Ulva* will gain more attention as biofuel materials due to its high growth, carbohydrate content and not occupying arable land. Another benefit of using *Ulva* as biofuels is that *Ulva* cultivation can remit climate change. Biofuels are carbon-zero energy as seaweeds absorb CO_2 by photosynthesis when they are grown and release the same amount of CO_2 when they are used as biofuels. The extensive use of biofuels would remit the carbon emissions and thus the climate change (e.g. global warming and ocean acidification) to a large extent. There are two ways to obtain *Ulva* materials for biofuel processing. One is to collect them from the field. This is especially feasible when green tides occur. The other way is to cultivate *Ulva*. Due to low demand on heavy metal and nutrition, *Ulva* grown to treat wastewater can be used to produce biofuels, which delivers dual functions of *Ulva*.

The main challenge for extracting biomethane from *Ulva* is that quite a proportion of H_2S can be produced during aerobic digestion which could do harm to both facility and human. Work on reducing the production of H_2S needs to be done in the future. One feasible method to deal with this problem is to add the sulphate reduction inhibitors into the anaerobic reactor, such as potassium molybdate, to reduce the H_2S production. But it will increase the cost of biomethane production. Another effective method is biological desulphurisation, in which some volume of air is introduced to the gasometer. Then specific sulpho-oxidizing microorganisms like *Thiomicrospira* sp. or *Thiobacillus* sp. transform hydrogen sulphide into elementary sulphur or sulphate with the oxygen contained in the air. One matter which needs attention is that the air and the hydrogen sulphide in the gasometer have to be properly mixed. The gas mixture can be explosive if too much air is input. Another possible way is to reduce sulphur content in *Ulva* by utilising the gene engineering technology. This needs complete understanding of sulphur metabolism and gene sequence.

7.2.4 Ulva *cultivation in the future*

To conduct *Ulva* cultivation, some fundamental work still needs to be done. The growth of *Ulva* under fluctuating light and temperature should be investigated if outdoor cultivation is to be carried out. A small scale outdoor cultivation can be conducted in order to make it. Considering the aeration rate and time may depend on the stock density, nutrient concentration and light intensity, the effects of aeration rate and

time should be investigated under various levels of stock density, nutrient, and light. With regard to preservation techniques, cryopreservation (at least –135^{o}C) should be a focus of future study as no significant chemical changes in a biological sample can occur at such low temperatures and thus cell deterioration can be prevented. How to enhance the tolerance of *Ulva* thalli, germlings, and swarmers to such low temperatures and thus improve viability is the key. A relatively new technique termed encapsulation-vitrification could be applied in *Ulva* cryopreservation since it has significantly increased the viability of higher plants and microalgae. In addition, longer time scales of (more than one year) preservation should be investigated using different methods and materials. The new strain in this study demonstrated significant differences from the wild one in terms of growth, reproduction, nutrient uptake, chemical composition, and functional properties. Unfortunately, it died due to improper preservation. Therefore, more research should be conducted into species selection and strain improvement according to the market demand. The priority is still to obtain a robust species which can maintain fast growth during long-term cultivation. Domestication is the simplest method of breeding but it is a time consuming process to find an ideal strain in the field. Mutation breeding with physical and chemical mutagens may be the most feasible to obtain robust strains. One disadvantage of mutation breeding is that the frequency of desirable mutations is usually very low. Therefore, genetic engineering could be a promising approach for strain improvement of *Ulva*. However, the whole genome sequences of *Ulva* species is currently unavailable, which leads to the difficulty for manipulating the functional gene sequences of *Ulva*.

Meanwhile, climate change will affect the pattern of *Ulva* cultivation in the world. There could be more *Ulva* cultivation conducted in cold areas due to the global warming, which would benefit the local farmers and market. On the other hand, green tides usually occur in the temperate zone (Ye et al., 2011) but it may be found more in cold areas in the future with the increasing global temperature. This would need more rigorous legislation and more effective measures to deal with the discharge of nitrate and phosphate, which may reduce the occurrence of green tides.

In order to increase the profit of *Ulva* cultivation, an integrated estimate of benefits in *Ulva* cultivation should be directed in future studies. This involves the value of the *Ulva* product along with the ecosystem services such as bioremediation and CO_2 removal that *Ulva* cultivation will bring. As environmental concerns about the problems of climate change increase, the role of *Ulva* cultivation in bioremediation and CO_2

removal could be highly valued. Furthermore, the integrated multi-trophic aquaculture has been considered as a mutually beneficial and environmentally friendly aquaculture system. Thus, the integrated cultivation of *Ulva* with marine animals, such as fishes, sea cucumbers, scallops and so on, should be developed to maximise the advantages of *Ulva* cultivation.

In summary, the present study has developed innovative techniques for *Ulva* cultivation, including conditions for optimizing growth, harvest methods, swarmers hatchery, strain improvement, and materials preservation etc. This study also investigated responses of *Ulva* to a changed/changing future ocean environment. These findings could make tangible contributions to *Ulva* cultivation now and in the future. In addition, a flexible *Ulva* farm mode was proposed and a framework for future research was produced.

Appendix A

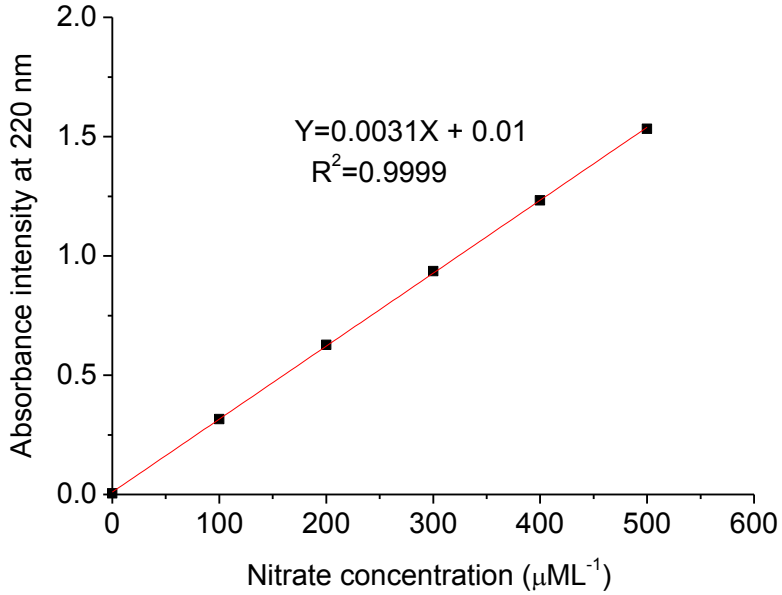

Figure A1.1 Relationship between nitrate concentration and absorbance intensity at 220 nm. Y, absorbance intensity; X, nitrate concentration (μM L^{-1}); R^2, the coefficient of determination.

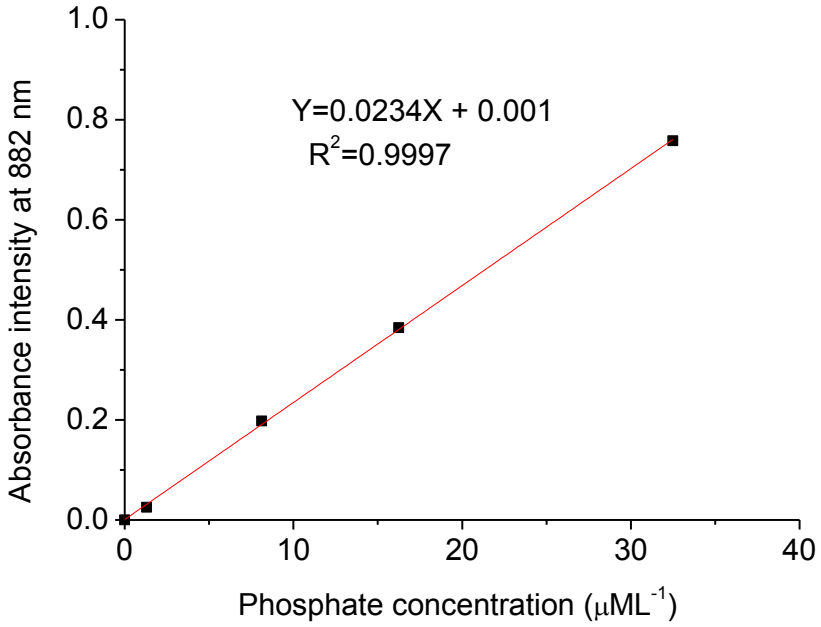

Figure A1.2 Relationship between nitrate concentration and absorbance intensity at 882 nm. Y, absorbance intensity; X, phosphate concentration (μM L^{-1}); R^2, the coefficient of determination.

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