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Effects of CO$_2$ Enrichment on Photosynthesis, Growth, and Biochemical Composition of Seagrass Thalassia hemprichii (Ehrenb.) Aschers

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Abstract

The effects of CO$_2$ enrichment on various ecophysiological parameters of tropical seagrass Thalassia hemprichii (Ehrenb.) Aschers were tested. T. hemprichii, collected from a seagrass bed in Xincun Bay, Hainan island of Southern China, was cultured at 4 CO$_2$(aq) concentrations in flow-through seawater aquaria bubbled with CO$_2$. CO$_2$ enrichment considerably enhanced the relative maximum electron transport rate (RETR$_{\text{max}}$) and minimum saturating irradiance ($E_{\text{s}}$) of T. hemprichii. Leaf growth rate of CO$_2$-enriched plants was significantly higher than that in unenriched treatment. Nonstructural carbohydrates (NSC) of T. hemprichii, especially in belowground tissues, increased strongly with elevated CO$_2$(aq), suggesting a translocation of photosynthate from aboveground to belowground tissues. Carbon content in belowground tissues showed a similar response with NSC, while in aboveground tissues, carbon content was not affected by CO$_2$ treatments. In contrast, with increasing CO$_2$(aq), nitrogen content in aboveground tissues markedly decreased, but nitrogen content in belowground was nearly constant. Carbon: nitrogen ratio in both tissues were obviously enhanced by increasing CO$_2$(aq). Thus, these results indicate that T. hemprichii may respond positively to CO$_2$-induced acidification of the coastal ocean. Moreover, the CO$_2$-stimulated improvement of photosynthesis and NSC content may partially offset negative effects of severe environmental disturbance such as underwater light reduction.


Introduction

The ocean has captured between 28 and 34% of the anthropogenic carbon dioxide (CO$_2$) emitted to the atmosphere between 1980 and 1994 (Sabine et al. 2004; Millero 2007). The ensuing increase in ocean CO$_2$(aq) concentration (Sabine et al. 2004; Millero 2007) has lead to a reduction of about 0.1 pH units in ocean surface waters compared to pre-industrial times (Caldeira and Wickett 2003), and a further decline by 0.3–0.5 pH units is expected by 2100 (Caldeira and Wickett 2005). Worst case scenario (continued, unabated usage of known fossil fuels reserves) estimates indicate that average surface ocean pH could fall by a maximum of 0.77 pH units by the year of 2300 (Caldeira and Wickett 2003). Such extents, and rates, of change can potentially affect the physiology of marine biota and thus influence their competitive interactions, community composition, and the biogeochemical cycling of key elements such as carbon (C), nitrogen (N), and phosphorus (P) (Vézina et al. 2008).

Seagrasses are flowering plants that thrive in shallow oceanic and estuarine waters around the world, and are ranked as one of the most ecologically and economically valuable
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Björk et al. 2008). They have been shown to raise pH values in dense stands or in isolated pools up to 9 (Beer et al. 2006b). At such high pH values, seagrasses are close to their upper limit of inorganic carbon (C$_i$) uptake, and photosynthetic rates are therefore lowered (Björk et al. 2008). Under increased CO$_2$(aq) concentrations in the future, the acidification of seawater could counter the high pH formed by photosynthesis in such dense seagrass stands (Björk et al. 2008), hence enhancing seagrass photosynthesis and productivity. For example, seagrass production and shoot density were higher at shallow coastal sites where volcanic CO$_2$ vents lowered the pH of the water column than anywhere else around Ischia (Hall-Spencer et al. 2008).

Seagrasses can use bicarbonate ions (HCO$_3^-$) as an additional C$_i$ source for their photosynthetic needs (Beer 1989). However, it has been considered that they do so less efficiently than macroalgae (Beer and Koch 1996), such that they are not saturated with C$_i$ in today’s atmosphere-equilibrated shallow coastal oceanic habitats where they grow (Beer and Koch 1996; Invers et al. 2001). This general notion of C$_i$-limitation was supported by the experimental finding that CO$_2$ enrichment increased growth, photosynthetic rates, and leaf sugar content of *Zostera marina* (Zimmerman et al. 1995; Thom 1996; Zimmerman et al. 1997); and CO$_2$ enrichment led to significantly higher reproductive output, belowground biomass and vegetative proliferation of new shoots in light-replete treatments for *Z. marina* (Palacios and Zimmerman 2007), increased CO$_2$ availability by acid titration also enhanced the photosynthesis of *Halophila johnsonii* (Torquemada et al. 2005) and the carbon budget of *Posidonia oceanica* (L.) Delile (Invers et al. 2002). In contrast, no difference was found in the photosynthetic performances of individual *P. oceanica* leaves between the four stations where pH ranged from 8.2 to 7.6 (Hall-Spencer et al. 2008), and there was no effect of increasing CO$_2$(aq) levels on the aboveground productivity of *Z. marina* (Palacios and Zimmerman 2007).

Accordingly, only a few studies have been carried out on the effects of CO$_2$ enrichment on seagrasses, and have only focused on the temperate seagrasses such as *Z. marina* (Zimmerman et al. 1995; Thom 1996; Zimmerman et al. 1997; Palacios and Zimmerman 2007) and *P. oceanica* (Invers et al. 2002; Hall-Spencer et al. 2008). No study has been reported on the response of the tropical seagrass *Thalassia hemprichii* to CO$_2$ enrichment. *T. hemprichii* is among the most widely-distributed seagrass species in an Indo-Pacific flora, dominating in many mixed meadows (Short et al. 2007). Furthermore, the elemental C and N content of seagrass may affect herbivores’ feeding strategies and frequency (Heck and Valentine 2006), while no attention has been paid to the effect of CO$_2$ enrichment on elemental C and N content of seagrass.

Consequently, the objectives of this study were to assess the effects of CO$_2$ enrichment on photosynthesis, growth rate, biochemical (soluble sugar, starch, C, N, chlorophyll content) characteristics of the tropical seagrass *T. hemprichii* at four different levels of CO$_2$(aq). The results are expected to help predict future responses of seagrasses to global climate change, and provide a scientific basis for seagrass conservation.

### Results

**Photosynthesis and growth**

The photosynthesis responses of *T. hemprichii* to CO$_2$ enrichment are illustrated in Figure 1. The plots of relative maximum
electron transport (RETR) as a function of photosynthetically active radiation (PAR) showed that RETR rose linearly with PAR when light was limiting, reached a maximum, and then there was some decline. Thus, shoots under small enrichment (ES), middle enrichment (EM) treatment as well as the enrichment control (EC), exhibited initial photoinhibition at 729 μmol photons/m²/s, while shoots under large enrichment (EL) treatment exhibited photoinhibition at a higher PAR (1563 μmol photons/m²/s). Moreover, RETR of CO₂-enriched plants was higher than that in unenriched treatment at the same irradiance beyond 179 μmol photons/m²/s (Figure 1A). When the above data were fit to the double exponential decay function, shoots under increased CO₂(aq) conditions displayed higher RETRₘₐₓ than shoots in EC (control of enrichment), with increases of 17%, 29% and 54% at ES, EM and EL, respectively (Figure 1B, Table 1). The pattern of response for Eₖ was very similar to that for RETRₘₐₓ. Eₖ also increased with increasing CO₂(aq) by 15%, 31% and 79% at ES, EM and EL, respectively, relative to Eₖ in the EC treatment (Figure 1B, P < 0.01, Table 1). Leaf growth rate of shoots growing at ES, EM and EL, was 16%, 23% and 40%, respectively, larger than that in EC (Figure 2, P < 0.01, Table 1).

Table 1. Statistical analysis for the effects of CO₂ on dynamic properties of Thalassia hemprichii. P < 0.05 (significant); P < 0.01 (highly significant)

<table>
<thead>
<tr>
<th>Variable</th>
<th>df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>RETRₘₐₓ</td>
<td>3</td>
<td>39.00</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Eₖ</td>
<td>3</td>
<td>47.25</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Leaf growth rate</td>
<td>3</td>
<td>22.20</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Soluble sugar-aboveground</td>
<td>3</td>
<td>8.34</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Soluble sugar-belowground</td>
<td>3</td>
<td>69.25</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Starch-aboveground</td>
<td>3</td>
<td>34.84</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Starch-belowground</td>
<td>3</td>
<td>50.90</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Nonstructural carbohydrate-aboveground</td>
<td>3</td>
<td>11.01</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Nonstructural carbohydrate-belowground</td>
<td>3</td>
<td>89.41</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>C content-aboveground</td>
<td>3</td>
<td>2.98</td>
<td>0.160</td>
</tr>
<tr>
<td>C content-belowground</td>
<td>3</td>
<td>36.11</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>N content-aboveground</td>
<td>3</td>
<td>56.02</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>N content-belowground</td>
<td>3</td>
<td>3.36</td>
<td>0.136</td>
</tr>
<tr>
<td>C/N ratio-aboveground</td>
<td>3</td>
<td>32.31</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>C/N ratio-belowground</td>
<td>3</td>
<td>30.68</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Chla</td>
<td>3</td>
<td>10.40</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Chlb</td>
<td>3</td>
<td>7.19</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Chla/chlb</td>
<td>3</td>
<td>9.84</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>pH compensation point</td>
<td>3</td>
<td>0.43</td>
<td>0.743</td>
</tr>
</tbody>
</table>

df, degrees of freedom; F, the ratio of among-group variance (treatment mean square) divided by the within-group variance (error mean square); RETRₘₐₓ, relative maximum electron transport rate.

Figure 2. The effect of CO₂ enrichment on leaf growth rate of Thalassia hemprichii.

Different letters on column indicate significant difference (P < 0.05) among means. Error bars represent SE (n = 5). EC, control of enrichment; EL, large enrichment; EM, middle enrichment; ES, small enrichment.

Biochemical content and pH compensation point

The soluble sugar was 2.5-fold higher than the starch content in aboveground parts (Figure 3A), while in belowground parts, soluble sugar was only slightly higher than the starch concentration. Soluble sugar levels of both aboveground (P < 0.05, Table 1) and belowground parts (P < 0.01, Table 1) were strongly enhanced by the CO₂ enrichment, with the highest contents at EL treatment, which were 24–31% higher than EC treatment. Simultaneously, raising the CO₂(aq) levels also caused starch to increase considerably in both tissues (Figure 3B), but starch levels in belowground parts increased to a greater extent (P < 0.01, Table 1) than those in aboveground tissues (P < 0.01, Table 1). Hence, comparing the nonstructural carbohydrates (soluble sugar and starch, NSC) contents in aboveground and belowground for each CO₂ treatment, NSC in belowground (P < 0.01, Table 1) showed a greater CO₂ stimulation than that in aboveground (Figure 3C, P < 0.05, Table 1). Additionally, the enhancements of NSC reserve in the whole plant were 17%, 24% and 53% (ES, EM and EL, respectively) above the values of control plants. The excess NSC was curiously similar to the excess RETRₘₐₓ found in the T. hemprichii leaves. There was a positive correlation between them (R² = 0.9887).

The impact of CO₂ enrichment on the C and N contents of T. hemprichii is shown in Figure 4. Enrichment of CO₂ significantly enhanced the C content of belowground parts (Figure 4A, P < 0.01, Table 1), while the C content of aboveground tissues did not change considerably (P > 0.05, Table 1). By contrast, N content in aboveground parts showed a decline trend (Figure 4B, P < 0.01, Table 1), and no significant differences
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Figure 3. The effect of CO₂ enrichment on (A) soluble sugar, (B) starch and (C) nonstructural carbohydrates (NSC) in aboveground and belowground tissues of *Thalassia hemprichii*.

Different letters on column indicate significant difference \((P < 0.05)\) among means. Error bars represent SE \((n = 3)\). DW, dry weight; EC, control of enrichment; EL, large enrichment; EM, middle enrichment; ES, small enrichment.

of N concentrations were observed in belowground tissues \((P > 0.05, \text{ Table 1})\). Therefore, there was a marked difference in C/N ratios at both photosynthetic and non-photosynthetic tissues with increased CO₂(aq) content (Figure 4C). The atomic ratio of C/N in photosynthetic tissues \((P < 0.01, \text{ Table 1})\) increased by 6%, 10% and 27% for ES, EM and EL treatment respectively, compared with that of EC; while in non-photosynthetic tissues \((P < 0.01, \text{ Table 1})\), C/N ratio increased to a lesser degree.

Figure 4. The effect of CO₂ enrichment on (A) carbon, (B) nitrogen content and (C) C/N ratio in aboveground and belowground tissues of *Thalassia hemprichii*.

Different letters on column indicate significant difference \((P < 0.05)\) among means. Error bars represent SE \((n = 3)\). EC, control of enrichment; EL, large enrichment; EM, middle enrichment; ES, small enrichment.
The chlorophyll content decreased significantly with decreasing pH (Figure 5, Table 1). Chla, Chlb and total chlorophyll contents of *T. hemprichii* followed a similar pattern, whereas chla/chlb ratio was not affected. pH compensation point of *T. hemprichii* responded inversely to CO₂ enrichment (Figure 6, \( P < 0.01 \), Table 2). However, pH compensation point did not differ considerably among EC, ES, and EM treatments, but decreased significantly at EL treatment.

**Discussion**

**Photosynthesis and growth responses to CO₂ enrichment**

The present results showed a significant increase in RETR\(_{\text{max}}\) and \( E_k \) under CO₂ enrichment, representing the ability to transfer more electrons and a greater energy investment in the biochemical machinery of *T. hemprichii* for CO₂ fixation. This observed response of RETR\(_{\text{max}}\) to CO₂ enrichment will be less than the response of net photosynthesis to CO₂ enrichment (measured by CO₂ uptake or \( O_2 \) evolution), since under limiting CO₂ levels there will be significant contribution of photorespiration to RETR\(_{\text{max}}\). Gross rates of \( O_2 \) evolution reflects both carbon assimilation and photorespiration, and photorespiratory activity is higher under ambient than elevated CO₂ levels (Krall and Edwards 1992; Proctor 2003; Ananyev et al. 2005). With additional CO₂(aq) concentration, an increased supply of CO₂(aq) for photosynthesis improved the ribulose-bisphosphate carboxylase carboxylation efficiency in *T. hemprichii*, resulting in maintaining a high photosynthetic rate despite leaf chlorophyll content having decreased. Thus, CO₂ enrichment stimulated the photosynthesis of *T. hemprichii*.

Similar responses were observed in *Z. marina* and *Thalassia testudinum* (Zimmerman et al. 1995; Beer and Koch 1996; Zimmerman et al. 1997). Collectively, photosynthesis of many seagrasses is frequently limited by the availability of \( C_i \) under natural conditions (Zimmerman et al. 1997). In contrast, some species of seagrasses such as *Cymodocea serrulata* were shown to be \( C_i \) saturated (Schwarz et al. 2000), hence irrespective of atmospheric CO₂ levels, those species would not have enhanced productivity as a result of elevated inorganic carbon. In addition, the responses to CO₂ are also dependent on interactions with other environmental controls, and would be reduced when factors such as nutrients, temperature, or light are limiting (Palacios and Zimmerman 2007; Zou and Gao 2009).

Likewise, the present results also showed that CO₂ enrichment enhanced the leaf growth rate of *T. hemprichii*. Therefore, *T. hemprichii* could benefit from the future rise of CO₂(aq) in the ocean. A similar result was noted in *Z. marina*, in which high CO₂(aq) in culture markedly increased the leaf growth rate (Thom 1996). The growth enhancement by elevated CO₂(aq) could be attributed to accelerated photosynthetic carbon fixation at increased \( C_i \) availability and/or depression of photorespiration at higher ratios of CO₂ to \( O_2 \) in the medium as well as within the cells (Wu et al. 2008). Israel and Hophy (2002) indicated that growth response also depended on the presence of carbon concentrating mechanisms (CCMs). These processes involved substantial energetic and metabolic costs. For *T. hemprichii*, HCO₃⁻ was “dehydrated” to CO₂ with the aid of extracellular carbonic anhydrase (CA) acting in acidic
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Table 2. Equilibrium distribution of dissolved inorganic carbon in seawater and enriched treatments (µmol/L). Parameters of the carbonate system were calculated from pH, TA (alkalinity = 2291 µmol/L), temperature (temperature = 25 °C) and salinity (salinity = 34) using the program CO2SYS (Lewis and Wallace 1998)

<table>
<thead>
<tr>
<th>CO$_2$ level</th>
<th>pH</th>
<th>[CO$_2$(aq)]</th>
<th>[HCO$_3^-$]</th>
<th>[CO$_3^{2-}$]</th>
<th>DIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>8.10</td>
<td>9.6</td>
<td>1 724.0</td>
<td>230.6</td>
<td>1 964.2</td>
</tr>
<tr>
<td>Year 2100</td>
<td>7.75</td>
<td>24.8</td>
<td>1 998.2</td>
<td>119.4</td>
<td>2 142.4</td>
</tr>
<tr>
<td>Year 2200</td>
<td>7.50</td>
<td>47.6</td>
<td>2 116.7</td>
<td>71.1</td>
<td>2 234.6</td>
</tr>
<tr>
<td>An extreme beyond current prediction</td>
<td>6.20</td>
<td>1 005.4</td>
<td>2 282.2</td>
<td>3.8</td>
<td>3 291.5</td>
</tr>
</tbody>
</table>

diffusion boundary layer zones (Beer et al. 2006a). Consequently, rising CO$_2$(aq) availability might partially reduce the need for CCMs activity in *T. hemprichii*, which was verified by the significant decline of the pH compensation point under CO$_2$ enrichment. This resulted in decreasing the allocation of energy or nutrients for carbon acquisition but increasing them for stimulating growth.

Biochemical response to CO$_2$ enrichment

The plant C balance has been implicated as a major factor determining the growth and seagrass depth limit (Ralph et al. 2007). Under CO$_2$ enrichment, increased carbon uptake resulting from this stimulation of photosynthesis altered the balance of supply and capacity to use carbohydrates in *T. hemprichii*, and photosynthate availability exceeded the need for growth and/or respiration (NSC storage). Thus, NSC concentrations invariably increased within *T. hemprichii* grown at elevated CO$_2$(aq). This is consistent with previous studies (Thom 1996; Zimmerman et al. 1997). Furthermore, the higher C and NSC concentrations in belowground parts of *T. hemprichii* under CO$_2$ enrichment, indicated an effective NSC translocation from the aboveground tissues to belowground tissues, and the belowground tissues of *T. hemprichii* became a sink for fixed C.

Aboveground tissue N content of *T. hemprichii* was higher than that in belowground tissues regardless of the CO$_2$(aq) level. N concentration in aboveground tissues decreased with increasing CO$_2$(aq). Similar results were observed in other higher plants (Körner 2000). The leaf N reduction under CO$_2$ enrichment may be explained by dilution processes, owing to the stimulated accumulation of NSC in the leaf under high CO$_2$ (Gifford et al. 2000), so that stored N resources are gradually diluted during growth (Peralta et al. 2002).

The elevation of CO$_2$(aq) availability significantly enhanced C/N ratios of *T. hemprichii*. Similar results were found in macroalgae (Kübler et al. 1999) and marine picocyanobacteria (Fu et al. 2007). Burkhardt et al. (Burkhardt et al. 1999) suggested that if HCO$_3^-$ is primarily involved in inorganic carbon acquisition, perhaps elemental composition is less sensitive to variations in CO$_2$, but if CO$_2$ can be taken up actively, a greater sensitivity of elemental ratios would be expected. Since *T. hemprichii* showed higher affinities for CO$_2$ than for HCO$_3^-$ (Beer 1989), it was not surprising that elemental ratios of *T. hemprichii* were greatly affected by variations in CO$_2$(aq).

Ecological implications

The enriched CO$_2$(aq) in water could lower the requirement of light energy for CCMs in *T. hemprichii*, and thus may stimulate growth under light reduction conditions. Similarly, CO$_2$ enrichment significantly increased RETR$_{max}$ and $E_k$ of *T. hemprichii* photosynthetic organ, which may partially offset negative effects of decreased light availability by improving saturating photosynthetically active radiation, and may stimulate the overall C balance of plants in low-light environments (Zimmerman et al. 1997). Therefore, the possibility of colonization beyond current seagrass depth limits is possible as a consequence of increased CO$_2$ availability (Invers et al. 2002). In addition, the increment of CO$_2$(aq) promoted NSC accumulation in seagrass belowground tissue. The stored NSC can be used to meet the C demands of plants during periods of low photosynthetic C fixation caused by severe environmental disturbance such as underwater light reduction (Ralph et al. 2007). NSC also gives way to a period of enhanced rhizome growth, flowering shoot production and vegetative proliferation, and may buffer the negative effects of transplant shock by increasing rhizome reserve capacity (Palacios and Zimmerman 2007). Furthermore, increasing atmospheric CO$_2$ concentrations results not only in an increase in dissolved CO$_2$ concentrations, but also an increase in the relative proportion of dissolved CO$_2$ to HCO$_3^-$ (Short and Neckles 1999); and CO$_2$ enrichment lowered the pH compensation point, suggests that it would reduce the ability of seagrass to use HCO$_3^-$ . Thus, more benefits are expected to occur in seagrass, which favors the uptake of dissolved CO$_2$ more than seagrass with efficient HCO$_3^-$ uptake systems. Where seagrass is currently carbon limited, species with CO$_2$ uptake systems will tend to dominate seagrass communities (Connolly 2009).

Accordingly, the globally increasing CO$_2$ may enhance seagrass survival in eutrophic coastal waters, where populations have been devastated by algal proliferation and reduced water column light transparency (Zimmerman et al. 1997). Meanwhile, positive photosynthetic responses to elevated CO$_2$
concentrations may also at least partially counteract negative responses to decreased light availability from the rise in sea level (Short and Neckles 1999). Moreover, ocean acidification will stimulate seagrass biomass and productivity, leading to more favorable habitat and conditions for associated invertebrate and fish species. The previous study had demonstrated that direct injection of industrial flue gas could significantly increase seagrass productivity, which might prove useful for restoration efforts in degraded environments (Palacios and Zimmerman 2007).

Together our results indicate that CO₂ enrichment enhances photosynthetic rate, growth rate and NSC concentrations of T. hemprichii, but reduces leaf N content and pH compensation point. Consequently, T. hemprichii seems to respond positively to CO₂-induced acidification of the coastal ocean, which may have significant implications for carbon dynamics in shallow water habitats and for seagrass conservation. However, more detailed analyses, including Rubisco and CA content, enzyme activity and gene determination work are needed to establish how seagrasses respond to increased CO₂(aq) in the shallow water, and further long-term or in situ experiments are required to determine if the organisms will evolve to take advantage of the increased CO₂(aq), and provide a better understanding of the importance of rising CO₂(aq) in the distribution and production of marine seagrass communities.

**Materials and Methods**

**Plant material**

Intact vegetative plants of T. hemprichii were collected from Xincun Bay (18°24′34″N-18°24′42″N, 109°57′42″E-109°57′58″E) located in the south of Hainan Island, Southern China. Plants were collected carefully to keep belowground structures intact, and transported immediately to the laboratory in buckets containing seawater. Plants were gently washed free of sediments, sorted, placed in a clear aquarium, and maintained for 7 d prior to the start of the experiments. Light availability was provided by timer-controlled quartz-halogen lamps. The light intensity at the upper surface of the seagrass leaves was 250 µmol photons/m²/s, which was well in excess of photosynthetic saturation for T. hemprichii, and this level can also prevent photoinhibition of the leaves. The temperature was kept at 25 °C. For standardization, transplant units consisting of a single apical shoot with two rhizome internodes and associated root, were selected.

**Experimental design**

*Thalassia hemprichii* was cultured at pH 8.10, 7.75, 7.50, and 6.20, which represents the present ocean pH (control of enrichment, EC), the projected ocean pH for 2100 (small enrichment, ES), 2200 (Middle Enrichment, EM) and an extreme beyond the current predictions (a 100-fold increase in free CO₂ and an almost twofold increase in total dissolved inorganic carbon, Large Enrichment, EL; the same pH with previous study (Zimmerman et al. 1997)), respectively. Three aquariums were bubbled with compressed CO₂, which was metered by pH-controlled solenoid valves that maintained seawater pH within ±0.1 unit. Because no other acidifying agents or buffers were added to the seawater, pH served as proxy for the concentration of CO₂(aq) in each aquarium. Parameters of the carbonate system were calculated from pH, TA (alkalinity = 2291 µmol/L), temperature (temperature = 25 °C) and salinity (salinity = 34) using the program CO2SYS (Lewis and Wallace 1998). CO₂ enrichment caused acidification of the water, an increase in the CO₂(aq) level and distributional changes in all other dissolved carbon forms according to the new pH, as listed in Table 2.

The pH electrodes were submerged in each growth aquarium 30 cm below the surface, near the seawater outlet at the end of the aquarium opposite the water input. The electrodes were calibrated weekly using standardized pH buffers. When a solenoid valve was open, CO₂ was delivered via plastic tubing running through the bottom of the aquarium (Palacios and Zimmerman 2007). Plants were maintained under these conditions for 21 d.

**Plant analysis**

**Photosynthetic performance analysis**

Since pulse amplitude modulated (PAM) fluorometry could accurately assess the photosynthetic characteristics of these plants, and some species also showed photosynthetic electron transport rates that could be quantitatively related with rates of O₂ evolution (Ralph et al. 1998; Beer and Björk 2000). A PAM fluorometer (Mini-PAM, WALZGmbH) was used to generate rapid light curves (RLCs). The RLCs were initiated by attaching a dark leaf clip to the middle part of the second innermost leaf of every shoot for reducing any within-shoot variability. Eight consecutive light levels of 88, 179, 328, 512, 729, 1121, 1563 and 2 400 µmol photons/m²/s were applied at 10 s intervals. An effective yield measurement (Φ<sub>PSⅡ</sub>) was taken using a saturation pulse of 0.8 s, before the actinic light was applied (~ quasi-darkness), and at the end of each 10 s irradiance step, resulting in nine Φ<sub>PSⅡ</sub> measurements (Ralph and Gademann 2005). All measurements were conducted between 1 000–1 130 h. The RLCs used a relative measure (because leaf absorbance was not directly measured) of electron transport rates (RETR) (Schwarz et al. 2000; Beer et al. 2001), calculated from the following equation:

\[
RETR = \Phi_{PSII} \times PAR \times 0.5 \times AF,
\]

Without knowledge of the actual amount of light being absorbed, fluorescence measurements can be used as an
approximation of electron transport rates (Ralph et al. 1998; Belshe et al. 2007). We used the instrument default AF value (AF = 0.84) to calculate RETR, as recommended by Beer et al. (2001). The relative maximum electron transport rate (RETRmax), representing photosynthetic capacity, was derived by fitting the RLCs to the double exponential decay function (Platt et al. 1980), using a least-squares non-linear curve-fitting algorithm:

\[ \text{RETR} = Ps(1 - e^{-\alpha/\text{PAR}})e^{-\beta/\text{PAR}}, \]

\( Ps \) value is a scaling factor, which was in turn used to calculate RETRmax. \( \alpha \) (initial slope) and \( \beta \) (downregulation).

\[ \text{RETR}_{\text{max}} = Ps[\alpha/(\alpha + \beta)]1[\beta/(\alpha + \beta)]^{\beta/\alpha}, \]

The minimum saturating irradiance (\( E_k \)) was calculated by dividing the RETRmax by the initial slope. All calculations were performed in Statistica 6.0 (Statsoft, Tulsa, OK, USA).

**Growth analysis**

Growth rates were estimated by a modification of the classical punching method described for seagrasses (Zieman 1974). Leaves from apical shoots were marked with fine plastic fibers 1 cm above the sheath. Apical shoots were marked and collected after 14 d. Leaf growth rates (\( G_L \), cm/plant/day) were estimated according to the equation (Peralta et al. 2000):

\[ GL = \left( \sum G_{nm} + \sum G_{m} \right)/t, \]

where \( G_{nm} \) is the growth rate of unmarked leaves (small and new leaves),

\[ G_{nm} = TLL(t_f) - TLL(t_0), \]

where TLL is total leaf length and \( t_0 \), \( t_f \) are the days of marking and collection, respectively. \( G_m \) is the growth rate of marked leaves,

\[ G_m = MLL(t_f) - MLL(t_0), \]

where MLL is the length from the leaf base to the punching mark.

\[ t = t_f - t_0 \]

**Biochemical parameters analysis**

At the end of the experiment, plants were carefully retrieved and separated into root, rhizome and leaves. Rhizomes, roots and shoots were oven-dried (60 °C) until constant weight. Subsamples of all fractions were powdered and stored for nutrient content analysis. The samples were twice extracted in hot 80% ethanol. Soluble sugar content was determined by the anthrone-sulfuric acid method (Yemm and Willis 1954).

Starch content of the remaining materials was also analyzed by anthrone assay, following gelatinization at 100 °C for 15 min and solubilization in 70% perchloric acid (Quarmby and Allen 1989). Powdered samples were digested for N content determination by sulfuric acid-hydrogen peroxide method (Jiang 2000), and then N content was determined with Kjeldahl (BUCHI Auto Kjeldahl Unit K-370); whilst tissue C was measured with the Walkley-Blank acid digestion method (Chen 1983).

A 30 mm section from the youngest mature leaf was consistently chosen for analysis to avoid senescent and/or necrotic tissue and to ensure fully developed pigment characteristics. In a darkened room, the leaf material was finely chopped with a razor blade, ground in a cold mortar and pestle and then combined in a centrifuge tube with 10 mL of chilled 80% acetone. Chlorophyll extraction was determined by spectrophotometric method. Concentrations of chlorophylls a and b were determined from the following equations (Dennison 1990):

\[ \text{Chlorophyll } a \text{ (mg/FW)} = (12.7A_{663} - 2.69A_{645}) \times v/1000/w. \]

\[ \text{Chlorophyll } b \text{ (mg/FW)} = (22.9A_{645} - 4.68A_{663}) \times v/1000/w. \]

Where \( v \) is the volume of chlorophyll extraction, and \( w \) is the leaf fresh weight. Chlorophyll levels are presented as the summed values of chlorophyll a and b.

**pH compensation point**

In order to obtain the pH compensation point (an indicator of the ability to use HCO\(_3\)^−) of T. hemprichii grown under different conditions, pH-drift experiments were conducted in sealed glass vials containing 0.8 g fresh leaf and 20 mL unbuffered natural seawater at the same light-temperature condition. The final pH values were determined until there were no further increases (after 5–8 h) (Zou and Gao 2009).

**Statistics**

Means and standard errors of all variables were calculated for each CO\(_2\) level. The effect of the CO\(_2\) enrichment was analyzed by one-way ANOVA using SPSS for Windows version 10. Treatment means were compared and separated by least significant difference (LSD) at \( P < 0.05 \).

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