Complex interactive effects of several abiotic factors on ecophysiology of two Japanese seaweeds

（日本産海藻類２種の生理生態形質に影響を与える非生物的要因の複合的交互作用）

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Contents

Chapter 1 Background and Introduction

The marine environment is composed of various abiotic factors that constantly combine and interact with each other to allow for proper functioning of organisms that call the ocean its habitat (Arnott & Ruxton, 2002; Harley et al., 2012; Poloczanska et al., 2016). Abiotic factors can be temperature, nutrients, irradiance (sunlight), salinity, wind and water currents (Grangeré et al., 2012; Bal et al., 2021; Hancock et al., 2021). The interaction of these important abiotic factors can have synergistic, antagonistic or additive effect on responses (Piggott et al., 2015; Côté et al., 2016; Endo & Gao, 2022;). Synergistic and antagonistic effects are known as non additive (interactive) effects. Synergistic effects occur when sum of factors responses are greater than the individual responses added together. Antagonistic effects occur as a result of sum of factors responses being less than the individual responses added together. However, the effects might be difficult to understand as they do not take into account the direction of the effect. For instance, a synergistic effect could be of a positive direction or it could be of a negative direction. Various schemes and classification have been developed throughout the years to elucidate these interaction based on the direction of effect (Piggott et al., 2015; Endo & Gao, 2022). Piggott et al. (2015) classifies interactions into four types; positive synergism (+S), where the cumulative effect is more positive than the additive sum (AD); negative antagonism (-A), where the cumulative effect is less negative than the additive sum; positive antagonism (+A), where the cumulative effect is less positive than the additive sum and negative synergism where (-S) where the cumulative effect is more negative than the additive sum refer to Figure 1.

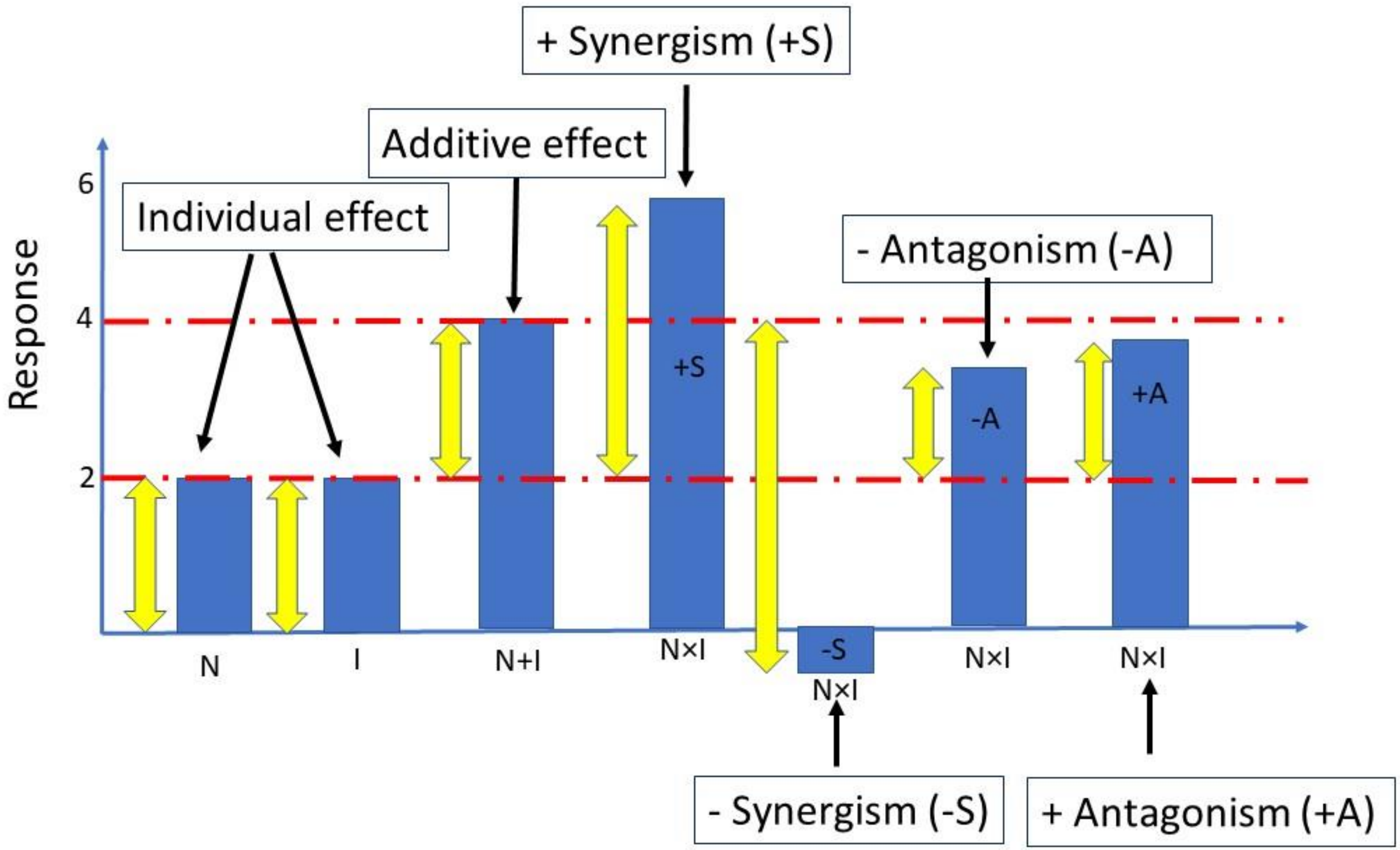

Figure 1. Schematic diagram of interaction effects where N and T represent nutrients and temperature respectively. Positive synergism indicates (+S), negative synergism indicates (-S), positive antagonism indicates (+A), negative antagonism indicates (-A).

Abiotic factors such as temperature, nutrients and irradiance are considered extremely important not only in the terrestrial environment, but also in the marine due to their ability to regulate important physiological processes in macroalgae (Flukes et al., 2015; Balfagón et al., 2019; Bal et al., 2021). Macroalgae are primary producers of the ocean and therefore are fundamental in maintaining ecosystem balance, being utilized for nutrient cycling, habitat and nursery but most importantly being used as food for humans (Coleman & Wernberg, 2017; Eger et al., 2020; FAO, 2021). However, an important abiotic factor, temperature is expected to cause global warming as a result of increased carbon dioxide presence in the atmosphere (Zandalinas et al., 2021).

The scenario of projected global warming of ca. 3 degrees Celsius in the next 100 years will affect all life forms including marine plants (IPCC, 2013). Studies have shown that increased oceanic temperatures affect kelp distribution by causing a decrease in abundance from temperate habitats to seek refuge in colder regions, mostly by movement towards the northern regions (Coleman & Wernberg, 2017; Smale, 2020). On the other hand, temperature and nutrient interaction have found that the negative effects of elevated temperature has antagonized growth of macroalgae in nutrient enriched conditions (Kay et al., 2016).

Various genus and species of macroalgae such as *Saccharina spp., Sargassum patens, Ulva* spp.*, Ecklonia cava, Hypnea* spp., *Undaria* spp.*, Acophyllum nodosum*, *Eisenia bicyclis* have been studied for interactive effects of temperature, nutrients and or irradiance with varying results of synergistic to antagonistic responses on growth (Endo et al., 2013; de Faveri et al., 2015; Gao et al., 2016; Kay et al., 2016; Endo et al., 2017; Gao et al., 2017; Endo et al., 2020). Anthropogenic effects have led to eutrophication and combined with increasing temperatures is predicted to have caused an overall decrease in marine biodiversity (Binzer et al., 2016).

Food security issues throughout the world is increasing due to the changing climate. Seaweeds have been used throughout many parts of the world with multiple purpose. They have been part of the diet of the Asian world for centuries. According to FAO (2016), within the fisheries industry, seaweed farming is quite prominent with an 8 % increase per year in the last decade. Likewise, 30% of the global aquaculture comes from seaweed production (FAO, 2021). Two seaweeds namely, *Sargassum fusiforme* and *Ulva prolifera* are commercially important edible seaweeds in Japan. However, China has the highest production of *S. fusiforme* (Hijiki) which is exported to Japan as degradation of coastal areas has decreased *S. fusiforme* cultivation in Japan (Kokubu et al., 2015).

Sargassum fusiforme is a brown macroalga of the phylum Ochrophyta, predominantly found in the intertidal regions with cultivation increasing gradually from 2005—2016 throughout the world with 190 tonnes /live weight production in 2016 (FAO, 2018). *S. fusiforme* is widely distributed in Japan, however they are from five different lineages (Horiuchi et al., 2017). *S. fusiforme* is also found in eulittoral to intertidal coastal zone in subtropics and temperate regions. *S. fusiforme* starts to become abundant from winter and starts reducing or disappearing by summer. *S. fusiforme* has perennial holdfast and stipes with annual shoots (Bast, 2014; Kim et al., 2014; Hwang et al., 2015). The life history is depicted in Figure 2.

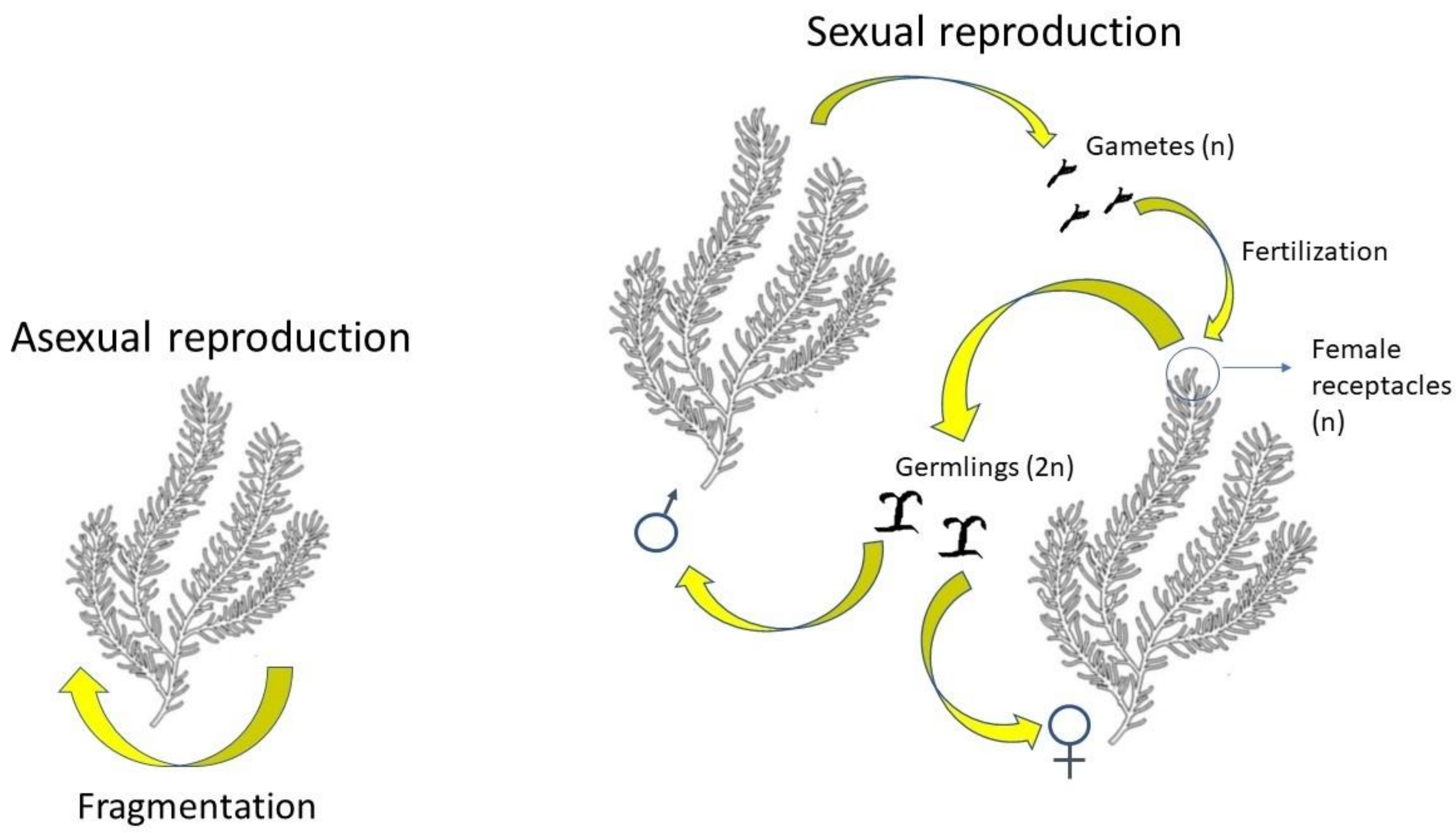

Figure 2. The life history of *Sargassum fusiforme*.

On the other hand, *U. prolifera* is a green macroalga of the phylum Chlorophyta which prefers to grows in brackish waters in Japan, becoming abundant during early spring (Ohno & Miyanoue, 1980). In Japan, consumption of *U. prolifera* has been made famous because of the strong flavour that it possesses and being rich in Vitamins (Ohno M, 1993; Watanabe et al., 1999).

Ulva prolifera is considered an annual species. (Mathieson & Hehre, 1983; Hiraoka et al., 2020). *U. prolifera* is widely distributed in Japan with three prominent lineages (Shimada et al., 2008). They have multiple life histories with the two prominent asexual and sexual life histories (Liu et al., 2022). Asexual life history contains zoids of same size from two successive generation that contains negative phototaxis (inability to move directionally in response to light) (Zhao et al., 2019). Asexual apomictic population is mostly cultivated for consumption in Japan as cultivation conditions can be controlled in a laboratory (Hiraoka & Oka, 2008). Sexual life history on the other hand is exhibited by two different size of zoids which have opposite phototactic response or zoids of the same size which have positive phototaxis (ability to move directionally in response to light) in two successive generation (Zhao et al., 2019). Figure 3 shows the life history of *U. prolifera*.

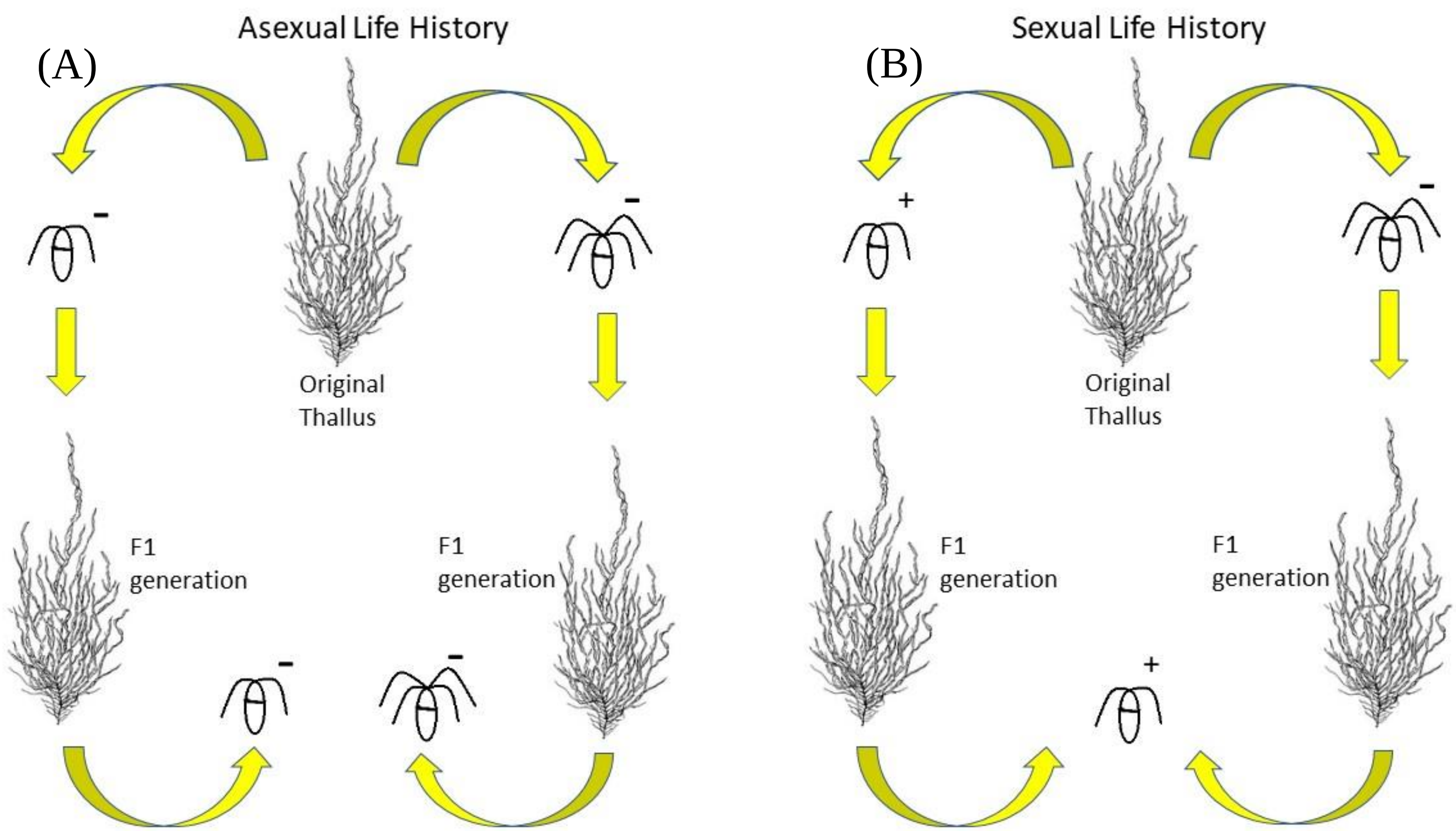

Figure 3. Asexual (A) and Sexual (B) life history of *Ulva prolifera*. Note: "+" represents positive phototaxis and "-" represents negative phototaxis.

These seaweeds are very different from each other however, both have preference for intertidal to subtidal regions in their natural habitat. Important conditions in the marine environment such as the oceanic temperature, nutrients and irradiance to name a few, play monumental roles in the governing of seaweed physiology.

Nutrients plays an important role in population dynamics and community structure onto which macroalgal species depend on for survival. Temperature with nutrients studies have mostly been performed on brown macroalgae with varying effects on biochemical composition. In fucoids and kelps, carbon content and growth are not affected by temperature and nutrients (Endo et al., 2017; Endo et al., 2021). *Sargassum fusiforme* is a brown macroalgae which might have similar biochemical performance to kelps and fucoids. On the contrary, *Ulva prolifera* is affected by both temperature and nutrients (Shi et al., 2015), therefore excess nutrients have resulted in *U. prolifera* transitioning from being an indicator species for pollution to harmful algal bloom (HAB). Due to the sensitive response to temperature and nutrients, *Ulva* species (*U. rigida* and *U. linza*) are expected to increase in abundance under eutrophication and warming (Gao et al., 2017, 2018; Lee & Kang, 2020). Nitrogen content is linked to high temperature tolerance in macroalgae while carbon content is related to carbon fixation in kelps (Gerard, 1997). Nutrients uptake is affected by temperatures effect on nitrogen assimilation in kelps (Gerard, 1997). However, in *Ulva linza,* under multiple temperature and nutrient conditions, carbon content does not vary although more nitrogen assimilation takes places under high nutrient environments resulting in higher growth (Lee & Kang, 2020). According to Sato et al (2021), carbon fixation does not vary among strains of *U. prolifera* in fluctuating temperature conditions. However, interactive effects of temperature, nutrients and irradiance on *U. prolifera* strains remain unknown.

Elevated irradiance mostly affects chlorophyll pigments such as Chl *a*, and accessory pigments by decreasing its contents due to excess light energy that produces the harmful reactive oxygen species (ROS) such as singlet oxygen in the chloroplast (Macintyre et al., 2002; Pintó-Marijuan & Munné-Bosch, 2014). This also results in chlorophyll degradation. However, xanthophyll cycle pigments such violaxanthin, antheraxanthin and zeaxanthin, beta carotene and neoxanthin increase in response to acclimatizing to elevated irradiance (Goss & Lepetit, 2015). This is because xanthophyll pigments help dissipate excess light energy as heat to evade photoinhibition. Increased reactive oxygen species production could occur under elevated irradiance and warming conditions (Murata et al., 2007). On the other hand, combined effect of multiple stressors on pigments of *S. fusiforme* and *U. prolifera* is unknown.

Purpose for this research

Many studies on these two seaweeds have been performed in regards to growth and physiology however, they mostly include two factors (environmental condition) studies. A three factors study becomes essential in further enhancing knowledge about the growth, physiology and biochemical composition of these particularly interesting seaweeds which are important yet very different from each other. The increasing ocean temperatures in the past and the further increase in 3 ºC that is expected to occur in the next 100 years will change the interaction relationship between the abiotic factors and macroalgae. Macroalgal cultivation is dependent on the environmental conditions in which it is cultured in to produce good yield and quality. *Sargassum fusiforme* and *Ulva prolifera* cultivation's prevalence will be affected due to the changing oceanic temperatures. The purpose of this study thus becomes to investigate and determine the effect of elevated temperature, nutrients, irradiance and possible interactions between these factors on the specific growth rate (SGR) and biochemical compositions of *S. fusiforme* and *U. prolifera*. The data from this study will not only enhance the knowledge of eco-physiological study but can also help improve commercial production of edible seaweeds in the changing environment condition as there are limited information available on interactive effects of temperature, nutrients and irradiance.

Chapter 2 The interactive effect of temperature, nutrient and irradiance on the specific growth rate (SGR) and biochemical composition of *Sargassum fusiforme*

Materials and Methods

Six different *Sargassum fusiforme* shoots (with nine dominant shoots) were collected from a depth of 1–2 m along the Kitsunezaki Coast (38° 21′ 01″N, 141° 25′ 06″E) located in Oshika Peninsula, Miyagi Prefecture, in Northern Japan on January 2020. This site had summer temperatures of ca. 23 °C between 2011-2017 (Suzuki, 2018). However, near the sampling site at about 0.85 m depth, a 3.1-3.3 °C degrees warming in summer temperatures were observed in 2012-2015. According to Suzuki (2018), the conditions at these sites were as follows; irradiance was 2.0–21.8 mol photons/m^{2}d, nitrate was 0.22–1.64 µM, ammonium was 0.22–3.76 µM and phosphorus was 0.11–0.28 µM at 0.85 m depth.

The samples were transported in insulated cool boxed and cleaned with sterile seawater to remove the epiphytes and diatoms. Fragments of 3 cm apical shoots were cut because apical shoots contain meristems which allow growth even after excision (Li et al., 2019). After excision, the shoots were placed into 1L flasks which contained artificial seawater (AW) to mitigate the negative effects of excision. AW was without nitrate or phosphate (LIVESea, DELPHIS Co., Hyogo, Japan). The 1L flasks with excised shoots were then placed in an incubator (FLI-2000A, Tokyo Rikakikai. Co. Ltd., Tokyo, Japan) with temperature of 20 °C and 70 µmol photon m^{-2} s^{-1} under 12 h L (light) and 12 h D (dark) for 2 days. After acclimation for 2 days, the shoots were transferred to the experimental treatment containers.

Experimental Treatment

Eight different treatments (2 × 2 × 2 treatments) were utilized which were composed of two temperature levels (23 and 26 °C), two irradiance levels (30 and 150 μmol photon m^{-2} s^{-1}) and two nutrient levels (enriched and non-enriched) (Figure 4). The sampled shoots were cultured for 9 days where and there were six flasks per treatment. Six shoots from different *Sargassum fusiforme* were used as replicates (n=6 per 8 treatments).

The reason for choosing the two temperature regimes were because ca. 23 °C represented average seawater temperature during summer while 26 °C was indicative of the higher seawater temperature in Kitsunezaki during summer (Endo et al., 2017; Suzuki, 2018). It is also expected that sea surface temperatures will increase in the next 100 years to ca. 3 °C if nothing is done limit carbon dioxide emission (IPCC, 2013). The irradiance of 30 μmol photon m^{-2} s^{-1} was used to represent compensation irradiance which is between 5-37 μmol photon m^{-2} s^{-1} (Kokubu et al., 2015) while 150 μmol photon m^{-2} s^{-1} was chosen to represent optimal growth irradiance which is between 100-180 μmol photon m^{-2} s^{-1} (Baba, 2007). Nutrient enrichment was prepared by using 5 % Provasoli's enrichment (5 % PESI) (Tatewaki, 1966) while AW was prepared by using LIVESea which was representative of non enrichment as it was free from nitrate and phosphate. *Sargassum* spp. grow well in less nutrient enrichment as opposed to high nutrient enrichment which is the reason for using 5 % PESI (Endo et al., 2013). The culture medium was changed every 3 days. The salinity of 5% PESI and AW was ca. 34 psu (n=5).

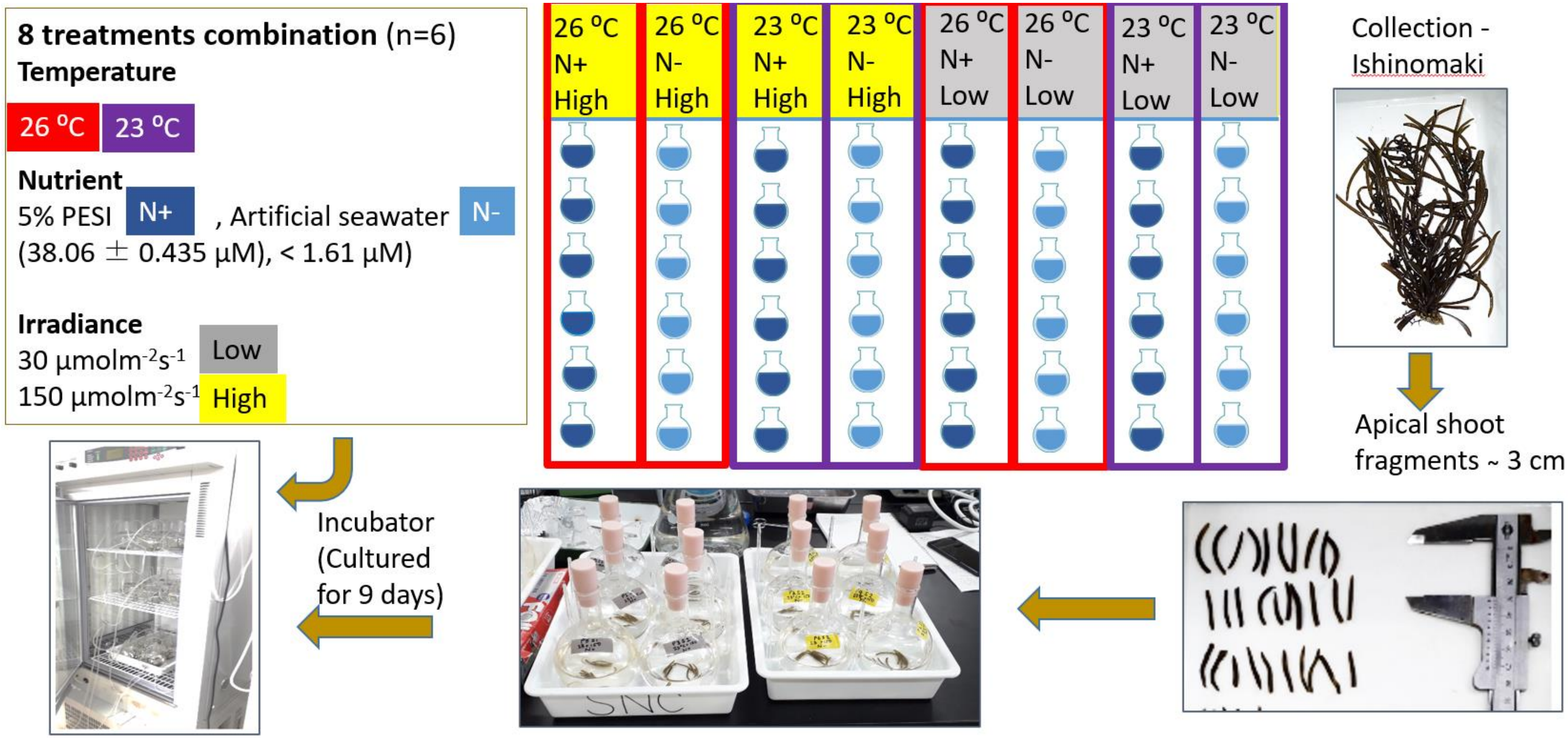

Figure 4. Eight experimental treatment conditions.

Specific growth rate (SGR) and biochemical composition determination

The samples were blotted dry on a paper towel to remove excess moisture and then the measurement of the initial weight (before culturing) and final weight (after culturing) using an electronic balance that had 0.001 g accuracy was done. The SGR was determined using the following equation;

SGRs (% d^{-1}) = 100 × ln (final wet weight/initial wet weight)/9 d

Half of the samples were oven dried for 12 days at 80 °C before carbon and nitrogen determination using the organic elemental analyzer (FLASH2000, ThermoFisher Scientific, Waltham, MA, USA) was done.

Pigment determination was performed using high-performance liquid chromatography (HPLC) after the samples were placed in 9 mL bottles containing 5 mL of dimethylformamide (DMF). Following methods by Zapata et al. (2000), 80% supernatant was achieved after diluting in distilled water. The guard column was placed between the injection valve and the analytical column (Symmetry C8, Waters Milford Massachusetts, USA). The pigments analysed included Chlorophyll *a* (Chl *a*), Chlorophyll c_2 (Chl c_2), fucoxanthin (Fuco), Violaxanthin (Viola) , Antheraxanthin (Anthera), and Zeaxanthin (Zea). Xanthophyll cycle pigments Viola (V), Anthera (A) and Zea (Z) were calculated by adding them (VAZ). In addition the ratio of (Chl c_2, Fuco, and VAZ was determined due to no difference being seen in Fuco, Viola, Anthera and Zea.

Statistical Analysis

Analysis of variance (ANOVA) was used to determine any difference in initial wet of the eight treatments. The normality and homoscedasticity was determined using the

Shapiro-Wilk's test and Bartlett's test. Data of Fuco/Chl *a* were not normally distributed thus were logarithmically transformed to stabilize variance. The combined interactive effects of SGR and biochemical composition was determined by a three way ANOVA. Tukey's multiple comparison test was determined if there were synergistic or antagonistic effects. John's Macintosh Project (JMP) software version 10 was used for all analysis.

Results

The mean initial wet weight ± standard deviation (SD) of shoots was 1.346 ± 0.205 g. There were no significant differences in initial wet weight among the treatments (df = 7, MS = 0.103, F = 3.304, $p > 0.05$). The initial carbon (C), nitrogen (N) contents and C/N ratio had a mean value of 22.827 ± 1.293%, 1.233 ± 0.135%, and 18.733 ± 2.483 respectively. The initial mean Chl *a*, Chl c_2, Fuco, Viola, Anthera and Zea contents were 349 ± 9, 43 ± 17, 83 ± 30, 44 ± 19, 28 ± 11 and 6 ± 3 µg g^{-1}ww respectively. The initial mean ratio of Chl c_2/ Chl *a*, Fuco/Chl *a* and VAZ/Chl *a* were 0.122 ± 0.014, 0.236 ± 0.022, and 0.225 ± 0.031 respectively.

Individual significant effects of temperature and irradiance was detected by ANOVA as well as an interaction between temperature and irradiance on SGR (Table 1). Tukey's multiple comparison test showed that SGR has increased under elevated irradiance and 23 °C however, it did not change under 26 °C (Figure 5). SGR showed a decreased response to elevated temperature and elevated irradiance however not in low irradiance. ANOVA had significant individual effects of temperature and irradiance on carbon content (C) (Table 1). However, there was an interaction between temperature and nutrients on C (Figure 6A). Nitrogen content (N) was significantly affected by nutrients (Table 1). There was an interaction between irradiance and nutrients on N (Figure 6B). The high N content under elevated irradiance and enriched conditions however, reduced under elevated irradiance but at non enriched conditions. ANOVA detected individual significant effect of irradiance and nutrients on C/N as well as an interaction between irradiance and nutrients (Table1).

Tukey's multiple comparison test showed that under non enrichment and elevated irradiance conditions, C/N is high however, C/N ratio decreases under enrichment and low irradiance (Figure 6C). ANOVA found significant individual effect of irradiance on Chl *a*, Chl c_2 and Fuco (Table 2). Individual significant effect of nutrients was also found in Chl c_2. There were no significant effects found on Viola, Anthera, Zea (Table 2).

Table 1. Results of a three-way ANOVA on the effects of temperature, nutrient availability and irradiance on SGR, C, N and C/N of *S. fusiforme*. Where $p < 0.05$, $p < 0.01$ and $p < 0.001$ are denoted as *, ** and ***, respectively.

Source	df	MS	F	P	
SGR					
Temperature (T)	1	15.256	8.628	0.005	**
Irradiance (I)	1	12.380	7.001	0.012	*
Nutrient (N)	1	1.532	0.866	0.358	
T × I	1	8.364	4.730	0.036	*
T × N	1	1.700	0.962	0.333	
I × N	1	0.497	0.281	0.599	
T × I × N	1	0.331	0.187	0.668	
Carbon					
Temperature (T)	1	40.829	9.413	0.004	*
Irradiance (I)	1	192.871	44.469	<0.001	***
Nutrient (N)	1	2.517	0.580	0.451	
T × I	1	0.041	0.009	0.923	
T × N	1	25.426	5.863	0.020	*
I × N	1	1.350	5.863	0.580	
T× I × N	1	2.403	0.554	0.461	
Nitrogen					
Temperature (T)	1	0.001	0.032	0.859	
Irradiance (I)	1	0.005	0.184	0.670	
Nutrient (N)	1	4.552	174.838	<0.001	***
T × I	1	0.006	0.233	0.632	
T × N	1	0.000	0.002	0.964	
I × N	1	0.202	7.772	0.008	**
T × I × N	1	0.051	1.961	0.169	
C/N					
Temperature (T)	1	0.069	3.306	0.077	
Irradiance (I)	1	0.364	17.383	<0.001	***
Nutrient (N)	1	4.131	197.012	<0.001	***
T × I	1	0.007	0.329	0.569	
T × N	1	0.068	3.244	0.079	
I × N	1	0.193	9.224	0.004	**
T × I × N	1	0.013	0.627	0.433	

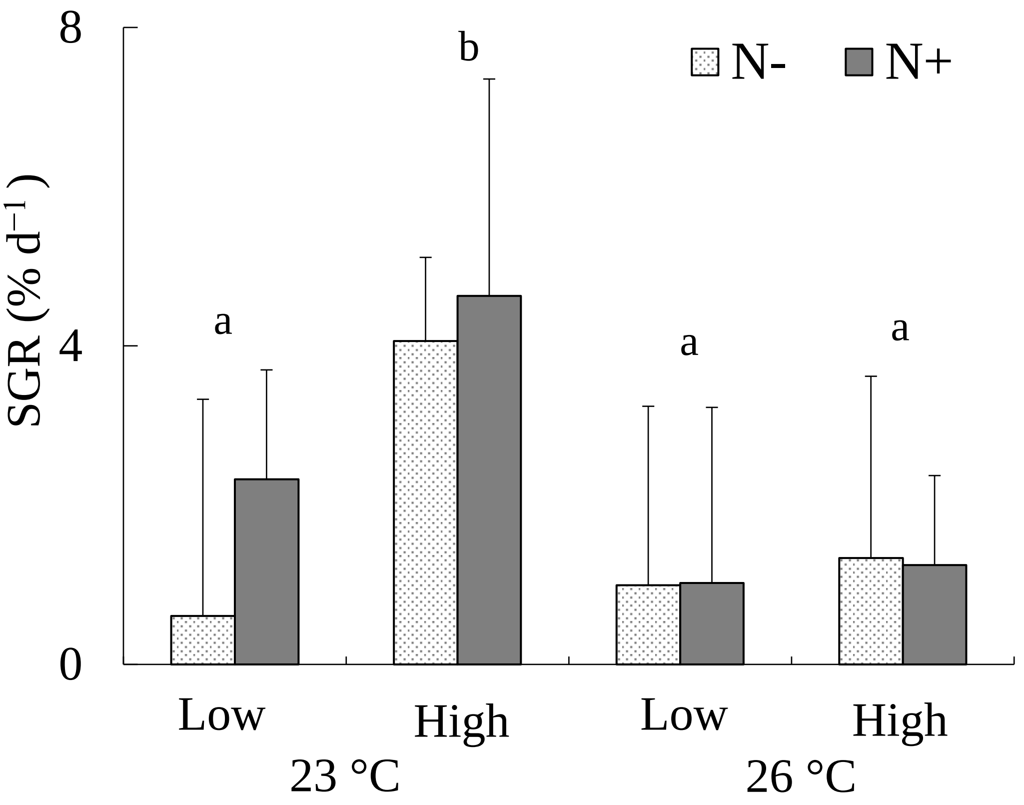

Figure 5. Specific growth of *Sargassum fusiforme* shoots cultured in eight different treatments (mean + SD, n = 6). Low and High indicate 30 and 150 (μmol photon m^{-2} s^{-1}) irradiance treatments, respectively. N+ and N- indicate nutrient enriched and non-enriched treatments, respectively. Different small letters indicate statistical significances among different treatments (P < 0.05).

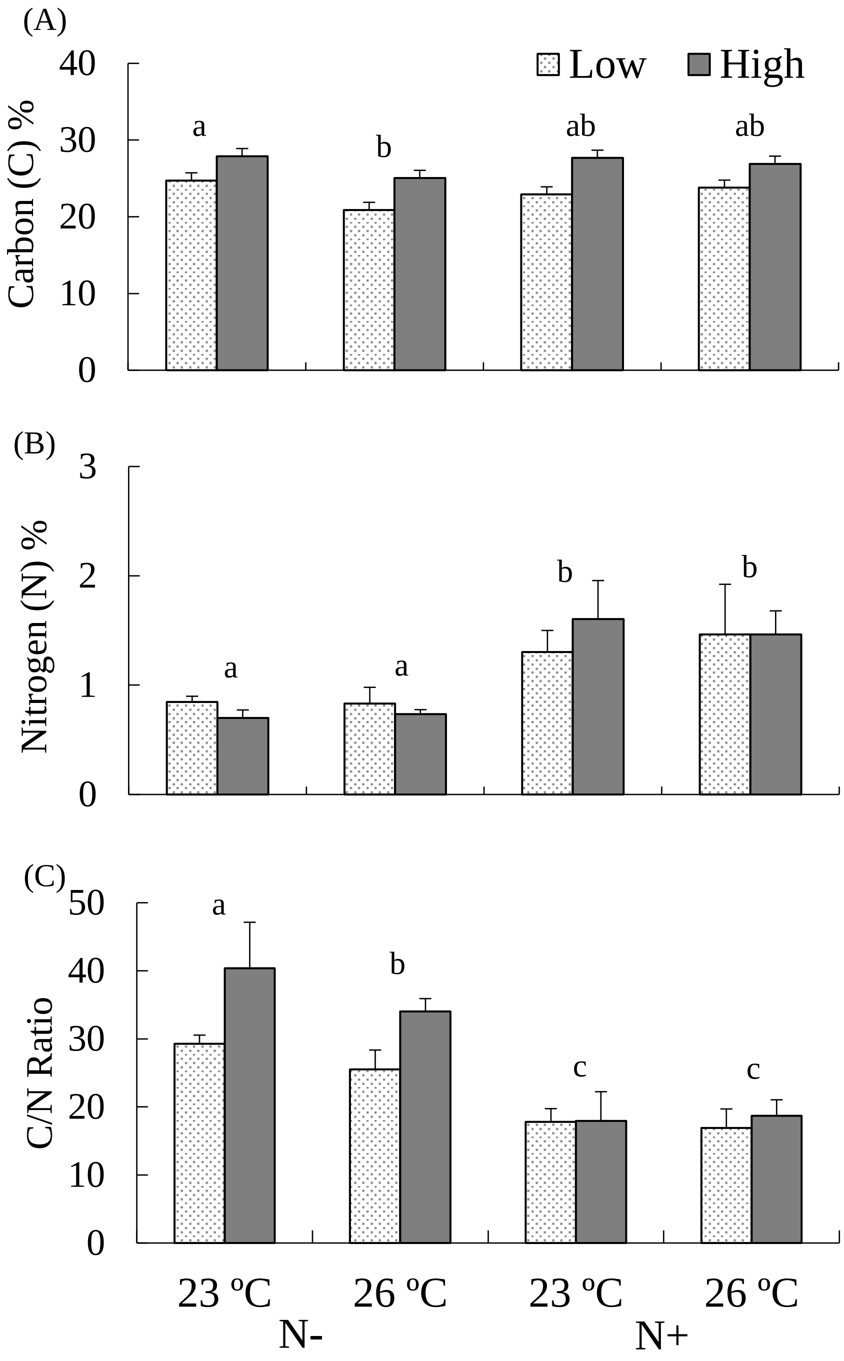

Figure 6. Carbon (A) and nitrogen (B) contents and C/N (C) of *Sargassum fusiforme* shoots cultured in eight different treatments (mean + SD, n = 6). Low and High indicate 30 and 150 (μmol photon m^{-2} s^{-1}) irradiance treatments, respectively. N+ and N- indicate nutrient enriched and non-enriched treatments, respectively. Different small letters indicate statistical significances among different treatments (P < 0.05).

Table 2. Results of a three-way ANOVA on the effects of temperature, nutrient availability and irradiance on pigments of *S. fusiforme*. Where $p < 0.05$ and $p < 0.01$ are denoted as * and **, respectively.

Source	df	MS	*F*	*P*	
Chl *a*					
Temperature (T)	1	0.0006	0.047	0.829	
Irradiance (I)	1	0.074	5.238	0.027	*
Nutrient (N)	1	0.054	3.878	0.055	
T × I	1	0.002	0.208	0.651	
T × N	1	0.007	0.539	0.467	
I × N	1	0.00002	0.001	0.973	
T × I × N	1	0.00077	0.054	0.815	
Chl c_2					
Temperature (T)	1	0.00004	0.193	0.662	
Irradiance (I)	1	0.002	7.638	0.009	**
Nutrient (N)	1	0.001	5.793	0.021	*
T × I	1	0.00001	0.062	0.804	
T × N	1	0.00009	0.403	0.529	
I × N	1	0.00003	0.176	0.676	
T × I × N	1	0.00004	0.202	0.655	
Fucoxanthin					
Temperature (T)	1	0.00009	0.092	0.762	
Irradiance (I)	1	0.004	4.811	0.034	*
Nutrient (N)	1	0.003	3.848	0.056	
T × I	1	0.00008	0.081	0.777	
T × N	1	0.0006	0.664	0.42	
I × N	1	0.0001	0.147	0.7068	
T × I × N	1	0.00007	0.074	0.785	
Violaxanthin					
Temperature (T)	1	0.000009	0.065	0.8	
Irradiance (I)	1	0.000002	0.014	0.904	
Nutrient (N)	1	0.000001	0.012	0.911	
T × I	1	0.0001	0.781	0.382	
T × N	1	0.00004	0.339	0.563	
I × N	1	0.000001	0.012	0.911	
T × I × N	1	0.0001	0.774	0.384	
Antheraxanthin					
Temperature (T)	1	0.0000004	0.069	0.794	
Irradiance (I)	1	0.0001	2.504	0.121	

Nutrient (N)	1	0.0002	3.092	0.086
T × I	1	0.00007	1.095	0.301
T × N	1	0.00005	0.712	0.403
I × N	1	0.00006	0.878	0.354
T × I × N	1	0.00009	1.361	0.25
Zeaxanthin				
Temperature (T)	1	0.000003	0.299	0.587
Irradiance (I)	1	0.000006	0.531	0.468
Nutrient (N)	1	0.00002	1.788	0.188
T × I	1	0.000002	0.104	0.748
T × N	1	0.000002	0.177	0.676
I × N	1	0.000007	0.64	0.428
T × I × N	1	0.000001	0.101	0.752

The individual significance of factors effects on Chl *a* (Figure 7A), Chl c_2 and Fuco allowed for ratio to be calculated for Chl c_2/Chl *a* and Fuco/ Chl *a*. ANOVA indicated an individual effect of nutrient and irradiance on the Chl c_2/Chl *a* with an interaction between temperature and irradiance (Table 3, Figure 7B). ANOVA did not indicate any significant effect of factors on Fuco/ Chl *a* (Table 3). ANOVA indicated that there were significant individual effects of nutrients and irradiance on VAZ/ Chl *a* (Table 3). However, there was interaction of temperature and nutrients (two factor interaction) and interestingly a three factor interaction between temperature, nutrient and irradiance on VAZ/ Chl *a* (Figure 7C) was found.

Table 3. Results of a three-way ANOVA on the effects of temperature, nutrient availability and irradiance on ratio of pigments of *S. fusiforme*. Where $p < 0.05$, $p < 0.01$ and $p < 0.001$ are denoted as *, ** and ***, respectively

Source	df	MS	*F*	*P*	
Chl *c2* /Chl *a*					
Temperature (T)	1	0.00009727	0.9521	0.3351	
Nutrient (N)	1	0.00098004	9.5931	0.0036	**
Irradiance (I)	1	0.00157344	15.4016	0.0003	***
T × I	1	0.00116258	11.3799	0.0017	**
T × N	1	0.0000692	0.6774	0.4154	
I× N	1	0.00024568	2.4048	0.1288	
T × N × I	1	0.0000835	0.8173	0.3714	
Fuco/Chl *a*					
Temperature (T)	1	0.0000439	0.1082	0.7439	
Nutrient (N)	1	0.00006093	0.1502	0.7004	
Irradiance (I)	1	0.0000521	0.1284	0.722	
T × I	1	0.0030943	0.7625	0.3877	
T× N	1	0.00006143	0.1514	0.6993	
I× N	1	0.00023984	0.5911	0.4465	
T × N × I	1	0.0000003	0.0007	0.9786	
VAZ/Chl *a*					
Temperature (T)	1	0.00335083	0.4829	0.4911	
Nutrient (N)	1	0.18666262	26.9012	<0.001	***
Irradiance (I)	1	0.54731186	78.8767	<0.001	***
T × I	1	0.01854718	2.673	0.1099	
T × N	1	0.05366527	7.7341	0.0082	**
I × N	1	0.00016772	0.0242	0.8772	
T × N × I	1	0.03584109	5.1653	0.0285	*

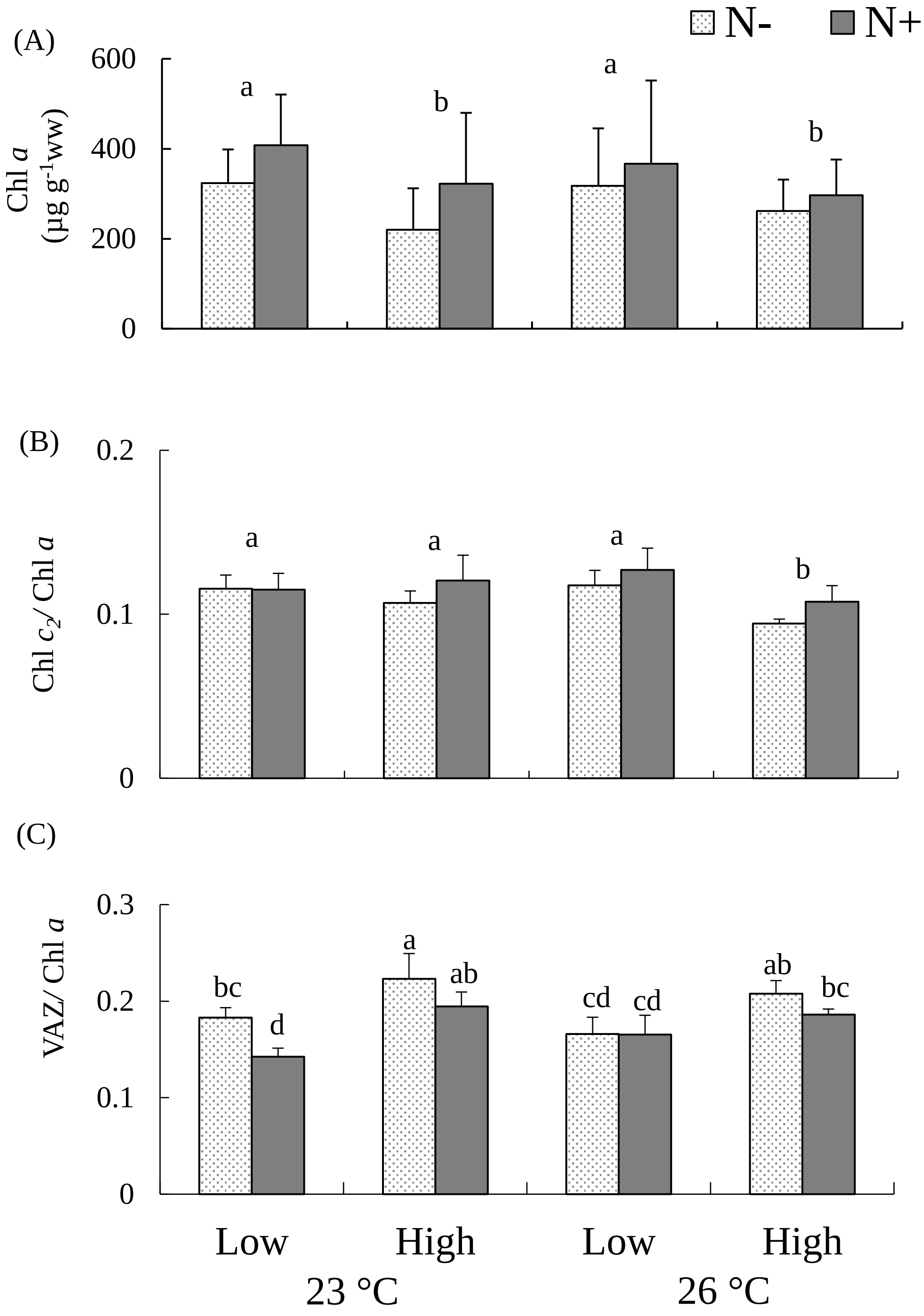

Figure 7. Chl *a* content (A), Chl c_2/ Chl *a* (B), and VAZ/Chl *a* (C) of *Sargassum fusiforme* shoots cultured in eight different treatments (mean + SD, n = 6). Low and High indicate 30 and 150 (μmol photon m^{-2} s^{-1}) irradiance treatments, respectively. N+ and N- indicate nutrient enriched and non-enriched treatments, respectively. Different small letters indicate statistical significances among different treatments ($P < 0.05$).

Discussion

In this current study, individual significance as well as interaction between temperature and irradiance was found. The effect of elevated temperature and irradiance reduced the SGR, however, SGR did not decrease under low irradiance treatments. At the same time, elevated irradiance increased SGR at 23 °C but not at elevated temperature of 26 °C. This indicated that a possible negative effect of warming on *Sargassum fusiforme* growth was synergized by elevated irradiance and/or a positive effect of elevated irradiance was antagonised by elevated temperature. This could be explained by the biochemical changes occurring in *S. fusiforme* as a result of multiple stressor interaction as similar interactive effects were observed on Chl c_2/ Chl *a*, where Chl c_2/ Chl *a* ratio decreased under elevated temperature and elevated irradiance conditions but not at lower irradiance condition. In addition, excess light energy production under warming condition triggers an increase in the production of reactive oxygen species (ROS) in chloroplast (Murata et al., 2007; Balfagón et al., 2019). ROS causes photoinhibition and chlorophyll degradation (Murata et al., 2007; Balfagón et al., 2019; Endo et al., 2020). In this current study, the photosystem II maximum efficiency (*Fv*/*Fm*) which is evidence of photoinhibition was not attained to conclusively prove photoinhibition. However, the Chl *a* content of *S. fusiforme* had reduced in response to elevated irradiance which was not synergised by elevated temperature. Therefore, elevated irradiance combined with elevated temperature could have decreased SGR through Chl c_2 degradation rather than Chl *a* degradation. This inference however, needs to be further validated.

In the current study, VAZ/ Chl *a* (xanthophyll cycle pigments to Chl *a*) was expected to increase under elevated temperature and elevated irradiance interaction, however, such as interaction was in observed. On the other hand, a three factor interaction among temperature, nutrients and irradiance was observed. This interaction indicated that

VAZ/ Chl *a* increased under elevated irradiance however not in enriched conditions combined with elevated temperature. It is possible that high light acclimation in VAZ/ Chl *a* could be inhibited under elevated temperatures and eutrophication.

In this present study, an interaction between temperature and nutrients was found on carbon content of *S. fusiforme.* The carbon content reduced under elevated temperature and non-enrichment, however, unaffected by temperature under enriched conditions. This showed that the negative effect of elevated temperature on carbon content was antagonized by enrichment. Further studies on metabolomics need to validate this. There were individual effects of nutrients on nitrogen content which had interacted with irradiance. However, there were no individual effects of irradiance on nitrogen contents. Past studies have shown individual effects of nutrient on kelps and fucoids resulted in increased nitrogen content (Endo et al., 2015; Gao et al., 2016; Kay et al., 2016; Endo et al., 2017; Gao et al., 2017; Franco et al., 2018; Mabin et al., 2019). However, interactions with irradiance have not been documented. This could mean that among fucoid species, exposure to irradiance has differences in nitrogen accumulation.

Paper from Chapter 2 was published as:

Charan H*, Inomata E, Endo H, Sato Y, Okumura Y and Aoki M N (2022) Decreased irradiance and nutrient enrichment mitigate the negative effect of ocean warming on growth and biochemical compositions of a canopy-forming marine macroalga. *Journal of Marine Science and Engineering* 10: 479 https://doi.org/10.3390/jmse10040479

Chapter 3 The interactive effect of temperature, nutrient and irradiance on the specific growth rate (SGR) and biochemical composition of two strains of *Ulva prolifera*

Materials and Methods

Origin of strains

Two *Ulva prolifera* strains, namely Sekiguchi and Takeshima were obtained from Yuriage Factory, Riken Food Co., Ltd., in Natori City. However, the strains were established at Usa Marine Biological Institute at Kochi University, Japan. The origin of the strains were Iwate Prefecture (39°28´28.5´´N, 141°57´03.3´´E) for Sekiguchi strain and Kochi Prefecture (32°57´44.5´´N, 132°58´34.0´´E) for Takeshima strain respectively (Sato et al., 2021).

Germling Cluster Method

"Germling Cluster Method" (GCM) is sieving the germlings first through 250 µm sieve. The collected germlings are then sieved through 40 µm sieve. The top germlings on 40 µm is then collected and cultured at 20 °C with Provasoli's Enriched Seawater medium at 200 µmol photons m^{-2} s^{-1} at half strength. This process is repeated two or three times depending on how well clusters of germlings were being formed. Once the ten clusters have attained the weight of 0.01 g, they are then cultivated in the eight experimental treatments in the incubator.

Experimental Treatment

There were ten clusters per flask in each treatment for each strain that were cultivated for 9 days. Similar to Chapter 2, this experiment design utilized a 2 × 2 × 2 factorial design, where two temperature levels of 23 and 26 ºC, two irradiance levels of 30 and 150 µmol photon m^{-2} s^{-1} and two nutrients levels of non enriched and enriched were used.

23 ºC was chosen due to optimum growth of *U. prolifera* being between 20 -25 ºC (Hiraoka et al., 2020; Sato et al., 2021). On the other hand, 26 ºC was utilized to represent the warming of 3 ºC that is expected to happen in the next 100 years (IPCC, 2013). From an earlier photosynthesis -irradiance experiment (PI or PE) experiment, E_c compensation was 4.3-40 µmol photon m^{-2} s^{-1} and thus, 30 µmol photon m^{-2} s^{-1} was used to represent low irradiance (Figure 8). However, E_k saturation was 116.2-200 µmol photon m^{-2} s^{-1} and therefore, 150 µmol photon m^{-2} s^{-1} was representative for higher irradiance (Figure 8). Non enrichment was represented by sterile seawater that was collected from Yuriage Factory, Riken Food Co., Ltd., in Natori City, Miyagi Prefecture. Enrichment was represented by 5% Provasoli's enriched seawater (5% PES) as *U. prolifera* naturally grow well in eutrophic waters. The nitrate, nitrite, ammonium and phosphate concentrations were measured n=5. The salinity was 33 psu n=5 which was measured with Horiba LAQUA act, HORIBA Advanced Techno Co., Ltd., Kyoto, Japan. The culture medium was changed every 2 days (Endo et al., 2017)..

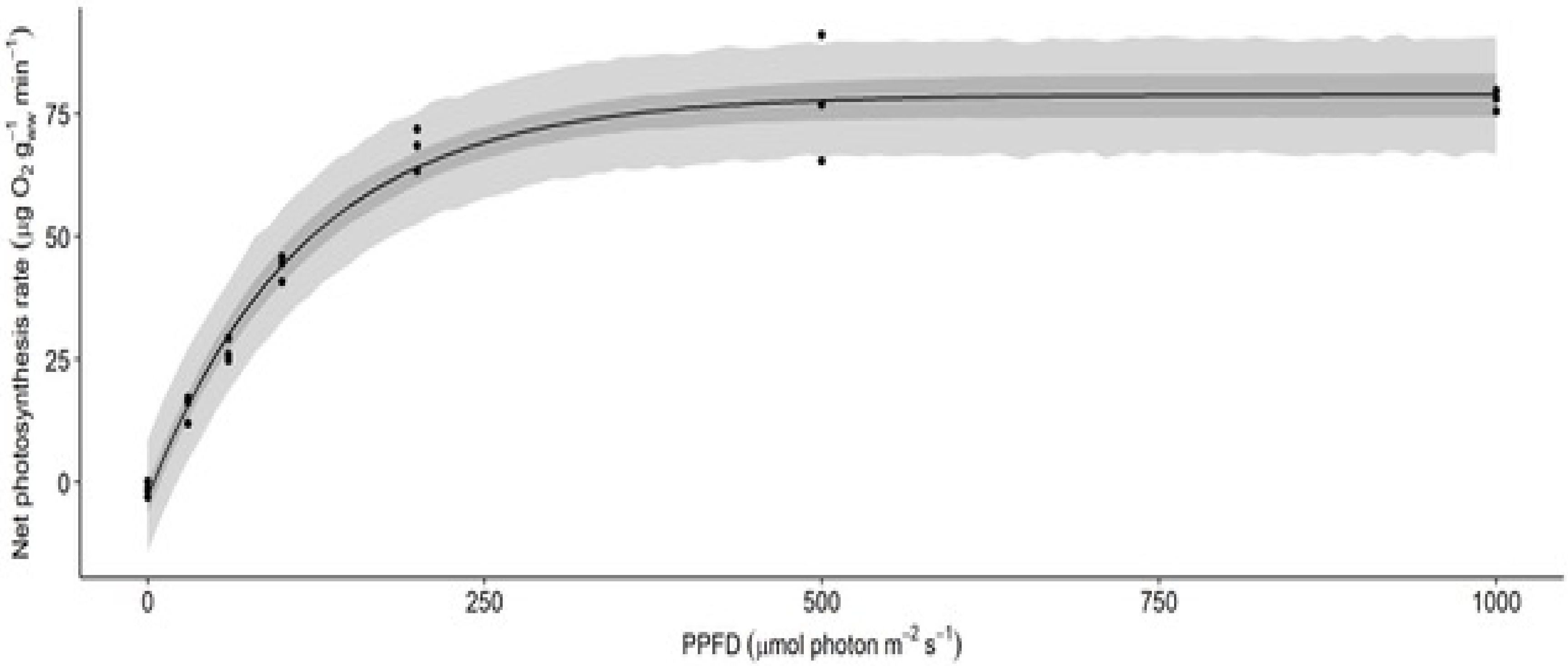

Figure 8. Response of net photosynthetic rate of *Ulva prolifera* to increasing photosynthetically active radiation (PAR). Dots indicate measured rates (n = 3) lines indicate expected value while shaded region indicates 95% Bayesian prediction interval (95% BPI).

The samples were blotted dry on a paper towel to remove excess moisture and the initial weight (before culturing) and final weight (after culturing) was measured using an electronic balance that had 0.001 g accuracy. The SGR was determined using the following equation;

SGRs (% d^{-1}) = 100 × ln (final wet weight/initial wet weight)/9 d

Five clusters from each experimental flask for both strains were oven dried for 12 days at 80 °C for carbon and nitrogen analysis by the organic elemental analyzer (FLASH2000, ThermoFisher Scientific, Waltham, MA, USA).

The pigment analysis was performed using high-performance liquid chromatography (HPLC) after the five clusters were placed in 9 mL bottles containing 4-5 mL of dimethylformamide (DMF). Method by Zapata et al. (2000) was used where 80% supernatant was achieved after diluting in distilled water. The guard column was placed between the injection valve and the analytical column (Symmetry C8, Waters Milford Massachusetts, USA). The pigments analysed for both strains included Chlorophyll *a* (Chl *a*), Chlorophyll *b* (Chl *b*), Violaxanthin (Viola), Antheraxanthin (Anthera), and Zeaxanthin (Zea), Neoxanthin (Neo) and Beta carotene (βcarotene).

Statistical Analysis

ANOVA analysis of variance was utilized on initial weight of the two strains. Normality and homoscedasticity were determined by Shapiro-Wilk test and Bartlett's test respectively. The combined effects of temperature, nutrients and irradiance on SGR and biochemical compositions were determined by a three-way ANOVA. Interactive effects of synergism and/or antagonism were determined by Tukey’s multiple comparison test. Comparison of SGR and biochemical compositions between the two strains in eight treatments was done using Welch’s t-test. Analysis was done by R software version 4.1.0.

Results

Sekiguchi strain had the initial mean ± SD wet weight as 0.014 ± 0.001 g while Takeshima strain has initial mean weight as 0.015 ± 0.002 g. For Sekiguchi strain, the Shapiro wilk test showed some differences (df = 7, MS= 6.339E-06, F = 2.3, P = 0.046). Also, for Sekiguchi strain, Bartlett's test (Bartlett's K squared = 5.933, df = 7, P = 0.547) showed no significant difference among treatments in initial wet weight. For Takeshima strain, the Shapiro-Wilks test (df = 7, MS= 8.552E-06, F = 2.123, P > 0.05) and Bartlett's test (Bartlett's K squared = 5.933, df = 7, P = 0.547) showed no significant difference among treatments in initial wet weight. Both strains had homogeneity of variance. The nitrate, nitrite, ammonium and phosphate concentration in sterile seawater were 1.463 ± 0.355 µM, < 0.010 µM, <0.550 µM, 0.113 ± 0.017 µM respectively. The nitrate, nitrite, ammonium and phosphate concentration in 5 % PES were 15.750 ± 2.108 µM, < 0.011 µM, 3.114 ± 0.160 µM and < 0.010 µM respectively. Figure 9 and 10 show the results from the experimental treatments. The growth over nine days is shown in Figure 11.

Specific growth rate

In Sekiguchi strain, there were significant individual effects of temperature, nutrients and irradiance on SGR in ANOVA (Table 4). However, the only interaction detected was between irradiance and nutrients where SGR increased in response to elevated irradiance and enriched conditions but not at low irradiance conditions (Figure 12A). In Takeshima strain, there were significant individual effects of temperature, nutrients and irradiance on SGR in ANOVA (Table 4). There were interaction between temperature and irradiance where SGR increased in response to high irradiance under both temperatures (Figure 12B). There was also an interaction between temperature and nutrients. The SGR for both temperatures, increased significantly under enriched conditions and not under non enriched conditions. There was also an interaction between irradiance and nutrients where the response of SGR increased under elevated irradiance and enriched conditions but not at low irradiance conditions. When comparing the two strains (Takeshima and Sekiguchi) under eight treatment conditions, Welch's t-test indicated there were differences in all treatments except in two treatment which were 23 ºC, low irradiance, non-enrichment and 26 ºC, high irradiance and enrichment combination (Table 5).

Table 4. Shows results of a three-way ANOVA on the effects of temperature, nutrient availability and irradiance on SGR of Takeshima and Sekiguchi strain of *U. prolifera*. Where $p < 0.05$, and $p < 0.001$ are denoted as * and ***, respectively.

Source	df	MS	*F*	*P*	
SGR Sekiguchi strain					
Temperature (T)	1	125	23.686	1.81E-05	***
Irradiance (I)	1	3700	699.877	<2E-16	***
Nutrient (N)	1	4522	855.426	<2E-16	***
T × I	1	8	1.594	2.14E-01	
T × N	1	21	3.89	0.0555	
I × N	1	1403	265.442	<2E-16	***
T × I × N	1	2	0.44	0.511	
SGR Takeshima strain					
Temperature (T)	1	109.4	13.163	0.0008	***
Irradiance (I)	1	2863.7	344.715	<2E-16	***
Nutrient (N)	1	1645.4	198.063	<2E-16	***
T × I	1	255.2	30.716	2.08E-06	***
T × N	1	48.8	5.869	0.02	*
I × N	1	389.5	46.88	3.07E-08	***
T × I × N	1	10.1	1.213	0.2774	

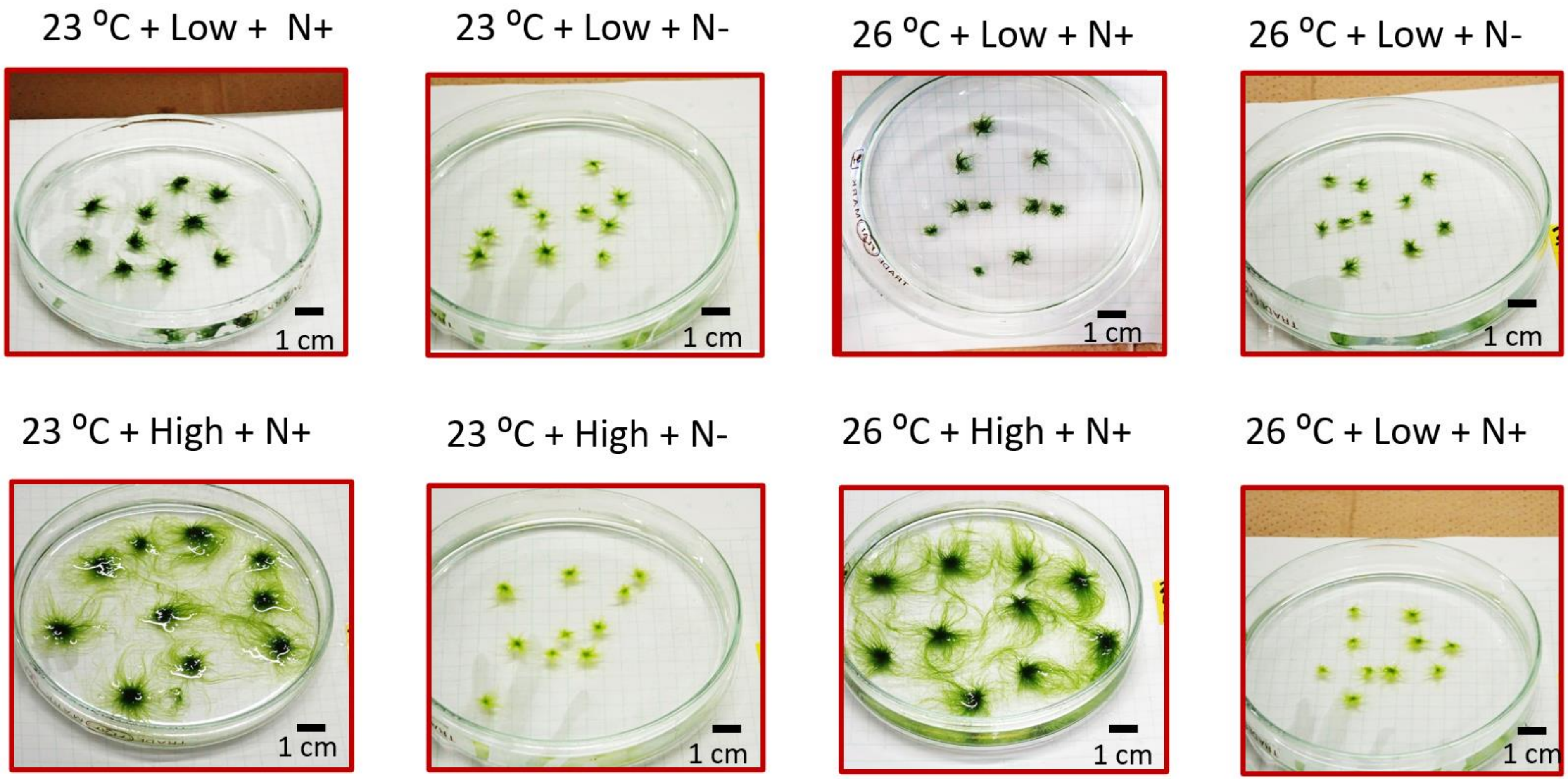

Figure 9. Growth of Sekiguchi strain after 9 days of cultivation.

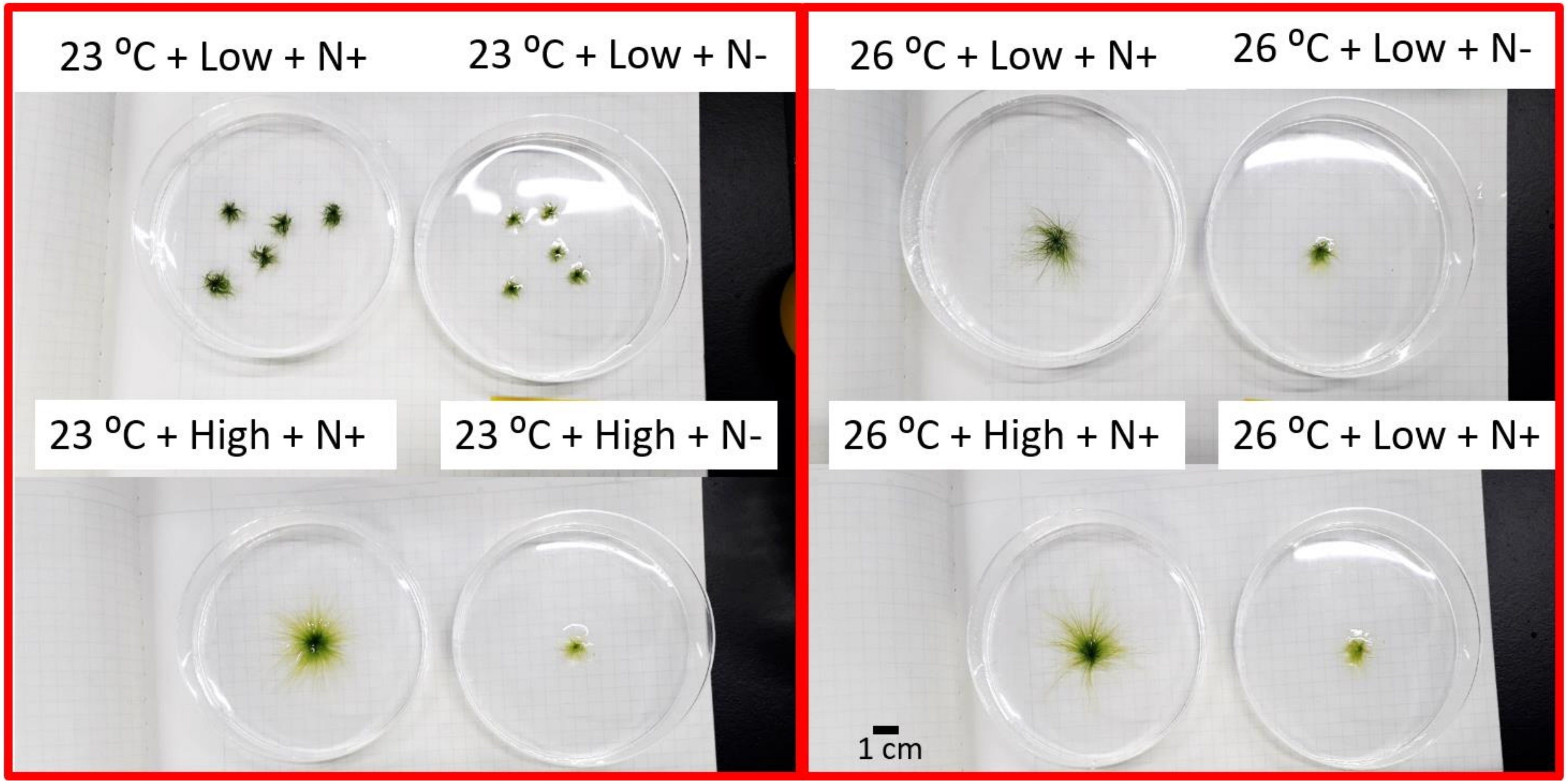

Figure 10. Growth of Takeshima strain after 9 days of cultivation.

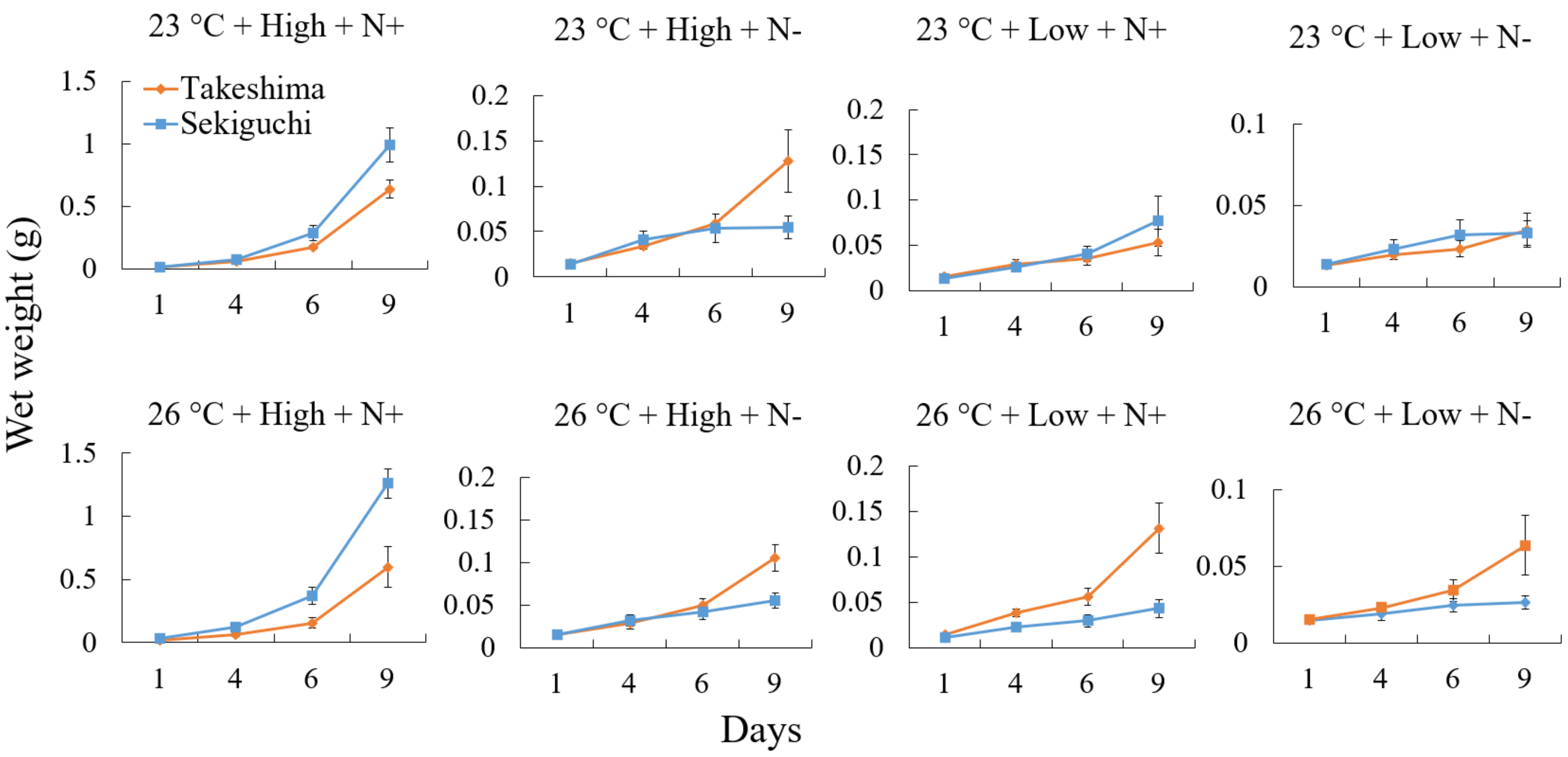

Figure 11. Wet weight growth over nine days for Sekiguchi and Takeshima strain under eight experimental conditions.

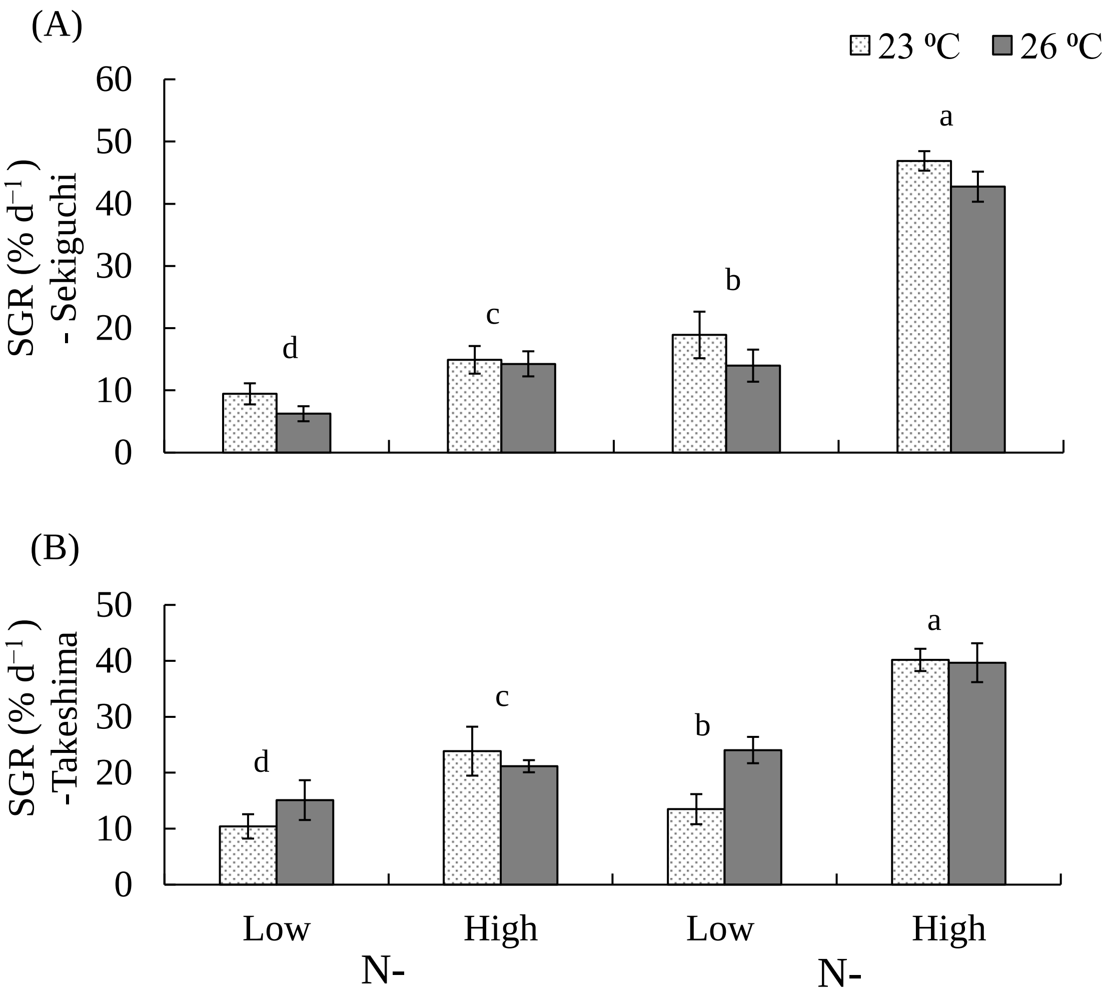

Figure 12. Specific Growth Rate (SGR) of Sekiguchi (A) and Takeshima (B) strain of *Ulva prolifera* cultured in eight different treatments (mean + SD, n = 6). Low and High indicate 30 and 150 (μmol photon m^{-2} s^{-1}) irradiance treatments, respectively. N+ and N- indicate nutrient enriched and non-enriched treatments, respectively. Different small letters indicate statistical significances among different treatments (P < 0.05).

Table 5. Results Welch's t-test on the comparison of SGR between Takeshima strain and Sekiguchi strain of *U. prolifera* under eight treatment conditions. Low and High indicate 30 and 150 (μmol photon m^{-2} s^{-1}) irradiance treatments, respectively. N+ and N- indicate nutrient enriched and non-enriched treatments, respectively. Where $p < 0.05$, and $p < 0.01$, and $p < 0.001$ are denoted as *, ** and ***, respectively.

Source	df	t	*P*	
SGR comparison				
23°C+ High+ N+	9.4776	-6.4972	8.85E-05	***
23°C+ High + N-	7.4242	4.4678	0.00251	**
23°C+ Low + N+	9.0717	-2.8826	0.01796	*
23°C+ Low + N-	9.4352	0.85832	0.412	
26°C+ High + N+	8.9217	-1.7793	0.1092	
26°C+ High + N-	7.6706	7.3841	9.59E-05	***
26°C+ Low + N+	9.9155	7.0696	3.57E-05	***
26°C+ Low + N-	6.135	5.779	0.001	**

Carbon, Nitrogen and C/N Content

For Sekiguchi strain, carbon content was significantly affected by nutrients and irradiance individually (Table 6). Similar to Takeshima strain Tukey's multiple comparison detected an interaction between irradiance and nutrients however, the carbon content had increased under high irradiance and enriched conditions (Figure 13A). In Takeshima strain, carbon content was affected by temperature, nutrients and irradiance individually (Table 6). Tukey's multiple comparison detected an interaction between irradiance and nutrients where the carbon content under higher irradiance had decreased under enrichment (Figure 13B). When comparing the two strains (Takeshima and Sekiguchi) under eight treatment conditions, Welch's t-test detected there were differences in all treatments between the strains (Table 7).

Table 6. Shows results of a three-way ANOVA on the effects of temperature, nutrient availability and irradiance on carbon content (%) of Takeshima and Sekiguchi strain of *U. prolifera*. Where $p < 0.05$, $p < 0.01$ and $p < 0.001$ are denoted as *, ** and ***, respectively.

Source	df	MS	*F*	*P*	
Carbon – Sekiguchi strain					
Temperature (T)	1	3.4	0.926	3.42E-01	
Irradiance (I)	1	91.2	25.168	1.12E-05	***
Nutrient (N)	1	334.2	92.257	6.09E-12	***
T × I	1	1.6	0.45	5.06E-01	
T × N	1	9.2	2.548	1.18E-01	
I × N	1	111.1	30.666	2.11E-06	***
T × I × N	1	3.3	0.916	3.44E-01	
Carbon - Takeshima strain					
Temperature (T)	1	12.850	4.830	0.034	*
Irradiance (I)	1	55.850	20.990	0.000	***
Nutrient (N)	1	27.970	10.513	0.002	**
T × I	1	0.110	0.041	0.841	
T × N	1	1.410	0.530	0.471	
I × N	1	18.810	7.068	0.011	*
T × I × N	1	6.670	2.505	0.121	

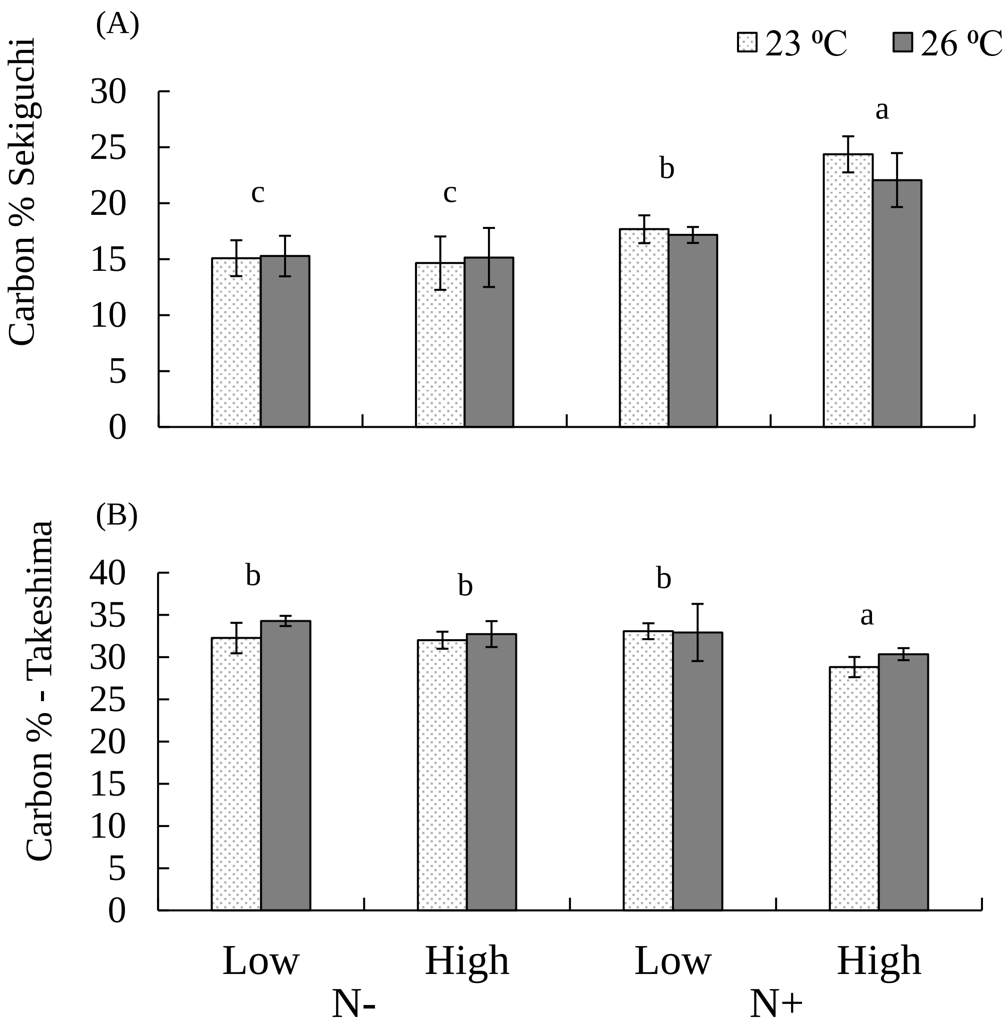

Figure 13. Carbon content (C %) of Sekiguchi (A) and Takeshima (B) strain of *Ulva prolifera* cultured in eight different treatments (mean + SD, n = 6). Low and High indicate 30 and 150 (μmol photon m^{-2} s^{-1}) irradiance treatments, respectively. N+ and N- indicate nutrient enriched and non-enriched treatments, respectively. Different small letters indicate statistical significances among different treatments (P < 0.05).

Table 7. Results Welch's t-test on the comparison of Carbon content (C%) between Takeshima strain and Sekiguchi strain of *U. prolifera* under eight treatment conditions. Low and High indicate 30 and 150 (μmol photon m^{-2} s^{-1}) irradiance treatments, respectively. N+ and N- indicate nutrient enriched and non-enriched treatments, respectively. Where $p < 0.001$ is denoted as ***.

Source	df	t	*P*	
Carbon- Comparison				
23°C+ High+ N+	9.2311	5.4256	0.000384	***
23°C+ High + N-	6.7378	16.417	1.10E-06	***
23°C+ Low + N+	9.3084	24.18	1.02E-09	***
23°C+ Low + N-	9.8642	17.418	9.83E-09	***
26°C+ High + N+	5.8669	8.07	2.17E-04	***
26°C+ High + N-	8.0348	14.102	5.96E-07	***
26°C+ Low + N+	5.4423	11.158	5.91E-05	***
26°C+ Low + N-	6.0924	24.381	2.63E-07	***

For Sekiguchi strain, individual effect of temperature, nutrient and irradiance had significant effect on the nitrogen content (Table 8). However, a three- factor interaction between temperature, nutrient and irradiance was detected by Tukey's test where elevated temperature and low irradiance resulted in higher nitrogen content under enriched conditions but not under non-enriched conditions (Figure 14A). For Takeshima strain, there were significant individual effects of temperature, nutrients and irradiance on nitrogen content detected by ANOVA (Table 8). There was an interaction detected between temperature and irradiance on nitrogen content by Tukey's test. It showed that nitrogen content had increased under low irradiance in both temperatures however, it decreased under higher irradiance. There was also an interaction between temperature and nutrient detected where nitrogen content increased under enriched conditions but not in non-enriched conditions. In addition, an interaction between irradiance and nutrients on nitrogen content was also detected (Figure 14B). Under low irradiance and enriched conditions, nitrogen content was high however, it reduced under high irradiance. A Welch's t-test was used to compare the nitrogen content between the two strains in eight treatments with significance in all treatment combination except in 26 ^{0}C, high irradiance and nutrient enrichment combination (Table 9).

Table 8. Shows results of a three-way ANOVA on the effects of temperature, nutrient availability and irradiance on nitrogen content (N%) of Takeshima and Sekiguchi strain of *U. prolifera*. Where $p < 0.05$, and $p < 0.001$ are denoted as * and ***, respectively.

Source	df	MS	*F*	*P*	
Nitrogen - Sekiguchi Strain					
Temperature (T)	1	0.017	4.281	4.50E-02	*
Irradiance (I)	1	1.079	64.802	6.76E-10	***
Nutrient (N)	1	26.86	1612.651	<2E-16	***
T × I	1	0.079	4.744	3.54E-02	*
T × N	1	0.013	0.798	3.77E-01	
I × N	1	0.783	46.981	2.99E-08	***
T × I × N	1	0.304	18.282	1.15E-04	***
Nitrogen - Takeshima Strain					
Temperature (T)	1	0.24	4.467	0.0408	*
Irradiance (I)	1	34.81	657.78	<2E-16	***
Nutrient (N)	1	6	113.445	3.04E-13	***
T × I	1	1.34	25.36	1.06E-05	***
T × N	1	0.39	7.298	0.0101	*
I × N	1	1.04	19.614	7.16E-05	***
T × I × N	1	0.06	1.078	3.05E-01	

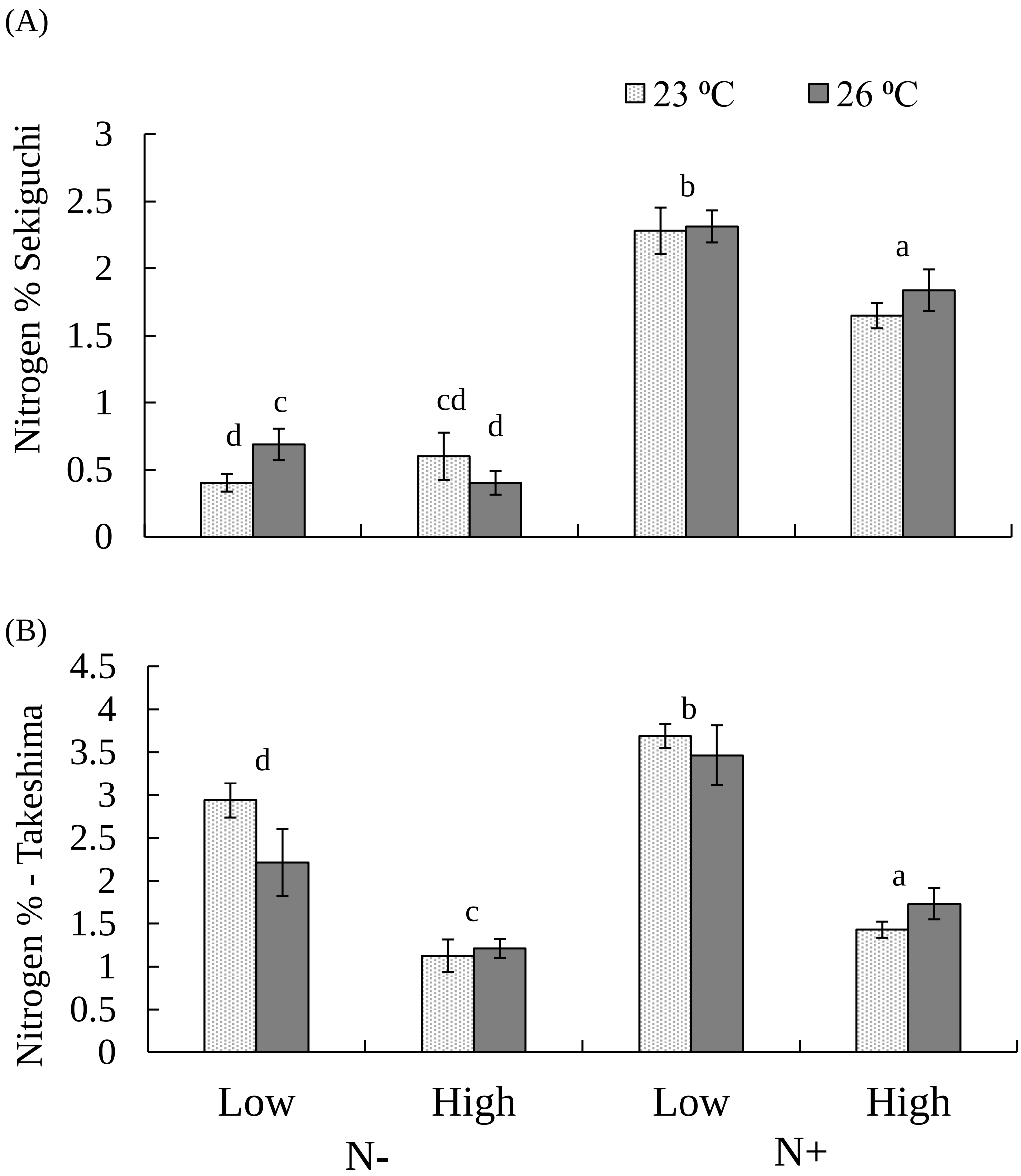

Figure 14. Nitrogen content (N %) of Sekiguchi (A) and Takeshima (B) strain of *Ulva prolifera* cultured in eight different treatments (mean + SD, n = 6). Low and High indicate 30 µmol and 150 (µmol photon m^{-2} s^{-1}) irradiance treatments, respectively. N+ and N- indicate nutrient enriched and non-enriched treatments, respectively. Different small letters indicate statistical significances among different treatments (P < 0.05).

Table 9. Results of Welch's t-test on the comparison of nitrogen content (N%) between Takeshima strain and Sekiguchi strain of *U. prolifera* under eight treatment conditions. Low and High indicate 30 and 150 (µmol photon m^{-2} s^{-1}) irradiance treatments, respectively. N+ and N- indicate nutrient enriched and non-enriched treatments, respectively. Where $p < 0.01$ and $p < 0.001$ are denoted as ** and ***, respectively.

Source	df	t	*P*	
Nitrogen - Comparisons				
23°C+ High+ N+	9.9991	-4.0775	2.22E-03	**
23°C+ High + N-	9.9512	4.9719	5.68E-04	***
23°C+ Low + N+	9.5706	15.605	3.99E-08	***
23°C+ Low + N-	6.0515	29.353	9.32E-08	***
26°C+ High + N+	9.7053	-1.0693	3.11E-01	
26°C+ High + N-	9.4329	13.831	1.41E-07	***
26°C+ Low + N+	6.1341	7.6067	2.42E-04	***
26°C+ Low + N-	5.9106	9.2414	9.89E-05	***

For Sekiguchi strain there was a three factor interaction on C/N where warming combined with high irradiance increased C/N however, this is reduced under enriched conditions (Figure 15A). In Takeshima, individual effects of irradiance and nutrients were significant on C/N based on ANOVA (Table 10). There were interactions between temperature and irradiance where C/N increased significantly under both temperatures and high irradiance. There was also temperature and nutrient interaction where C/N under both temperatures were higher under non enrichment than enrichment. An interaction between irradiance and nutrient on C/N showed that C/N increased under high irradiance and enrichment but not at low irradiance (Figure 15B). A Welch's t-test was used to compare the nitrogen content between the two strains in eight treatments with significance in all treatment combination except in 23 ^{o}C, high irradiance and non- enrichment combination (Table 11).

Table 10. Shows results of a three-way ANOVA on the effects of temperature, nutrient availability and irradiance on Carbon/Nitrogen (C/N) of Takeshima and Sekiguchi strain of *U. prolifera*. Where $p < 0.05$, and $p < 0.001$ are denoted as * and ***, respectively.

Source	df	MS	*F*	*P*	
Carbon/Nitrogen (C/N) - Sekiguchi strain					
Temperature (T)	1	21	2.513	1.21E-01	
Irradiance (I)	1	161	19.121	8.52E-05	***
Nutrient (N)	1	4966	590.908	<2e-16	***
T × I	1	484	57.621	2.86E-09	***
T × N	1	1	0.079	7.81E-01	
I × N	1	56	6.695	1.34E-02	*
T × I × N	1	690	82.159	3.03E-11	***
Carbon/Nitrogen (C/N)- Takeshima strain					
Temperature (T)	1	0.9	0.221	0.640	
Irradiance (I)	1	1787.9	426.371	<2E-16	***
Nutrient (N)	1	534	127.354	5.28E-14	***
T × I	1	68.2	16.256	2.42E-04	***
T × N	1	19.4	4.637	3.74E-02	*
I × N	1	74.3	17.714	1.41E-04	***
T × I × N	1	8.6	2.05	1.60E-01	

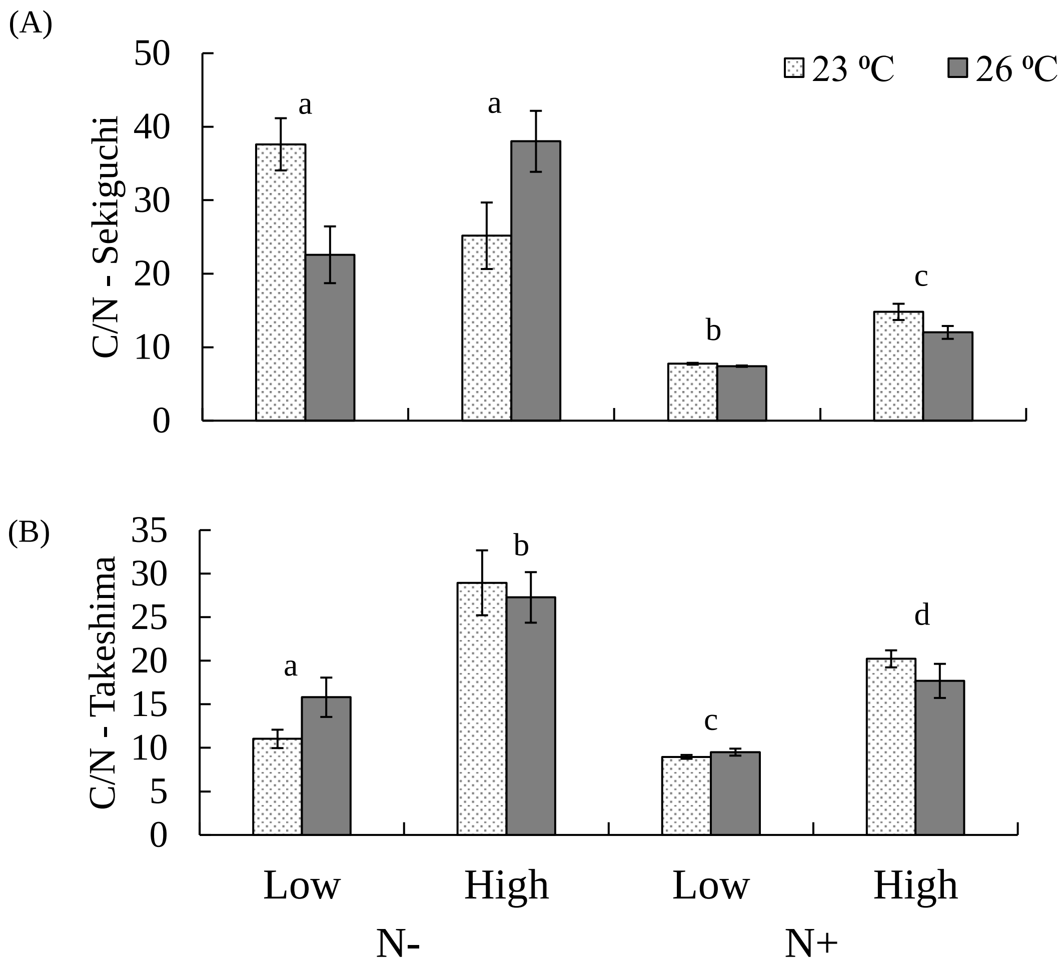

Figure 15. Carbon/Nitrogen (C/N) of Sekiguchi (A) and Takeshima (B) strain of *Ulva prolifera* cultured in eight different treatments (mean + SD, n = 6). Low and High indicate 30 and 150 (μmol photon m^{-2} s^{-1}) irradiance treatments, respectively. N+ and N- indicate nutrient enriched and non-enriched treatments, respectively. Different small letters indicate statistical significances among different treatments (P < 0.05).

Table 11. Results of Welch's t-test on the comparison of carbon/nitrogen (C/N) between Takeshima strain and Sekiguchi strain of *U. prolifera* under eight treatment conditions. Low and High indicate 30 and 150 (μmol photon m^{-2} s^{-1}) irradiance treatments, respectively. N+ and N- indicate nutrient enriched and non-enriched treatments, respectively. Where $p < 0.01$ and $p < 0.001$ are denoted as ** and ***, respectively.

Source	df	t	*P*	
C/N comparison				
23°C+ High+ N+	9.8569	8.9964	4.59E-06	***
23°C+ High + N-	9.6449	1.584	1.45E-01	
23°C+ Low + N+	7.841	11.708	3.05E-06	***
23°C+ Low + N-	5.8787	-17.574	2.64E-06	***
26°C+ High + N+	6.9239	6.4837	3.55E-04	***
26°C+ High + N-	8.9471	-5.1911	5.82E-04	***
26°C+ Low + N+	5.5374	12.371	3.04E-05	***
26°C+ Low + N-	8.0681	-3.7041	0.005918	**

Nitrogen accumulation was higher under enriched and low irradiance in both strains however, growth did not increase with nitrogen accumulation in Takeshima strain (Figure 16C). Growth increased with nitrogen accumulation in Sekiguchi strain (16A). The relationship between carbon content and growth in Sekiguchi strain showed that carbon was higher at high irradiance and enrichment when growth increased (Figure 16B). The carbon content was high at low irradiance and enrichment but decreased as growth increased at high irradiance and enrichment combination in Takeshima strain (Figure 16D).

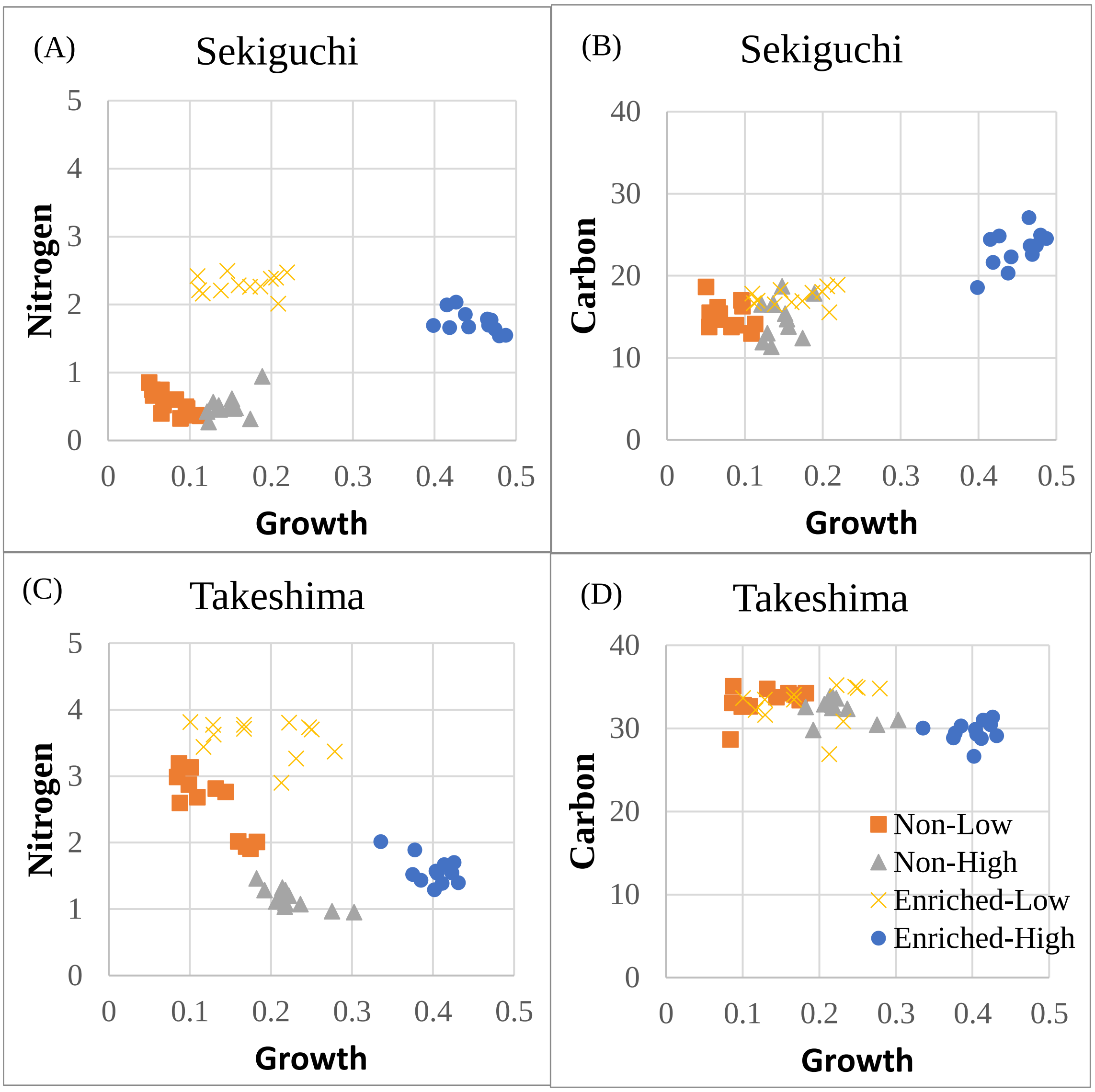

Figure 16. Shows the relationship between Nitrogen (A), Carbon (B) and growth o Sekiguchi and Nitrogen (C) and Carbon (D) and growth of Takeshima strain.

Pigment Contents

In Sekiguchi strain, there were significant individual effects of temperature, irradiance and nutrients (Table 12). However, there were no three- factor interactions. Instead, two factor interaction between temperature and irradiance was detected where higher temperature and lower irradiance had resulted in increased Chl *a* (Figure 17A) and Chl *b* (18A). There was also a temperature and nutrient interaction in Sekiguchi strain where higher temperature and enrichment resulted in higher Chl *a* and Chl *b*. In addition, Tukey's also detected irradiance and nutrients interaction on Chl *a* and Chl *b* of Sekiguchi strain where lower irradiance and enrichment had higher Chl *a* and Chl *b*. ANOVA had detected significant individual effects of temperature, irradiance and nutrients on Chl *a* and Chl *b* of Takeshima strain (Table 12). ANOVA showed a three factor interaction between temperature, irradiance and nutrients where warming and low irradiance conditions with nutrient enrichment resulted in higher Chl *a* (Figure 17B) and Chl *b* (18B). A comparison of Chl *a* between Sekiguchi and Takeshima strain showed significant difference among all treatments except 23 °C + Low + N+, 26°C+ Low + N+, 26°C+ Low + N- treatment combinations (Table 13). On the other hand, a comparison of Chl *b* between Sekiguchi and Takeshima strain showed significant difference among all treatments except 23°C+ High + N-, 23°C+ Low + N+, 26°C+ Low + N+, 26°C+ Low + N- (Table 13).

Table 12. Shows results of a three-way ANOVA on the effects of temperature, nutrient availability and irradiance on Chl *a* and Chl *b* of Takeshima and Sekiguchi strain of *U. prolifera*. Where $p < 0.05$, $p < 0.01$ and $p < 0.001$ are denoted as *, ** and ***, respectively.

Source	df	MS	*F*	*P*	
Chl *a* - Sekiguchi strain					
Temperature (T)	1	1.705	8.652	0.00541	**
Irradiance (I)	1	6.443	32.687	1.18E-06	***
Nutrient (N)	1	11.799	59.853	1.81E-09	***
T × I	1	1.807	9.164	0.00430	**
T × N	1	1.174	5.956	0.01919	*
I × N	1	1.902	9.648	0.00348	**
T × I × N	1	0.343	1.742	0.194	
Chl *a* -Takeshima Strain					
Temperature (T)	1	1.147	10.959	0.00198	**
Irradiance (I)	1	20.981	200.541	< 2E-16	***
Nutrient (N)	1	15.58	14800917	4.60E-15	***
T × I	1	0.323	3.087	8.68E-02	
T × N	1	1.974	18.864	9.33E-05	***
I × N	1	6.27	59.926	1.78E-09	***
T × I × N	1	2.06	19.689	6.97E-05	***
Chl *b* - Sekiguchi strain					
Temperature (T)	1	0.082	11.223	0.001772	**
Irradiance (I)	1	0.360	49.329	1.74e-08	***
Nutrient (N)	1	0.447	61.289	1.35E-09	***
T × I	1	0.116	15.869	0.000279	***
T × N	1	0.041	5.555	0.023412	*
I × N	1	0.155	21.239	4.08E-05	***
T × I × N	1	0.005	0.702	0.407	
Chl *b* -Takeshima Strain					
Temperature (T)	1	0.027	7.146	0.010821	*
Irradiance (I)	1	0.858	223.840	< 2E-16	***
Nutrient (N)	1	0.443	115.565	2.30E-13	***
T × I	1	0.007	1.813	0.186	
T × N	1	0.070	18.275	0.000115	***
I × N	1	0.205	53.607	6.71E-09	***
T × I × N	1	0.076	19.868	6.55E-05	***

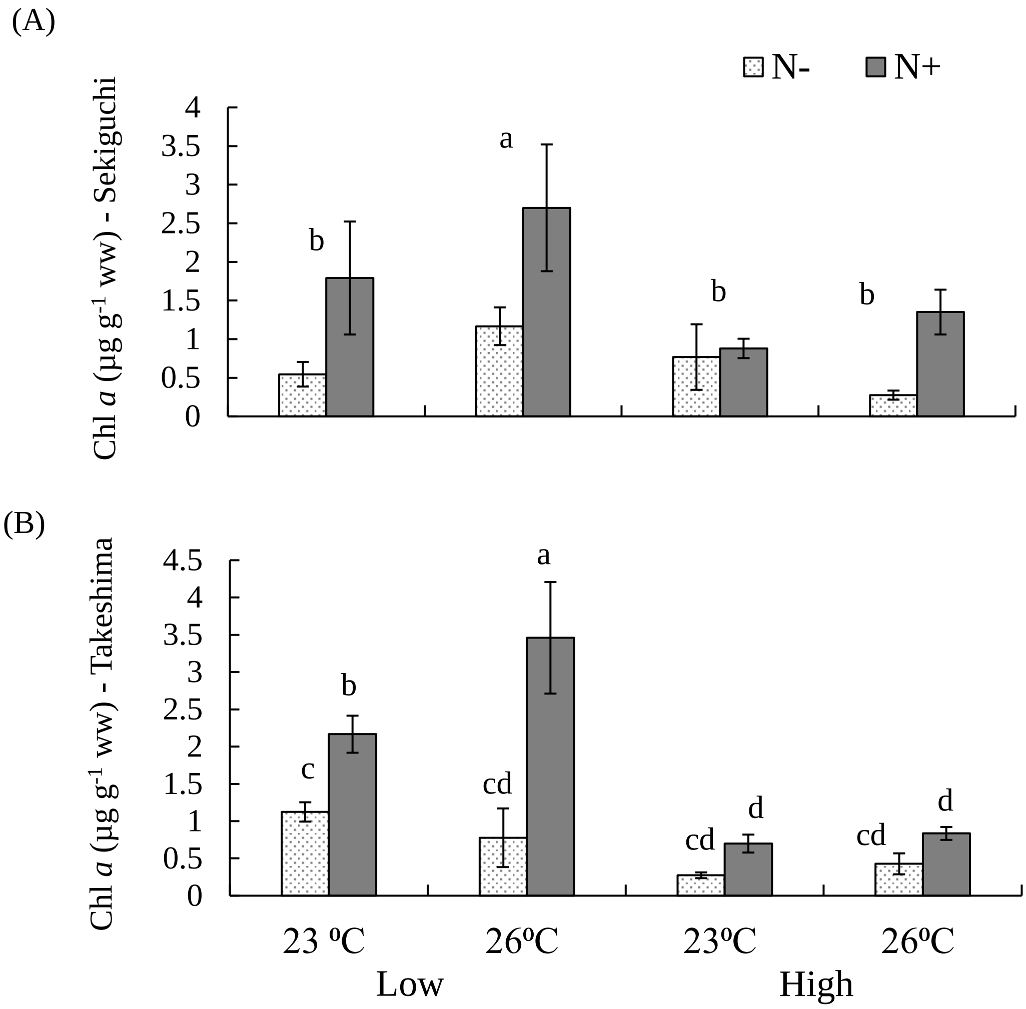

Figure 17. Chl *a* content of Sekiguchi (A) and Takeshima (B) strain of *Ulva prolifera* cultured in eight different treatments (mean + SD, n = 6). Low and High indicate 30 and 150 (µmol photon m^{-2} s^{-1}) irradiance treatments, respectively. N+ and N- indicate nutrient enriched and non-enriched treatments, respectively. Different small letters indicate statistical significances among different treatments (P < 0.05).

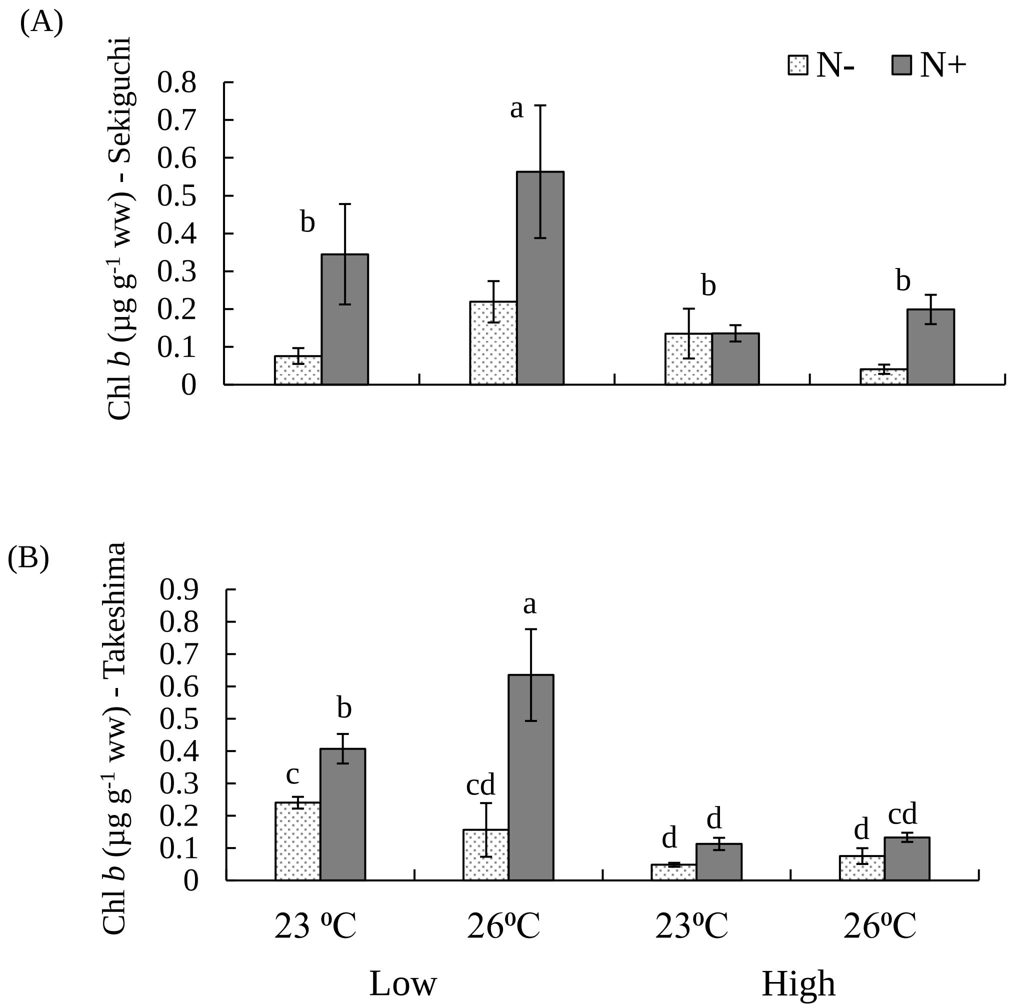

Figure 18. Chl *b* content of Sekiguchi (A) and Takeshima (B) strain of *Ulva prolifera* cultured in eight different treatments (mean + SD, n = 6). Low and High indicate 30 and 150 (µmol photon m^{-2} s^{-1}) irradiance treatments, respectively. N+ and N- indicate nutrient enriched and non-enriched treatments, respectively. Different small letters indicate statistical significances among different treatments (P < 0.05).

Table 13. Results of Welch's t-test on the comparison of Chl *a* and Chl *b* between Takeshima strain and Sekiguchi strain of *U. prolifera* under eight treatment conditions. Low and High indicate 30 and 150 (μmol photon m^{-2} s^{-1}) irradiance treatments, respectively. N+ and N- indicate nutrient enriched and non-enriched treatments, respectively. Where $p < 0.05$, $p < 0.01$ and $p < 0.001$ are denoted as *, ** and ***, respectively.

Source	df	t	*P*	
Chl *a* comparison				
23°C+ High+ N+	9.998	2.538	0.029	*
23°C+ High + N-	5.083	2.840	0.035	*
23°C+ Low + N+	6.141	-1.18	0.279	
23°C+ Low + N-	9.597	-6.884	5.241E-05	***
26°C+ High + N+	5.893	4.166	0.006	**
26°C+ High + N-	6.715	-2.451	0.045	*
26°C+ Low + N+	9.915	-1.673	0.125	
26°C+ Low + N-	8.351	2.061	0.071	
Chl *b* comparison				
23°C+ High+ N+	5.085	3.217	0.022	*
23°C+ High + N-	9.834	1.958	0.079	
23°C+ Low + N+	6.167	-1.085	0.318	
23°C+ Low + N-	9.799	-14.574	5.773E-08	***
26°C+ High + N+	6.337	3.893	0.007	**
26°C+ High + N-	7.374	-3.080	0.016	*
26°C+ Low + N+	9.579	-0.779	0.454	
26°C+ Low + N-	8.659	1.552	0.156	

In Sekiguchi strain, there were individual effects of temperature, nutrients and irradiance on Violaxanthin (Table 14). Tukey's had detected two factor interactions but not a three factor interaction (Figure 19D). ANOVA had detected individual significant effects of nutrients and irradiance on Violaxanthin in Takeshima strain (Table 14). However, A factor interaction between temperature, nutrient and irradiance on Violaxanthin was detected were elevated temperature, nutrient enrichment and low irradiance had resulted in higher Viola content on Takeshima strain (Figure 19A).

In Sekiguchi strain ANOVA did not have any individuals effects of temperature, nutrients and irradiance on Antheraxanthin content (Table 14). However, a three factor interaction was detected. The three factor interaction indicated that Antheraxanthin under optimum temperature of 23 °C under low irradiance conditions is exacerbated in non enriched conditions (Figure 19E). However, ANOVA had detected significant individual effects of temperature, nutrients and irradiance on Antheraxanthin in Takeshima strain (Figure 19B) with two factor interaction between the factors but not three factor interaction (Table 14).

Significant effects of individual effects of temperature, nutrients and irradiance on Zeaxanthin in Takeshima strain and Sekiguchi strain respectively was detected by ANOVA (Figure 19C; Figure 19F) with two factor interaction (Table 13). However, only temperature and nutrient interaction was detected in Sekiguchi strain where elevated temperature and enrichment yielded higher Zeaxanthin content.

When compared by Welch's t-test. The two strains differed in their Violaxanthin content in all experimental treatment except under high irradiance in both temperatures (23 and °C) and enrichment and non enrichment conditions combinations (Table 15). However, antheraxanthin different between the strains in treatments of 23°C, high irradiance and non enrichment treatment , 23 °C, low and non enrichment and 26 °C, high non enrichment (Table 15). However, zeaxanthin between the strains differed in two treatment conditions (26 °C, high irradiance, enrichment, nutrient enrichment combination and 26 °C, low irradiance and non enrichment combination) (Table 15).

Table 14. Shows results of a three-way ANOVA on the effects of temperature, nutrient availability and irradiance on Violaxanthin (Viola), Antheraxanthin (Anthera) and Zeaxanthin (Zea) of Takeshima and Sekiguchi strain of *U. prolifera*. Where $p < 0.05$, $p < 0.01$ and $p < 0.001$ are denoted as *, ** and ***, respectively.

Source	df	MS	*F*	*P*	
Viola - Sekiguchi					
Temperature (T)	1	0.001	7.784	0.008	**
Irradiance (I)	1	0.003	23.992	1.64E-05	***
Nutrient (N)	1	0.002	17.236	0.0001	***
T × I	1	0.000	2.319	0.136	
T × N	1	0.001	5.950	0.019	*
I × N	1	0.000	2.635	0.112	
T × I × N	1	0.000	1.217	0.277	
Anthera - Sekiguchi					
Temperature (T)	1	0.000	0.283	0.598	
Irradiance (I)	1	0.000	0.114	0.738	
Nutrient (N)	1	0.000	1.170	0.286	
T × I	1	0.000	35.605	5.26E-07	***
T × N	1	0.000	8.817	0.005	**
I × N	1	0.000	25.619	9.73E-06	***
T × I × N	1	1.36E-04	18.544	0.0001	***
Zea - Sekiguchi					
Temperature (T)	1	0.00977	6.326	0.016	*
Irradiance (I)	1	0.04162	26.949	6.42E-06	***
Nutrient (N)	1	0.04927	31.901	1.48E-06	***
T × I	1	0.00407	2.637	0.112	
T × N	1	0.01032	6.679	0.013	*
I × N	1	0.00486	3.145	0.083	
T × I × N	1	0.00015	0.099	0.754	
Viola- Takeshima					
Temperature (T)	1	0.000	3.299	0.077	
Irradiance (I)	1	0.006	146.376	6.04E-15	***
Nutrient (N)	1	0.004	92.411	5.95E-12	***
T × I	1	0.000	0.899	0.349	
T × N	1	0.000	7.578	0.008	**
I × N	1	0.002	48.951	1.90E-08	***
T × I × N	1	0.001	24.741	1.29E-05	***

Anthera - Takeshima					
Temperature (T)	1	0.000	31.365	1.72E-06	**
Irradiance (I)	1	0.000	59.806	1.82E-09	***
Nutrient (N)	1	0.000	61.326	1.34E-09	***
T × I	1	0.000	13.377	0.0007	***
T × N	1	0.000	20.817	4.72E-05	***
I × N	1	0.000	21.979	3.18E-05	***
T × I × N	1	5.16E-06	2.441	0.126	
Zea - Takeshima					
Temperature (T)	1	0.00862	13.480	0.0007	***
Irradiance (I)	1	0.10274	160.720	1.36E-15	***
Nutrient (N)	1	0.08127	127.140	5.42E-14	***
T × I	1	0.00116	1.810	0.186059	
T × N	1	0.0136	21.280	4.03E-05	***
I × N	1	0.03172	49.620	1.63E-08	***
T × I × N	1	0.01466	22.940	2.31E-05	***

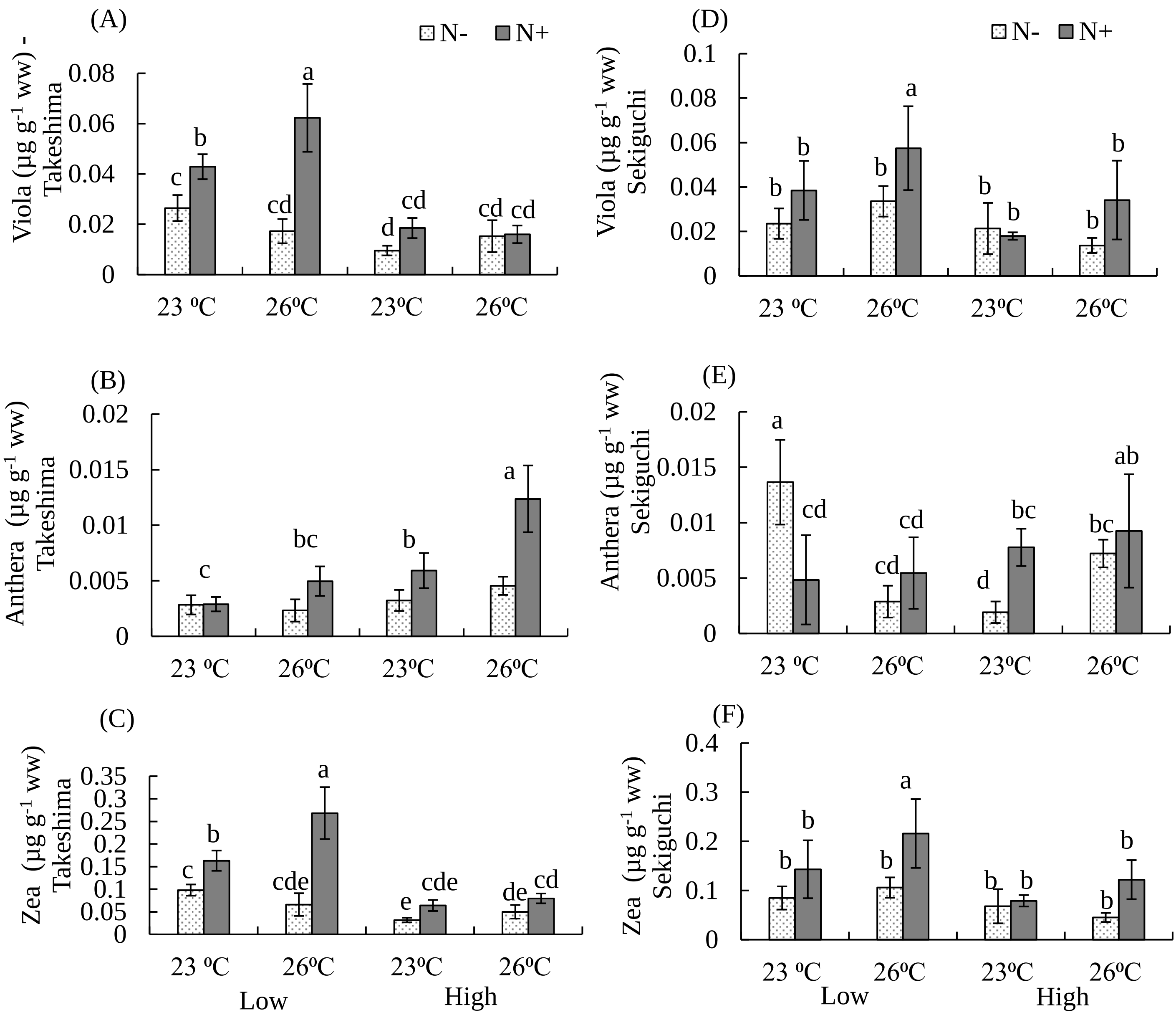

Figure 19. Violaxanthin (A), Antheraxanthin (B), Zeaxanthin (C) of Takeshima and Violaxanthin (D), Antheraxanthin (E), Zeaxanthin (F) of Sekiguchi strain of *Ulva prolifera* cultured in eight different treatments (mean + SD, n = 6). Low and High indicate 30 and 150 (μmol photon m^{-2} s^{-1}) irradiance treatments, respectively. N+ and N- indicate nutrient enriched and non-enriched treatments, respectively. Different small letters indicate statistical significances among different treatments ($P < 0.05$).

Table 15. Results of Welch's t-test on the comparison of Violaxanthin, Antheraxanthin, Zeaxanthin between Takeshima strain and Sekiguchi strain of *U. prolifera* under eight treatment conditions. Low and High indicate 30 and 150 (μmol photon m^{-2} s^{-1}) irradiance treatments, respectively. N+ and N- indicate nutrient enriched and non-enriched treatments, respectively. Where $p < 0.05$, $p < 0.01$ and $p < 0.001$ are denoted as *, ** and *** respectively.

Source	df	t	*P*	
Viola comparison				
23°C+ High+ N+	6.697	-0.324	0.755	
23°C+ High + N-	5.279	2.474	0.053	
23°C+ Low + N+	5.838	-18.991	1.8E-06	***
23°C+ Low + N-	6.399	-27.46	7.05E-08	***
26°C+ High + N+	5.387	2.455	0.054	
26°C+ High + N-	7.641	-0.539	0.605	
26°C+ Low + N+	5.1766	-9.882	0.0001	***
26°C+ Low + N-	5.068	-3.607	0.015	*
Antheraxanthin comparison				
23°C+ High+ N+	9.962	1.961	0.078	
23°C+ High + N-	9.994	-2.375	0.038	*
23°C+ Low + N+	5.256	1.170	0.292	
23°C+ Low + N-	5.509	6.761	0.0007	***
26°C+ High + N+	9.970	-0.789	0.449	
26°C+ High + N-	8.622	4.373	0.001	**
26°C+ Low + N+	6.642	0.338	0.745	
26°C+ Low + N-	8.669	0.688	0.509	
Zeaxanthin comparison				
23°C+ High+ N+	8.669	0.688	0.052	
23°C+ High + N-	5.221	2.532	0.0503	
23°C+ Low + N+	6.417	-0.771	0.468	
23°C+ Low + N-	7.580	-1.211	0.262	
26°C+ High + N+	5.742	2.523	0.046	*
26°C+ High + N-	8.289	-0.661	0.526	
26°C+ Low + N+	9.637	-1.416	0.188	
26°C+ Low + N-	9.641	3.008	0.013	*

For Sekiguchi strain, ANOVA detected significant individual effects of temperature, nutrients and irradiance on neoxanthin (Table 16). There were significant interactions between temperature and irradiance and temperature and nutrients (Figure 20A). Temperature and irradiance interaction indicated that warming and low irradiance resulted in higher neoxanthin content. Temperature and nutrient interaction indicated that warming and enrichment resulted in a higher neoxanthin content. Similarly, ANOVA detected significant interaction between temperature, nutrients and irradiance on beta carotene content (Table 16). However, interaction were on detected between temperature and nutrients and irradiance and nutrients (Figure 20B). Temperature and nutrient interaction indicated that warming and enrichment resulted in a higher beta carotene content. Irradiance and nutrients interaction indicated that low irradiance and enrichment resulted in higher beta carotene content.

ANOVA found significant individual effects of temperature, nutrients and irradiance on neoxanthin of Takeshima strain with significant two factor interactions as well as three factor interaction (Table 16). This three factor interaction among temperature, nutrient and irradiance indicated that warming combined with lower irradiance and enrichment had better neoxanthin content (Figure 20C). Furthermore, Takeshima strain had significant individual interactions with three factor interaction among temperature, nutrient and irradiance on beta carotene content (Table 16, Figure 20D). This indicated that warming combined with lower irradiance and enrichment had better beta carotene content.

Welch's t test indicated significant difference in the neoxanthin content between the two strains among the following treatments; 23 °C, high irradiance and enrichment combination, 23 °C, high irradiance and non enrichment combination, 26°C, high irradiance and enrichment combinations and 26 °C, low irradiance and non enrichment combinations (Table 17). For beta carotene, there were significant difference among the two strains in 23 °C, high irradiance and enrichment combination, 23 °C, high irradiance and non enrichment combination and 26 °C, low irradiance and non enrichment combinations (Table 17).

Table 16. Shows results of a three-way ANOVA on the effects of temperature, nutrient availability and irradiance on Neoxanthin (Neo) and Beta carotene (βcarotene) and Zeaxanthin (Zea) of Takeshima and Sekiguchi strain of *U. prolifera*. Where $p < 0.05$, $p < 0.01$ and $p < 0.001$ are denoted as *, **and ***, respectively.

Source	df	MS	*F*	*P*	
Neo - Sekiguchi Strain					
Temperature (T)	1	0.000537	9.109	0.00441	**
Irradiance (I)	1	0.001333	22.603	2.58e-05	***
Nutrient (N)	1	0.003341	56.651	3.50e-09	***
T × I	1	0.000351	5.953	0.01922	*
T × N	1	0.000278	4.707	0.03603	*
I × N	1	0.000144	2.444	0.12588	
T × I × N	1	0.000109	1.847	0.18176	
βcarotene - Sekiguchi strain					
Temperature (T)	1	0.01022	7.599	0.008756	**
Irradiance (I)	1	0.06272	46.65	3.24e-08	***
Nutrient (N)	1	0.02795	20.784	4.77e-05	***
T × I	1	0.00247	1.837	0.182944	
T × N	1	0.01033	7.681	0.008427	**
I × N	1	0.02128	15.825	0.000284	***
T × I × N	1	0.00233	1.736	0.195137	
Neo -Takeshima Strain					
Temperature (T)	1	0.0001464	10.445	0.00246	**
Irradiance (I)	1	0.0020738	147.936	5.11e-15	***
Nutrient (N)	1	0.0018516	132.084	3.01e-14	***
T × I	1	0.000066	4.711	0.03596	*
T × N	1	0.0002834	20.22	5.80e-05	***
I × N	1	0.0004261	30.393	2.28e-06	***
T × I × N	1	0.0002901	20.695	4.92e-05	***
βcarotene – Takeshima strain					
Temperature (T)	1	0.00263	8.21	0.00661	**
Irradiance (I)	1	0.03583	111.738	3.80e-13	***
Nutrient (N)	1	0.04749	148.1	5.02e-15	***
T × I	1	0.00032	0.989	0.32594	
T × N	1	0.00268	8.347	0.00621	**
I × N	1	0.01298	40.47	1.47e-07	***
T × I × N	1	0.00295	9.198	0.00424	**

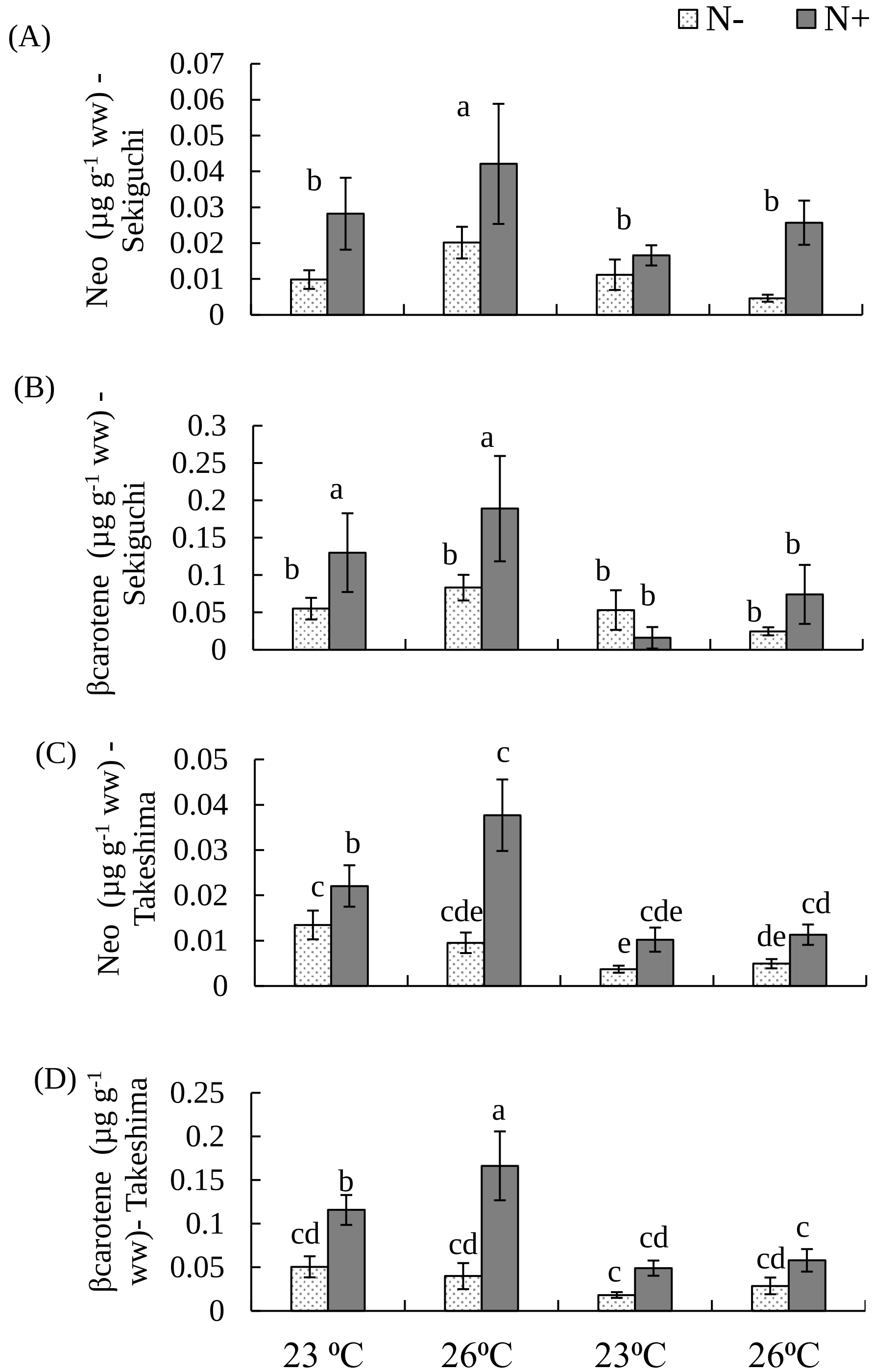

Figure 20. Neoxanthin (A), Beta carotene (B) of Sekiguchi and Neoxanthin (C) and Beta carotene (D) of Takeshima strain of *Ulva prolifera* cultured in eight different treatments (mean + SD, n = 6). Low and High indicate 30 and 150 (µmol photon m^{-2} s^{-1}) irradiance treatments, respectively. N+ and N- indicate nutrient enriched and non-enriched treatments, respectively. Different small letters indicate statistical significances among different treatments (P < 0.05).

Table 17. Results of Welch's t-test on the comparison of Neoxanthin and Beta carotene between Takeshima strain and Sekiguchi strain of *U. prolifera* under eight treatment conditions. Low and High indicate 30 and 150 (µmol photon m^{-2} s^{-1}) irradiance treatments, respectively. N+ and N- indicate nutrient enriched and non-enriched treatments, respectively. Where $p < 0.05$, $p < 0.01$ and $p < 0.001$ are denoted as *, ** and ***, respectively.

Source	df	t	*P*	
Neo comparison				
23°C+ High+ N+	9.966	4.017	0.002	**
23°C+ High + N-	5.336	4.246	0.007	**
23°C+ Low + N+	6.991	1.362	0.215	
23°C+ Low + N-	9.629	-2.164	0.056	
26°C+ High + N+	6.309	5.367	0.002	**
26°C+ High + N-	9.975	-0.454	0.659	
26°C+ Low + N+	7.118	0.583	0.577	
26°C+ Low + N-	7.454	5.241	0.0009	***
βcarotene comparison				
23°C+ High+ N+	8.216	-4.797	0.001	**
23°C+ High + N-	5.152	3.200	0.023	*
23°C+ Low + N+	6.045	0.633	0.549	
23°C+ Low + N-	9.679	0.588	0.569	
26°C+ High + N+	6.054	0.956	0.375	
26°C+ High + N-	7.889	-0.875	0.407	
26°C+ Low + N+	7.854	0.687	0.511	
26°C+ Low + N-	9.810	4.666	0.0009	***

Discussion

Takeshima strain indicated a temperature and irradiance interaction where the positive effect of elevated temperature on SGR was synergised by high irradiance. However, a past study on *Ulva prolifera* had shown temperature and irradiance interaction where the negative effect of 26 ºC on relative growth rate was synergised by elevated irradiance of 280 µmol photon m^{-2} s^{-1} (Wu et al., 2018) under a 3 × 3 (3 temperature levels with 3 irradiance levels) experimental design. Furthermore, in Takeshima strain, the positive effect of elevated temperature on SGR was synergised by enrichment or the positive effect of enrichment on SGR was synergised by elevated temperature. Past studies show that positive effect of enrichment is synergised from optimum to higher temperature on *Ulva* spp. (Steen, 2004; Gao et al., 2017, 2018).

However, the higher temperature used within these studies were ca. < 20 °C and can be regarded as optimum temperature condition. On the other hand, warming from 30 °C under eutrophication would cause no further change in growth in *Ulva* spp (Lee & Kang, 2020). From this, it can be inferred that warming occurring in cold seasons (< 30 °C) when waters are rich in nutrients could result in *Ulva* spp. blooms (Endo & Gao, 2022). Both Takeshima and Sekiguchi strains had positive effect of irradiance on SGR being synergised by enrichment. Xu et al., (2014), also found that under enrichment and irradiance of 130 µmol photon m^{-2} s^{-1} in a two irradiance levels and two nutrients level design experiment, relative growth rate increased.

The SGR in Sekiguchi strain under 23 ºC, high irradiance and enriched treatment combination was higher and significantly different from SGR of Takeshima strain under the same treatment condition. This meant that the condition of 23 ºC, high irradiance and enriched was more related to its original habitat. Sekiguchi strain from Iwate prefecture (northern Japan) is adapted to temperatures of 20 ºC while Takeshima from Koichi prefecture (southern Japan) is adapted to 20-25ºC (Sato et al., 2021). In addition, under warming of 26 ºC, high irradiance and enriched treatment combination, there was no difference between the SGR of the two strains. This means that Sekiguchi strain does have the capability to grow and thrive to the same level of growth as Takeshima under multiple environmental conditions. Previously, strains that were found in close proximity to each other were thought to have similar thermosensitivity.

However, Sato et al (2021) showed that strains are not dependent on site specific conditions. Therefore, under extreme conditions with multiple stressors, plasticity could allow for adaptation to new conditions. Tolerance to increased temperature is synonymous to *hsp* 90 gene in *U. prolifera* (Ogawa et al., 2014) that could allow for genotype to phenotypic changes where all potential heritable phenotypic changes are possible (Zabinsky et al., 2019).

The negative effect of high irradiance on carbon content (C) was synergised by enrichment in Takeshima strain. However, in Sekiguchi strain, the positive effect of high irradiance on C was synergised by enrichment. The differences between the C of *U. prolifera* strains could indicate a difference in carbon concentrating mechanism (CCM) (Xu et al., 2012; Valiela et al., 2018; Liu et al., 2020). *U. prolifera* is know to assimilate carbon through two CCM namely C_3 and C_4 pathway (Beer & Israel, 1986). C_3 pathway (Calvin- Benson cycle) where carbon dioxide and ribulose biphosphate are converted into 3- phosphoglyceric acid by RuBP carboxylase enzyme by a process called carboxylation).

On the other hand, in C_4 pathway, an addition of enzyme phosphoenolpyruvate carboxylase (PEPCase) helped in carboxylation enhancing carbon fixation at rubisco site which is not present in C_3 (Liu et al., 2020). This allows for C_4 plants to undergo more carbon fixation. It is possible that *Ulva* spp. could have a combination of both pathways that could arise from changing environmental conditions thus promoting evolution of this complex trait (Xu et al., 2012) . Further research on this is required for *U. prolifera* strains on CCM to confirm this. Takeshima strain overall had a better carbon content under all treatment combinations than Sekiguchi strain. Takeshima strain could be a better candidate for contributing towards a blue carbon economy since it is able to store higher amounts of carbon under varied environmental conditions.

Both strains had the positive effect of enrichment on nitrogen content (N) being synergised by low irradiance. Both strains also showed temperature and irradiance interaction. Takeshima strain showed that the negative effect of elevated temperature on N was antagonised by high irradiance while Sekiguchi strain showed that the positive effect of temperature on N was antagonised by high irradiance. Takeshima strain also showed that the negative effect of temperature on N became synergised by non -enrichment. This is related to the nutrients uptake kinetics which could be different among strains in various environmental conditions.

U. prolifera is a fast growing generalist species that has been found to have increased uptake rate between 20-25 °C under enriched condition (Fan et al., 2014). Takeshima strain was better at storing nitrogen than Sekiguchi under all experimental condition. However, the carbon and nitrogen storage in relation to growth was different among the strains. This could be due to the differences in nutrient environment of the two strains. Takeshima strain is from Kochi prefecture which is affected by the nutrient poor Kuroshio current could be better adapted to grow in oligotrophic condition. However, Sekiguchi strain which is from Iwate prefecture is affected by nutrient-rich Oyashio cold current and accumulation of nitrogen could be in preparation of maturation (Sato et al., 2016).

Ulva spp. share eco-physiological parameters such as nutrient uptake with *U. pinnatifida* that becomes adapted to site specific conditions (Sato et al., 2016). According to Kang et al (2011), C/N ratio are an indicator of physiological performance. C/N ratio are low under conditions where nutrient are abundant (Kang & Chung, 2017 ; Reidenbach et al., 2017). In Sekiguchi strain, a 3 factor interaction showed that the positive effect of warming and high irradiance was antagonized by enrichment which means that Sekiguchi strain might be more sensitive than Takeshima strain.

Takeshima strain, showed a 3 factor interaction where the positive effect of elevated temperature and low irradiance on Chl *a*, Chl *b*, violaxanthin, zeaxanthin, neoxanthin and beta carotene content was synergized by enrichment or the positive effect of nutrient enrichment and higher temperature was antagonised by high irradiance. Such a three factor interaction was not observed in Sekiguchi strain, however, two factor interaction of temperature with irradiance, temperature with nutrients and irradiance with nutrients where observed.

The three factor interaction in Takeshima strain means even under warming conditions it had higher Chl *a*, Chl *b*, violaxanthin, zeaxanthin, neoxanthin and beta carotene content however, with high irradiance, pigment deterioration could have occurred. When comparing the Chl *a* and Chl *b* pigment content between the two strains. Under warming, high irradiance with and without nutrients combinations, Takeshima strain's Chl *a* content was higher when compared with Sekiguchi strain therefore, it probably has a better adapted photosynthetic system. The growth did not decrease with increased irradiance under warming, it is possible that stress to PSII is mitigated by cyclic electron flow from PSI which is less sensitive to stress than PSII by providing energy from ATP to repair PSII (Zhao et al., 2016). However, further research on this required.

Chapter 4 General Discussion

Global warming is causing kelps and fucoids to decline or migrate towards the northern or colder regions of the world (Smale, 2020). The interaction of temperature and nutrients on growth is common in most brown macroalgae. For instance in *Ecklonia cava* (Gao et al., 2016), *Laminaria ochrolleuca* (Franco et al., 2018) and *Macrocystic pyrifera* (Mabin et al., 2019). However, in this study, temperature and nutrient interaction on growth was not found in *Sargassum fusiforme* which is prominent in brown macroalgae. Instead irradiance and temperature was prominent in *S. fusiforme* growth and pigment content. Irradiance combination with these factors are important in *Eisenia bicyclis* (Endo et al., 2020) since submerged species do to have access to high irradiance. High irradiance can induce photoinhibition to which some species especially intertidal to sub tidal species are vulnerable to.

This study also showed that Takeshima strain of *Ulva prolifera* was affected by the interaction of the two or three factors more than Sekiguchi strain was. Sekiguchi strain was an elite strain in terms of growth under multiple environmental conditions where warming did not decrease growth. Sekiguchi strain also did not have three factor interaction which suggests that they are not affected by multiple environmental stressors used in this study.

As commercially important species, *S. fusiforme* and *U. prolifera*, are different from each other. *S. fusiforme* is a slow growing, large, brown macroalgae growing 20-100 cm and living in marine environment while *U. prolifera* is a fast growing, green macroalgae with blades growing 10-50 cm in length and mostly found in brackish environments (Titlyanov et al., 2017). *S. fusiforme* is more widely distributed vertically however, *U. prolifera* appears to be widely horizontally distributed due to the size. *U. prolifera* is found in brackish waters where it more exposed to rocky shores than the marine *S. fusiforme*. Higher exposure to sunlight has possibly allowed photoinhibition to act as a protective mechanism in *U. prolifera*. On the contrary to brown macroalga, warming with high irradiances can cause heat induced photoinhibition (Endo et al., 2020). When growth decreases as a result of warming and irradiance working synergistically to negatively affect the PSII then the PSII could be damaged which could be indicative of decreased pigments contents. In this study, *S. fusiforme* growth decreased in additions to ratio of pigments Chl c_2/Chl *a* pigments which indicated that there could be degradation of Chl c_2 or damage to the PSII. On the other hand, *U. prolifera* did not decrease growth with increasing irradiance and warming and possible recovered from photoinhibition. However, further study of Photosystems are required to ascertain this.

In addition, *U. prolifera* belongs to the *linza, procera, prolifera* (LPP) complex clade where these three species are able to inter-breed together (Shimada et al., 2008). Moreover, *U. prolifera* has multiple life cycles as a fast growing species. This suggests that *U. prolifera* is more diverse than *S. fusiforme*. On the other hand, further study is required on the effect of multiple environmental stressor on S. *fusiforme* as only one strain was studied this this current research. However, both of these macroalga grow in abundance from autumn until spring before disappearing by summer which is one of their similarities.

In *S. fusiforme,* carbon content appeared more conservative than *U. prolifera* under warming, enrichment and higher irradiance as carbon content does not vary considerably in brown macroalgae. Carbon content is related to the efficiency of the photosynthetic process where pigments also play an important role in driving photosynthesis. On the other hand, for *S. fusiforme* although growth had halted under warming and higher irradiance and chlorophyll degradation possibly had occurred, the carbon content did not decrease. This possibly amplifies the role of brown macroalgae as carbon sinks long after they become detritus and contributing towards the blue carbon economy. Carbon content differed between the strains of *U prolifera* with Takeshima strain reducing carbon content under higher irradiance with enrichment while Sekiguchi strain increased carbon content under the same condition. Thus, carbon fixation not only differs among different types of macroalgae but also differs between strains. Nitrogen content variability was considerable under enrichment in both macroalga as nitrogen variation is short term.

Application based research on edible macroalgae's physiology under changing conditions not only contributes towards development of their role as food source for people but helps in the understanding of a larger ecological role they might play. Establishment of integrated multiple tropic aquaculture for intertidal to subtidal *S. fusiforme* under high irradiance while providing enrichment from shellfish or oysters might improve cultivation. Similarly, for *U. prolifera*, land based cultivation under optimum irradiance and enriched conditions is more feasible and controllable due to the fast growing nature of this species. However, since Sekiguchi strain grew optimally under higher temperature, there is potential for this strain to become more prominent under the changing environmental conditions.

In Pacific island countries where temperature has drastically affected the ecosystem of edible seaweeds. Introducing slow release fertiliser in areas of optimum light as mariculture could help mitigate the warming effect from heatwaves which is putting a pressure on edible marine plants. Application based research as such could provide nature based solutions (Nbs) in marine protected areas (MPA) not only for developed countries but also developing countries.

Chapter 5 Conclusion

Sargassum fusiforme is a large, brown and slow growing macroalga while *Ulva prolifera* is a small, green and fast growing macroalga. As commercially important macroalga under changing conditions, newer management strategies would need to be developed.

Warming in the form of heat waves has a negative effect on SGR of *S. fusiforme* however, increased irradiance would be able to mitigate this negative effect on *S. fusiforme*. In addition, the carbon content of *S. fusiforme* was negatively affected by warming however, this was antagonized by nutrient enrichment. This could mean that under warming and enriched conditions (eutrophic), carbon fixation can be improved. However, adding nutrients *in situ* can cause phytoplankton blooms due to eutrophication. It is very difficult to decide on satisfactory level of nutrient addition to improve macroalgal production without damaging the ecosystem. Introduction of integrated multitrophic aquaculture where fish, shellfish or oyster excrement may provide natural enrichment to macroalgae and could be sustainable. *S. fusiforme* is an important economical and ecological macroalga which based on results is dependent on local nutrient and light environment that could offset the negative effects of warming leading to conserving them and contributing towards carbon sequestration.

U. prolifera strains on the other hand appeared to thrive under warming and combined effects of elevated irradiance and enrichment. However, there were physiological differences between the strains. Based on the biochemical performance, Takeshima strain had higher carbon fixation, nitrogen assimilation and contained higher pigments contents when compared to Sekiguchi strain. However, Sekiguchi strain had higher growth than Takeshima strain, where Sekiguchi strain showed no interaction with temperatures which means that temperature variation does not affect its growth under multiple environmental conditions. On the other hand, Takeshima strain showed a two factor interaction of temperature with nutrients and temperature with irradiance on SGR. Sekiguchi strain thrived under eutrophic and higher irradiance conditions and cultivation of this strain under these conditions would produce higher yield.

Under oligotrophic conditions and higher irradiance, Takeshima strain would thrive in conditions with low nutrients. However, for blue carbon economy it becomes important that macroalgae remain in the ocean therefore removing macroalgae for consumption or other purposes would be hindering contribution towards a blue carbon economy. Solutions for this is to invest in macroalgae that is fast growing and *U. prolifera* is a fast growing species and can be easily cultivated or replaced.

S. fusiforme and *U. prolifera* are very different in their life cycle. *U. prolifera* has multiple cycles which allows it to evolve faster under the rapidly changing environmental conditions.

Further research is required on these two macroalga under multiple environmental conditions to not only understand their persistence but also how biological characteristics could play a role in determining better strains and developing newer management strategies.

Acknowledgements

This research would not have been possible without the patience, guidance, kindness and encouragement from the members of my supervisory team.

I would like to express my sincere gratitude and appreciation to Professor Masakazu Aoki and Emeritus Professor Yukio Agatsuma of Marine Plant Ecology Laboratory at Tohoku University for their continuous support and guidance throughout the duration of my PhD study.

I am extremely grateful to Dr. Hikaru Endo of Kagoshima University for his immense suggestions, advice and continuous guidance during the course of my study to mould me into a better researcher. His pioneering work on multiple stressor effects on Japanese macroalgae had always peaked my interest since my University days in Fiji. I truly came to understand this work under his tutelage.

I am enormously thankful to the training and support from Dr. Yoichi Sato of Yuriage Research Institute in Riken that provided me an insight into *U. prolifera* cultivation. Under his guidance, I also learnt about macroalgae cultivation and management in Japan. I would also like to express my sincere gratitude towards everyone at Yuriage Research Institute in Riken who had helped me.

I would also like to show my sincere appreciation towards Dr. Yutaka Okumura of Tohoku National Fisheries Research Institute for his expertise in pigment analysis.

To Dr. Eri Inomata and Dr. Haruka Suzuki, thank you for your guidance in C/N analysis. I would also like to extend my sincere appreciation to the members of the Applied Marine Plant Ecology Laboratory for being a wonderful family in Japan.

Finally, I would like to thank my loved ones, especially my late grandmother, whose kindness and warmth, I shall never forget. I was looking forward to meeting you upon my return, sadly that could not happen. I hope you are at peace.

Dedication

For my beloved grandmother (नानी), the late Mrs Parvati Sen. You are dearly missed by your children and grandchildren.

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