Journal of Experimental Marine Biology and Ecology 443 (2013) 114–122

Contents lists available at SciVerse ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

Effect of nitrate enrichment and salinity reduction on the seagrass *Thalassia hemprichii* previously grown in low light

Zhijian Jiang [a,b], Xiaoping Huang [a,*], Jingping Zhang [a,b]

[a] State Key Laboratory of Tropical Oceanography, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, PR China
[b] Tropical Marine Biological Research Station in Hainan, Chinese Academy of Sciences, Sanya 572200, PR China

ARTICLE INFO

Article history:
Received 16 November 2012
Received in revised form 8 February 2013
Accepted 21 February 2013
Available online 25 March 2013

Keywords:
Enzymatic activity
Growth
Nitrate enrichment
Salinity reduction
Seagrass
Thalassia hemprichii

ABSTRACT

A sub-acute experiment that tested carbon and nitrogen metabolisms and the plant's antioxidant defence system was used to investigate the effects of nitrate enrichment and salinity reduction on the tropical seagrass *Thalassia hemprichii* that had previously been grown in low light conditions. Shading significantly reduced leaf growth rate, nitrate reductase activity and non-structural carbohydrate (NSC) content in both aboveground and belowground tissues, but enhanced superoxide dismutase and guaiacol peroxidase (POD) activities and free amino acids in aboveground tissue. After shade removal, salinity reduction with pre-shading decreased minimum fluorescence and maximum fluorescence under all nitrate conditions. Pre-shading aggravated the negative effect of nitrate enrichment and salinity reduction on leaf growth rate. Nitrate enrichment led to a greater reduction in NSC in aboveground tissues of pre-shading plants compared to the control. Furthermore, salinity reduction with pre-shading even induced effective NSC translocation from belowground to aboveground tissues. Nitrate enrichment and salinity reduction both increased POD activity in *T. hemprichii* grown previously in low light. Therefore, pre-shading increases the vulnerability of *T. hemprichii* to eutrophication and hyposalinity, which must be considered when predicting future responses and effective management of seagrass resources.

1. Introduction

Seagrasses are keystone species in many shallow lagoons and estuaries, providing a complex habitat and high rates of primary production for ecologically and economically important higher consumers (Bloomfield and Gillanders, 2005). Despite their importance, seagrass beds are rapidly declining due to a variety of natural and human-induced disturbances (Björk et al., 2008; Burkholder et al., 2007; Duarte, 2008; Manzanera et al., 1998; Neckles et al., 2005; Waycott et al., 2007). Seagrasses are particularly sensitive to chronic and temporary light reduction (Biber et al., 2009). A small decrease in light intensity can cause significant declines in seagrass growth and distribution (Ralph et al., 2007). Temporary light reduction may occur due to dredging, boating activities, altered land-use patterns and shoreline hardening (Biber et al., 2009; Ralph et al., 2007). Similarly, proliferation of drifting macroalgae also leads to a temporary decrease in light intensity (Biber et al., 2009; Brun et al., 2003; Lamote and Dunton, 2006). Many studies have examined the response of seagrasses to light reduction (Brun et al., 2008; Christianen et al., 2011; Collier et al., 2009; Serrano et al., 2011) and their subsequent recovery dynamics (Biber et al., 2009; Bité et al., 2007; Collier et al., 2009; Longstaff and Dennison, 1999; Longstaff et al., 1999). During periods of depressed photosynthesis caused by light reduction, seagrasses mobilise stored non-structural carbohydrates (NSCs) to maintain metabolic processes (Alcoverro et al., 2001; Ralph et al., 2007). The decline in NSC induced by shading may change the response of seagrasses to other environmental stress since NSC reserves play an important role in determining seagrass growth (Ralph et al., 2007).

In shallow coastal areas, rapidly expanding low-salinity aquaculture activity may discharge low-salinity water with a high concentration of nutrients (Cao et al., 2007; Funge-Smith and Briggs, 1998; Heil, 2000). In addition, climate change has promoted an increase in pulse-type heavy rainfall events (IPCC, 2007), which often dramatically increase high-nutrient freshwater loads (Faxneld et al., 2010; Waycott et al., 2007). Thus, low-salinity water discharge is frequently occurring in synergy with significant increases in nutrient loads, possibly imposing interactive effects on coastal seagrasses (Waycott et al., 2007). Freshwater discharge may adversely affect photosynthesis and seagrass growth by diluting seawater to a lower salinity (Björk et al., 2008). Such an extreme event has caused large-scale losses of seagrass habitats in Eastern Africa (Bandeira and Gell, 2003), Queensland of Australia (Campbell and McKenzie, 2004) and Venezuela (Chollett et al., 2007).

Abbreviations: DW, dry weight; FAA, free amino acid; F_m, maximum fluorescence; F_o, minimum fluorescence; F_v/F_m, maximum quantum yield; FW, fresh weight; G_L, leaf growth rate; G_m, the growth rate of marked leaves; G_{nm}, the growth rate of unmarked leaves; HL, high light; LL, low light; MLL, the length from the leaf base to the punching mark; NBT, nitro-blue tetrazolium; NE, nitrate enrichment; NE + RS, nitrate enrichment and salinity reduction; NRA, nitrate reductase activity; NSCs, nonstructural carbohydrates; POD, guaiacol peroxidase; PS II, photosynthetic system II; PVP, polyvinylpyrrolidone; ROS, reactive oxygen species; RS, salinity reduction; SOD, superoxide dismutase; t_f, the days of collection; TLL, total leaf length; t_0, the days of marking.

* Corresponding author. Tel./fax: +86 02 89023210.
E-mail address: xphuang@scsio.ac.cn (X. Huang).

0022-0981/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jembe.2013.02.034

Furthermore, under excessive nitrogen conditions, carbon requirements for synthesising amino acids may exceed carbon fixation capacity, causing a decrease in NSC reserves and growth rates (Leoni et al., 2008). The effects of salinity reduction on seagrasses have been examined together with nitrogen enrichment (Kahn and Durako, 2006; Van Katwijk et al., 1999). Nevertheless, salinity reduction and high nutrient inputs may exert greater impact on seagrasses that have previously been grown in low light due to a shortage in carbon reserves and energy from decreased assimilation at low light (Ralph et al., 2007). However, information on this is not currently available, and it must be considered for predicting future responses and effective management of seagrass resources (Biber et al., 2009).

We conducted a sub-acute experiment to examine the effects of nitrate enrichment and salinity reduction on the tropical seagrass *Thalassia hemprichii* previously grown in low light by evaluating carbon and nitrogen metabolism (nitrate reductase activity (NRA)) and antioxidant defence systems (superoxide dismutase (SOD) and guaiacol peroxidase (POD)) in the plant. To this aim, we tested the following hypotheses: (1) pre-shading promotes sensitivity of *T. hemprichii* to nitrate enrichment and salinity reduction and (2) the responses of the plant's antioxidant enzyme activities to the combined stresses may be stronger than those to individual stressors. The results obtained in this study may help to predict responses of seagrasses to future global climate change and anthropogenic stress and strengthen the understanding needed to improve the management and protection of these environmentally important marine angiosperms. To the best of our knowledge, this is the first report on the effects of nitrate enrichment and salinity reduction on seagrasses previously grown in low light.

2. Materials and methods

2.1. Plant material

Healthy clumps 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 southeast of Hainan Island, Southern China. Individual shoots were carefully removed by hand to keep belowground structures intact. Plants were subsequently transported to the laboratory and cultured with in situ seawater under saturating light conditions (ca. 250 μmol photons m^{-2} s^{-1}) at 25 °C for 7 days. Prior to being used in the experiments, transplant units were standardised so that each shoot consisted of a single apical shoot with two rhizome internodes and associated roots.

2.2. Experimental design

The experiment was divided into two phases. The first phase consisted of the shading pre-culture. After shade removal, the second phase began to examine the effect of nitrate enrichment and salinity reduction on *T. hemprichii* during its recovery from shading (Fig. 1).

At the beginning of the first phase, approximately 450 shoots of *T. hemprichii* were randomly reallocated into ten, 40-l aquaria, with 45 shoots in each aquarium. They were incubated for 10 days under two different light intensities. During this pre-culture phase, 5 of the 10 aquaria were covered with neutral density screens (25 μmol photons m^{-2} s^{-1}), and the remaining aquaria were not covered with neutral density screens (250 μmol photons m^{-2} s^{-1}).

At the end of the pre-culture phase, approximately 9 shoots of fresh plants from each shaded and non-shaded aquarium were collected as corresponding replicate samples, which were used to determine chlorophyll fluorescence, leaf growth rate, NRA, SOD and POD. Plants were retrieved and separated into root, rhizome and leaves to determine their non-structural carbohydrates (NSC, sum of soluble sugars and starch) and free amino acid (FAA) content.

The remaining shaded and non-shaded plants (approximately 360 shoots) were reallocated into 24, 15-l aquaria for the second phase of the experiment, with 15 shoots in each aquarium (three replicates for each treatment). This consisted of eight treatments: two light levels (25 μmol photons m^{-2} s^{-1}, LL; and 250 μmol photons m^{-2} s^{-1}, HL) × two nitrate concentrations (3.0 and 50.0 μmol l^{-1} added as KNO_3) × two salinity levels (20 and 34). In situ nitrate concentration and salinity of the seawater in the seagrass bed were approximately

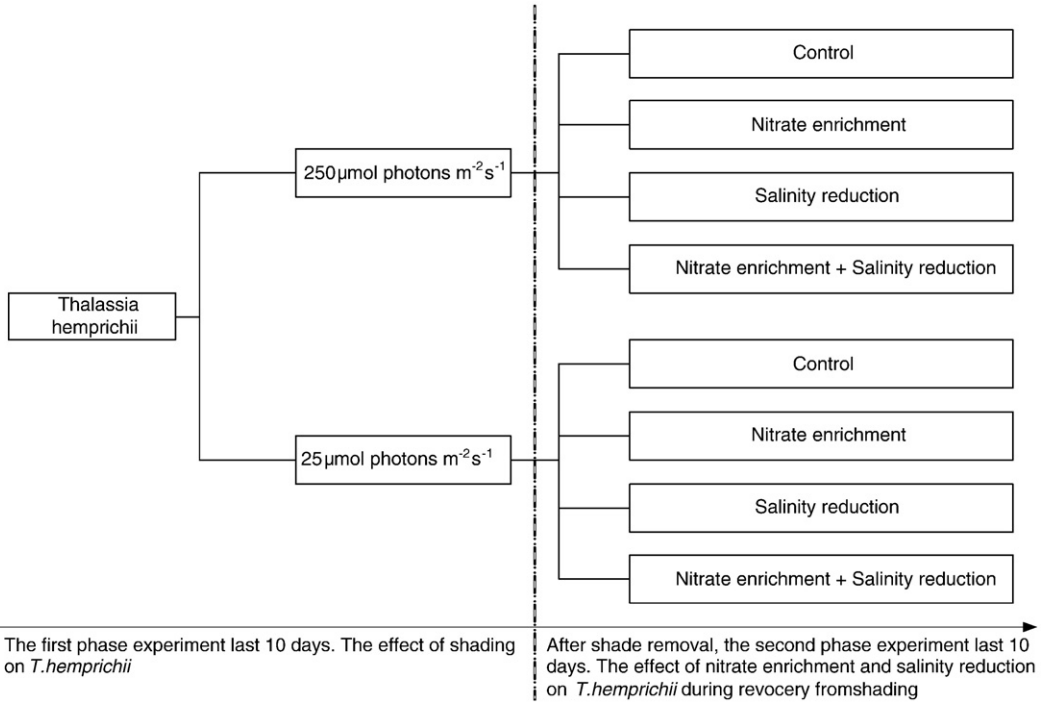

Fig. 1. Experimental set-up of the laboratory treatment. The experiment was divided into two phases, with each phase lasting 10 days. The first phase was the shading pre-culture phase. After shade removal, the second phase began to examine the effect of nitrate enrichment and salinity reduction on *Thalassia hemprichii* during recovery from shading.

3.0 μmol l^{-1} and 34, respectively, acting as control levels in the laboratory experiments.

The medium was carefully renewed daily. Lower salinities were obtained by diluting seawater with demineralised freshwater (Torquemada et al., 2005). Salinity was monitored with a dissolved oxygen/conductivity meter (YSI, model 85, USA). Once the aquaria were filled with treatment water, they were placed in a larger water-filled container to keep temperatures as constant as possible during each experiment. Plants were maintained under these conditions for another 10 days.

2.3. Plant analysis

2.3.1. Chlorophyll fluorescence analysis

At the end of the first and second phases, plants were dark-adapted for 20 min before measurement. Chlorophyll fluorescence of leaves was measured with a MINI-PAM fluorometer (Walz, Effeltrich, Germany). Fluorescence was determined on the middle of the blades using a dark-clip (DLC-8, modified to minimise boundary layer restrictions as described by Beer et al. (1998)) to ensure constant distance between the optical fibre and leaf. The plant material being measured was subjected to a pulse of saturating light. Two fluorescence measurements were made: one before (F_o, minimum fluorescence) and one during (F_m, maximal fluorescence) the pulse. Maximum quantum yield was calculated as F_v/F_m (where $F_v = F_m - F_o$) using dark-acclimated leaves, which allows for all of the reaction centres to open and all primary electron acceptors to be oxidised (Beer et al., 2001). The F_v/F_m ratio has been applied as an indicator of seagrass stress, with an optimal range for seagrass of approximately 0.7–0.8 (Ralph, 1999). Therefore, we used an F_v/F_m ratio of 0.7 as a lower "stress" threshold in the interpretation of our results (Koch et al., 2007).

2.3.2. 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 fibres 1 cm above the sheath. Apical shoots were marked at the beginning of the first and second phases of the experiment and collected after 10 days. Leaf growth rates (G_L, cm $plant^{-1}$ day^{-1}) were estimated according to the following equations (Peralta et al., 2000):

$$G_L = (\sum G_{nm} + \sum G_m)/t, \quad (1)$$

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

$$G_{nm} = TLL(t_f) - TLL(t_0), \quad (2)$$

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

$$G_m = MLL(t_f) - MLL(t_0), \quad (3)$$

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

$$t = t_f - t_0. \quad (4)$$

2.3.3. Biochemical parameter analysis

At the end of the first and second experimental phases, all of the plants in each aquarium were carefully retrieved and separated into root, rhizome and leaves. Rhizomes, roots and shoots were oven-dried (60 °C) until they reached a constant weight. Subsamples of all fractions were individually powdered with a grinder to pass through an 80-mesh sieve (the mesh diameter was 0.18 mm) and stored for nutrient content analysis. The samples (0.1 g) were extracted twice in hot 80% ethanol. The soluble sugar content was determined by the anthrone–sulfuric acid method (Yemm and Willis, 1954). The starch content of the remaining materials was also analysed by the anthrone assay, following gelatinisation at 100 °C for 15 min and solubilisation in 70% perchloric acid (Quarmby and Allen, 1989). FAA measurements were performed by the ninhydrin method (Yemm et al., 1955).

In vivo NRA was assayed in leaves by the method of Srivastava (1974). The excised leaf tissue (0.5 g) was incubated in a 10-ml reaction mixture containing 50 mM phosphate buffer (pH, 7.5), 10 mM KNO_3 and 0.5% *n*-propanol (v/v). The samples were vacuum-filtered in a dessicator and incubated in the dark at 30 °C for 30 min. The mixture was boiled in a water bath, and the concentration of NO_2^- formed was estimated by adding 1.0 ml of 1% sulphanilamide in 1 N HCl (w/v) and 1.0 ml of 0.02% naphthylethylenediamine dihydrochloride (w/v) to a 1-ml aliquot of the above mixture. After centrifugation for 15 min (10,000 rpm), the absorbance of the supernatant was read at 540 nm. NR activity was calculated as μmol NO_2^- g^{-1} FW h^{-1}.

The 0.5-g fresh weight leaves were homogenised in 50 mM cold sodium phosphate buffer (pH 7.8) including 0.1 mM EDTA and 8.75% (w/v) polyvinylpyrrolidone (PVP, Mw 10,000) using a pre-chilled mortar and pestle in an ice bath. The homogenate was centrifuged at 10 500 rpm for 30 min at 4 °C and the supernatant was used for the enzyme assays. SOD activity was assayed on leaves by monitoring the inhibition of the photochemical reduction of nitro-blue tetrazolium (NBT) according to the method of Beauchamp and Fridovich (1971). The 3-ml reaction mixture contained 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 0.1 mM EDTA, 2 μM riboflavin and 0.2 ml of the enzyme extract. The reaction mixtures were illuminated at an intensity of 4 500 lx for 20 min. The absorbance of the supernatant at 560 nm was then measured. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT. POD activity in leaves was determined as the oxidation of guaiacol by H_2O_2 (Upadhyaya et al., 1985). The reaction mixture consisted of 3 ml of 200 mM potassium phosphate buffer (pH 6.0), 0.18% (v/v) H_2O_2, 1% guaiacol and 0.5 ml of the enzyme extract. The oxidation of guaiacol was measured by the increase in absorbance at 470 nm every 30 s (extinction coefficient 26.6 mM^{-1} cm^{-1}). One unit of POD activity was defined as units per min and mg of protein.

2.4. Statistics

The means and standard errors of all variables were calculated for each factor. Data for the end of phase one (Time 0) was analysed by a *T*-test. 2-Way ANOVA was used to analyse the effects of nitrate enrichment and salinity reduction on shaded and non-shaded plants. Fisher's LSD *post hoc* analysis was performed when appropriate. Furthermore, the main effects of pre-shading, nitrate enrichment and salinity reduction were analysed by a multifactorial ANOVA. The interactions between pre-shading, nitrate enrichment and salinity reduction were tested in the following way: (pre-shading) × (nitrate enrichment), (pre-shading) × (salinity reduction) and (nitrate enrichment) × (salinity reduction). Treatment means were compared and separated by the least significant difference (LSD) at $p < 0.05$. All statistical analyses were carried out using SPSS for Windows version 10.

3. Results

3.1. Chlorophyll fluorescence and growth

At the end of the first phase (Time 0), F_o (Fig. 2A), F_m (Fig. 2B) and F_v/F_m were not markedly affected by shading ($p > 0.05$). Similarly, at the end of the second phase, there were no significant effects of pre-shading, nitrate enrichment and salinity reduction on F_o, F_m or F_v/F_m in *T. hemprichii* (Table 1). No changes in F_o, F_m, and F_v/F_m were detected under nitrate enrichment, irrespective of the pre-shading treatment. However, a negative effect on F_o was observed when salinity reduction was combined with nitrate enrichment and pre-shading. The reduction

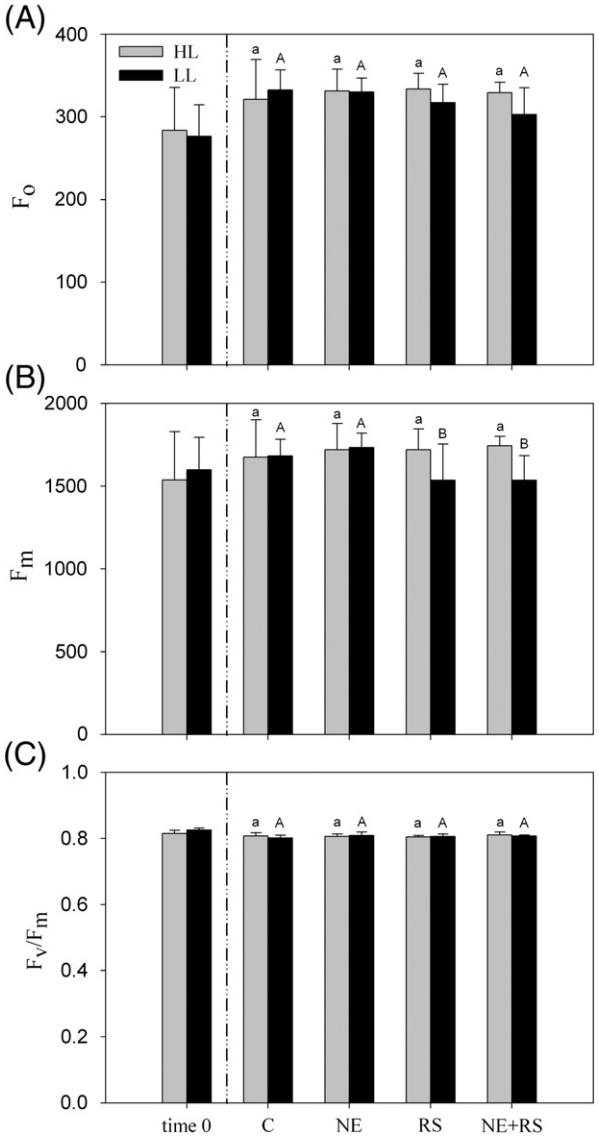

Fig. 2. Effects of pre-shading, nitrate enrichment and salinity reduction on F_o (A), F_m (B) and F_v/F_m (C). Error bars represent SE (n = 5). Time 0 denotes seagrass harvested once the pre-shading phase ended (preculture phase lasted 10 days). C, control; NE, nitrate enrichment; RS, salinity reduction; NE + RS, nitrate enrichment and salinity reduction; HL, high light; LL, low light. Asterisk indicates statistical significance ($p < 0.05$) for the end of phase one data. Different small and capital letters on columns indicate significant differences ($p < 0.05$) for the end of phase two data.

in F_o was accompanied by a decline in F_m, resulting in no change in F_v/F_m.

At the end of the pre-culture phase (Time 0), shading produced a significant reduction in the leaf growth rate of *T. hemprichii* ($p < 0.01$). After shading removal, leaf growth rate in *T. hemprichii* that had been pre-shaded quickly recovered, and was even higher than that without shading (Fig. 3, Table 1). Leaf growth rate was unaffected by nitrate enrichment without pre-shading, but it was reduced by nitrate enrichment with pre-shading. Furthermore, salinity reduction at two light levels during pre-culture both decreased leaf growth rate, with greater reduction in shaded pre-cultures of *T. hemprichii* ($p < 0.01$, Table 1). In contrast, salinity reduction caused greater decrease in leaf growth rate than nitrate enrichment.

3.2. Biochemical content and nitrate reductase activity

Shading significantly decreased NSC in the aboveground ($p < 0.01$, Fig. 4A) and belowground ($p < 0.05$, Fig. 4B) tissues of *T. hemprichii*. Similarly, NSC in both tissues of *T. hemprichii* was depressed by nitrate enrichment both with and without pre-shading but this depression was higher in pre-shaded aboveground plant tissues. Nevertheless, salinity reduction without pre-shading did not change NSC in both tissues of *T. hemprichii*. Salinity reduction with a shaded pre-culture slightly enhanced NSC in aboveground tissues, but diminished NSC in belowground tissues. Nitrate enrichment combined with salinity reduction decreased NSC in both tissues, irrespective of the pre-culture conditions. Overall, pre-shading and nitrate enrichment had significant effects on NSC in both tissues, whereas salinity reduction only considerably affected NSC in belowground tissue (Table 1).

Pre-culturing with shading considerably increased the FAA content in aboveground tissues ($p < 0.01$, Fig. 5A) of *T. hemprichii*, but did not change the FAA content in belowground tissues ($p > 0.05$, Fig. 5B, Table 1). Nitrate enrichment, salinity reduction and their combined interaction all increased FAA content in both aboveground and belowground tissues both with and without pre-shading. The combined effect of nitrate enrichment and salinity reduction resulted in a greater enhancement in FAA in aboveground tissues than for either treatment alone. FAA in both tissues was significantly affected by nitrate enrichment and salinity reduction (Table 1).

Pre-shading apparently diminished leaf NRA ($p < 0.05$) in *T. hemprichii*, but NRA quickly returned to a normal level after shading was removed (Fig. 6). Nitrate enrichment, salinity reduction and their combined interaction all markedly enhanced NRA in both pre-shaded and not pre-shaded plants (Table 1). The highest NRA was observed for *T. hemprichii* grown under reduced salinity without pre-shading.

3.3. Antioxidant enzyme activity

POD ($p < 0.05$, Fig. 7A) activity in the leaves of *T. hemprichii* was considerably enhanced following pre-shading. A shaded pre-culture did not significantly affect SOD activity ($p > 0.05$, Fig. 7B), but did result in greater activity quantitatively. Furthermore, SOD activity quickly returned to initial values after shading was removed. Nitrate enrichment, salinity reduction and their combined interaction all caused positive effects on POD and SOD activities, with a greater enhancement in POD activity for pre-shaded *T. hemprichii*. Pre-shaded *T. hemprichii* grown with nitrate enrichment and salinity reduction showed the highest POD and SOD activities. Nitrate enrichment increased SOD activity to a greater extent than salinity reduction. Moreover, only pre-shading and nitrate enrichment as single stressors considerably affected POD and SOD activities, respectively ($p < 0.01$) (Table 1).

4. Discussions

4.1. Shade-induced responses and recovery

The present results show that chlorophyll fluorescence (F_o, F_m, F_v/F_m) failed to detect the onset of shading mortality in *T. hemprichii*, which was in agreement with findings of Biber et al. (2009). However, shading clearly reduced leaf growth rate, NRA and NSC content in both aboveground and belowground tissues of *T. hemprichii*. Such responses suggest that shading makes it difficult for *T. hemprichii* to balance its carbon budgets. During shading, the availabilities of light, energy and carbon skeletons were insufficient to support nitrate reduction as well, leading to a decrease in the NRA. In contrast, an increase in FAA in aboveground tissues of *T. hemprichii* was observed for low light conditions, which could be explained by the reduced leaf growth rate. Assimilated nitrogen does not need to be used for growth and was thus stored (Ruíz and Romero, 2001). Peralta et al. (2002) also observed that shading stimulated leaf nitrogen content in *Zostera noltii* Hornem. The results of the present study showed that shading enhanced SOD and POD activities in the leaves of *T. hemprichii*. A mechanism to account for the enhancement in oxidative

Table 1
Statistical analysis of the effects of pre-shading, nitrate enrichment and salinity reduction on the properties of *T. hemprichii*. $p < 0.05$ (significant); and $p < 0.01$ (highly significant). df: degrees of freedom; F: the ratio of among-group variance divided by the within-group variance.

Variable	df	F	p
1. F_o			
Pre-shading (L)	1	0.91	0.348
Nitrate enrichment (N)	1	0.11	0.738
Salinity reduction (S)	1	0.86	0.360
L × N	1	0.44	0.513
L × S	1	2.35	0.135
N × S	1	0.61	0.442
L × N × S	1	0.003	0.954
2. F_m			
Pre-shading (L)	1	2.99	0.093
Nitrate enrichment (N)	1	0.70	0.409
Salinity reduction (S)	1	1.51	0.228
L × N	1	0.01	0.907
L × S	1	3.80	0.060
N × S	1	0.03	0.871
L × N × S	1	0.002	0.962
3. F_v/F_m			
Pre-shading (L)	1	0.27	0.610
Nitrate enrichment (N)	1	1.71	0.200
Salinity reduction (S)	1	0.13	0.723
L × N	1	0.16	0.694
L × S	1	0.03	0.875
N × S	1	0.01	0.937
L × N × S	1	1.51	0.228
4. Leaf growth rate			
Pre-shading (L)	1	3.44	0.078
Nitrate enrichment (N)	1	0.75	0.395
Salinity reduction (S)	1	10.63	<0.01
L × N	1	0.59	0.453
L × S	1	2.93	0.102
N × S	1	0.27	0.610
L × N × S	1	0.93	0.345
5. NSC aboveground			
Pre-shading (L)	1	48.67	<0.01
Nitrate enrichment (N)	1	59.69	<0.01
Salinity reduction (S)	1	2.60	0.126
L × N	1	2.68	0.121
L × S	1	5.84	<0.05
N × S	1	0.37	0.553
L × N × S	1	0.003	0.956
6. NSC belowground			
Pre-shading (L)	1	135.34	<0.01
Nitrate enrichment (N)	1	15.88	<0.01
Salinity reduction (S)	1	22.92	<0.01
L × N	1	3.30	0.088
L × S	1	18.81	<0.01
N × S	1	0.01	0.909
L × N × S	1	2.59	0.127
7. FAA aboveground			
Pre-shading (L)	1	506.59	<0.01
Nitrate enrichment (N)	1	171.97	<0.01
Salinity reduction (S)	1	98.78	<0.01
L × N	1	9.70	<0.01
L × S	1	0.77	0.393
N × S	1	3.59	0.076
L × N × S	1	5.69	<0.05
8. FAA belowground			
Pre-shading (L)	1	0.23	0.637
Nitrate enrichment (N)	1	19.69	<0.01
Salinity reduction (S)	1	37.30	<0.01
L × N	1	39.57	<0.01
L × S	1	3.71	0.072
N × S	1	123.45	<0.01
L × N × S	1	44.34	<0.01
9. NRA			
Pre-shading (L)	1	3.03	0.101
Nitrate enrichment (N)	1	22.20	<0.05
Salinity reduction (S)	1	167.52	<0.01
L × N	1	10.09	<0.01
L × S	1	20.77	<0.01
N × S	1	138.03	<0.01
L × N × S	1	0.96	0.342
10. POD			
Pre-shading (L)	1	9.80	<0.01
Nitrate enrichment (N)	1	2.38	0.143
Salinity reduction (S)	1	2.95	0.105
L × N	1	0.22	0.649
L × S	1	0.18	0.682
N × S	1	1.17	0.296
L × N × S	1	0.09	0.771
11. SOD			
Pre-shading (L)	1	0.06	0.804
Nitrate enrichment (N)	1	33.39	<0.01
Salinity reduction (S)	1	1.69	0.212
L × N	1	1.81	0.197
L × S	1	0.20	0.658
N × S	1	2.36	0.144
L × N × S	1	0.97	0.339

stress due to shading might involve the increased imbalance in carbon–nitrogen metabolism under low light conditions, which favoured the accumulation of reactive oxygen species (ROS) (Zhang et al., 2010).

Removal of the shade screens induced rapid and detectable responses in a number of physiological parameters. After 10 days, leaf growth rate and NRA and SOD activities in the leaves of *T. hemprichii* quickly returned to control levels, indicating that *T. hemprichii* could quickly recovery from short-term shading. Collier et al. (2009) also observed that removal of the shade screens induced leaf growth in *Posidonia sinuosa* with rates returning to control levels after two weeks. In contrast, Longstaff and Dennison (1999) reported little recovery of seagrasses after light attenuation. Interestingly, the leaf growth rate of *T. hemprichii* with pre-shading was even higher than that without pre-shading after imposed shading. It is possible that light availability was not limiting seagrass growth due to the removal of shading, and an increase in tissue nutrient availability (higher leaf FAA) could lead to an enhancement in seagrass growth (Udy et al., 1999).

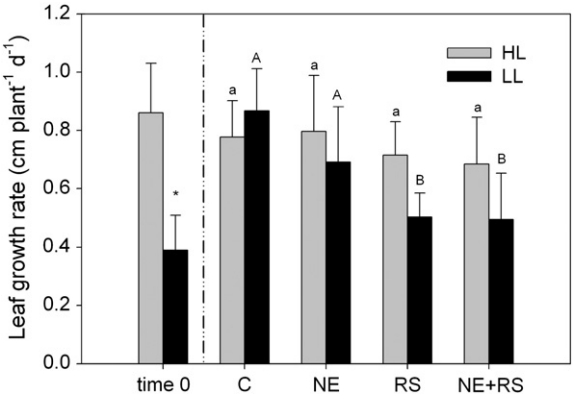

Fig. 3. Effect of pre-shading, nitrate enrichment and salinity reduction on leaf growth rate. Error bars represent SE (n = 3). Time 0 denotes seagrass harvested once the pre-shading phase ended (preculture phase lasted 10 days). C, control; NE, nitrate enrichment; RS, salinity reduction; NE + RS, nitrate enrichment and salinity reduction; HL, high light; LL, low light. Asterisk indicates statistical significance ($p < 0.05$) for the end of phase one data. Different small and capital letters on columns indicate significant differences ($p < 0.05$) for the end of phase two data.

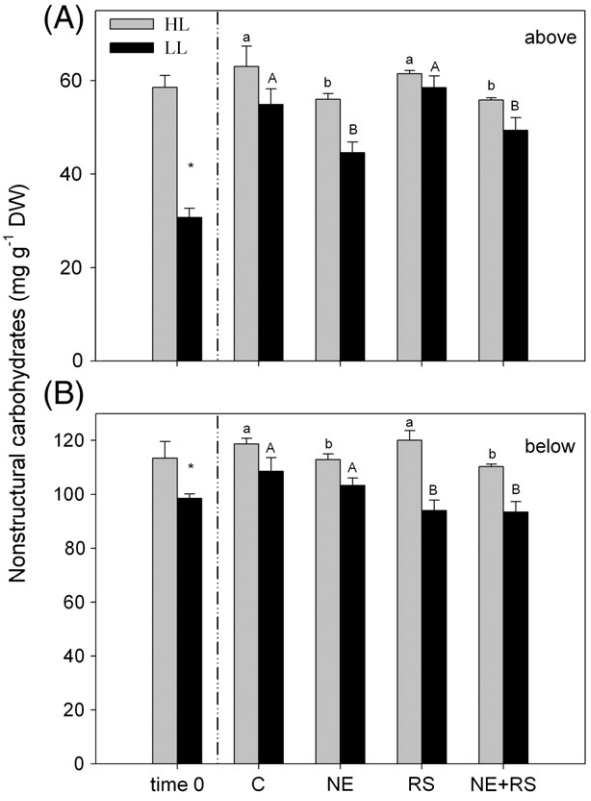

Fig. 4. Effect of pre-shading, nitrate enrichment and salinity reduction on non-structural carbohydrates (NSC) in aboveground (A) and belowground (B) tissues of *Thalassia hemprichii*. Error bars represent SE (n = 3). Time 0 denotes seagrass harvested once the pre-shading phase ended (preculture phase lasted 10 days). C, control; NE, nitrate enrichment; RS, salinity reduction; NE + RS, nitrate enrichment and salinity reduction; HL, high light; LL, low light; DW, dry weight. Asterisk indicates statistical significance ($p < 0.05$) for the end of phase one data. Different small and capital letters on columns indicate significant differences ($p < 0.05$) for the end of phase two data.

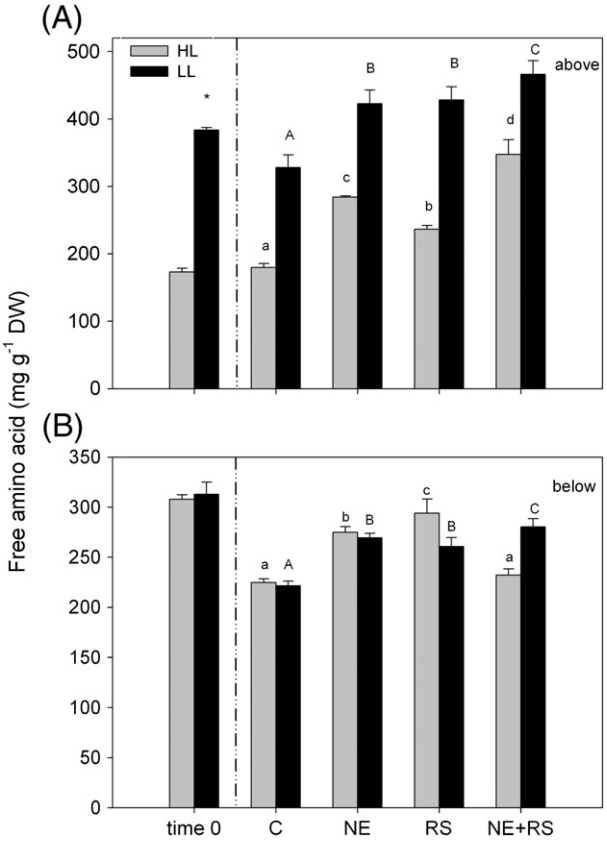

Fig. 5. Effect of pre-shading, nitrate enrichment and salinity reduction on free amino acid (FAA) in aboveground (A) and belowground (B) tissues of *Thalassia hemprichii*. Error bars represent SE (n = 3). Time 0 denotes seagrass harvested once the pre-shading phase ended (preculture phase lasted 10 days). C, control; NE, nitrate enrichment; RS, salinity reduction; NE + RS, nitrate enrichment and salinity reduction; HL, high light; LL, low light; DW, dry weight. Asterisk indicates statistical significance ($p < 0.05$) for the end of phase one data. Different small and capital letters on columns indicate significant differences ($p < 0.05$) for the end of phase two data.

4.2. Effects of nitrate enrichment and salinity reduction on T. hemprichii previously grown in low light

The data obtained here indicate that both nitrate enrichment and salinity reduction did not change F_o and F_m in pre-shaded plants, while salinity reduction with pre-shaded plants led to a decrease in F_o and F_m at the two nitrate concentrations. The decline in F_o and F_m under hyposaline conditions with pre-shading may be due to changes in the ionic steady state, compatible solute synthesis or water content of the leaf tissues (Murphy et al., 2003). A decrease in F_o is an indication of a high-energy dissipation in the minor antenna (Pietrini et al., 2005), suggesting that non-reversible damage occurred in the leaves of *T. hemprichii*. Also, reduction in F_m reflects a decrease in the ability of photosystem II (PS II) to reduce the primary quinone acceptor (Mathur et al., 2011). Thus, salinity reduction caused osmotic shock (Simon et al., 1999) and elevated energy costs to maintain an internal ionic balance due to changes in intracellular ionic concentrations (Sibly and Calow, 1989; Torquemada et al., 2005), while the shade-induced decline of organic solutes (NSC) within the leaf cells could not sustain stable membrane potentials under hyposaline conditions (Murphy et al., 2003), resulting in much higher degree of damage to PS II.

Salinity reduction decreased leaf growth rate in *T. hemprichii*. Other field and laboratory studies have also supported the adverse impact of salinity reduction on seagrass shoot production (Doering and Chamberlain, 2000; Irlandi et al., 2002). Salinity reduction resulted in mortality of *Halophila johnsonii* (Kahn and Durako, 2008), *Halophila ovalis* (Ralph, 1999) and *Thalassia testudinum* (Kahn and Durako, 2006). Thalassia is generally known to be less tolerant to salinity reduction than several other seagrass genera, such as Halodule, Halophila and Ruppia (Thorhaug et al., 2006). However, the combination of salinity reduction and nitrate enrichment did not further decrease leaf growth rate in *T. hemprichii*, indicating no additive negative

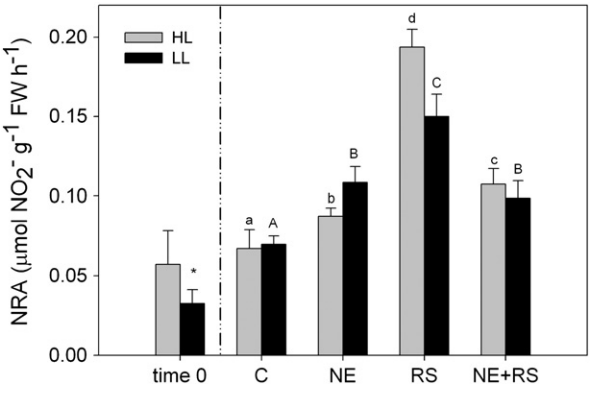

Fig. 6. Effect of pre-shading, nitrate enrichment and salinity reduction on NRA in *Thalassia hemprichii*. Error bars represent SE (n = 3). Time 0 denotes seagrass harvested once the pre-shading phase ended (preculture phase lasted 10 days). C, control; NE, nitrate enrichment; RS, salinity reduction; NE + RS, nitrate enrichment and salinity reduction; HL, high light; LL, low light; FW, fresh weight. Asterisk indicates statistical significance ($p < 0.05$) for the end of phase one data. Different small and capital letters on columns indicate significant differences ($p < 0.05$) for the end of phase two data.

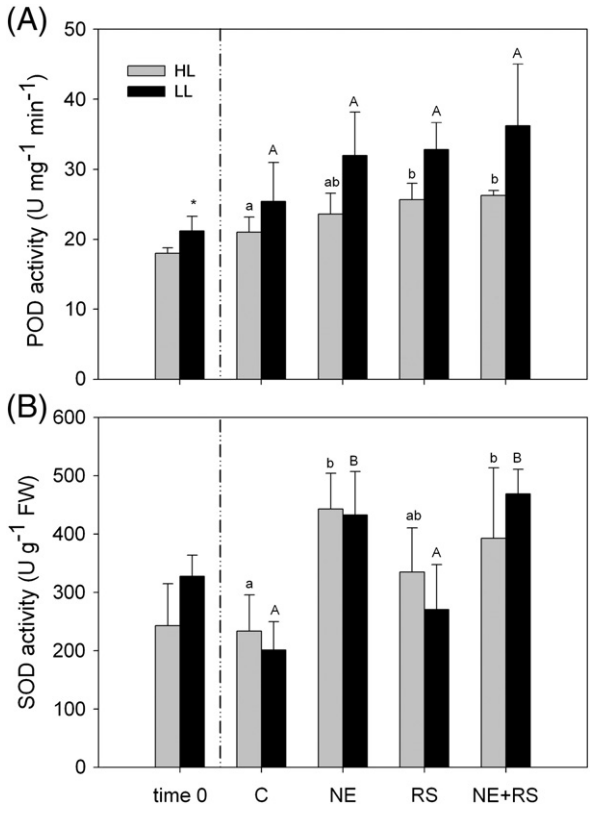

Fig. 7. Effects of pre-shading, nitrate enrichment and salinity reduction on POD (A) and SOD (B) activities in *Thalassia hemprichii*. Error bars represent SE (n = 3). Time 0 denotes seagrass harvested once the pre-shading phase ended (preculture phase lasted 10 days). C, control; NE, nitrate enrichment; RS, salinity reduction; NE + RS, nitrate enrichment and salinity reduction; HL, high light; LL, low light; FW, fresh weight. Asterisk indicates statistical significance ($p < 0.05$) for the end of phase one data. Different small and capital letters on columns indicate significant differences ($p < 0.05$) for the end of phase two data.

effect. Increased levels of ammonium further decreased growth in *T. testudinum* in the lower salinity treatment (Kahn and Durako, 2006). However, the temperate seagrass *Zostera marina* was positively influenced by high nutrient loads at reduced salinity levels (Van Katwijk et al., 1999). Such differences might be ascribed in part to differences in the experimental setup and salinity tolerance of seagrass species. Moreover, nitrate enrichment with pre-shading decreased leaf growth rate in *T. hemprichii*. Salinity reduction alone and combined with nitrate enrichment both resulted in a greater reduction in leaf growth rate for plants with pre-shading than those without pre-shading. Accordingly, the above results indicate that pre-shading aggravated the adverse influence of nitrate enrichment and salinity reduction on leaf growth rate in *T. hemprichii*. This corroborates our first hypothesis.

The lower salinities obtained by adding demineralised freshwater decreased the inorganic carbon content, resulting in less carbon synthesis (Hellblom and Björk, 1999). But salinity reduction without pre-shading did not modify NSC in both tissues of *T. hemprichii*. This might be due to the conversion of total carbohydrates to soluble carbohydrates that was induced for osmoregulation (Murphy et al., 2003). Furthermore, salinity reduction with pre-shading slightly enhanced NSC in aboveground tissues, but decreased NSC in belowground tissues, indicating an effective translocation of NSC from belowground to aboveground tissues. Similarly, osmotic stress treatments also decreased soluble carbohydrates in the rhizomes of *T. testudinum* (Koch et al., 2007) and *Posidonia oceanica* (Ruíz et al., 2009), which mobilised to supply the osmoregulatory demands of epidermal leaf cells. Our data indicate that NSC acted as one of the main organic solutes to maintain osmotic potential for *T. hemprichii* under hyposaline conditions, along with other seagrasses (Touchette, 2007). Additionally, nitrate enrichment with pre-shading resulted in a greater reduction of NSC in the aboveground tissues of *T. hemprichii* than nitrate enrichment without pre-shading, revealing that nitrate enrichment had a greater negative effect on NSC in pre-shading. Nitrate enrichment significantly stimulated leaf NRA and FAA content in aboveground and belowground tissues of *T. hemprichii*, indicating that NO_3^- absorption, reduction and assimilation were enhanced (Touchette and Burkholder, 2001). Similarly, the consumption of energy and carbohydrates to assimilate FAA was high for submersed macrophytes in eutrophic waters (Zhang et al., 2010). The carbohydrate reserves are thus reallocated to decrease carbohydrate content and internal carbon-limited conditions (Invers et al., 2004; Touchette and Burkholder, 2000, 2007). The FAA content of the aboveground tissues of *T. hemprichii* was also enhanced by salinity reduction and further enhanced when salinity reduction was combined with nitrate enrichment. Such responses suggest that FAA might be one of the main osmotics for *T. hemprichii* under salinity reduction. Increased amino acids at low salinity have been observed in other plants (Parida and Das, 2005). Rai (2002) also reported that soluble nitrogen containing compounds like other amino acids could protect plant tissues against osmotic stress. Specifically, NRA increased the most under salinity reduction, indicating that this stressor might induce higher nitrogen metabolism in *T. hemprichii*. Nitrate metabolite derivatives might be utilised in osmotic adjustment (Aslam et al., 1984), and the maintenance of leaf NSC induced under hyposaline conditions would supply energy and carbon skeletons to enhance nitrate reductase activity (Leoni et al., 2008). Therefore, stimulation of leaf NRA by salinity reduction would be propitious to accumulate FAA to adjust osmosis pressure.

SOD and POD act on scavenging ROS, and the increase in their activities is indicative of oxidative stress in plants (Slooten et al., 1995). In the present study, nitrate enrichment, salinity reduction and their combination all caused a greater POD activity in the leaves of *T. hemprichii* previously grown in low light. This suggests that more severe oxidative stress was triggered by nitrate enrichment and salinity reduction in pre-shaded plants. The greatest increase in POD and SOD activities was observed with the combination of nitrate enrichment and salinity reduction for *T. hemprichii* cultured previously in low light, which supports our second hypothesis. Typically, ROS produced by salinity reduction with pre-shading can ultimately affect the quantum yield of PS II fluorescence (Rodrigues et al., 2008), resulting in a decline in F_o and F_m. Overall, the greater enhancement of POD and SOD reconfirmed that pre-shading can amplify the negative effects of nitrate enrichment and salinity reduction in *T. hemprichii*.

5. Conclusions

Changes in the abovementioned physiological responses indicate that pre-shading increases the vulnerability of *T. hemprichii* to stress caused by short-term nitrate enrichment and salinity reduction. Thus, when we examine the recovery dynamics of seagrasses from temporary shading, we should consider the effects of other environmental stressors encountered during recovery. This is an important consideration when managing areas to maintain a suitable habitat for seagrasses.

Acknowledgements

This research was supported by the National Natural Science Foundation of China (nos. 41076069 and 40776086), the Project of Environmental Quality Evaluation of Seagrass Bed in South China Sea (no. DOMEP (MEA)-01-03), the Public Science and Technology Research Funds Projects of Ocean (no. 201305030), and the Knowledge Innovation Program of the Chinese Academy of Sciences (no. SQ201219). We express our thanks to Mr Li Lei and Feng Shaohui for their help in field sampling. We also would like to thank the

editors and reviewers for their comments and suggestions to the manuscript. **[ST]**

References

Alcoverro, T., Manzanera, M., Romero, J., 2001. Annual metabolic carbon balance of the seagrass *Posidonia oceanica*: the importance of carbohydrate reserves. Mar. Ecol. Prog. Ser. 211, 105–116.

Aslam, M., Huffaker, R.C., Rains, D.W., 1984. Early effects of salinity on nitrate assimilation in barley seedlings. Plant Physiol. 76, 321–325.

Bandeira, S., Gell, F., 2003. The seagrasses of Mozambique and southeastern Africa. In: Green, E., Short, F. (Eds.), World Atlas of Seagrasses. University of California Press, Berkeley, pp. 93–100.

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 44, 276–287.

Beer, S., Vilenkin, B., Weil, A., Veste, M., Susel, L., Eshel, A., 1998. Measuring photosynthetic rates in seagrasses by pulse amplitude modulated (PAM) fluorometry. Mar. Ecol. Prog. Ser. 174, 293–300.

Beer, S., Björk, M., Gademann, R., Ralph, P., 2001. Measurements of photosynthetic rates in seagrasses. In: Short, F., Coles, R. (Eds.), Global Seagrass Research Methods. Elsevier Publishing, The Netherlands, pp. 183–198.

Biber, P.D., Kenworthy, W.J., Paerl, H.W., 2009. Experimental analysis of the response and recovery of *Zostera marina* (L.) and *Halodule wrightii* (Ascher.) to repeated light-limitation stress. J. Exp. Mar. Biol. Ecol. 369, 110–117.

Bité, J.S., Campbell, S.J., McKenzie, L.J., Coles, R.G., 2007. Chlorophyll fluorescence measures of seagrasses *Halophila ovalis* and *Zostera capricorni* reveal differences in response to experimental shading. Mar. Biol. 152, 405–414.

Björk, M., Short, F., McLeod, E., Beer, S., 2008. Managing Seagrasses for Resilience to Climate Change. IUCN, Switzerland.

Bloomfield, A., Gillanders, B., 2005. Fish and invertebrate assemblages in seagrass, mangrove, saltmarsh, and nonvegetated habitats. Estuar. Coasts 28, 63–77.

Brun, F.G., Vergara, J.J., Navarro, G., Hernández, I., Pérez-Lloréns, J.L., 2003. Effect of shading by *Ulva rigida* canopies on growth and carbon balance of the seagrass *Zostera noltii*. Mar. Ecol. Prog. Ser. 265, 85–96.

Brun, F.G., Olive, I., Malta, E.J., Vergara, J.J., Hernández, I., Perez-Llorens, J.L., 2008. Increased vulnerability of *Zostera noltii* to stress caused by low light and elevated ammonium levels under phosphate deficiency. Mar. Ecol. Prog. Ser. 365, 67–75.

Burkholder, J.M., Tomasko, D.A., Touchette, B.W., 2007. Seagrasses and eutrophication. J. Exp. Mar. Biol. Ecol. 350, 46–72.

Campbell, S., McKenzie, L., 2004. Flood related loss and recovery of intertidal seagrass meadows in southern Queensland, Australia. Estuar. Coast. Shelf Sci. 60, 477–490.

Cao, L., Wang, W., Yang, Y., Yang, C., Yuan, Z., Xiong, S., Diana, J., 2007. Environmental impact of aquaculture and countermeasures to aquaculture pollution in China. Environ. Sci. Pollut. Res. 14 (7), 452–462.

Chollett, I., Bone, D., Pérez, D., 2007. Effects of heavy rainfall on *Thalassia testudinum* beds. Aquat. Bot. 87, 189–195.

Christianen, M., van der Heide, T., Bouma, T., Roelofs, J., van Katwijk, M., Lamers, L., 2011. Limited toxicity of NH_x pulses on an early and late successional tropical seagrass species; interactions with pH and light level. Aquat. Toxicol. 104, 73–79.

Collier, C., Lavery, P., Ralph, P., Masini, R., 2009. Shade-induced response and recovery of the seagrass *Posidonia sinuosa*. J. Exp. Mar. Biol. Ecol. 370, 89–103.

Doering, P., Chamberlain, R., 2000. Experimental studies in the salinity tolerance of turtle grass *Thalassia testudinum*. In: Bortone, S. (Ed.), Seagrasses: Monitoring, Ecology, Physiology and Management. CRC Press, Floridia, pp. 81–98.

Duarte, C.M., 2008. Seagrass meadows. In: Cleveland, C.J. (Ed.), Encyclopedia of Earth. Enviroemental Information Coalition, National Council for Science and the Environment, Washington DC.

Faxneld, S., Jörgensen, T.L., Tedengren, M., 2010. Effects of elevated water temperature, reduced salinity and nutrient enrichment on the metabolism of the coral *Turbinaria mesenterina*. Estuar. Coast. Shelf Sci. 88 (4), 482–487.

Funge-Smith, S.J., Briggs, M.R.P., 1998. Nutrient budgets in intensive shrimp ponds: implications for sustainability. Aquaculture 164 (1), 117–133.

Heil, L., 2000. Impact of shrimp farming on mangroves along India's east coast. Unasylva 203 (512), 48–55.

Hellblom, F., Björk, M., 1999. Photosynthetic responses in *Zostera marina* to decreasing salinity, inorganic carbon content and osmolality. Aquat. Bot. 65 (1), 97–104.

Invers, O., Kraemer, G.P., Pérez, M., Romero, J., 2004. Effects of nitrogen addition on nitrogen metabolism and carbon reserves in the temperate seagrass *Posidonia oceanica*. J. Exp. Mar. Biol. Ecol. 303, 97–114.

IPCC, 2007. Climate change 2007: the physical science basis. In: Solomon, S., Qin, M., Manning, M., Chen, Z., Marquis, M., Averyt, K.B., Tignor, M., Miller, H.L. (Eds.), Contribution of Working Group 1 to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY USA.

Irlandi, E., Orlando, B., Maciá, S., Biber, P., Jones, T., Kaufman, L., Lirman, D., Patterson, E.T., 2002. The influence of freshwater runoff on biomass, morphometrics, and production of *Thalassia testudinum*. Aquat. Bot. 72, 67–78.

Kahn, A., Durako, M., 2006. *Thalassia testudinum* seedling responses to changes in salinity and nitrogen levels. J. Exp. Mar. Biol. Ecol. 335, 1–12.

Kahn, A., Durako, M., 2008. Photophysiological responses of *Halophila johnsonii* to experimental hyposaline and hyper-CDOM conditions. J. Exp. Mar. Biol. Ecol. 367, 230–235.

Koch, M., Schopmeyer, S., Kyhn-Hansen, C., Madden, C., Peters, J., 2007. Tropical seagrass species tolerance to hypersalinity stress. Aquat. Bot. 86, 14–24.

Lamote, M., Dunton, K.H., 2006. Effects of drift macroalgae and light attenuation on chlorophyll fluorescence and sediment sulfides in the seagrass *Thalassia testudinum*. J. Exp. Mar. Biol. Ecol. 334, 174–186.

Leoni, V., Vela, A., Pasqualini, V., Pergent-Martini, C., Pergent, G., 2008. Effects of experimental reduction of light and nutrient enrichments (N and P) on seagrasses: a review. Aquat. Conserv. 18, 202–220.

Longstaff, B.J., Dennison, W.C., 1999. Seagrass survival during pulsed turbidity events: the effects of light deprivation on the seagrasses *Halodule pinifolia* and *Halophila ovalis*. Aquat. Bot. 65, 105–121.

Longstaff, B.J., Loneragan, N.R., O'Donohue, M.J., Dennison, W.C., 1999. Effects of light deprivation on the survival and recovery of the seagrass *Halophila ovalis* (R. Br.) Hook. J. Exp. Mar. Biol. Ecol. 234, 1–27.

Manzanera, M., Pérez, M., Romero, J., 1998. Seagrass mortality due to oversedimentation: an experimental approach. J. Coast. Conserv. 4, 67–70.

Mathur, S., Jajoo, A., Mehta, P., Bharti, S., 2011. Analysis of elevated temperature-induced inhibition of photosystem II using chlorophyll a fluorescence induction kinetics in wheat leaves (*Triticum aestivum*). Plant Biol. 13, 1–6.

Murphy, L., Kinsey, S., Durako, M., 2003. Physiological effects of short-term salinity changes on *Ruppia maritima*. Aquat. Bot. 75, 293–309.

Neckles, H.A., Short, F.T., Barker, S., Kopp, B.S., 2005. Disturbance of eelgrass *Zostera marina* by commercial mussel *Mytilus edulis* harvesting in Maine: dragging impacts and habitat recovery. Mar. Ecol. Prog. Ser. 285, 57–73.

Parida, A.K., Das, A.B., 2005. Salt tolerance and salinity effects on plants: a review. Ecotoxicol. Environ. Saf. 60 (3), 324–349.

Peralta, G., Pérez-Lloréns, J., Hernández, I., Brun, F., Vergara, J., Bartual, A., Gálvez, J., García, C., 2000. Morphological and physiological differences between two morphotypes of *Zostera noltii* Hornem. from the south-western Iberian Peninsula. Helgol. Mar. Res. 54, 80–86.

Peralta, G., Pérez-Lloréns, J., Hernández, I., Vergara, J., 2002. Effects of light availability on growth, architecture and nutrient content of the seagrass *Zostera noltii* Hornem. J. Exp. Mar. Biol. Ecol. 269, 9–26.

Pietrini, F., Chaudhuri, D., Thapliyal, A., Massacci, A., 2005. Analysis of chlorophyll fluorescence transients in mandarin leaves during a photo-oxidative cold shock and recovery. Agric. Ecosyst. Environ. 106, 189–198.

Quarmby, C., Allen, S.E., 1989. Organic constituents. In: Allen, S.E. (Ed.), Chemical Analysis of Ecological Materials. Wiely, New York, pp. 160–201.

Rai, V., 2002. Role of amino acids in plant responses to stresses. Biol. Plant. 45, 481–487.

Ralph, P., 1999. Photosynthetic response of *Halophila ovalis* (R. Br.) Hook. f. to combined environmental stress. Aquat. Bot. 65, 83–96.

Ralph, P., Durako, M., Enriquez, S., Collier, C., Doblin, M., 2007. Impact of light limitation on seagrasses. J. Exp. Mar. Biol. Ecol. 350, 176–193.

Rodrigues, L.J., Grottoli, A.G., Lesser, M.P., 2008. Long-term changes in the chlorophyll fluorescence of bleached and recovering corals from Hawaii. J. Exp. Biol. 211, 2502–2509.

Ruíz, J.M., Romero, J., 2001. Effects of in situ experimental shading on the Mediterranean seagrass *Posidonia oceanica*. Mar. Ecol. Prog. Ser. 215, 107–120.

Ruíz, J.M., Marín-Guirao, L., Sandoval-Gil, J., 2009. Responses of the Mediterranean seagrass *Posidonia oceanica* to in situ simulated salinity increase. Bot. Mar. 52, 459–470.

Serrano, O., Mateo, M.A., Renom, P., 2011. Seasonal response of *Posidonia oceanica* to light disturbances. Mar. Ecol. Prog. Ser. 423, 29–38.

Sibly, R.M., Calow, P., 1989. A life-cycle theory of responses to stress. In: Calow, P., Berry, R.J. (Eds.), Evolution Ecology and Environmental Stress: Biol. J. Linnean. Soc, 37, pp. 101–116.

Simon, C., Ar Gall, E., Levavasseur, G., Deslandes, E., 1999. Effects of short-term variations of salinity and temperature on the photosynthetic response of the red alga *Grateloupia doryphora* from Brittany (France). Bot. Mar. 42, 437–440.

Slooten, L., Capiau, K., Van Camp, W., Van Montagu, M., Sybesma, C., Inzé, D., 1995. Factors affecting the enhancement of oxidative stress tolerance in transgenic tobacco overexpressing manganese superoxide dismutase in the chloroplasts. Plant Physiol. 107, 737–750.

Srivastava, H., 1974. In vivo activity of nitrate reductase in maize seedlings. Indian J. Biochem. Biophys. 11, 230–232.

Thorhaug, A., Richardson, A., Berlyn, G., 2006. Spectral reflectance of *Thalassia testudinum* (Hydrocharitaceae) seagrass: low salinity effects. Am. J. Bot. 93, 110–117.

Torquemada, Y., Durako, M., Lizaso, J., 2005. Effects of salinity and possible interactions with temperature and pH on growth and photosynthesis of *Halophila johnsonii* Eiseman. Mar. Biol. 148, 251–260.

Touchette, B.W., 2007. Seagrass-salinity interactions: physiological mechanisms used by submersed marine angiosperms for a life at sea. J. Exp. Mar. Biol. Ecol. 350, 194–215.

Touchette, B.W., Burkholder, J.M., 2000. Overview of the physiological ecology of carbon metabolism in seagrasses. J. Exp. Mar. Biol. Ecol. 250, 169–205.

Touchette, B.W., Burkholder, J.M., 2001. Nitrate reductase activity in a submersed marine angiosperm: controlling influences of environmental and physiological factors. Plant Physiol. Biochem. 39, 583–593.

Touchette, B.W., Burkholder, J.M., 2007. Carbon and nitrogen metabolism in the seagrass, *Zostera marina* L.: environmental control of enzymes involved in carbon allocation and nitrogen assimilation. J. Exp. Mar. Biol. Ecol. 350, 216–233.

Udy, J.W., Dennison, W.C., Lee Long, W.J., McKenzie, L.J., 1999. Responses of seagrass to nutrients in the Great Barrier Reef, Australia. Mar. Ecol. Prog. Ser. 185, 257–271.

Upadhyaya, A., Sankhla, D., Davis, T.D., Sankhla, N., Smith, B., 1985. Effect of paclobutrazol on the activities of some enzymes of activated oxygen metabolism and lipid peroxidation in senescing soybean leaves. J. Plant Physiol. 121, 453–461.

Van Katwijk, M., Schmitz, G., Gasseling, A., Van Avesaath, P., 1999. Effects of salinity and nutrient load and their interaction on *Zostera marina*. Mar. Ecol. Prog. Ser. 190, 155–165.

Waycott, M., Collier, C., McMahon, K., Ralph, P., McKenzie, L., Udy, J., Grech, A., 2007. Vulnerability of seagrasses in the Great Barrier Reef to climate change. In: Johnson, J.E., Marshall, P.A. (Eds.), Climate Change and the Great Barrier Reef Great Barrier Reef. Great Barrier Marine Park Authority and Australian Greenhouse Office, Australia, pp. 193–235.

Yemm, E., Willis, A., 1954. The estimation of carbohydrates in plant extracts by anthrone. Biochem. J. 57, 508–514.

Yemm, E., Cocking, E., Ricketts, R., 1955. The determination of amino-acids with ninhydrin. Analyst 80, 209–214.

Zhang, M., Cao, T., Ni, L., Xie, P., Li, Z., 2010. Carbon, nitrogen and antioxidant enzyme responses of *Potamogeton crispus* to both low light and high nutrient stresses. Environ. Exp. Bot. 68, 44–50.

Zieman, J., 1974. Methods for the study of the growth and production of turtle grass, *Thalassia testudinum* König. Aquaculture 4, 139–143.