

ORIGINAL ARTICLE

The Effects of Elevated CO₂ and Ammonium Levels in Seawater on the Physiology of *Gracilariopsis chorda* (Holmes) Ohmi

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Abstract

We examined the effects of ocean acidification (OA) and eutrophication on the physiology of a red alga, *Gracilariopsis chorda*, using specimens collected at Wando Island, Korea, in July of 2015. The samples were transported to a laboratory and placed on growth media for treatments involving low or high levels of ammonium (4 μM or 60 μM NH₄⁺) and low or high pH (7.5 or 8.2). The control treatment used filtered seawater (pH 8.2 and 4 μM NH₄⁺). All experiments were conducted at 20°C and under a lighting intensity of 80 μmol photons m⁻² s⁻¹, with or without an injection of CO₂ (pH 7.5). In addition, we calculated rates of respiration under darkness, at a pH of 7.5 and 60 μM NH₄⁺. Fluctuations in pH as well as the evolution of photosynthetic oxygen and NH₄⁺ uptake rates were monitored for 6 h. The greatest increase in pH levels, from 7.50 to 8.65, occurred in response to 60 μM NH₄⁺, whereas the largest decrease, from 7.50 to 7.42, was associated with elevated respiration rates. At a pH of 7.5, rates of oxygen evolution were higher (236% saturation) for samples treated with 60 μM NH₄⁺ than for the control (121% saturation). Ammonium uptake was highest at pH 7.5 and 60 μM NH₄⁺, with a rate of 0.526±0.002 μmol g⁻¹ FW h⁻¹, followed in order by the treatments of pH 8.2/60 μM NH₄⁺, pH 7.5/4 μM NH₄⁺, and the control (pH 8.2/4 μM NH₄⁺). We speculated that the rates of photosynthesis and NH₄⁺ uptake could be enhanced at a higher ammonium concentration and lower pH because CO₂ concentrations were increased through greater photosynthetic activity. Therefore, these findings suggest that the physiology of *G. chorda* populations can be improved by the interaction of optimized CO₂ concentrations and an adequate supply of essential nutrients such as ammonium.

Key words : CO₂, Eutrophication, *Gracilariopsis chorda*, NH₄⁺, Ocean acidification (OA)

1. Introduction

Since the Industrial Revolution in the mid-18th to mid-19th centuries, atmospheric CO₂ concentrations have risen from 280 to 400 ppm, and levels are predicted to double by the end of the 21st Century (Roleda et al., 2012; Zou, 2005). As those concentrations continue to increase, global warming is significantly affecting marine ecosystems worldwide (Halpern et al., 2008). Approximately 30% of the

CO₂ that is released from the atmosphere is dissolved in the oceans, where it causes the pH of seawater to decline (Feely et al., 2004). Those reductions in pH are estimated to be 0.3 to 0.4 units annually and 0.8 units by 2300 (Caldeira and Wickett, 2003, 2005). Associated with this decrease in pH is the phenomenon of ocean acidification (OA), which potentially threatens communities of marine life because of its influence on the physiology and ecology of marine species (Caldeira and Wickett, 2003; Cornwall and Hurd, 2015).

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Results from earlier investigations of OA have demonstrated that, although it has a negative effect on the growth of calcifying organisms, acidification has a positive effect on almost all tested seaweed species (Kuffner et al., 2008; Xu and Gao, 2012; Zou, 2005). However, most of the previous studies were conducted in the laboratory. In fact, laboratory study could control the experimental conditions more easily compared to those of field survey. But laboratory studies were less realistic than mesocosm and field scale experiments.

The process of eutrophication introduces excessive nutrient inputs and is intensified by anthropogenic activities such as aquaculture industries that also cause serious problems for OA in a coastal environment (Fei, 2004; Wu et al., 2015). Continuous eutrophication leads to harmful algae blooms that can harm the food chain for marine ecosystems (Flynn et al., 2015). A surplus of nutrients in the ocean influences the primary production of phytoplankton and seaweeds and can alter the composition of marine communities (Alvera-Azcárate et al., 2003; Deng et al., 2012).

Seaweed species are the main components of habitats in marine ecosystems (Harley et al., 2012). They also play roles in bioremediation and the mitigation of nutrient toxicities, and their beds can potentially sequester CO₂. Numerous research projects concerning those aspects have focused on *Ecklonia cava*, *E. stolonifera*, and various *Gracilaria* species (Abreu et al., 2011; Chung et al., 2013; Yang et al., 2015). The genus *Gracilaria* belongs to the family Gracilariaceae, Rhodophyta. While distributed throughout the world, most of its species are found in tropical and subtropical areas, including the southern and western coasts of Korea (Choi et al., 2006; Lee and Kang, 2001). These species serve as valuable sources of agar, which is cultivated worldwide, especially in Chile, China, and Taiwan (Armisen, 1995; Tseng, 2001; Yang et al., 2015). Although the genus has been extensively surveyed (Kumar et al., 2013; Samocha et

al., 2015; Yang et al., 2015), only a few studies of *Gracilaria* cultivation have been conducted in Korea, even though it could possibly become one of the most important topics in future discussions about global warming.

Various cultivation techniques have been preliminarily evaluated (Choi et al., 2006; Kim et al., 2001; Kim, 2009) and some molecular phylogeny have been examined (Yang et al., 2013). However, data are lacking about how increases in CO₂ concentrations and nutrient levels might influence the physiology of *G. chorda* in particular. Therefore, the premise of the research presented here was to examine the effects of elevated CO₂ and nutrient supplies on the pH of seawater, as well on the rates of photosynthetic evolution of oxygen and the uptake of ammonium by this alga. Moreover, our objective was to assess the possibility of using this species for mass cultivation and as a key component in systems for integrated multi-trophic aquaculture (IMTA) under ocean acidification and eutrophication state.

2. Materials and Methods

Samples of *Gracilariopsis chorda* were collected at Wando Island, Korea (34°19'N, 126°45'E) in July of 2015. They were then transported to the biological oceanography laboratory at Pusan National University and washed several times with filtered seawater (0.45 μm) to remove any epiphytes.

Several treatments were used to evaluate how *G. chorda* responds physiologically to elevated CO₂ concentrations and excess levels of ammonium. Samples (6 g each) were placed on culture media (500 mL each) representing various test combinations: pH, low of 7.5 or high of 8.2; and NH₄⁺, low of 4 μM or high of 60 μM. Low pH culture media were injected CO₂ gas by CO₂ tank and controlled CO₂ regulator. Low-pH and high-NH₄⁺ media were made by mixing CO₂ gas and 60 μM ammonium chloride (NH₄Cl) in

filtered seawater. Culturing conditions in the chambers during the 6-h monitoring period included 20°C and a lighting intensity of 80 μmol photons m⁻² s⁻¹, as provided from LED fixtures and checked with an LI-250 light meter (LI-COR, USA). The control treatment involved samples exposed to filtered seawater at a pH of 8.2 and an NH₄⁺ level of 4 μM. In a separate experiment, we measured respiration rates for samples held under dark conditions at a pH of 7.5 and NH₄⁺ level of 60 μM.

The physiological activities of *G. chorda* were determined based on alterations in pH as well as rates calculated for photosynthetic oxygen evolution and NH₄⁺ uptake. The change in pH was measured at 30-min intervals with an Orion-250A pH meter (Thermoscientific, USA). To study whether pH might be affected by factors other than oxygen evolution, we also used a non-biomass culture chamber (blank). Photosynthesis rates were analyzed continuously over a 6-h period with a Clark-type oxygen microelectrode (Unisense, Denmark), which was linked to a microsensor multimeter. The microelectrode was calibrated with a mixed solution of C₆H₇NaO₆ (sodium ascorbate) and NaOH (sodium hydroxide) that detected response times of less than 1 s. Relative rates of oxygen saturation were calculated from real-time data, according to the following equation :

$$\text{Relative oxygen saturation rate (X)} \\ = C_T / C_I \times 100 (\%)$$

Where C_I is the initial concentration of dissolved oxygen, C_T is the final concentration of dissolved oxygen after T hours, and X is the relative oxygen saturation rate (%) after T hours.

Estimates for rates of NH₄⁺ uptake (in units of μmol ammonium per gram fresh weight per hour) were made based upon the amount of NH₄⁺ that was lost from the culture media during each 2-h period. The following equation was applied(Phillips and

Hurd, 2004) :

$$\text{Uptake rate (V)} = (S_i - S_f) \times \text{vol} \times W^{-1} \times T^{-1}$$

Where V is the uptake rate (μmol g⁻¹ FW h⁻¹), S_i is the initial concentration of NH₄⁺, S_f is the final concentration of NH₄⁺ after T hours, vol is the volume of the culture media, W is the fresh weight of the alga sample, and T is the culture period (in hours).

Rates of NH₄⁺ uptake were plotted against the corresponding average NH₄⁺ concentration, as calculated per the Michaelis-Menten equation(Pedersen, 1994) :

$$V = V_{\max} \times S / (K_s + S)$$

Where V is the uptake rate, V_{max} is the maximum uptake rate, S is the substrate concentration, and K_s is the half-saturation constant. In addition, uptake affinity was computed as V_{max}/K_s.

One-way ANOVAs were conducted after the NH₄⁺ uptake data were tested for normality and homogeneity. Tukey's tests were used to compare the results among culture treatments, with P-values <0.05 indicating that the differences were statistically significant. All analyses were performed with SPSS software (version 21.0).

3. Results

3.1. Changes in pH of the culture media

After 6 h of treatment, pH values increased by 0.24 units in response to the high pH/low NH₄⁺ combination, by 0.27 units for low pH/low NH₄⁺, by 0.41 units for high pH/high NH₄⁺, and by 1.15 units for the low pH/high NH₄⁺ combination (Fig. 1). By contrast, exposure to dark conditions caused the pH level to decrease by 0.08 units due to respiration by the *G. chorda* samples during the 6-h period. The pH levels in the non-biomass culture chamber ranged

from 7.50 to 7.51, indicating that pH was affected only by photosynthetic oxygen evolution. These data showed that changes in pH occurred more rapidly in the first half of the experiment, i.e., Hours 0 to 3, than in the second half, Hours 3 to 6.

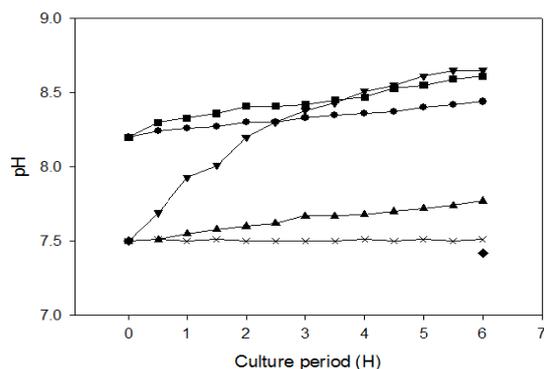


Fig. 1. Variations in pH levels in response to different treatment combinations. Values are means for 3 replicates. ●, High pH / Low NH₄⁺; ▲, Low pH / Low NH₄⁺; ■, High pH / High NH₄⁺; ▼, Low pH / High NH₄⁺; ◆, Low pH / High NH₄⁺ (Dark); x, Non-biomass.

3.2. Photosynthetic oxygen evolution in the culture media

Relative rates of oxygen saturation were compared between the high pH/low NH₄⁺ and low pH/high NH₄⁺ treatments (Fig. 2). Over time, those rates increased by 121.29% for the former and by 235.65% for the latter combination. Two phases of response were noted, with the slope of the change being approximately 5.0 times (high pH/low NH₄⁺) and 6.6 times (low pH/high NH₄⁺) steeper in the first half (Hours 0-3) than in the second half (Hours 3-6).

3.3. Rates of NH₄⁺ uptake in the culture media

Over the 6-h experimental period, the NH₄⁺ concentrations and uptake rates were 0.963±0.045 μM and 0.044±0.001 μmol g⁻¹ FW h⁻¹, respectively, for the high pH/low NH₄⁺ combination; 0.172±0.076 μM and 0.055±0.001 μmol g⁻¹ FW h⁻¹ for low pH/low

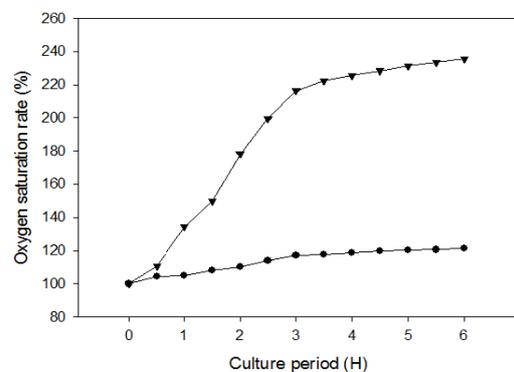


Fig. 2. Relative oxygen saturation rates (%) in response to different treatment combinations. ●, High pH / Low NH₄⁺; ▼, Low pH / High NH₄⁺.

NH₄⁺; 44.493±0.154 μM and 0.215±0.002 μmol g⁻¹ FW h⁻¹ for high pH/high NH₄⁺; and 22.119±0.166 μM and 0.526±0.002 μmol g⁻¹ FW h⁻¹ for low pH/high NH₄⁺ (Fig. 3; Table 1). This demonstrated an inverse relationship between initial pH and the rate at which ammonium was accumulated over time. In fact, the amount taken up was 12 times greater for the low pH/high NH₄⁺ treatment than for the high pH/low NH₄⁺ combination. For all treatments, the reduction in NH₄⁺ levels was more rapid in the first two 2 h than

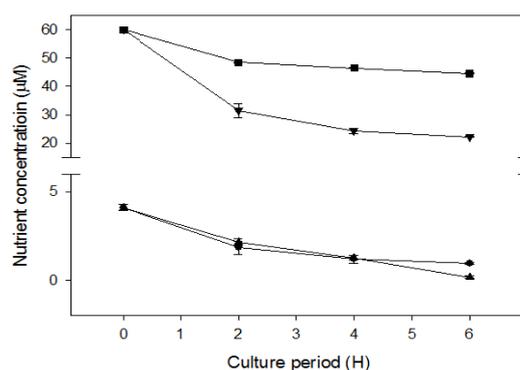


Fig. 3. NH₄⁺ depletions from samples of *Gracilariopsis chorda* in response to different pH and NH₄⁺ combinations. Data are means ± SD (n=3). ●, High pH / Low NH₄⁺; ▲, Low pH / Low NH₄⁺; ■, High pH / High NH₄⁺; ▼, Low pH / High NH₄⁺.

Table 1. Rates of NH₄⁺ uptake (μmol NH₄⁺ g⁻¹ FW h⁻¹) by *Gracilariopsis chorda* samples under different combinations of pH and ammonium concentrations

pH	NH ₄ ⁺ uptake rate (μmol NH ₄ ⁺ g ⁻¹ FW h ⁻¹)	
	Low	High
Low	0.055±0.001 ^b	0.526±0.002 ^d
High	0.044±0.001 ^a	0.215±0.002 ^c

Data are means ± SD (n=3 replicates). Values not followed by same letter are significantly different at *P*<0.05

Table 2. Rates of NH₄⁺ uptake (μmol NH₄⁺ g⁻¹ FW h⁻¹) by *Gracilariopsis chorda* samples, measured at 2-h intervals

Culture period (H)	Conditions			
	High pH / Low NH ₄ ⁺	Low pH / Low NH ₄ ⁺	High pH / High NH ₄ ⁺	Low pH / High NH ₄ ⁺
0 - 6	0.044±0.001 ^a	0.055±0.001 ^b	0.215±0.002 ^c	0.526±0.002 ^d
0 - 2	0.095±0.015 ^a	0.082±0.008 ^a	0.480±0.007 ^a	4.754±0.406 ^b
2 - 4	0.027±0.014 ^a	0.037±0.012 ^a	0.082±0.009 ^a	0.298±0.049 ^b
4 - 6	0.009±0.003 ^a	0.045±0.006 ^a	0.084±0.006 ^b	0.092±0.038 ^b

Data are means ± SD (n=3 replicates). Within each row, values not followed by same letter are significantly different at *P*<0.05

Table 3. Maximum ammonium uptake rates (V_{max}), half-saturation constant (K_s), and V_{max}/K_s ratios calculated for *Gracilariopsis chorda* samples treated with different pH/NH₄⁺ combinations

	High pH/Low NH ₄ ⁺	Low pH/Low NH ₄ ⁺	High pH/High NH ₄ ⁺	Low pH/High NH ₄ ⁺
V _{max}	0.044±0.001 ^a	0.055±0.001 ^b	0.215±0.002 ^c	0.526±0.002 ^d
K _s	1.970±0.026 ^a	2.479±0.045 ^b	9.676±0.090 ^c	23.736±0.104 ^d
V _{max} / K _s	0.022	0.022	0.022	0.022

Data are means ± SD (n=3 replicates). Within each row, values not followed by same letter are significantly different at *P*<0.05

in the second 4 h. In addition, NH₄⁺ uptake rates calculated every 2 h that, under all treatments, NH₄⁺ absorption was more active in the first 2 h (Table 2).

The maximum NH₄⁺ uptake rate, V_{max}, was highest for the low pH/high NH₄⁺ treatment, followed in order by high pH/high NH₄⁺, low pH/low NH₄⁺, and high pH/low NH₄⁺ (Table 3). The K_s values increased in response to additional CO₂.

4. Discussion

We used fresh samples of *Gracilariopsis chorda* to determine how pH, photosynthetic oxygen evolution,

and rates of NH₄⁺ uptake respond to different pH/ammonium combinations. Here, pH levels were altered under all treatment conditions due to CO₂ utilization and oxygen evolution. Atmospheric CO₂ is dissolved in the oceans, and is converted according to the following chemical reaction: CO₂(gas) ↔ CO₂(aq)+H₂O ↔ H₂CO₃ ↔ H⁺+HCO₃⁻ ↔ 2H⁺+CO₃²⁻ (Falkowski and Raven, 2007). In this process, dissolved H⁺ generally causes the seawater pH to decrease. However, in a marine environment, *G. chorda* absorbs dissolved inorganic carbon (DIC) and uses it as a material for photosynthesis. Accordingly,

we found that the pH of our culture media increased over time. A rise in CO₂ concentrations in seawater means that more DIC is available (CO₂(aq), HCO₃⁻, CO₃²⁻) for photosynthesis, thereby enhancing seaweed growth (Zou, 2005). Our analysis revealed that the largest increase in pH values was associated with the initially low pH/high NH₄⁺ treatment combination. This demonstrated that the physiological response by this species is complex and depends upon an integration of factors rather than being influenced by only one. The pH values measured for pH were also higher for the high pH/high NH₄⁺ treatment than for the low pH/low NH₄⁺ combination, indicating that photosynthesis by these organisms is not improved by higher CO₂ concentrations in the ocean if the nutrient supply is not sufficient. Finally, our test conditions of low pH and darkness led to a decline in photosynthesis for *G. chorda* because of an increase in respiration. This result is in accord with the general pattern reported previously for seaweeds (Suárez-Álvarez et al., 2012; Zou and Gao, 2009).

Higher CO₂ concentrations in the ocean accelerate physiological activity by seaweeds, thereby leading to CO₂ consumption and oxygen evolution. In a study of the brown seaweed *Sargassum fusiforme*, Zou (2005) showed that activity is enhanced when CO₂ levels are elevated in the culture medium. Similarly, we found that when CO₂ was utilized for photosynthesis, the pH values increased. When photosynthesis by seaweed is constrained by CO₂ concentrations, several biological strategies can be used to overcome that challenge (Larsson and Axelsson, 1999). For example, seaweed can use bicarbonate or directly assimilate CO₂ via carbon-concentrating mechanisms (CCM; Raven, 1997). However, greater amounts of CO₂ in the ocean cause DIC levels to rise, possibly increasing physiological responses such as photosynthesis (Gordillo et al., 2001). Such scenarios have been reported with *Gracilaria chilensis*, *Lomentaria articulata*, *Sargassum fusiforme*, *G.*

lemaniformis, *Macrocystis pyrifera*, and *Hypnea spinella* (Gao et al., 1993; Kübler et al., 1999; Roleda et al., 2012; Suárez-Álvarez et al., 2012; Xu et al., 2010; Zou, 2005). In contrast, declines in seaweed growth due to increased CO₂ have been observed for *G. tenuistipitata*, *Porphyra leucosticta*, and *G. gaditana* (Andría et al., 1999; García-Sánchez et al., 1994; Mercado et al., 1999). However, some researches have shown that a rise in CO₂ concentrations does not significantly affect seaweed growth (Chen and Zou, 2014; Israel and Hophy, 2002; Liu and Zou, 2015). These contrasting results demonstrate the diversified physiological responses by such organisms. Finally, an investigation of *G. lemaniformis* by Zou and Gao (2009) found that, regardless of an increase in the CO₂ concentration, growth could be improved if light levels were sufficient. All of these findings provide evidence that the physiological activities of seaweeds depend upon the interaction between CO₂ concentrations and other environmental factors, e.g., temperature, light intensity, and nutrient levels (Suárez-Álvarez et al., 2012).

Nutrient assimilation by seaweeds varies in response to increasing CO₂ levels. For example, elevated CO₂ in the culture media stimulates NH₄⁺ uptake rates for *G. chilensis* and *P. leucosticta* (Gao et al., 1993; Mercado et al., 1999) but decreases those rates for *G. tenuistipitata* and *G. gaditana* (Andría et al., 1999; García-Sánchez et al., 1994). Therefore, it is apparent that additional investigations are needed that focus on how OA influences seaweed physiology. Currently, some of the most critical environmental challenges are related to acidification of the oceans and eutrophication. Gracilariales species are cultivated in tropical and subtropical regions but are not yet used for mass production in Korea. However, the rise in seawater temperatures due to global warming will affect the distribution of seaweed populations, potentially shifting the range of tropical and subtropical species to more northern regions (Wernberg et al.,

2011). In case of Korea, aquaculture production of *Pyropia sp.*, *Undaria sp.*, and *Laminaria sp.* may decrease in the future. Therefore, we propose that *G. chorda* could serve as a good alternative for aquaculture systems under climate change conditions, based on preliminary studies of its suitability (Abreu et al., 2011; Choi et al., 2006; Yang et al., 2006) and our results.

5. Conclusion

This study examined physiology of *Gracilariopsis chorda* under elevated CO₂ and ammonium concentrations. The physiological activity of *G. chorda* is affected by increases in CO₂ concentrations and nutrient levels in the ocean. Enhancing the amount of CO₂ can improve the availability of essential nutrients and the physiological responses of this seaweed. Because we predict that *G. chorda* will become an even more valuable source of raw material for agar production, this species should be incorporated into future IMTA experiments to assess its role in mass cultivation. To date, experiments with this species have focused on short-term responses and small scales. Further investigations will require long-term surveys on a larger scale, e.g., as mesocosm or field studies, that will provide researchers with more realistic data related to the metabolism of *G. chorda*.

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