Ocean Sci. J. (2020) 55(1):115–127
http://dx.doi.org/10.1007/s12601-020-0005-y

Available online at http://link.springer.com

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Article

Ocean Science Journal

pISSN 1738-5261
eISSN 2005-7172

Impact of Temperature, Low pH and NH_4^+ Enrichment on Ecophysiological Responses of a Green Tide Species *Ulva australis* Areschoug

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Received 27 September 2019; Revised 22 November 2019; Accepted 24 November 2019

Abstract – *Ulva* are ubiquitous and opportunistic green algae species that easily adapt to various environmental conditions. These algae are responsible for the green tides that cause many environmental and ecological problems in coastal waters. We investigated the physiological responses of *Ulva australis* under warming, acidification, and eutrophication conditions. The physiological changes in the algae were observed under various combinations of temperature, pH, and NH_4^+ levels. Combinations of three temperatures (10°C, 20°C, and 30°C), two pH levels (7.80 and 8.20), and two NH_4^+ concentrations (4 μM and 120 μM) were considered under laboratory conditions. Temperature, NH_4^+, and pH had significant impact on the photosynthetic and nutrient uptake rates. However, the 12 h observation could not stimulate the seaweed to change the pH in the cultured media. Changes in relative growth rates, photosynthetic efficiency, and variations in tissue C and N were not affected by the interactions between temperature, pH level, and nutrient concentration. It is probable that, due to global warming, the bloom of *Ulva australis* may continue in warm, acidic, coastal waters with high nutrient levels.

Keywords – *Ulva*, temperature, pH, NH_4^+, ecophysiology, green tides

1. Introduction

Seaweeds play an important role in the coastal environment. They provide many ecosystem services, such as providing habitats for marine organisms, serving as primary producers, absorbing nutrients and carbon, and are also useful resources for food, pharmaceuticals, and other functional materials for human use (Graham 2004; Harley et al. 2012; Koch et al. 2013; Hurd et al. 2014, Sondak et al. 2017). However, due to global warming, seaweeds are experiencing severe environmental changes, including warming seawater temperatures, acidification, and eutrophication. The atmospheric CO_2 concentration has increased from 280 to over 400 ppm, seawater temperatures have increased by nearly 0.13°C, and pH levels have decreased by 0.06–0.32 due to elevated anthropogenic atmospheric CO_2 concentrations (IPCC 2014). The current climate change models predict that seawater temperature will increase by more than 4°C, and consequently the pH may decrease to 7.70 if the targeted reduction in carbon emissions is not met (Bartsch et al. 2012; IPCC 2014). Moreover, increased nutrient concentrations, promoted by anthropogenic activities, such as commercial industrialization, rapid aquaculture practices, and untreated municipal sewage near coastal areas, may increase the nutrient levels in the water and cause eutrophication and hypoxia (Rabalais et al. 2009; Smetacek and Zingone 2013). Severe eutrophication may increase the acidity of seawater (ocean acidification: OA) because active microorganisms synthesize large amounts of organic and inorganic nutrients and produce CO_2, consequently reducing the pH level (Cai et al. 2011; Wallace et al. 2014).

Many researchers have investigated different physiological responses of marine organisms under ocean acidification conditions. As low pH levels decrease the availability of CO_3^{-2}, it has negative effects on organisms with $CaCO_3$ body structures, which can hinder their survival, growth, and reproduction (Gazeau et al. 2013; Koch et al. 2013; Kroeker

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et al. 2013; Reymond et al. 2013; McCoy and Kamenos 2015; Kram et al. 2015).

At the same time, investigations of seaweeds show increases in their metabolism, growth, and photosynthesis (Brown et al. 2014: Zou and Gao 2014a, 2014b; Gao et al. 2017a, 2017b; Kang and Chung 2017; Ober and Thornber 2017; Xu et al. 2017). However, the growth and survival of *Ecklonia cava* and *Saccharina japonica* sporophytes fluctuates under increased temperature and nutrient concentrations (Gao et al. 2016, 2017b). The excessive nutrient levels caused by eutrophication drives the acidification of coastal water with increasing pCO_2 reducing the pH level and lowering dissolved oxygen concentrations (Wallace et al 2014; Flynn et al. 2015).

The response of the seaweed genus, *Ulva*, to high nutrient concentrations and high water temperatures causes green tide bloom in eutrophic coastal water. Growth and photosynthesis of *U. conglobate*, *U. prolifera,* and other *Ulva* spp. are increased at high temperatures and nutrient concentrations (Taylor et al. 2001; Kim et al. 2011). Various experiments to observe the physiological responses of seaweeds have been carried out only under single-factor experiments (Widdicombe et al. 2010; Wernberg et al. 2012). However, a limited number of studies investigated the physiology of *Ulva* spp. under a combination of environmental conditions, such as elevated temperature, CO_2 levels, PAR and UV radiation, nutrient concentrations, photoperiod and salinity (Suárez-Álvarez et al. 2012; Figueroa et al. 2014a, 2014b; Stengel et al. 2014; Gao et al. 2017a, 2017b, 2018; Li et al. 2018; Bao et al. 2019; Ma et al. 2019; Yue et al. 2019; Zheng et al. 2019). Other aspects of OA concerning the trophic relationships (Duarte et al. 2016; Chen et al. 2019), metabolites (Gao et al. 2019) and multiple-contamination by heavy metal toxicity (Ge et al. 2017) were also studied, while its effects under the concurrent influences of warming, UV radiation and deoxygenation were reviewed by Gao et al. (2019).

We carried out a multi-factorial experiment (Koch et al. 2013), to determine the ecophysiological responses of *U. australis* Areschoug to multiple environmental conditions, representative of future ocean warming and eutrophication events, including temperatures, CO_2 levels, nutrient concentrations, and the interactions between them (Boyd et al. 2018). The changes in pH, photosynthetic rates, NH_4^+ uptake rates, relative growth rates, chlorophyll fluorescence, and C and N contents within seaweed tissues were measured to determine the effects of the treatments on the ecophysiological responses of *U. australis*.

2. Materials and Methods

Samples of *Ulva australis* seaweed were collected during the summer, in July 2016, at Cheongsapo, South Korea (35°09'N, 129°11'E). The environmental parameters were monitored in situ. Temperature and salinity were measured using dissolved oxygen, conductivity, salinity instrument (YSI Pro 2030-US, YSI Inc., USA) while pH units were measured using a YSI Pro 10 pH meter (YSI Inc., USA). Seaweed samples were transported to Pusan National University Biological Oceanography Laboratory in a cool box. In the laboratory, seaweeds were cleaned with filtered seawater (0.20 μm pore size) several times to remove epiphytes and unwanted organisms. Prior to observation, samples were acclimated for three days at an optimal growth temperature of 20°C (Kang et al. 2011; Kim et al. 2011) and light intensity of 80 μmol m^{-2} s^{-1} with 12:12h (light:dark) photoperiod. The light intensity was checked with a LI-250 light meter (LI-COR, USA). To evaluate the physiological responses of *U. australis*, one gram of fresh sample was placed in a beaker with 500 mL filtered seawater. They were treated under different incubation conditions: three temperatures of 10°C (L_T), 20°C (M_T), and 30°C (H_T); two pH levels of 7.80 (L_{pH}) and 8.20 (H_{pH}); and two NH_4^+ concentrations of 4 μM (L_{NH4}) and 120 μM (H_{NH4}). The light intensity and photoperiod were the same as those of the acclimation stage.

The L_T of 10°C was chosen according to the minimum monthly seawater temperature (mean value 11.0 ± 0.80°C) based on KHOA (2016). The M_T of 20°C was selected as the optimal growth condition of *Ulva* species (Kim and Lee 1996; Taylor et al. 2001), and the H_T of 30°C was selected according to the maximum monthly seawater temperature (mean value 23.74 ± 2.36°C) plus the expected seawater temperature increase of 4°C as predicted for global warming (IPCC 2014; KHOA 2016). The L_{pH} of 7.80 was set as a prediction value by Caldeira and Wickett (2005) and H_{pH} followed natural ambient conditions. The L_{pH} in cultured media was created using the method by Gattuso et al. (2010), which is based on a CO_2 bubbling method of enriching filtered seawater with pure CO_2 gas from a CO_2 tank.

The total alkalinity (TA) of the cultured media was measured using an electro-titration method (Gran 1952; precision ± 4 μmol kg^{-1}), while the rest of the inorganic carbonate parameters were calculated using CO2SYS software (Lewis and Wallace 1998). The dissociation constant and KSO_4 values were based on those of Millero et al. (2006) and Dickson (1990)

Table 1. Seawater carbonate system parameters across different culture treatments

Culture conditions	TA	pCO_2	CO_2	CO_3^{2-}	HCO_3^-	DIC
$L_TH_{pH}L_{NH4}$	2207.67 ± 13.79	458.82 ± 5.54	6.54 ± 2.10	124.77 ± 5.67	1830.44 ± 13.38	1961.75 ± 14.07
$M_TH_{pH}L_{NH4}$	2311.01 ± 17.81	460.41 ± 4.92	8.48 ± 0.98	139.09 ± 6.85	1823.57 ± 16.11	1971.14 ± 16.9
$H_TH_{pH}L_{NH4}$	2319.67 ± 14.73	452.55 ± 3.45	11.13 ± 1.02	170.04 ± 4.30	1882.34 ± 13.18	2063.51 ± 13.50
$L_TL_{pH}L_{NH4}$	2314.05 ± 13.61	817.07 ± 8.09	20.66 ± 3.20	62.49 ± 3.61	2049.13 ± 20.28	2132.29 ± 22.10
$M_TL_{pH}L_{NH4}$	2330.15 ± 11.14	818.85 ± 3.81	55.36 ± 1.23	83.34 ± 4.55	2152.24 ± 10.03	2290.94 ± 20.71
$H_TL_{pH}L_{NH4}$	2341.33 ± 23.50	819.60 ± 6.19	91.73 ± 0.45	116.79 ± 5.13	2121.76 ± 13.35	2330.27 ± 13.53
$L_TH_{pH}H_{NH4}$	2211.33 ± 16.29	459.88 ± 2.70	6.57 ± 0.07	126.10 ± 3.39	1837.11 ± 17.03	1969.78 ± 20.49
$M_TH_{pH}H_{NH4}$	2314.59 ± 18.14	460.76 ± 1.94	8.49 ± 1.13	139.42 ± 4.87	1825.92 ± 16.26	1973.83 ± 17.16
$H_TH_{pH}H_{NH4}$	2329.54 ± 23.81	452.63 ± 7.12	11.15 ± 0.62	170.33 ± 3.31	1885.42 ± 23.68	2066.91 ± 15.07
$L_TL_{pH}H_{NH4}$	2322.67 ± 19.87	813.63 ± 8.55	20.57 ± 0.25	61.81 ± 6.59	2040.50 ± 21.45	2122.88 ± 23.36
$M_TL_{pH}H_{NH4}$	2329.67 ± 16.20	818.73 ± 5.54	55.36 ± 1.18	83.32 ± 6.81	2151.94 ± 14.59	2290.62 ± 15.57
$H_TL_{pH}H_{NH4}$	2431.33 ± 24.85	822.46 ± 3.26	91.85 ± 4.14	117.09 ± 4.35	2129.81 ± 9.17	2338.76 ± 19.66

Values are means ± SD. Both pH and TA (μmol kg^{-1}) were measured directly for each scenario, while pCO_2 (ppm), CO_2 (μmol kg^{-1}), CO_3^{2-} (μmol kg^{-1}), HCO_3^- (μmol kg^{-1}), and DIC (μmol kg^{-1}) were calculated according to the CO2SYS program (Lewis and Wallace 1998)

(Table 1). The L_{NH4} and H_{NH4} of 4 and 120 μM, respectively, were created by adding NH_4Cl to the culture media following the method of Kang and Chung (2017). To avoid nutrient depletion, the media were changed every day. Four replicates were run for each of the experimental treatments.

Measurements of pH change in culture media

The pH change in the culture media was measured with an Orion-250A pH meter (Thermo Fisher Scientific Inc., Waltham, MA, USA) at initial, 2, 4, 6, and 12 h intervals. As our controls, flasks with no seaweed (blank) were used for each seawater treatment to seaweed for any pH and oxygen changes not due to seaweed metabolism. Prior to the experiment, the initial pH values in the culture media were recorded.

Photosynthetic rates

To measure the photosynthetic rates as oxygen production per FW of seaweed (μmol g^{-1} h^{-1}), we used a Clark-type microelectrode oxygen sensor (Unisense, Aarhus, Denmark) after a 12 h culture period. Prior to the observation, the oxygen sensor was calibrated using a mixed solution of $C_6H_7NaO_6$ and NaOH with distilled water. The changing of O_2 in the water media was recorded in each medium treatment.

NH_4^+ uptake rates

The rates of NH_4^+ uptake per FW of seaweed (μmol g^{-1} h^{-1}) were estimated by calculating the decrease in NH_4^+ concentrations in the water media after 12 h of observation. NH_4^+ measurements were performed according to the method of Parsons et al. (1984). NH_4^+ uptake rates were calculated using the following equation:

$$V = (S_i - S_f) \times Vol \times W^{-1} \times T^{-1} \quad (1)$$

where S_i and S_f represent the initial and final concentration of NH_4^+ (μmol NH_4^+), respectively, Vol is the volume of the medium (mg), W is the weight of each sample (g FW), and T is the incubation time used in observation (h).

Measurements of the relative growth rates (RGR)

Seaweeds were grown under the treatment conditions for 14 d. The growth of the seaweed was measured at the final day of the culturing period. The relative growth rates (RGR) were calculated as:

$$RGR\ (\%\ day^{-1}) = (\ln W_2 - \ln W_1) \times 100 \times T^{-1} \quad (2)$$

where W_1 and W_2 are the initial and final weight of seaweed (g FW) and T is the incubation period (14 d).

Measurements of chlorophyll fluorescence

Chlorophyll fluorescence was measured to determine the photosynthetic efficiency of the seaweed. We used a pulse amplitude modulation fluorometer (DIVING-PAM, Walz, Germany) to measure the chlorophyll fluorescence after 14 d in culture. The maximum quantum yield of thalli was calculated using the method of Cosgrove and Borowitzka (2011) using the following equation:

$$F_v/F_m = (F_m - F_o) / F_m \quad (3)$$

where F_m is maximum fluorescence after dark-adaptation, F_0 is minimum fluorescence after dark-adaptation, and F_v/F_m

is photosynthetic efficiency. Samples of *U. australis* were placed in leaf-clip holders and F_v/F_m was measured by saturating pulse. Prior to the measurement, the samples were dark-adapted for 15 min.

Measurement of C/N ratio

C and N content in the seaweed tissues was determined at the end of the culture period (14 d). Samples were dried (at 60°C for 48h) and ground using a Tissuelyser LT (Qiagen, Hilden, Germany), after which 2–3 mg of the ground tissue was analyzed with an elemental analyzer (Flash 2000 Series; Thermo Fisher Scientific, Waltham, MA, USA). The C/N ratio was calculated on a molar basis.

Data analyses

A three-way analysis of variance (ANOVA) was performed. Prior to statistical analyses, all data were tested for normality and homogeneity. Tukey's tests were used for multiple comparisons. A p-value < 0.05 indicated significant differences. All statistical analyses were conducted with SPSS, version 23.0 (IBM, USA).

3. Results

pH changes

The pH levels in the media were monitored for 12 h. Variation of pH in each treatment (ΔpH) was significantly affected by temperature and pH level with $p < 0.05$ (Table 2). However, the interaction between temperature, pH level, and nutrient concentrations was not significant and did not prompt the seaweed to change the pH of the media ($p > 0.05$; $p = 0.53$; $F = 0.65$). The ΔpH in the cultured media was higher in $M_TL_{pH}L_{NH4}$ and $M_TL_{pH}H_{NH4}$ than that of the other treatments (Fig. 1), but not significantly.

Photosynthetic Rates

Photosynthetic rates (μmol g^{-1} h^{-1}) were significantly influenced by temperature, NH_4^+ concentration, and combinations of temperature, pH, and nutrient concentrations ($p < 0.05$; F = 5.01; Table 2, Fig. 2). Photosynthetic rates were high at M_T and H_T. The maximum photosynthetic rate of 124.24 ± 1.42 μmol g^{-1} h^{-1} was found at $M_TL_{pH}H_{NH4}$, while the minimum of 74.99 ± 2.84 μmol g^{-1} h^{-1} was at $L_TH_{pH}L_{NH4}$. The photosynthetic rates of seaweed at similar pH levels combined with different NH_4^+ concentrations were higher at MT than those at H_T, but not significantly so ($L_{pH}L_{NH4}$; $F_{1,4}$ =

Table 2. Results of three-way ANOVA on the effect of temperature (T), pH level (pH), and NH_4^+ concentration (N) on the physiological activities of *Ulva australis* samples. Extent of variation in pH (ΔpH), photosynthetic rate (μmol g^{-1} h^{-1}), and NH_4^+ uptake rate (μmol g^{-1} h^{-1}), relative growth rates (% day^{-1}), chlorophyll fluorescence, tissue carbon contents (% DW), tissue nitrogen contents (% DW), and C/N molar ratio

Source	df	MS	*F*-value	*p*-value
Extent of variation in pH				
T	2	0.22	30.91	< 0.05
pH	1	1.59	227.49	< 0.05
N	1	< 0.05	< 0.05	0.86
T × pH	2	< 0.05	1.79	0.19
T × N	2	< 0.05	0.33	0.72
pH × N	1	< 0.05	1.03	0.32
T × pH × N	2	< 0.05	0.65	0.53
Photosynthetic rates				
T	2	1567.15	104.80	< 0.05
pH	1	2.83	0.19	0.67
N	1	5473.04	365.99	< 0.05
T × pH	2	61.48	4.11	< 0.05
T × N	2	248.69	8.11	< 0.05
pH × N	1	15.97	1.07	0.31
T × pH × N	2	74.79	5.01	< 0.05
NH_4^+ uptake rates				
T	2	0.38	61.77	< 0.05
pH	1	< 0.05	7.49	< 0.05
N	1	348.08	56210.52	< 0.05
T × pH	2	0.07	11.37	< 0.05
T × N	2	0.35	55.68	< 0.05
pH × N	1	0.06	8.89	< 0.05
T × pH × N	2	0.06	10.23	< 0.05
Relative growth rates				
T	2	8.41	30.57	< 0.05
pH	1	7.08	25.74	< 0.05
N	1	94.87	345.04	< 0.05
T × pH	2	0.94	3.42	0.05
T×N	2	0.03	0.11	0.89
pH × N	1	0.22	0.82	0.38
T × pH × N	2	0.20	0.74	0.49
Chlorophyll fluorescence				
T	2	< 0.05	1.77	0.19
pH	1	< 0.05	0.26	0.62
N	1	< 0.05	1.50	0.23
T × pH	2	< 0.05	1.80	0.18
T × N	2	< 0.05	< 0.05	0.99
pH × N	1	< 0.05	< 0.05	0.84
T × pH × N	2	< 0.05	1.20	0.32

Table 2. Continued

Source	df	MS	*F*-value	*p*-value
Tissue C content				
T	2	1.67	1.10	0.35
pH	1	1.12	0.74	0.40
N	1	1.94	1.27	0.27
T × pH	2	0.43	0.28	0.76
T × N	2	3.51	2.30	0.12
pH × N	1	0.27	0.18	0.68
T × pH × N	2	1.19	0.78	0.47
Tissue N content				
T	2	0.11	1.95	0.16
pH	1	< 0.05	< 0.05	0.96
N	1	3.26	56.95	< 0.05
T × pH	2	< 0.05	< 0.05	0.97
T × N	2	0.06	1.09	0.35
pH × N	1	< 0.05	0.17	0.68
T × pH × N	2	< 0.05	0.27	0.76
C/N molar ratio				
T	2	6.58	1.26	0.30
pH	1	0.49	0.09	0.76
N	1	304.44	58.71	< 0.05
T × pH	2	0.22	< 0.05	0.96
T × N	2	4.74	0.91	0.42
pH × N	1	0.99	0.19	0.67
T × pH × N	2	0.41	0.08	0.93

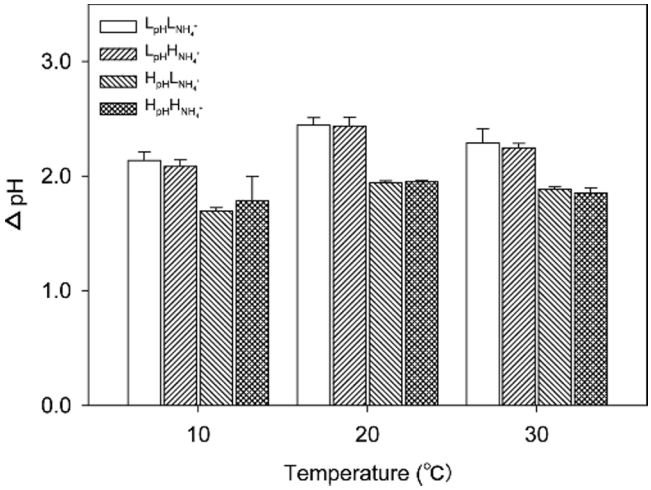

Fig. 1. Change in pH levels in the culture medium over 12 h. Vertical bars are means ± SD (n = 3). L_T = Low temperature (10°C); M_T = Medium temperature (20°C); H_T = High temperature (30°C); L_{pH} = Low pH (7.80); H_{pH} = High pH (8.20); L_{NH4} = Low NH_4^+ (4 μM); H_{NH4} = High NH_4^+ (120 μM)

1.08, $p = 0.36$: $L_{pH}H_{NH4}$; $F_{1,4} = 4.76$, $p = 0.10$: $H_{pH}L_{NH4}$; $F_{1,4} = 5.38$, $p = 0.08$: $H_{pH}H_{NH4}$; $F_{1,4} < 0.01$, $p = 0.99$). Moreover, the

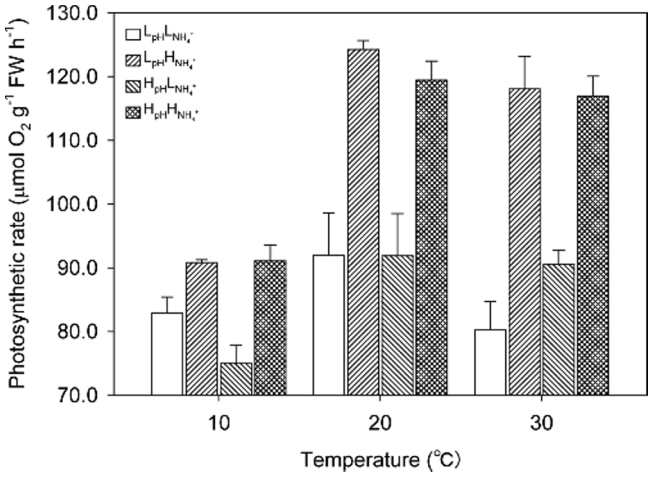

Fig. 2. Photosynthetic rates (μmol g^{-1} h^{-1}) of *Ulva australis* under different culture conditions. Vertical bars are means ± SD (n = 3). L_T = Low temperature (10°C); M_T = Medium temperature (20°C); H_T = High temperature (30°C); L_{pH} = Low pH (7.80); H_{pH} = High pH (8.20); L_{NH4} = Low NH_4^+ (4 μM); H_{NH4} = High NH_4^+ (120 μM)

photosynthetic rates of seaweeds at similar temperatures and NH_4^+ concentrations did not differ significantly between L_{pH} and H_{pH} (L_TL_{NH4}; $F_{1,4} = 5.92$, $p = 0.07$: L_TH_{NH4}; $F_{1,4} = 4.66$, $p = 0.10$: M_TL_{NH4}; $F_{1,4} < 0.01$, $p = 0.99$: M_TH_{NH4}; $F_{1,4} = 3.42$, $p = 0.14$: H_TL_{NH4}; $F_{1,4} = 3.27$, $p = 0.15$: H_TH_{NH4}; $F_{1,4} = 1.05$, $p = 0.36$). The photosynthetic rates of *U. australis* seaweed treated under similar temperatures combined with pH differed significantly between NH_4^+ concentrations ($p < 0.05$). All statistical analyses in each physiological response of *U. australis* are shown in Table 2.

NH_4^+ uptake rates

Rates of NH_4^+ uptake (μmol g^{-1} h^{-1}) by *U. australis* were significantly influenced by temperature, pH, and NH_4^+ concentration (Table 2). The maximum uptake rate of 6.62 ± 0.04 μmol g^{-1} h^{-1} was found at $M_TH_{pH}H_{NH4}$ treatment (Fig. 3), while L_{NH4} rates were low regardless of temperature and pH. The uptake rates of seaweed at the same pH level combined with NH_4^+ were higher at M_T than those at H_T or L_T, but not significantly ($L_{pH}L_{NH4}$; $F_{1,4} = 1.18$, $p = 0.34$: $L_{pH}H_{NH4}$; $F_{1,4} < 0.05$, $p = 0.87$: $H_{pH}L_{NH4}$; $F_{1,4} = 4.28$, $p = 0.12$: $H_{pH}H_{NH4}$: $F_{1,4} = 0.60$, $p = 0.48$).

Relative growth rate (RGR)

The relative growth rates (% day^{-1}) were influenced by temperature, pH, and NH_4^+ concentration. However, the RGRs

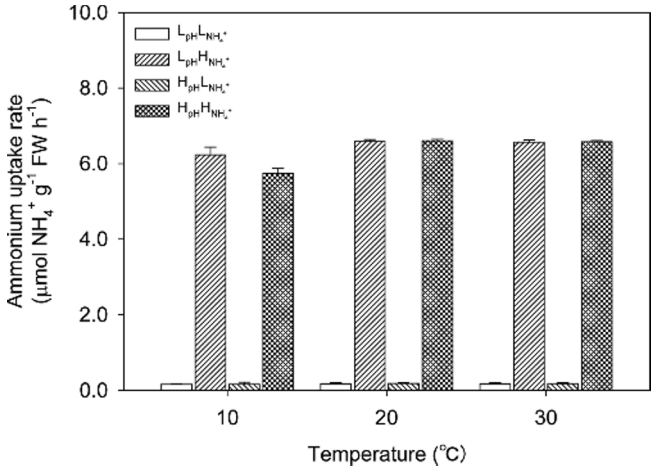

Fig. 3. Rates of NH_4^+ uptake ($\mu mol_4^+ g^{-1} h^{-1}$) of *Ulva australis* under different culture conditions. Vertical bars are means ± SD (n = 3). L_T = Low temperature (10°C); M_T = Medium temperature (20°C); H_T = High temperature (30°C); L_{pH} = Low pH (7.80); H_{pH} = High pH (8.20); L_{NH4} = Low NH_4^+ (4 μM); H_{NH4} = High NH_4^+ (120 μM)

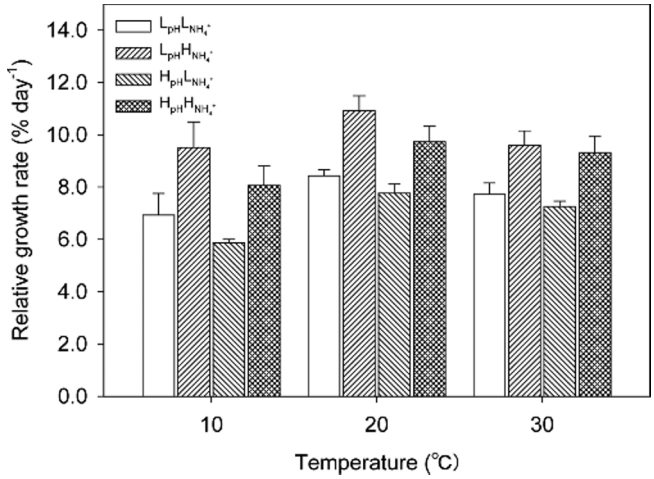

Fig. 4. Relative growth rates (% day^{-1}) of *Ulva australis* under different culture conditions. Vertical bars are means ± SD (n = 3). L_T = Low temperature (10°C); M_T = Medium temperature (20°C); H_T = High temperature (30°C); L_{pH} = Low pH (7.80); H_{pH} = High pH (8.20); L_{NH4} = Low NH_4^+ (4 μM); H_{NH4} = High NH_4^+ (120 μM)

rates were not affected by interactions among the treatments (i.e. combinations of temperature, pH, and nutrient concentrations) (Table 2). Maximum RGR was 10.92 ± 0.57% day^{-1} at $M_TL_{pH}H_{NH4}$, while other conditions showed similar RGR values and did not differ significantly (L_T; $F_{1,4} = 0.78$, $p = 0.43$: H_T; $F_{1,4} < 0.01$, $p = 0.95$: Fig. 4). The minimum RGR of 5.88 ± 0.13% day^{-1} was found at $L_TH_{pH}L_{NH4}$, but it did not differ significantly from $L_TL_{pH}L_{NH4}$ ($F_{1,4} = 2.90$, $p = 0.16$).

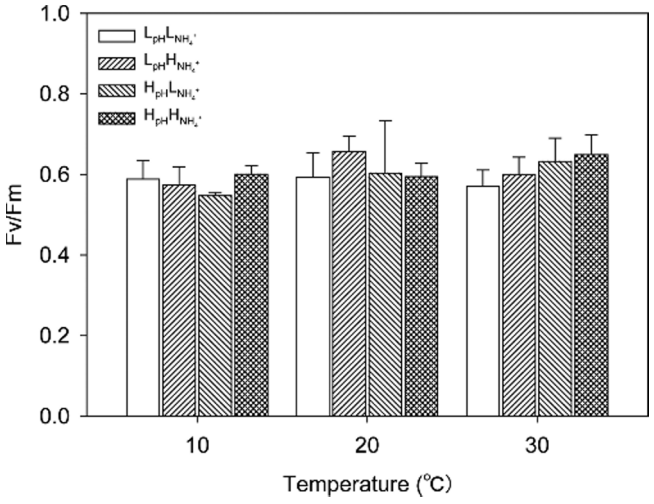

Fig. 5. Photosynthetic efficiency (F_v/F_m) of *Ulva australis* under different culture conditions. Vertical bars are means ± SD (n = 3). L_T = Low temperature (10°C); M_T = Medium temperature (20°C); H_T = High temperature (30°C); L_{pH} = Low pH (7.80); H_{pH} = High pH (8.20); L_{NH4} = Low NH_4^+ (4 μM); H_{NH4} = High NH_4^+ (120 μM)

Chlorophyll fluorescence

Photosynthetic efficiency, as measured by chlorophyll fluorescence (F_v/F_m) was not influenced by the three factors together (Table 2). The chlorophyll fluorescence ranged from 0.55 ± 0.01 to 0.66 ± 0.04 after 14 d of culture (Fig. 5). The maximum and minimum F_v/F_m were at $M_TL_{pH}H_{NH4}$ and $L_TH_{pH}L_{NH4}$, respectively, but they did not differ significantly from the other treatments ($p > 0.05$).

Tissue content of carbon and nitrogen

The tissue C content (% DW) was not affected by any of the culture conditions. However, tissue N content (% DW) was affected by the NH_4^+ concentrations (Table 2). The maximum tissue C and N contents of 25.57 ± 1.45% and 2.11 ± 0.23% were found at $H_TH_{pH}H_{NH4}$ and $M_TH_{pH}H_{NH4}$, respectively, and were significantly different from that of $M_TH_{pH}L_{NH4}$ ($F_{1,4} = 7.21$, $p < 0.05$) (Figs. 6 and 7). Significant N values affected the C/N ratio, but overall, the treatment conditions did not affect the seaweed C and N contents (Fig. 8). The lowest and highest C/N ratios of 11.66 ± 0.47 and 19.86 ± 3.27 were found at $M_TH_{pH}H_{NH4}$ and $L_TH_{pH}L_{NH4}$, respectively, but were not significantly different from the other treatment combinations ($p > 0.05$).

Interactive relationships

Temperatures, pH levels and NH_4^+ concentrations had

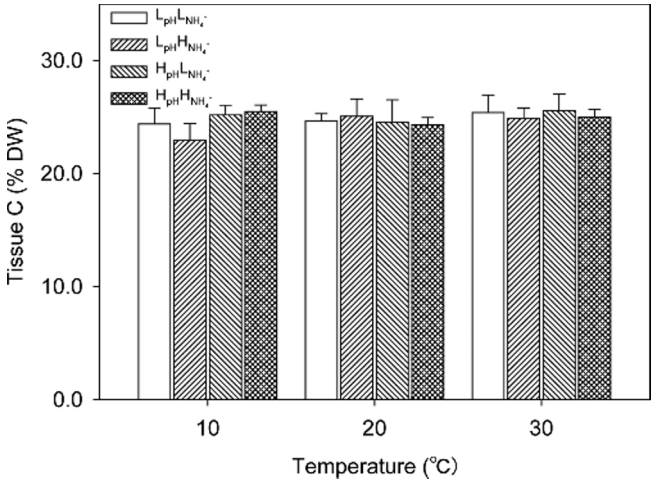

Fig. 6. Carbon contents of *Ulva australis* tissues under different culture conditions. Vertical bars are means ± SD (n = 3). L_T = Low temperature (10°C); M_T = Medium temperature (20°C); H_T = High temperature (30°C); L_{pH} = Low pH (7.80); H_{pH} = High pH (8.20); L_{NH4} = Low NH_4^+ (4 μM); H_{NH4} = High NH_4^+ (120 μM)

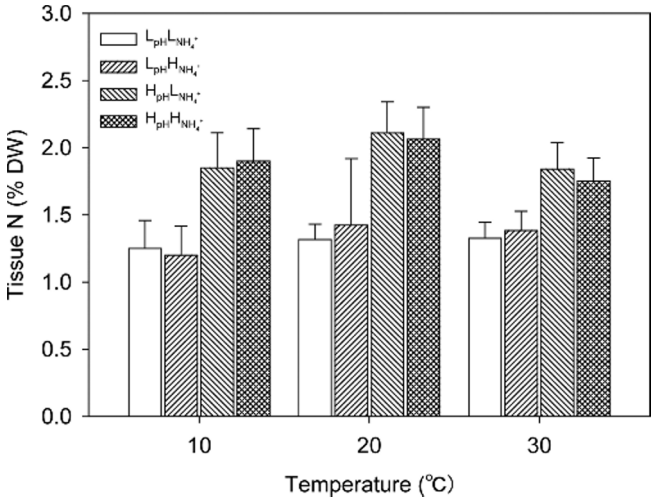

Fig. 7. Nitrogen contents of *Ulva australis* tissues under different culture conditions. Vertical bars are means ± SD (n = 3). L_T = Low temperature (10°C); M_T = Medium temperature (20°C); H_T = High temperature (30°C); L_{pH} = Low pH (7.80); H_{pH} = High pH (8.20); L_{NH4} = Low NH_4^+ (4 μM); H_{NH4} = High NH_4^+ (120 μM)

significant impacts on the photosynthetic rates, NH_4^+ uptake rates, and RGRs but chlorophyll fluorescence and tissue C contents were not affected. Tissue N contents and C/N ratios were affected by NH_4^+ only. Significant interactive effects between temperatures and pH levels were found in the photosynthetic rates, NH_4^+ uptake rates and RGRs. Interactions between temperatures and NH_4^+ concentrations were found

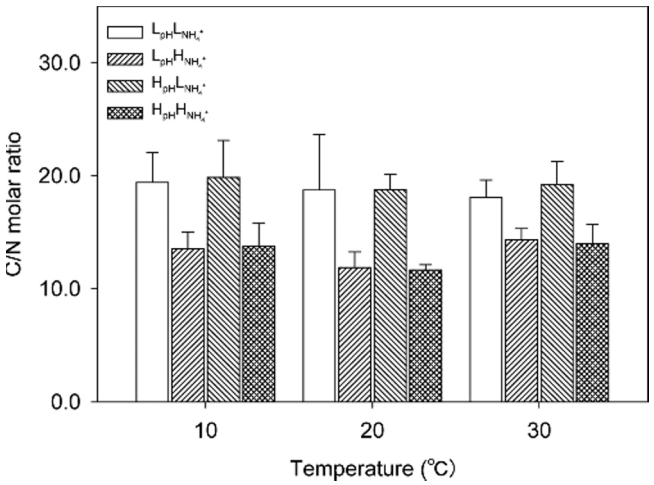

Fig. 8. Carbon to nitrogen ratios (C/N) within tissues of *Ulva australis* under different culture conditions. Vertical bars are means ± SD (n = 3). L_T = Low temperature (10°C); M_T = Medium temperature (20°C); H_T = High temperature (30°C); L_{pH} = Low pH (7.80); H_{pH} = High pH (8.20); L_{NH4} = Low NH_4^+ (4 μM); H_{NH4} = High NH_4^+ (120 μM)

on the photosynthetic rates and NH_4^+ uptake rates, while significant interactions among all the three factors were found for the photosynthetic rates and NH_4^+ uptake rates (Table 2).

4. Discussion

The effects of these three conditions (temperatures, pH levels and NH_4^+ concentrations) as proxies of global warming, ocean acidification and eutrophication are summarized in Table 2. Although some of our results showed significant interactive impacts (Table 2), due to the short-term nature of our experiments, we could not confirm whether these effects were synergistic or antagonistic. The impacts of each condition in connection with pH change, photosynthetic rates, NH_4^+ uptake rates, RGRs, chlorophyll fluorescence, tissue carbon and nitrogen contents were considered as follows.

Temperature and pH change

The temperature and pH of the culture media were affected by the initial temperatures and pH levels. Changes in pH values differed significantly between L_T and M_T, except at $H_{pH}H_{NH4}$. The L_T of 10°C was chosen according to the minimum monthly seawater temperature (mean value 11.0 ± 0.80°C) based on KHOA (2016), while the M_T of 20°C was selected as the optimal growth condition. Over a short period, elevated temperatures may enhance the metabolism of *U. autralis,* with additional supply of CO_2 and nutrients in the culture

media, which was also found in *U. fasciata* (Mantri et al. 2011). However, changes in the pH of the culture media were not significantly affected by L_T or H_T at the same pH and NH_4^+ concentration. During photosynthesis, seaweeds take up not only nutrients such as N and P, but also dissolved inorganic carbons (DICs) such as HCO_3^- and CO_2 (aq). The elevated DIC concentrations indicated that seaweeds could easily take up DICs for photosynthesis. Zhang et al. (2012) and Kang and Chung (2017) mentioned that the pH level can increase under low pH conditions due to large amounts of available DICs for photosynthesis. Therefore, ΔpH at L_{pH} was higher than that at H_{pH}. Additionally, seaweeds can increase the pH of seawater, and may therefore mitigate the rate of ocean acidification (Zou 2005; Moazami-Goudarzi and Colman 2012). The appropriate nutrient concentrations can enhance the utilization of DICs by algae (Huppe and Turpin 1994; Young and Beardall 2005). Therefore, the relationship between pH variation and nutrient concentrations requires further consideration.

Photosynthetic rates

The photosynthetic rates (μmolg^{-1} h^{-1}) of *U. australis* differed at each temperature or NH_4^+ concentration and combined temperature and pH, or temperature and NH_4^+. Additionally, the photosynthetic rates were influenced by the interaction of temperature, pH, and NH_4^+ concentration. High photosynthetic rates were shown at M_T and H_T. Davison (1991) indicated that temperature is a major factor controlling the rate of photosynthesis in algae. Murase et al. (1994) mentioned that optimal temperature conditions for photosynthesis of *U. australis* ranges from 20 to 25°C. A fluctuation in temperature can change the rate of photosynthesis (Ji et al. 2016). However, Kang and Kim (2016) indicated that the photosynthesis of *U. australis* was not affected by elevated temperatures.

Elevated nutrient concentrations can enhance the photosynthetic rate in seaweeds (Dawes and Koch 1990; Kang et al. 2016; Li et al. 2016; Kang and Chung 2017; Reidenbach et al. 2017). Zou and Gao (2014a, 2014b) mentioned that the photosynthetic rates of *Ulva* spp. are affected at elevated temperatures and high nutrient concentrations. Many studies indicate that the photosynthesis of *U. australis* is not enhanced by elevated CO_2 levels (Kang and Kim 2016; Kang and Chung 2017). Furthermore, the photosynthetic pigment contents of *Ulva* spp. are decreased under elevated CO_2 levels (Gordillo et al. 2003; Gao et al. 2018a). Liu and Zou (2015) mentioned that the photosynthetic rates of *U. lactuca* increase at high CO_2 and temperature conditions but are not affected under enriched CO_2 levels only. Temperature could be a major factor determining the photosynthesis of this alga and might enhance the photosynthesis of *U. australis* in response to ocean warming, acidification, and eutrophication.

NH_4^+ uptake rates

NH_4^+ uptake (μmol g^{-1} h^{-1}) rates were significantly affected when all three factors were tested. The nutrient uptake rates of *U. rigida* are also enhanced under high temperature, CO_2, and nutrient concentrations during a cultivation period of seven days (Gao et al. 2018b). Elevated temperatures affect the nutrient uptake of algae (Fan et al. 2014; Gao et al. 2018b). Abreu et al. (2011) indicated that temperature is the main factor controlling nutrient usage rates in seaweeds. Moreover, Fan et al. (2014) mentioned that the nutrient requirements of seaweeds vary significantly with temperature. The results of the present study indicated that *U. australis* could positively maintain nutrient uptake rates at high temperatures. Multiple studies have reported that elevated CO_2 levels increase the nutrient uptake of seaweeds such as *Sargassum fusiforme*, *Hypnea spinella*, and *Gracilariopsis lemaneiformis* (Zou 2005; Suárez-Álvarez et al. 2012; Kang et al. 2017). Enhanced CO_2 levels increase the availability of nutrients and elevate nitrate reductase activity (Gordillo et al. 2001; Hofmann et al. 2012; Xu et al. 2017). Moreover, increased nutrient concentrations can enhance nutrient uptake by *Ulva* spp. (Luo et al. 2012; Li et al. 2016). When nutrient availability increases, *Ulva* spp. can take up nutrients more efficiently (Pérez-Mayorga et al. 2011) and nutrient enrichments can enhance the utilization of DICs (Huppe and Turpin 1994; Young and Beardall 2005). In addition, the sheet-like thallus with its high surface/volume ratio enhances the possibility of nutrient uptake (Littler 1980; Wallentinus 1984).

Measurements of RGR

The relative growth rates (% day^{-1}) of *U. australis* were enhanced at each elevated temperature, pH, and NH_4^+ concentration. Temperature is a vital factor for the growth of *Ulva* spp. (Taylor et al. 2001; Liu et al. 2013). *Ulva* species grow in a wide range of temperatures (ranging from 10 to 30°C) and the highest growth rates occur between 15 and 20°C (Taylor et al. 2001; Kim et al. 2004). The maximum growth rates of the algae in the present study were also reported at 20°C. Mantri et al. (2011) indicated that seaweed growth can be accelerated at elevated temperatures because

of increased metabolism. Furthermore, the growth of *Ulva* spp. is enhanced by elevated CO_2 levels more than at ambient CO_2 concentrations (Xu and Gao 2012; Young and Gobler 2017; Ober and Thornber 2017; Gao et al. 2018b). Under enhanced CO_2 levels, this species can save energy by down-regulating the activity of carbon concentrating mechanisms (CCMs) and reallocate the energy towards growth (Beardall and Giordano 2002; Xu and Gao 2012). However, Kang and Kim (2016) indicated that RGR of *U. australis* was not affected by elevated CO_2 conditions (1,000 μatm). Axelsson et al. (1999) mentioned that the RGR of *U. lactuca* is not affected by elevated CO_2 concentrations because this alga may have efficient CCMs and its photosynthetic rate could be saturated under current CO_2 levels.

The growth of this alga was also affected by elevated NH_4^+ concentrations. Several researches indicated that NH_4^+ is the preferred N source for seaweeds because it requires lower energy for N assimilation compared to NO_3^- (McGlathery et al. 1996; Pedersen and Borum 1996; Runcie et al. 2003). Nutrient enrichment can stimulate the growth of seaweeds (Teichberg et al. 2010; Gao et al. 2017a; Young and Gobler 2017). *Ulva* spp. can respond quickly to excess nutrients when the temperature for their growth is optimal (Hernández et al. 1997; Morand and Merceron 2005). If the nutrient concentrations of coastal waters continuously increase, slow-growing species could be replaced by fast-growing species, such as *Ulva* spp. (Pedersen and Borum 1996).

Chlorophyll fluorescence

Chlorophyll fluorescence was measured as the proxy of photosynthetic efficiency and it was not affected by temperature, pH, or NH_4^+ concentrations. Previous studies mentioned that chlorophyll fluorescence of *Ulva rigida* is not enhanced by elevated temperature, CO_2, or nutrient concentrations compared to that of the control culture conditions (Figueroa et al. 2014a, b). Kang and Kim (2016) also indicated that the photosynthetic efficiency of *U. australis* is not affected by ocean warming and acidification conditions. Moreover, many studies indicated that the F_v/F_m values are not affected by elevated CO_2 concentrations (Hofmann et al. 2012; Kram et al. 2015). However, chlorophyll fluorescence of *U. lactuca* significantly increases under elevated CO_2 levels (Chen et al. 2015). Photosynthetic efficiency is enhanced by increased N concentrations in seaweeds (Dawes and Koch 1990; Kang and Chung 2017). Maxwell and Johnson (2000) have shown that a reduction in F_v/F_m values can indicate stress or photoinhibition in tissues under culture conditions. However, in our study, the chlorophyll fluorescence remained constant, so the seaweed might not experience any stress under future ocean conditions.

Tissue carbon and nitrogen

The tissue C contents (% DW) was not affected by temperature, pH, or NH_4^+ concentration. Similar results were found in *Hypnea spinella* at different CO_2 levels (Suárez-Álvarez et al. 2012) and in *U. australis* at CO_2 with NH_4^+ treatments (Reidenbach et al. 2017). However, Ober and Thornber (2017) showed that the tissue C contents of *U. lactuca* and *U. australis* are enhanced by elevated CO_2 concentrations with increased nutrient treatments. Gao et al. (2018b) indicated that the tissue C content of *U. rigida* is significantly affected by interactions between temperature, CO_2, and nutrient concentrations. Tissue N content (% DW) was affected by nutrient levels. Reidenbach et al. (2017) indicated that the tissue N contents of *U. australis* is also affected by elevated NH_4^+ concentrations. They found that the enriched NH_4^+ treatment enhanced nutrient assimilation compared to the ambient NH_4^+ treatment (Reidenbach et al. 2017). If nutrients are abundant, seaweeds can store the N in their tissues (Gómez-Pinchetti et al. 1998). The C/N ratio was also affected by nutrient concentrations. Generally, the C/N ratio is a good indicator of the physiological status of seaweeds (Chen and Johns 1991; Vergara et al. 1993). The C/N ratio of *U. australis* decreased when NH_4^+ was enriched. Previous studies indicated that the C/N ratio of seaweeds decreases when nutrients are abundant (Kim et al. 2007; Kang et al. 2011; Ober and Thornber 2017). This phenomenon could increase the tissue quality of *U. australis*. Ober and Thornber (2017) indicated that nutrient level is a key component of the tissue C/N ratio of *Ulva* spp. The elevated nutrient concentrations can benefit fast-growing species, such as *Ulva* spp. (Pedersen and Borum 1996). However, Kang and Kim (2016) mentioned that the C/N ratios of *U. australis* are not affected by elevated ocean temperatures, acidification, or by both warming and acidification conditions.

5. Conclusion

Our study demonstrated that several ecophysiological characteristics, such as pH variation in the culture media, photosynthetic rates, NH_4^+ uptake rates, relative growth rates, tissue N contents, and the C/N ratios of *U. australis* were affected by ocean warming, acidification, and eutrophication

conditions. However, chlorophyll fluorescence and tissue C contents did not show significant changes under those conditions. In the future, environmental changes due to anthropogenic activities could have multiple impacts on seaweeds. Our study indicated that the physiological responses of the green tide species, *U. australis*, might be enhanced under ocean warming, acidification, and eutrophication.

Acknowledgements

We would like to thank Editage (www.editage.co.kr) for the English language editing.

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