

The effect of ulvoid macroalgae on the inorganic carbon utilization by an intertidal seagrass *Thalassia hemprichii*

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Abstract. Blooms of ulvoid macroalgae (mainly *Enteromorpha* and *Ulva*) have covered 80% of the intertidal seagrass bed at Wanlitung, southern Taiwan, effectively shading the seagrass species *Thalassia hemprichii* resulting in a decrease in photosynthetic performance and low inorganic carbon (C_i) uptake. We looked for evidence of C_i limitation and investigated the C_i utilization characteristics of ulvoid-free and ulvoid-covered *T. hemprichii*. The rapid light curve (RLC) function of the Diving-PAM (Diving-PAM, Walz, Germany) was used to measure *in situ* photosynthetic performance (based on the effective quantum yield of PSII [Y] values) of intact seagrasses that were placed in small incubating chambers. Significantly, a lower $RETR_{max}$ (maximum relative electron transport rate) and E_k (light intensity at the onset of saturation) were noted in the ulvoid-covered compared to the ulvoid-free *T. hemprichii*, suggesting that the former has acclimatized to the low light environment becoming a “shade type” plant. The ulvoid-covered *T. hemprichii* showed some evidence of C_i limitation since a significant increase in $RETR_{max}$ (up to 46%; $P < 0.05$) was noted after an increase in the concentration of $NaHCO_3$ from 2.2 (normal seawater) to 6.2 mM. In terms of C_i utilization characteristics, *T. hemprichii* could directly absorb HCO_3^- as the major C_i source but partially depended on the extracellular carbonic anhydrase (CA) to convert HCO_3^- to CO_2 prior to uptake in the ulvoid-free, high light-adapted populations. A wastewater stream with a high nutrient load coming from the urbanized area may have caused the frequent blooms of ulvoid macroalgae.

Keywords: Bicarbonate; Photosynthesis; Taiwan; Ulvoid bloom.

Abbreviations: **AF**, absorption factor; **AZ**, acetazolamide; **CA**, carbonic anhydrase; **C_i** , inorganic carbon; **DIN**, dissolved inorganic nitrogen; **DIP**, dissolved inorganic phosphate; **E_k** , the light intensity at the onset of photosynthetic saturation; **RETR**, relative electron transport rate; **HEPES**, N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; **F**, minimal chlorophyll-a fluorescence in the light-adapted state; **Fm'**, maximum chlorophyll-a fluorescence in the light-adapted state; **PAM**, pulse amplitude modulated; **PPFD**, photosynthetic photon flux density; **$RETR_{max}$** , maximum relative electron transport rate; **PSII**, photosystem II; **Y**, the effective quantum yield of PSII; **α_{RETR}** , the initial slope of the light-limited relative electron transport rate.

Introduction

Seagrasses are submerged marine angiosperms that comprise about 60 species worldwide (Den Hartog, 1970; Green and Short, 2003), seven of which have been recorded from Taiwan (Yang et al., 2002). To date, very few studies on seagrasses in Taiwan have appeared (Mok et al., 1993; Shieh and Yang, 1997; Lin and Shao, 1998; Yang et al., 2002). Lin and Shao (1998) reported that *Thalassia hemprichii* (Ehrenberg) Ascherson from southern Taiwan was threatened by anthropogenic activities due to excessive nutrient input. The nutrient over-enrichment of coastal waters can lead to the proliferation of bloom-forming “ephemeral” macroalgae which end up shading seagrass populations and eventually displacing seagrass as the dominant benthic autotrophs (McGlathery, 2001).

Manipulative experiments on a naturally occurring bloom of *Ulvaria obscura* (Kützting) Gayral in Armitage Bay, Blakely Island, Washington State, USA resulted in reduced seagrass shoot density (Nelson and Lee, 2001). Competition with other macrophytes for one or more resources, such as light, O_2 , or inorganic carbon (C_i) could be behind the seagrass growth reduction (Coffaro and Bocci, 1997) suggesting some form of resource limitation.

At Wanlitung (N 21°99'60", E 120°70'50") in southern Taiwan frequent blooms of ulvoid macroalgae (mainly *Enteromorpha* spp. and *Ulva* spp.) were observed in the intertidal habitat covering some portion of the flourishing seagrass, *Thalassia hemprichii*. A continuous wastewater stream from the urbanized area was observed to drain into the seagrass habitat. Dissolved inorganic nitrogen (DIN) was present at 0.51 ppm during low tide and 0.49 ppm during high tide. Dissolved inorganic phosphorus (DIP) was 0.24 ppm during low tide and 0.03 ppm during high tide (Liu and Wang, unpublished data). The DIN:DIP

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ratio was 2.1 at low tide and 16.3 at high tide. Clearly, DIP was very high during low tide. This could partly explain the bloom of ulvoid macroalgae that covered about 80% of the seagrass bed.

Schwarz et al. (2000) observed that photosynthesis in *Cymodocea serrulata* (R. Brown) Ascherson et Magnus and *Halophila ovalis* (R. Brown) Hooker f. inhabiting the subtidal habitat (10–12 m depth) had photosynthetic rates limited by ambient C_i concentrations depending on the irradiance available. The same species in the intertidal habitat were not limited by C_i concentration at any irradiance up to $1500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. The latter result was contrary to a previous conclusion regarding C_i limitation in intertidal plants (Björk et al., 1997), and Schwarz et al. (2000) highlighted the need for performing photosynthetic investigation *in situ*.

We hypothesized that the shading of seagrass by ulvoid macroalgae could have detrimental effects on *T. hemprichii*, lowering its photosynthetic performance and thus its C_i requirement. In addition, an excessive amount of ulvoid algae could deplete the surrounding C_i concentration, thereby also affecting the pH of the surrounding medium. Beer (1996) suggested two possible mechanisms for C_i transport in seagrass: (1) direct uptake of HCO_3^- and (2) extracellular dehydration of HCO_3^- to CO_2 as catalyzed by membrane-associated carbonic anhydrase (CA).

In the ulvoid-covered population, we expect *T. hemprichii* to exhibit some degree of C_i limitation and adopt a mechanism of C_i utilization slightly different to what it would use in an ulvoid-free population. In this study, we tested for evidence of C_i limitation and investigated differences in C_i utilization characteristics between ulvoid-free and ulvoid-covered *T. hemprichii*. We also used an underwater PAM fluorometer (Diving-PAM) for *in situ* measurements of photosynthetic performance of *T. hemprichii*.

Materials and Methods

Study Site

Our study area was on the intertidal reef flats at Wanlitung (N $21^{\circ}99'60''$, E $120^{\circ}70'50''$), southern Taiwan (Figure 1). The seagrass bed covered about 0.002 ha and was found at 0–50 cm depth between low tide and high tide. To get a sense of the ambient environmental conditions around the ulvoid-free and ulvoid-covered seagrass populations during low tide and high tide, irradiance and pH was determined with a spherical quantum sensor and pH meter, respectively. To get a rough idea on the coverage of *T. hemprichii* and the ulvoid algae, the transect quadrat method after Lin and Shao (1998) was used. A 0.25 m^2 quadrat ($50 \times 50 \text{ cm}$) divided into 25 squares ($10 \times 10 \text{ cm}$) was placed on the substratum at five-meter intervals along a 50-m transect line, and the coverage of *T. hemprichii* and ulvoid algae was estimated. In each quadrat, only five squares ($10 \times 10 \text{ cm}^2$) were randomly selected for shoot density estimation of *T. hemprichii*.

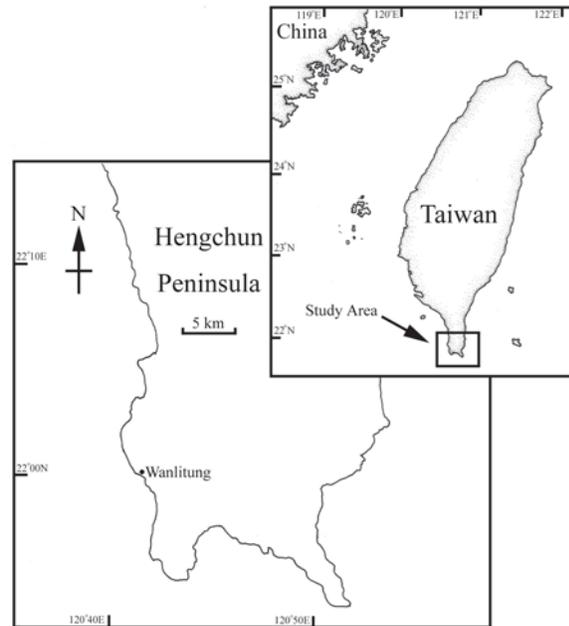


Figure 1. Map showing the study site at the southern tip of Taiwan.

Counting of shoot density was done manually. Simultaneously, five squares ($10 \times 10 \text{ cm}^2$) were also randomly selected for estimating the biomass of the ulvoid algae (*Enteromorpha-Ulva* spp.). The ulvoid algal samples were collected and dried using silica gel. The silica gel was changed every 2 h for 24 h. The use of silica gel was done to facilitate the drying of algal samples while in the field.

Experimental Protocol

The *in situ* experimental protocol for studying the C_i utilization characteristics of *T. hemprichii* (not covered and covered with ulvoid macroalgae) followed that of Schwarz et al. (2000). The rapid light curve (RLC) function of the Diving-PAM (Diving-PAM, Walz, Germany) was used to measure the *in situ* photosynthetic performance of seagrass samples (Beer et al., 1998). Materials were placed in Perspex incubating chambers (20 ml) (Schwarz et al., 2000) and tested for evidence of C_i limitation by adding a known amount of NaHCO_3 in the incubating chamber and measuring the photosynthetic performance of the seagrass sample before and after the addition. All RLC's were performed under normal seawater conditions. An initial RLC was performed under ambient HCO_3^- concentrations (ca. $2.2 \text{ mM } C_i$, mainly in the form of HCO_3^-). Then, 0.4 ml of 100 mM NaHCO_3 solution was injected from a 2.5 ml syringe to increase by about 2 mM the C_i concentration in the chamber (4.2 mM), and the second RLC was measured. Prior to the third RLC measurement, a further 0.4 ml of 100 mM NaHCO_3 was injected to give a final C_i concentration of 6.2 mM in the chamber.

To determine whether the mechanism of C_i transport for *T. hemprichii* was direct HCO_3^- uptake or external CA-me-

diated HCO_3^- utilization, photosynthesis of the seagrass sample inside an incubating chamber was measured (using the RLC function of the diving PAM) before and after the addition of 1 ml 1 M HEPES buffer solution (pH 8.8, final concentration 50 mM). The aim was to change the equilibrium between the different C_i forms within the chamber, resulting in a CO_2 concentration that was 0.11% and an HCO_3^- concentration that was 67% of the total seawater C_i content. In addition, to demonstrate evidence for an external CA-mediated HCO_3^- uptake, photosynthesis of a separate seagrass sample inside an incubating chamber was measured (using the RLC function of the Diving-PAM) before and after the addition of 0.2 ml 0.001 mM acetazolamide (AZ) solution (a membrane-impermeable CA inhibitor, final concentration 100 μM). All experimental protocols were performed thrice.

Chlorophyll Fluorescence Measurements

The photosynthetic performance of seagrass samples was assessed based on the effective quantum yield of PSII (Y) values provided by the Diving-PAM's RLC function. Y was measured in darkness, and eight irradiance intensities were provided by the internal halogen lamp of the Diving-PAM. Photosynthetic photon flux density (PPFD) ranged from non-saturating to saturating intensities (139, 213, 299, 450, 612, 943, 1376, and 2097 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$) at 10s-intervals. The light intensities we used were within the range established by Ralph and Burchett (1995) in their study on the photosynthetic responses of the seagrass *Halophila ovalis*. In order to avoid other factors (i.e. age, position, and any lesions of the leaf) and the diurnal variations of fluorescence parameters that might affect the fluorescence yield of the samples, all measurements were done at the middle fresh portion of the second ranked leaf between 10:00 and 16:00 hours (Durako and Kunzelman, 2002, Durako et al., 2003). Due to lack of measurements for leaf absorbance, only relative transport rate was estimated. The RETR at each irradiance intensity of RLCs was estimated by the equation: $\text{RETR} = Y * \text{PPFD}$, where Y was the effective quantum yield of PSII ($\Phi_{\text{PSII}} = (F_m' - F)/F_m'$) and PPFD was the photosynthetically available irradiance reaching the leaf ($\mu\text{mol photon m}^{-2} \text{s}^{-1}$). From the RETR-irradiance data, the maximal RETR at light saturation (RETR_{max}) and the initial slope of the light-limited relationship (α_{RETR}) was calculated using the hyperbolic tangent equation: $\text{RETR} = \text{RETR}_{\text{max}} \tanh(\alpha_{\text{RETR}} \cdot I / \text{RETR}_{\text{max}})$, where RETR was the relative electron transport rate, RETR_{max} was the maximum relative electron transport rate, I was the irradiance and α_{RETR} the initial slope of the light-limited relationship (Jassby and Platt, 1976). An additional parameter, E_k , defined as the light intensity at the onset of saturation was also computed as: $E_k = \text{RETR}_{\text{max}} / \alpha_{\text{RETR}}$.

Statistical Analysis

Means of photosynthetic parameters (i.e., RETR_{max} , E_k and α_{RETR}) between ulvoid-free and ulvoid-covered *T. hemprichii* were compared using a t -test for independent samples at the 95% significance level. We used ANOVA

with repeated measures to test the effect of RETR_{max} before and after adding the various chemicals (NaHCO_3 , HEPES buffer or AZ) to the incubating medium. Initially the data were tested for normality and homoscedasticity. Appropriate transformations were carried when data (i.e., data on effects of C_i concentration and AZ treatment were converted to log base 2) were not normal and heteroscedastic (Zar, 1984).

Results

In seagrass covered with ulvoid algae, the ambient light intensity at the bottom was 168 and 24 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ during low tide and high tide, respectively. In ulvoid-free seagrass, the ambient light intensity was 780 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ during low tide and 425 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ during high tide. The pH of the ambient water in ulvoid-covered seagrass was 8.75 and 8.32 during high tide and low tide, respectively, while in ulvoid-free seagrass, the ambient pH was 8.42 and 8.19, respectively. The coverage of the ulvoid algae was about 80% of the coverage of *T. hemprichii*. The total number of shoot counts in *T. hemprichii* was 52 ± 4 in five $10 \times 10 \text{ cm}^2$ quadrats, and the total biomass of the ulvoid algae was about $15 \pm 4 \text{ g}$ dry weight in five $10 \times 10 \text{ cm}^2$ quadrats.

Significantly lower RETR_{max} and E_k values were recorded for the ulvoid-covered compared to the uncovered *T. hemprichii* populations (Figure 2, Table 1). There was no significant difference between populations for α_{RETR} (initial slope of the RETR-irradiance curve). However, it should be noted that our starting light intensity (i.e., 193 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$) was relatively high. There were also few data points below the onset of light saturation (E_k), which may have led to a less precise estimate of α_{RETR} . Hence, these values should be treated as preliminary and subject to further confirmation in future studies.

Our results also indicated a certain degree of C_i limitation for ulvoid-covered *T. hemprichii*. A significant in-

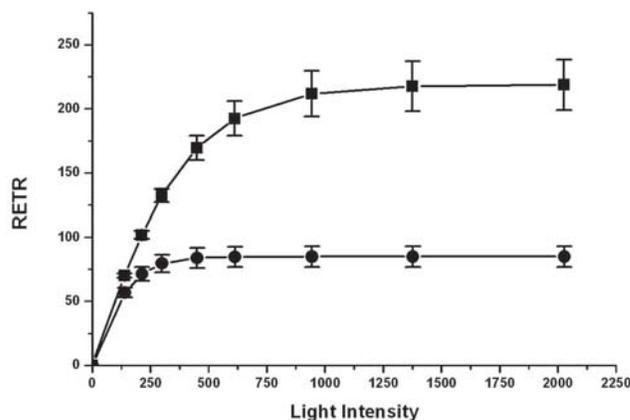


Figure 2. RETR curves of *Thalassia hemprichii* at different photosynthetic photon flux density (PPFD, $\mu\text{mol photon m}^{-2} \text{s}^{-1}$) for ulvoid-covered populations (solid circle) and ulvoid-free populations (solid square). Data were obtained under ambient seawater conditions. Bars are standard errors ($n = 9$).

Table 1. T-test results comparing means of maximum rate of relative electron transport rate ($RETR_{max}$), the initial slope of the light-limited relative electron transport rate (α_{RETR}), and the onset of light saturation (E_k) in the ulvoid algae-covered and uncovered *Thalassia hemprichii*, Wanlitung, southern Taiwan.

Parameters	Covered with ulvoid algae, mean \pm SE (n = 9)	Without ulvoid algae, mean \pm SE (n = 9)	t-value	df	P-value
$RETR_{max}$	85 \pm 8	219 \pm 20	-7.10	16	0.000*
α_{RETR}	0.21 \pm 0.01	0.22 \pm 0.01	-1.08	16	0.297
E_k	250 \pm 5	453 \pm 33	-8.80	16	0.000*

*Significant lower values using t-test ($P < 0.05$).

crease in $RETR_{max}$ (up to 46%; $P = 0.043$; Tukey HSD test) was noted when ulvoid-covered *T. hemprichii* were supplied with increased $NaHCO_3$, from a concentration of 2.2 (normal seawater) to 6.2 mM (Figure 3, Table 2). On the other hand, the 4.4 mM and 6.2 mM HCO_3^- concentrations showed no significant difference. The $RETR_{max}$ of ulvoid-free *T. hemprichii* remained within the same levels with the addition of $NaHCO_3$.

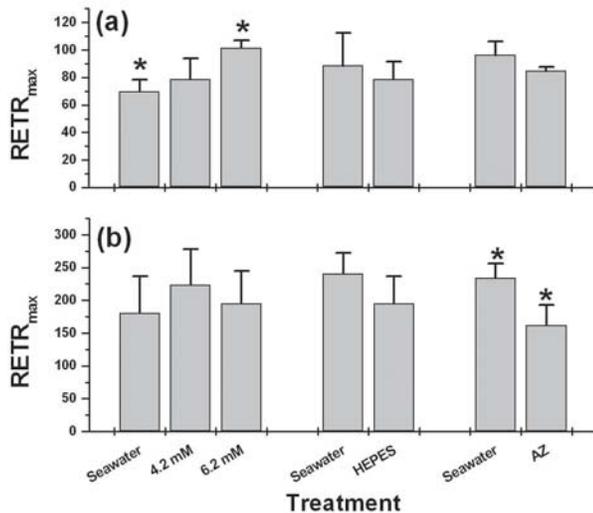


Figure 3. $RETR_{max}$ (mean and SE, n = 3) of (a) ulvoid-covered and (b) ulvoid-free *Thalassia hemprichii* under different inorganic carbon (C_i) concentrations and after the addition of HEPES buffer solution and acetazolamide (AZ). Identical icons are significant at $P < 0.05$.

Table 2. Post-hoc comparison before and after addition of $NaHCO_3$ in ulvoid-covered and ulvoid-free *T. hemprichii* showing the statistical P value.

	2.2 mM	4.2 mM	6.2 mM
Ulvoid-covered			
2.2 mM	-	0.887	0.043*
4.2 mM	0.887	-	0.174
6.2 mM	0.043*	0.174	-
Ulvoid-free			
2.2 mM	-	0.298	0.927
4.2 mM	0.298	-	0.751
6.2 mM	0.927	0.751	-

*Significant at $P < 0.05$.

Generally, $RETR_{max}$ in the HEPES-added medium was higher than in the AZ-added medium. These results seemed to suggest that *T. hemprichii* was able to directly absorb HCO_3^- as the major C_i source but partially depended on the extracellular carbonic anhydrase (CA) to convert HCO_3^- to CO_2 prior to uptake in the high light-adapted populations. However, there was no significant difference in $RETR_{max}$ between ulvoid-covered and ulvoid-free *T. hemprichii* after HEPES buffer was added to the medium (Table 3). A lower $RETR_{max}$ value in the ulvoid-covered and ulvoid-free seagrass was observed, indicating a lower affinity for direct HCO_3^- uptake. On the other hand, the $RETR_{max}$ of the ulvoid-free *T. hemprichii* showed a significant ($P = 0.049$; LSD test) decrease (about 31%) after the enzyme AZ was added to the medium, suggesting the presence of an external CA-mediated HCO_3^- utilization pathway.

Discussion

The $RETR$ -light curves in Figure 1 strongly suggest that the ulvoid-covered *T. hemprichii* in the intertidal area at Wanlitung, southern Taiwan have acclimatized to the low light environment and become “shade type” plants as opposed to the “sun type” *T. hemprichii* living in intertidal habitats. Seagrasses adapted to high light conditions have been shown to have a higher maximum photosynthesis and a higher capacity to use HCO_3^- as a source of C_i (Mercado et al., 2003). Similarly, the $RETR_{max}$ of ulvoid-free *T. hemprichii* in this study were higher than those of the ulvoid-covered variety, indicating higher photosynthetic capacity.

Table 3. Post-hoc comparison before and after addition of HEPES buffer and AZ in ulvoid-covered and ulvoid-free *T. hemprichii* showing the statistical P value.

	HEPES addition		AZ addition	
	Before	After	Before	After
Ulvoid-covered				
Before	-	0.878	-	0.678
After	0.878	-	0.678	-
Ulvoid-free				
Before	-	0.087	-	0.049*
After	0.087	-	0.049*	-

*Significant at $P < 0.05$.

In this study, the factors affecting the photosynthetic rates of the ulvoid-free and ulvoid-covered *T. hemprichii* were irradiance level and C_i concentration, suggesting similarity to other studies (Mercado et al., 2003; Schwarz et al., 2000). Seagrasses in subtidal habitats subjected to low light conditions had photosynthetic rates that were limited by the ambient C_i concentration while intertidal seagrass of the same species exhibited less limitation at any irradiance (Schwarz et al., 2000). Light reduction could result in increased carbon demand by seagrasses for their daily metabolic energy requirements in an effort to compensate for the rapid depletion of stored carbohydrate reserves and low productivity (Lee and Dunton, 1997; Kraemer and Hanisak, 2000). Lower light levels may, therefore, explain why the ulvoid-covered *T. hemprichii* had lower a $RETR_{max}$ and suffered more C_i limitations. Aside from irradiance level and C_i concentration, diffusion across the boundary layer (or DBL) could also influence the photophysiology of the ulvoid-covered and ulvoid-free seagrasses. Mercado et al. (2003) showed that DBL had an effect on the two morphotypes (sun and shade growth forms) of *Zostera noltii*. The presence of ulvoid algae in the seagrass habitat could lessen the flow of the water (i. e., fluid dynamics), increasing the boundary layer and reducing the rate of exchange between the seagrass and the surrounding medium.

Although $RETR_{max}$ increased significantly in the ulvoid-covered *T. hemprichii* in relation to the increase of C_i concentration, the degree was still relatively small compared to what other studies have reported (Durako, 1993; Beer and Koch, 1996; Beer and Rehnberg, 1997; Björk et al., 1997; Hellblom et al., 2001; Invers et al., 2001) involving C_i limitation of other species of seagrass using detached leaves in the laboratory. In one such study, Abel (1984) and Björk et al. (1997) indicated that the photosynthetic rates of *T. hemprichii* were totally inhibited at pH 8.6, and assumed that HCO_3^- could not be used as a C_i source for photosynthesis by this entity. Our results contradict this finding, and the discrepancy may be due to the difference between the artificial media and detached leaves in their studies and our *in situ* study. Our results are more similar to the *in situ* measurements made by Schwarz et al. (2000) and suggest only minor C_i limitation for ulvoid-covered plants.

In the ulvoid-free population, *T. hemprichii* could directly absorb HCO_3^- as the major C_i source but partially depended on extracellular carbonic anhydrase (CA) to convert HCO_3^- to CO_2 prior to uptake. *Ulva lactuca* has been shown to preferentially take up HCO_3^- directly under a high external pH value (e.g. under conditions conducive to high photosynthetic rate, such as high irradiance, temperature, and nutrient loading) (Axelsson et al., 1995), and it can also use HCO_3^- via extracellular CA-mediated conversion to CO_2 under a low external pH value. Since elevated external pH values (9.0-9.5) have been observed in accumulated ulvoid mats (Invers et al., 1999), similarly high levels might be expected in the ulvoid-covered *T. hemprichii* population (i. e. up to 8.75 during low tide in our observations). This

could explain why the ulvoid-covered *T. hemprichii* populations preferentially take up HCO_3^- without the aid of carbonic anhydrase (CA), enabling maintenance of a high photosynthetic rate. The difference between the ulvoid-covered and the ulvoid-free populations seems to indicate the existence of a dual mechanism in *T. hemprichii* similar to that reported for *U. lactuca* (Axelsson et al., 1995). However, for now, such a hypothesis must be treated as preliminary and should be verified by further investigations. Furthermore, testing whether such physiological regulation exists in other species of macroalgae and seagrasses would be worthwhile.

The worldwide decline in seagrass habitats has been attributed mainly to a eutrophication-induced degradation of water quality leading to reduced light for submerged vegetation (Koch and Beer, 1996; McGlathery, 2001). Analogous situations exist for the seagrass habitat at Wanlitung. The eutrophication-induced blooms of the ulvoid green algae seemed to cause two main stresses: namely, light limitation and C_i limitation. Comparing other seagrass sites in southern Taiwan, Lin and Shao (1998) estimated the seagrass beds at Nanwan and Dakwan at about 0.3-0.4 ha while only about 0.0002 ha was observed at Wanlitung. Our data are not sufficient to estimate the extent to which the photosynthetic response of the plants to shade conditions quantitatively affects plant health or to which extent C_i limitation reduces the photosynthetic potential of these plants. Further research on a wider spatial scale and with a seasonal component would help uncover these problems.

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利用沉水性葉綠素螢光分析儀實測石蓴類海藻對潮間帶海草 *Thalassia hemprichii* 於無機碳源使用的影響

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台灣南部萬里桐地區大型石蓴類海藻藻華覆蓋 *Thalassia hemprichii* 海草床面積約近 80%，覆蓋的遮蔽效應造成了海草光合作用和無機碳源吸收都降低。本研究利用沉水性葉綠素螢光分析儀的快速光曲線 (rapid light curve, RLC) 功能在野外實測被遮蔽和未遮蔽海草間的無機碳使用情形，探討其相互間是否有無差異。研究結果顯示，相較於未遮蔽海草族群，被遮蔽族群有顯著較低的 $RETR_{max}$ 和 E_k 值 ($P < 0.05$)，顯示其已馴化成為適應低光的族群。當無機碳濃度從海水的 2.2 mM 增加到 6.2 mM 時，被遮蔽海草的光合作用顯著增加 46% ($P < 0.05$)，顯示在其生長地遭遇無機碳源的限制。在無機碳源使用情況的研究上，發現適應高光未遮蔽的 *T. hemprichii* 能夠直接吸收重碳酸鹽作為光合作用的無機碳源，同時它也部分依賴胞外碳酸酐酶的幫助來吸收重碳酸鹽。因此，廢水的注入被發現造成萬里桐地區大型石蓴類海藻的藻華，並對海草造成光合生理的影響。

關鍵詞：重碳酸鹽；窒息；光合作用；台灣；石蓴類藻華。