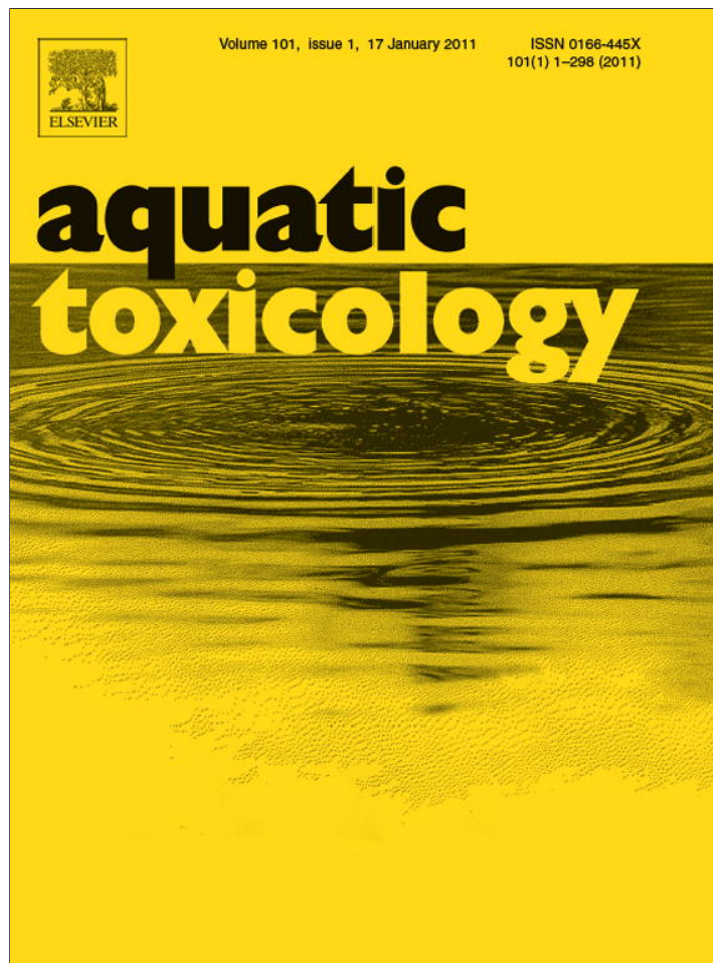


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## Aquatic Toxicology

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## Limited toxicity of NH<sub>x</sub> pulses on an early and late successional tropical seagrass species: Interactions with pH and light level

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### ABSTRACT

Seagrasses have declined at a global scale due to light reduction and toxicity events, caused by eutrophication and increased sediment loading. Although several studies have tested effects of light reduction and toxicants on seagrasses, there is at present no information available on their interacting effects. In a full-factorial 5-day laboratory experiment, we studied short-term interactive effects of light conditions, pH and reduced nitrogen (NH<sub>x</sub>) in the water layer, mimicking pulses of river discharge, on the tropical early successional species *Halodule uninervis* and the late successional species *Thalassia hemprichii*.

In contrast to recent results reported for the temperate species *Zostera marina*, increased NH<sub>x</sub> supply did not affect leaf mortality or photochemical efficiency in *H. uninervis* and in 7 out of 8 treatments for *T. hemprichii*. However, both tropical species demonstrated striking differences in nitrogen accumulation, free amino acid composition and free NH<sub>3</sub> accumulation. The increase in tissue nitrogen content was two times higher for *H. uninervis* than for *T. hemprichii*. Nitrogen stored as free amino acids (especially asparagine) only increased in *H. uninervis*. High pH only affected *T. hemprichii*, but only when not shaded, by doubling its free NH<sub>3</sub> concentrations, concomitantly decreasing its photosynthetic efficiency.

Our results indicate that the early successional *H. uninervis* has higher tolerance to high NH<sub>x</sub> loads as compared to the late successional *T. hemprichii*. *H. uninervis* was better able to avoid toxic internal NH<sub>x</sub> levels by further assimilating glutamine into asparagine in contrast to *T. hemprichii*. Moreover, both tropical species seem to cope much better with high NH<sub>x</sub> than the temperate *Z. marina*. The implications for the distribution and succession of seagrass species under high nutrient loads are discussed.

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### 1. Introduction

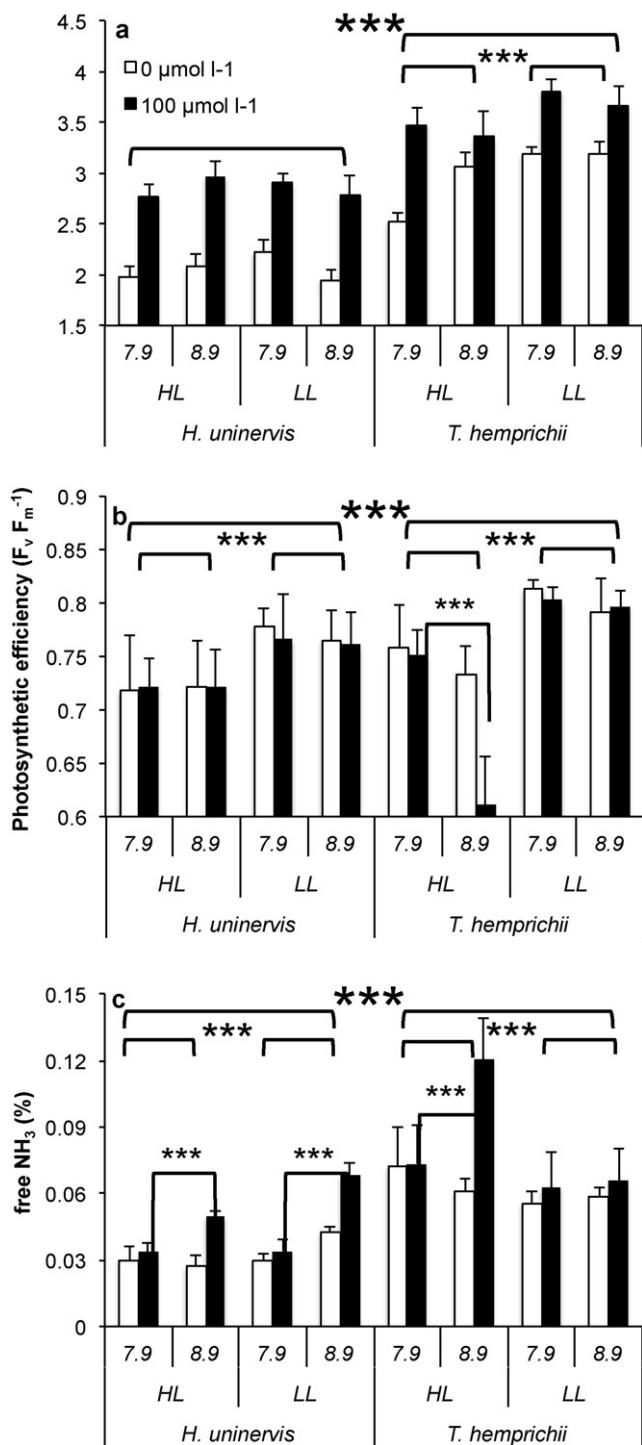
Seagrasses (marine angiosperms) play an essential role in tropical estuarine and marine ecosystems. They are very sensitive to changes in the environment and can therefore be used as early indicators of system change (Orth et al., 2006). A global decline of seagrasses has been reported in past decades, which has predominantly been ascribed to light reduction and toxicity events caused by increased sediment loading or algal overgrowth following eutrophication (Orth et al., 2006; Short and Wyllie-Echeverria, 1996). In addition, direct physiological stress caused by eutrophication-related toxicity of sulfide (Carlson et al., 1994; Goodman et al., 1995), nitrate (Burkholder et al., 1992), and in particular ammonium (Van Katwijk et al., 1997; Brun et al., 2002, 2008;

Van der Heide et al., 2008), has been reported for temperate seagrasses. Unraveling the interacting effects of direct toxicity and light reduction is therefore vital to understand the drastic decline of seagrass meadows.

This is particularly the case for the tropics, where such studies are lacking even though the potential for toxicity events increases dramatically due to ongoing rapid development and change of land use of coastal zones (Green and Short, 2003). In shallow coastal areas, river run-off can have a major impact on seagrass (Freeman et al., 2008). Here, increased sediment and nutrient loading originates from human activities such as coastal land development (Coles et al., 2003), terrestrial forestry (Terrados et al., 1998), mangrove clearing (Jakobsen et al., 2007), mining (Walling, 2006; Yuhi, 2008), aquaculture (Williams, 2007), and dredging (Neckles et al., 2005; Ertfemeijer and Lewis, 2006). Events of high sediment loading strongly decrease light availability in nearby coastal seagrass meadows (Jakobsen et al., 2007; Freeman et al., 2008). In addition, the rapidly expanding aquaculture may cause pulses of high con-

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**Fig. 1.** Effects of two NH<sub>x</sub> concentrations (0 μmol l<sup>-1</sup> vs. 100 μmol l<sup>-1</sup>) on *Halodule uninervis* and *Thalassia hemprichii* leaf tissue (mean ± SE), under pH 7.9 vs. 8.9 and higher (HL) vs. lower light (LL): (a) total nitrogen (N) content, (b) photosynthetic efficiency ((F<sub>m</sub> - F<sub>0</sub>)/F<sub>m</sub>), (c) free ammonia (NH<sub>3</sub>) content. Asterisks show the level of significance of differences between treatments or pairs of treatments connected by horizontal bars or double horizontal bars respectively; \*0.01 ≤ P ≤ 0.05, \*\*0.001 ≤ P ≤ 0.01, \*\*\*P < 0.001.

centrations of ammonium/ammonia (NH<sub>x</sub>) in the water column. Shrimp farming, for example, can result in ammonium concentrations up to 440 μmol l<sup>-1</sup> (Briggs and Funge-Smith, 1994) or fluxes up to 182 mmol m<sup>-2</sup> d<sup>-1</sup> from farm sediments (Burford and Longmore, 2001).

Based on observations for temperate seagrass species, such NH<sub>x</sub> pulses can be expected to have major impact on tropical seagrass meadows. In *Zostera noltii* and *Z. marina* exposure to NH<sub>x</sub> pulses can result in leaf mortality, photosynthetic inhibition, oxidative stress and internal carbon-nitrogen imbalance (Rudolph and Voigt, 1986; Britto and Kronzucker, 2002; Cao et al., 2004; Burkholder et al., 2007). These negative effects of high NH<sub>x</sub> loading can be aggravated by light deficiency (Brun et al., 2002, 2008) and high pH due to the increasing NH<sub>3</sub>-NH<sub>4</sub><sup>+</sup> ratio (pH 9; Van der Heide et al., 2008). The actual physiological response to NH<sub>x</sub> may vary between species when they have contrasting growth strategies. Compared to late successional species, early successional species have relatively higher nitrogen tissue contents, and higher production rates, which might result in faster nitrogen uptake rates and a faster recovery after disturbances (Duarte, 1991; Vermaat et al., 1995; Rollon et al., 1998). Whether NH<sub>x</sub> pulses also have major impact on tropical seagrass species with possible differences regarding contrasting growth strategies remains to be tested.

The aim of this study was twofold. *Firstly*, we assessed short-term effects of elevated NH<sub>x</sub>, and the combined effects of NH<sub>x</sub>, pH and light for two tropical seagrass species, *Halodule uninervis* (Forsskal) Ascherson, and *Thalassia hemprichii* (Ehrenberg) Ascherson. Low light conditions were used to mimic the main effect of high turbidity events, and high pH to simulate effects of high photosynthetic rates in dense seagrass beds or shallow pools (Suzuki et al., 1998; Beer et al., 2006; Semesi et al., 2009). *Secondly*, we compared the response to NH<sub>x</sub>, pH and light between the two species, *H. uninervis* and *T. hemprichii*. These species were selected because they: (a) represent an early-successional (*H. uninervis*) and late successional (*T. hemprichii*) species (Birch and Birch, 1984), (b) are two of the most common species along the coast of the Indo-Pacific (Mukai, 1993), and (c) often grow together in multispecies meadows (Verheij and Erfemeijer, 1993). The experiment was carried out as a controlled 5-day, full factorial laboratory experiment. We monitored leaf tissue survival, leaf tissue nitrogen accumulation and free amino-acid accumulation, which are known indicators for NH<sub>x</sub>-toxicity. In addition, we measured photosynthetic yield as a sub-lethal indicator for leaf fitness (Prange and Dennison, 2000).

## 2. Materials and methods

### 2.1. Plant material and culture conditions

Apical shoots of *Halodule uninervis* (HU, Ehrenberg, Ascherson) and *Thalassia hemprichii* (TH, Forsskal, Ascherson) were collected in Banten Bay, Indonesia (58°60'20"S, 106°09'07"E). Plants were directly transported to the laboratory in the Netherlands in dark, cooled boxes. The plants were acclimatized for 2 days in 100 L glass containers that were placed in a climate-controlled room at 25 °C. Light intensity was 200 μmol m<sup>-2</sup> s<sup>-1</sup>, with a 12:12 h day:night cycle. The photosynthetic efficiency (F<sub>v</sub>/F<sub>m</sub>) measured 0.80 ± 0.03 indicating high vitality. The culture medium for acclimatization, culture and experiments had a salinity of 32 PSU (similar to field conditions) and was composed of Classic synthetic sea salt (Tropic Marin, Wartenberg Germany, background NH<sub>4</sub><sup>+</sup> < 1 μmol) and deionized water. During culture, pH (8.2 ± 0.2), temperature (25 ± 3 °C), water level and ammonia concentration (< 1 μmol l<sup>-1</sup>) were monitored twice daily, and corrected if necessary.

### 2.2. Treatment

To test the short-term effect of NH<sub>x</sub>, pH, light level and their interactions on physiological responses, leaf survival and photosynthetic performance, we conducted a 5-day experiment under controlled conditions in glass containers (height:diameter = 45:16 cm).

To allow for a general comparison to temperate species, we used a similar experimental set-up, the same laboratory, and similar  $\text{NH}_x$  concentrations ( $\text{NH}_4\text{Cl}$ ) as Van der Heide et al. (2008), who tested the effects of  $\text{NH}_4^+$  and pH on the temperate species *Z. marina*. Per container, one experimental unit was attached to a stainless steel nut to keep shoots vertically in the water column (cf. Van der Heide et al., 2008). Similar to Van der Heide et al. (2008) plants could be maintained without sediment due to the short experimental period (e.g., Thursby and Harlin, 1982; Rubio et al., 2007). To correct for the differences in leaf biomass between the two species, experimental units of *T. hemprichii* and *H. uninervis* consisted of one shoot with 3 cm of rhizome, and a rhizome fragment with five shoots including the apical one, respectively. We selected plants with at least two healthy leaves without any tissue damage.

The experiment consisted of five replicates for eight treatments: two  $\text{NH}_x$  concentrations (0 and  $100 \mu\text{mol l}^{-1}$  added as  $\text{NH}_4\text{Cl}$ )  $\times$  two light levels ( $200 \mu\text{mol m}^{-2} \text{ s}^{-1}$ , HL; and  $50 \mu\text{mol m}^{-2} \text{ s}^{-1}$ , LL) (Van der Heide et al., 2008)  $\times$  two pH levels (7.9 and 8.9). A shading screen above and around the containers was used to create low light conditions of  $50 \mu\text{mol m}^{-2} \text{ s}^{-1}$ . pH was monitored twice a day and adjusted to either 7.9 or 8.9 using HCl and NaOH. The medium was carefully renewed daily. The concentration of  $\text{NH}_x$  in the medium was determined daily just before replenishment.

### 2.3. Leaf tissue fitness and photosynthetic performance

Leaf mortality was measured at the end of the 5-day experiment by counting leaves that had been discarded from the shoot or showed brown discoloration of more than 30% of its total surface (Van Katwijk et al., 1997). To check if high  $\text{NH}_x$  levels lowered photosynthetic performance, fluorescence measurements using a diving PAM (Walz Co., Effeltrich, Germany) were performed on all plants on the 3rd and last day of the experiment. We used a fixed distance of 1 cm, perpendicular to the apical side of the middle of the youngest full-grown leaf. Plants were adapted to dark conditions for 30 min before the saturating pulse was given after which the maximum photochemical efficiency of PS II was calculated ( $F_v F_m^{-1}$  or photochemical quantum yield) (Kitajima and Butler, 1975; Genty et al., 1989).

### 2.4. Nutrient and free amino acid analysis

Directly after photosynthetic performance measurements on the 5th day of the experiment, we separated leaves from roots and stored the plants at  $-20^\circ\text{C}$ . Free amino acids in the youngest mature leaves were extracted according to Van Dijk and Roelofs (1988). Free amino acid and free  $\text{NH}_3$  concentrations from this extract were measured after pre-column derivatisation with 9-fluorenylmethyl-chloroformate (FMOC-Cl) using high-pressure liquid chromatography (HPLC) (Tomassen et al., 2003). Total contents of carbon and nitrogen of the same sample were determined in freeze-dried leaf tissue using a CNS analyzer (type NA1500; Carlo Erba Instruments, Italy). Water  $\text{NH}_x$  concentrations were measured colorimetrically, using salicylate (Lamers et al., 1998).

### 2.5. Statistics

A multifactorial ANOVA (SPSS 11, 2002, Chicago IL) was used to test main effects and interactions of  $\text{NH}_x$ , pH, light, and species (*H. uninervis* vs. *T. hemprichii*). Prior to analysis, data were checked for normality (Shapiro–Wilk test), homogeneity of variance (Levene's test), and transformed using a method that fitted the type of skewness. For positively skewed data we used Log ( $x$ ) transformations and for negative skewed data we used the square root of the maximum of  $x$  minus  $x$  (Field, 2005) (Table 1; type of transformation used). No outliers were detected. For post hoc tests we used inde-

pendent  $t$ -tests. For both seagrass species we evaluated the effect of  $\text{NH}_x$  in all pH and light treatments (12 tests per species). For interspecific comparison we evaluated the effect of species on all treatments (8 tests). Type I error in the testing procedures was controlled using the false discovery rate method ( $P^* = 0.05$ ) (Benjamini and Hochberg, 1995; Verhoeven et al., 2005).

## 3. Results

### 3.1. Leaf tissue nutrient content

Several physiological effects were detected as a result of the treatments.  $\text{NH}_x$  addition increased total nitrogen content and decreased C:N ratio significantly in leaf tissue of both *H. uninervis* and *T. hemprichii* (Fig. 1a, Table 1,  $P < 0.001$ ). Effects of  $\text{NH}_x$  treatment were highly significant (Table 1). Multi-comparison post hoc tests on  $\text{NH}_x$  effect in all pH and light treatments for nitrogen content and C:N were significant in 10 out of 16 cases. These included the pH 7.9 treatments for *T. hemprichii*, and the low light (LL)–pH 7.9 and pH 8.9 treatments for *H. uninervis*.

Between species, total nitrogen content was significantly higher for *T. hemprichii* (Table 2) and ranged from  $2.5 \pm 0.07\%$  (control) to  $3.5 \pm 0.08\%$  ( $100 \mu\text{mol l}^{-1} \text{NH}_x$  – HL – pH 8.9 treatment). Total N content in *H. uninervis* ranged from  $2.0 \pm 0.05\%$  (control) to  $3.0 \pm 0.06\%$  ( $100 \mu\text{mol l}^{-1} \text{NH}_x$  – HL – pH 8.9 treatment).  $\text{NH}_x$  addition had a stronger effect on *H. uninervis* than on *T. hemprichii*; nitrogen contents increased with  $39.9\% (\pm 0.3)$  and  $21.2\% (\pm 0.2)$ , respectively, as compared to their respective controls (Table 2,  $P < 0.001$ ). There was, however, no effect of light availability and pH on this nitrogen response in the  $\text{NH}_x$  treatment for both species. A doubling in free ammonia ( $\text{NH}_3$ ) levels in plant tissue to  $0.12 \pm 0.02\%$  was observed in one out of eight treatments for *T. hemprichii*, namely  $100 \mu\text{mol NH}_x$  – HL – pH 8.9 (Fig. 1c). Free ammonia levels were two times higher on average for *T. hemprichii* than for *H. uninervis*.

Neither carbon nor phosphate contents were affected by any of the treatments, nor did they differ between species ( $P < 0.05$ ). Average carbon contents amounted to  $33.16 \pm 0.29\%$  and  $38.83 \pm 0.2\%$  for *H. uninervis* and *T. hemprichii*, respectively, and phosphate contents were 0.22% and 0.21% for *T. hemprichii* and *H. uninervis*, respectively. When compared with literature values these nutrient values are above the suggested levels of N- and P-limitation in seagrass species (1.8% N and 0.2% P, Duarte, 1990).

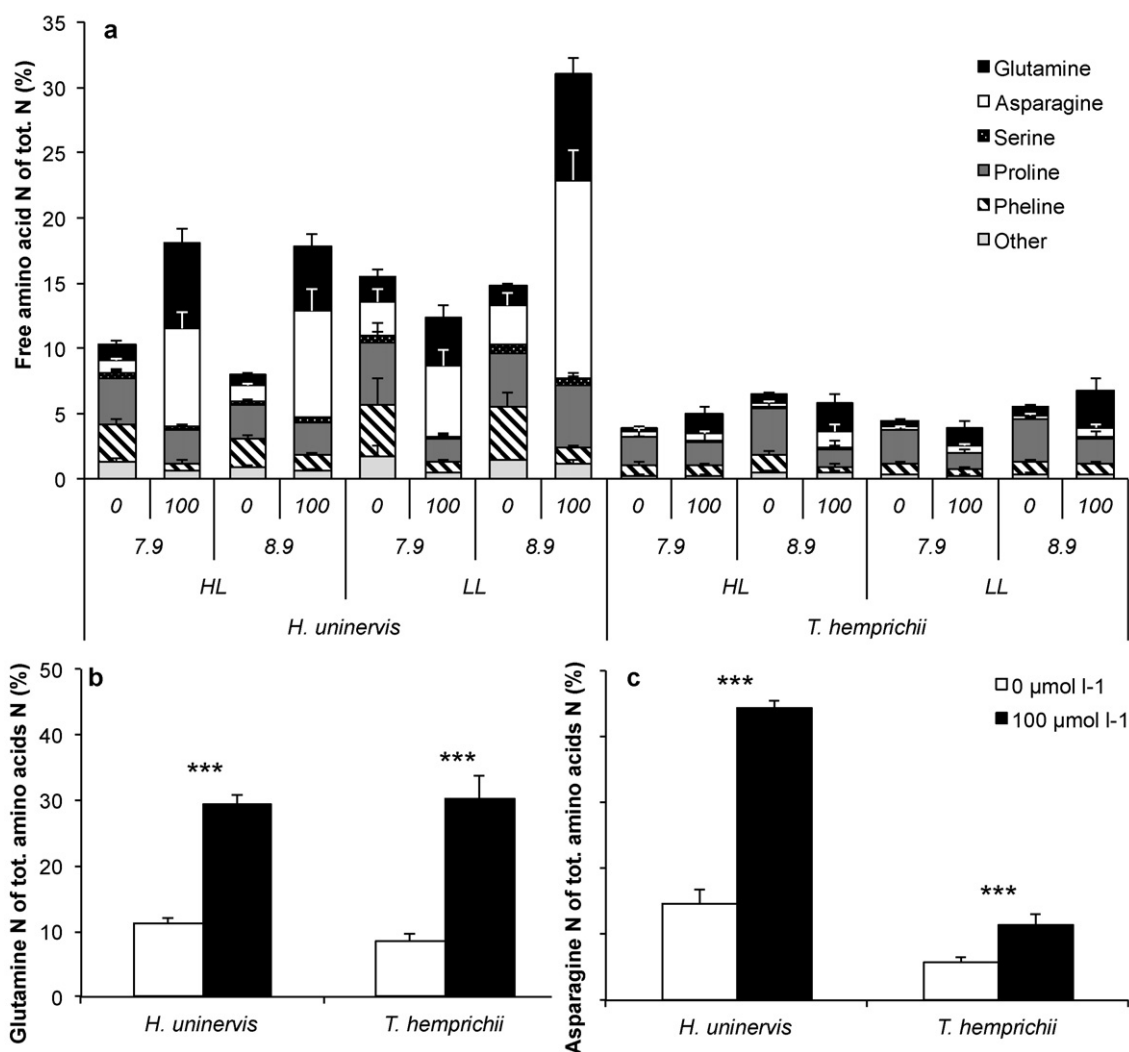
### 3.2. Free amino acid content

*H. uninervis* took up two times more nitrogen than *T. hemprichii* at  $100 \mu\text{mol NH}_x$  addition (Fig. 1a, Table 2,  $P < 0.001$ ), and was the only species that showed a significant increase of nitrogen in free amino acids relative to total %N (Table 1).

There was a clear difference between species with respect to the type of amino acid produced when metabolizing their excess nitrogen. In *H. uninervis* the percentage of nitrogen stored in free amino acids relative to total %N increased from  $12.1 \pm 1.6\%$  in the control to  $19.8 \pm 1.8\%$  in the  $\text{NH}_x$ -treatment, with asparagine being the dominant free amino acid (Fig. 2a). In *T. hemprichii*, however, this value was much lower ( $5.2 \pm 0.5\%$ ) and had not increased significantly in the  $\text{NH}_x$  treatment as compared to the control. Proportions of the three free amino acids glutamine, asparagine and proline also differed between the  $\text{NH}_x$  treatment and the control (Table 1). Both species significantly increased the amount of nitrogen stored as glutamine (Table 1, Fig. 2b), but only *H. uninervis* stored excess nitrogen as asparagine proportionately to the  $\text{NH}_x$  treatment (Table 1, Fig. 2c). In the control treatment, proline was the dominant amino acid in both species (Fig. 2a). There was no

**Table 1**  
Results of the multi-factor ANOVAs on all relevant variables for *Halodule uninervis* and *Thalassia hemprichii* separately. For data that was not normally distributed, the type of transformation is given. *F* values and significance levels are shown for all main effects and their interactions \* $0.01 \leq P \leq 0.05$ , \*\* $0.001 \leq P \leq 0.01$ , \*\*\* $P < 0.001$ , ns: not significant.

	NH <sub>x</sub>	pH	L (light)	L × NH <sub>x</sub>	L × pH	NH <sub>x</sub> × pH	L × pH × NH <sub>x</sub>	Transformation
<i>Halodule uninervis</i>								
Leaf mortality (%)	0.0 ns	0.0 ns	0.1 ns	0.0 ns	0.0 ns	0.1 ns	0.1 ns	
Photosynthetic efficiency ( <i>F<sub>v</sub>F<sub>m</sub></i> <sup>-1</sup> )	0.1 ns	0.3 ns	74.5***	1.7 ns	0.1 ns	0.5 ns	0.5 ns	
Total N (%)	24.9***	0.0 ns	0.0 ns	0.2 ns	2.1 ns	0.5 ns	0.0 ns	
Total C (%)	0.5 ns	1.1 ns	3.7 ns	0.0 ns	0.8 ns	0.4 ns	0.2 ns	
C:N (g:g)	43.3***	0.1 ns	0.7 ns	0.3 ns	1.8 ns	1.6 ns	0.2 ns	
Free NH <sub>3</sub> (%)	18.9**	23.2 ns	7.2 ns	0.1 ns	6.8**	9.5*	0.0 ns	
Free amino acid N of tot. N (%)	10.7**	2.7 ns	4.2 ns	0.2 ns	4.8 ns	5.1 ns	3.5 ns	log (x)
Glutamine N of tot. amino acids N (%)	203.7***	5.9 ns	5.0 ns	2.0 ns	2.2 ns	4.5 ns	2.4 ns	
Asparagine N of tot. amino acids N (%)	103.4***	0.1 ns	5.2 ns	3.4 ns	0.0 ns	0.0 ns	2.9 ns	log (x)
Proline N of tot. amino acids N (%)	171.6***	0.5 ns	0.6 ns	0.3 ns	0.2 ns	1.4 ns	0.4 ns	log (x)
Pheline N of tot. amino acids N (%)	53.7***	0.0 ns	0.1 ns	0.5 ns	0.5 ns	0.1 ns	1.0 ns	
<i>Thalassia hemprichii</i>								
Leaf mortality (%)	0.0 ns	0.1 ns	0.1 ns	0.1 ns	0.1 ns	0.0 ns	0.2 ns	
Photosynthetic efficiency ( <i>F<sub>v</sub>F<sub>m</sub></i> <sup>-1</sup> )	9.4**	20.8***	125.7***	2.0 ns	2.7 ns	2.4 ns	10.3**	
Total N (%)	28.9***	0.4 ns	10.7**	0.1 ns	1.7 ns	3.5 ns	1.4 ns	
Total C (%)	0.0 ns	0.1 ns	4.2*	0.2 ns	0.0 ns	0.0 ns	0.0 ns	
C:N (g:g)	45.6***	2.1 ns	13.0**	1.8 ns	4.5 ns	8.1**	4.4*	
Free NH <sub>3</sub> (%)	11.2***	3.3***	14.1*	4.1*	1.8 ns	6.8***	3.7 ns	
Free amino acid N of tot. N (%)	0.7 ns	2.6 ns	1.4 ns	0.0 ns	0.0 ns	0.2 ns	0.1 ns	log (x)
Glutamine N of tot. amino acids N (%)	139.3***	3.6 ns	1.0 ns	0.3 ns	0.2 ns	0.6 ns	0.0 ns	
Asparagine N of tot. amino acids N (%)	32.5***	0.1 ns	0.1 ns	1.0 ns	0.9 ns	0.0 ns	1.8 ns	log (x)
Proline N of tot. amino acids N (%)	10.4**	0.7 ns	1.2 ns	0.7 ns	0.9 ns	1.0 ns	0.8 ns	log (x)
Pheline N of tot. amino acids N (%)	25.8***	3.5 ns	0.2 ns	2.8 ns	0.0 ns	0.0 ns	2.7 ns	



**Fig. 2.** Effects of two NH<sub>x</sub> concentrations (0 μmol l<sup>-1</sup> vs. 100 μmol l<sup>-1</sup>) on *Halodule uninervis* and *Thalassia hemprichii* leaf tissue (mean ± SE), under pH 7.9 vs. 8.9 and higher (HL) vs. lower light (LL): (a) free amino acid nitrogen relative to total nitrogen (N) content for all treatments, (b) glutamine (GLN) nitrogen relative to total nitrogen content in free amino acids (FAA), and (c) asparagine (ASN) nitrogen relative to total nitrogen content in free amino acids; \* $0.01 \leq P \leq 0.05$ , \*\* $0.001 \leq P \leq 0.01$ , \*\*\* $P < 0.001$ .

**Table 2**  
Results of the multi-factor ANOVAs on all relevant variables, between species. For data that was not normally distributed, the type of transformation is shown for all main effects and their interactions \*0.01 ≤ P ≤ 0.05, \*\*0.001 ≤ P ≤ 0.01, \*\*\*P < 0.001, ns: not significant, L: light treatment.

Inter-species	Species	L × species	NH <sub>x</sub> × species	pH × species	L × NH <sub>x</sub> × species	L × Ph × species	NH <sub>x</sub> × pH × species	L × pH × NH <sub>x</sub> × species	Transformation
Leaf tissue survival (%)	0.0 ns	0.1 ns	0.1 ns	0.1 ns	0.1 ns	0.0 ns	0.1 ns	0.1 ns	
Photosynthetic efficiency (F <sub>v</sub> F <sub>m</sub> <sup>-1</sup> )	20.3***	21.5***	5.6*	12.0***	3.2 ns	4.7*	1.2 ns	4.7*	SQRT (Maxxx - x)
Total N (%)	135.6***	5.8*	2.2 ns	0.5 ns	0.0 ns	0.1 ns	3.4 ns	0.7 ns	
Total C (%)	0.0 ns	0.0 ns	0.4 ns	0.3 ns	0.0 ns	0.0 ns	0.7 ns	0.3 ns	
C:N (g:g)	39.4***	0.6 ns	28.8***	7.9***	0.2 ns	1.5 ns	0.5 ns	0.9 ns	log (x)
Free NH <sub>3</sub> (%)	102.5***	12.7***	13.5***	8.7***	3.2*	3.2*	7.7**	5.1**	
Free amino acid N of tot. N (%)	83.3***	3.7 ns	3.9*	0.02 ns	0.6 ns	2.7 ns	2.1 ns	0.3 ns	log (x)
Glutamine N of tot. amino acids N (%)	1.9 ns	4.1*	10.8**	8.6**	1.5 ns	1.3 ns	3.1 ns	0.6 ns	
Asparagine N of tot. amino acids N (%)	175.8***	5.6*	56.4***	3.1 ns	0.4 ns	0.2 ns	0.3 ns	2.7 ns	
Proline N of tot. amino acids N (%)	3.1 ns	3.4 ns	0.0 ns	0.8 ns	1.0 ns	0.9 ns	2.4 ns	0.1 ns	
Pheline N of tot. amino acids N (%)	2.0 ns	2.6 ns	5.4 ns	0.4 ns	0.0 ns	0.0 ns	0.4 ns	0.0 ns	

significant effect of light availability and pH on the accumulation of free amino acids in both species (Table 1). The responses of total free amino acid content and asparagine content after NH<sub>x</sub> addition were significantly different between both species (Table 2, Fig. 2c).

### 3.3. Leaf survival and photosynthesis

Surprisingly, none of the seagrass species showed any visual signs of toxicity. Leaf survival of both *T. hemprichii* and *H. uninervis* did not differ between treatments and averaged around 91 (± 0.9) and 90% (± 0.9), respectively (Table 3). No discoloration of leaves was observed in either species. Both species showed a slight increase in photosynthetic efficiency under low light (LL) conditions (Table 1, Fig. 1b). Post-hoc tests showed that a significant decrease in photosynthetic efficiency (F<sub>v</sub>F<sub>m</sub><sup>-1</sup>) to 0.6 ± 0.04 was only observed in one out of eight treatments for *T. hemprichii*, namely 100 μmol NH<sub>x</sub> - HL - pH 8.9 (P < 0.0001). It was noted that in this particular treatment, also the free ammonia (NH<sub>3</sub>) was significantly increased in comparison to all other treatments (Fig. 1c).

## 4. Discussion

Our study on the short-term effects of reduced nitrogen (NH<sub>x</sub>) pulses, and their interactive effects with increased pH (mimicking high production in dense shallow seagrass or algal stands) and reduced light (mimicking high riverine sediment loads) showed that while (1) nitrogen was taken up by both species, (2) leaf mortality and photochemical efficiency were largely unaffected. Furthermore, (3) effects of pH and light were not significant in most cases in contrast to earlier studies in temperate seagrass species (Van Katwijk et al., 1997; Van der Heide et al., 2008), and (4) the early successional species *Halodule uninervis* was well able to regulate its NH<sub>x</sub> levels through the synthesis of free amino acids glutamine and asparagine in contrast to the late successional species *Thalassia hemprichii*. The latter had lower nitrogen accumulation rates and synthesized surplus nitrogen predominantly into glutamine, but also showed significantly decreased photosynthetic efficiency in the high light, high pH treatment. This coincided with the accumulation of free ammonia, which indicates a higher sensitivity to NH<sub>x</sub> pulses as compared to *H. uninervis*. These findings are important, as anthropogenic pressure along tropical coasts has recently increased dramatically (e.g. as a result of fertilizer use, deforestation, shrimp farming; Green and Short, 2003). To our knowledge, the present study is the first to address the effects of NH<sub>x</sub> pulses on tropical seagrass plants, and to examine possible interactions with pH and light, which are environmentally relevant.

### 4.1. *Halodule uninervis*

The relative amount of nitrogen stored as free amino acids in *H. uninervis* was about two times higher in the NH<sub>x</sub> treatments compared to controls, except for the low light–low pH treatment. Storage of nitrogen in free amino acids is a well-known mechanism to prevent ammonia (NH<sub>3</sub>) toxicity (Smolders et al., 1996; Udy and Dennison, 1997; Britto and Kronzucker, 2002). The plant assimilates ammonium (NH<sub>4</sub><sup>+</sup>) rapidly into free amino acids. Synthesis of glutamine is the first essential step (Märschner, 1995), because all other amino acids are synthesized from glutamine. *H. uninervis* showed elevated glutamine, but also increased asparagine levels. The latter compound was probably formed to prevent glutamine accumulation. The substantial increase in free amino acids for *H. uninervis* after only 5 days could, however, imply increasing toxicity after long-term exposure to increased NH<sub>x</sub>.

**Table 3**  
Comparison of responses after addition of 100  $\mu\text{mol l}^{-1}$   $\text{NH}_x$  for three seagrass species; *Halodule uninervis*, *Thalassia hemprichii*, *Zostera marina*.  $\pm$ : no change,  $-$ : decrease,  $+$ : increase,  $++$ : strong increase, nm: not measured.

			Leaf tissue survival (%)	Photosynthetic efficiency ( $F_v F_m^{-1}$ )	N accumulation	FAA increase (%FAA-N of total %N)	Increase glutamine (%N of FAA-N)	Increase asparagine (%N of FAA-N)
<i>H. uninervis</i>	Tropical	Early successional	$\pm$	$\pm$	$++$	$++$	$++$	$++$
<i>T. hemprichii</i>	Tropical	Late successional	$\pm$	$-$	$+$	$+$	$++$	$+$
<i>Z. marina</i> <sup>a</sup>	Temperate	Intermediate	$-$	nm	$+$	$+$	$++$	$+$

<sup>a</sup> Van der Heide et al. (2008).

#### 4.2. *Thalassia hemprichii*

In contrast to *H. uninervis*, *T. hemprichii* did not substantially increase nitrogen stored in free amino acids after  $\text{NH}_x$  addition. This species increased absolute glutamine concentrations but did not process glutamine further into other amino acids. Although effects of light treatment and pH were not significant in most cases, *T. hemprichii* showed a decreased photosynthetic efficiency and accumulation of free  $\text{NH}_3$  in the high  $\text{NH}_x$  – pH 8.9 – high light treatment. A high light intensity generally results in higher photosynthetic rates, which has been found to increase  $\text{NH}_3$  toxicity in several higher plants including seagrasses (Pulich, 1986; Britto and Kronzucker, 2002; Guo et al., 2007; Brun et al., 2008). A possible explanation could be an increase of intracellular  $\text{NH}_x$  concentration due to  $\text{NH}_4^+$  being released in the conversion of glycine to serine during photorespiration (Pulich, 1986; Guo et al., 2007). A second explanation could be that increased photosynthesis at high light would lead to even higher pH at the leaf boundary layer due to enhanced  $\text{OH}^-$  efflux related to  $\text{HCO}_3^-$  uptake (Beer et al., 2002), an idea that is supported by our observation of white  $\text{CaCO}_3$  or  $\text{MgCO}_3$  precipitation on some of the leaf surfaces during the experiments. A higher pH pushes the equilibrium between  $\text{NH}_4^+$  and  $\text{NH}_3$  towards the more toxic  $\text{NH}_3$  (Johansson and Wedborg, 1980). At high concentrations,  $\text{NH}_3$  can diffuse rapidly across the outer membranes of chloroplasts (Heber et al., 1974; Britto and Kronzucker, 2002), where it may cause uncoupling of photophosphorylation (Märschner, 1995). As a result, photosynthetic efficiency is reduced, which negatively affects amino acid synthesis and  $\text{NH}_x$  detoxification.

Finally, it should be noted that the observed decreased photosynthetic efficiency did not suggest lethal effects because it dropped by only 20% (from 0.76 to 0.61  $F_v F_m^{-1}$ ). A field study on *T. hemprichii* (Lan et al., 2005) described photosynthetic recovery even from a value of 0.45  $F_v F_m^{-1}$  after 1.5 h of air exposure. This indicates that the photosynthetic performance of this species can be expected to be resilient to a five-day exposure to high  $\text{NH}_x$ . However, a longer term exposure to  $\text{NH}_x$ , particularly at high pH can be expected to result in toxicity due to decreased photosynthetic performance and the accumulation of free  $\text{NH}_3$  (Smolders et al., 1996; Britto and Kronzucker, 2002; Van der Heide et al., 2008).

#### 4.3. Differential sensitivities among seagrass species

Although total nitrogen content were found to increase in both species, only *H. uninervis* showed increased nitrogen assimilation into free amino acids in the  $\text{NH}_x$  treatments. The relative contributions of different free amino acids to  $\text{NH}_x$  detoxification are strikingly different among species. *T. hemprichii* (this study), *Zostera capricornis* (Udy and Dennison, 1997) and *Z. marina* (Van der Heide et al., 2008) showed only glutamine accumulation and therefore seem to be unable to process  $\text{NH}_4^+$  further into other amino acids. *H. uninervis*, in contrast, seemed better equipped to regulate its  $\text{NH}_x$  levels further through the glutamate synthase–glutamine syn-

thetase route assimilating glutamine into asparagine. This suggests that the latter species seems better able to avoid internal  $\text{NH}_x$  toxicity by the synthesis of N-rich amino acids. This hypothesis is supported by the fact that reduced photosynthetic performance of *T. hemprichii* was already detectable after five days, in contrast to *H. uninervis*, which implies that *T. hemprichii* is more vulnerable to  $\text{NH}_x$  pulses.

#### 4.4. Ecological implications

We speculate here that differences in growth strategies could explain the differences in nitrogen uptake and metabolism. *H. uninervis* is an early successional species that is characterized by a higher production, and thus a higher nutrient metabolism than late successional species such as *T. hemprichii* (Fourqurean et al., 1995; Uku et al., 2005), therefore possibly allowing the former species to colonize disturbed areas more successfully compared to late successional species.

The moderate toxicity effects found for both tropical species are in strong contrast to earlier findings on the effect of high  $\text{NH}_x$  concentrations on temperate seagrass species *Z. marina* tested in an equal setup (Van der Heide et al., 2008), and other studies on *Z. marina* and *Z. noltii* (Van Katwijk et al., 1997, 1999; Brun et al., 2002, 2008). Where *Z. marina* showed tissue damage and high leaf mortality, the two tropical species did not show any visual signs of toxicity. *T. hemprichii* only decreased its photosynthetic efficiency under high pH and light, and *H. uninervis* effectively detoxified high  $\text{NH}_x$  inputs by the synthesis of free amino acids. This could suggest that tropical species might be able to cope better with high  $\text{NH}_x$  loads than temperate species, although a greater range of species from both climate regions, spanning the range of successional stages, should still be tested.

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#### References

- Beer, S., Bjork, M., Hellblom, F., Axelsson, L., 2002. Inorganic carbon utilization in marine angiosperms (seagrasses). *Funct. Plant Biol.* 29, 349–354.
- Beer, S., Mtolera, M., Lyimo, T., Bjork, M., 2006. The photosynthetic performance of the tropical seagrass *Halophila ovalis* in the upper intertidal. *Aquat. Bot.* 84, 367–371.
- Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate—a practical and powerful approach to multiple testing. *J. R. Stat. Soc. B: Met.* 57, 289–300.

- Birch, W.R., Birch, M., 1984. Succession and pattern of tropical intertidal seagrasses in Cockle Bay, Queensland, Australia—a decade of observations. *Aquat. Bot.* 19, 343–367.
- Briggs, M.R.P., Funge-Smith, S.J., 1994. A nutrient budget of some intensive marine shrimp ponds in Thailand. *Aquacult. Fish. Manage.* 5, 809–811.
- Britto, D.T., Kronzucker, H.J., 2002.  $\text{NH}_4^+$  toxicity in higher plants: a critical review. *J. Plant Physiol.* 159, 567–584.
- Brun, F.G., Hernandez, I., Vergara, J.J., Peralta, G., Perez-Llorens, J.L., 2002. Assessing the toxicity of ammonium pulses to the survival and growth of *Zostera noltii*. *Mar. Ecol. Prog. Ser.* 225, 177–187.
- Brun, F.G., Olive, I., Malta, E.J., Vergara, J.J., Hernandez, 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.
- Burford, M.A., Longmore, A.R., 2001. High ammonium production from sediments in hypereutrophic shrimp ponds. *Mar. Ecol. Prog. Ser.* 224, 187–195.
- Burkholder, J.M., Mason, K.M., Glasgow, H.B., 1992. Water-column nitrate enrichment promotes decline of Eelgrass *Zostera marina*—evidence from seasonal mesocosm experiments. *Mar. Ecol. Prog. Ser.* 81, 163–178.
- Burkholder, J.M., Tomasko, D.A., Touchette, B.W., 2007. Seagrasses and eutrophication. *J. Exp. Mar. Biol. Ecol.* 350, 46–72.
- Cao, T., Ni, L.Y., Xie, P., 2004. Acute biochemical responses of a submersed macrophyte, *Potamogeton crispus* L., to high ammonium in an aquarium experiment. *J. Freshwater Ecol.* 19, 279–284.
- Carlson, P.R., Yarbro, L.A., Barber, T.R., 1994. Relationship of sediment sulfide to mortality of *Thalassia testudinum* in Florida Bay. *Bull. Mar. Sci.* 54, 733–746.
- Coles, R., McKenzie, L., Campbell, S., Fortes, M., Short, F., 2003. The seagrasses of the Western Pacific islands. In: Green, E.P., Short, F.T. (Eds.), *World Atlas of Seagrasses*. University of California Press, Berkeley, pp. 161–168.
- Duarte, C.M., 1990. Seagrass nutrient content. *Mar. Ecol. Prog. Ser.* 67, 201–207.
- Duarte, C.M., 1991. Allometric scaling of seagrass form and productivity. *Mar. Ecol. Prog. Ser.* 77, 289–300.
- Erfteimeijer, P.L.A., Lewis, R.R.R., 2006. Environmental impacts of dredging on seagrasses: a review. *Mar. Pollut. Bull.* 52, 1553–1572.
- Field, A.P., 2005. *Discovering Statistics Using SPSS: And sex and drugs and rock n roll*, second ed. Sage publications, London.
- Fourqurean, J.W., Powell, G.V.N., Kenworthy, W.J., Zieman, J.C., 1995. The effects of long-term manipulation of nutrient supply on competition between the seagrasses *Thalassia testudinum* and *Halodule wrightii* in Florida Bay. *Oikos* 72, 349–358.
- Freeman, A.S., Short, F.T., Isnain, I., Razak, F.A., Coles, R.G., 2008. Seagrass on the edge: land-use practices threaten coastal seagrass communities in Sabah, Malaysia. *Biol. Conserv.* 141, 2993–3005.
- Genty, B., Briantais, J.M., Baker, N.R., 1989. The relationship between the quantum yield of photosynthetic electron-transport and quenching of chlorophyll fluorescence. *Biochim. Biophys. Acta* 990, 87–92.
- Goodman, J.L., Moore, K.A., Dennison, W.C., 1995. Photosynthetic responses of Eelgrass (*Zostera marina*) to light and sediment sulfide in a shallow barrier island lagoon. *Aquat. Bot.* 50, 37–47.
- Green, E.P., Short, F.T., 2003. *World Atlas of Seagrasses*, first ed. University of California Press, Berkeley.
- Guo, S.W., Zhou, Y., Gao, Y.X., Li, Y., Shen, Q.R., 2007. New insights into the nitrogen form effect on photosynthesis and photorespiration. *Pedosphere* 17, 601–610.
- Heber, U., Kirk, M.R., Gimmler, H., Schafer, G., 1974. Uptake and reduction of glycinate by isolated-chloroplasts. *Planta* 120, 31–46.
- Jakobsen, F., Hartstein, N., Frachisse, J., Golingi, T., 2007. Sabah shoreline management plan (Borneo, Malaysia): ecosystems and pollution. *Ocean Coast. Manage.* 50, 84–102.
- Johansson, O., Wedborg, M., 1980. Ammonia-ammonium equilibrium in seawater at temperatures between 5 and 25-Degrees-C. *J. Solution Chem.* 9, 37–44.
- Kitajima, M., Butler, W.L., 1975. Quenching of chlorophyll fluorescence and primary photochemistry in chloroplasts by Dibromothymoquinone. *Biochim. Biophys. Acta* 376, 105–115.
- Lamers, L.P.M., Tomassen, H.B.M., Roelofs, J.G.M., 1998. Sulfate-induced eutrophication and phytotoxicity in freshwater wetlands. *Environ. Sci. Technol.* 32, 199–205.
- Lan, C.Y., Kao, W.Y., Lin, H.J., Shao, K.T., 2005. Measurement of chlorophyll fluorescence reveals mechanisms for habitat niche separation of the intertidal seagrasses *Thalassia hemprichii* and *Halodule uninervis*. *Marine Biol.* 148, 25–34.
- Märtschner, H., 1995. *The Mineral Nutrition of Higher Plants*, second ed. Academic Press, London.
- Mukai, H., 1993. Biogeography of the tropical seagrasses in the Western Pacific. *Aust. J. Mar. Freshw. Res.* 44, 1–17.
- 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.
- Orth, R.J., Carruthers, T.J.B., Dennison, W.C., Duarte, C.M., Fourqurean, J.W., Heck, K.L., Hughes, A.R., Kendrick, G.A., Kenworthy, W.J., Olyarnik, S., Short, F.T., Waycott, M., Williams, S.L., 2006. A global crisis for seagrass ecosystems. *Bioscience* 56, 987–996.
- Prange, J.A., Dennison, W.C., 2000. Physiological responses of five seagrass species to trace metals. *Mar. Pollut. Bull.* 41, 327–336.
- Pulich, W.M., 1986. Variations in leaf soluble amino-acids and ammonium content in subtropical seagrasses related to salinity stress. *Plant Physiol.* 80, 283–286.
- Rollon, R.N., Van Steveninck, E.D.D., Van Vierssen, W., Fortes, M.D., 1998. Contrasting recolonization strategies in multi-species seagrass meadows. *Mar. Pollut. Bull.* 37, 450–459.
- Rubio, L., Linares-Rueda, A., García-Sánchez, M.J., Fernández, J.A., 2007. Ammonium uptake kinetics in root and leaf cells of *Zostera marina* L. *J. Exp. Mar. Biol. Ecol.* 352 (2), 271–279.
- Rudolph, H., Voigt, J.U., 1986. Effects of  $\text{NH}_4^+$ -N and  $\text{NO}_3^-$ -N on growth and metabolism of *Sparganium magellanicum*. *Physiol. Plantarum* 66, 339–343.
- Semesh, I.S., Beer, S., Bjork, M., 2009. Seagrass photosynthesis controls rates of calcification and photosynthesis of calcareous macroalgae in a tropical seagrass meadow. *Mar. Ecol. Prog. Ser.* 382, 41–47.
- Short, F.T., Wyllie-Echeverria, S., 1996. Natural and human-induced disturbance of seagrasses. *Environ. Conserv.* 23, 17–27.
- Smolders, A.J.P., den Hartog, C., van Gestel, C.B.L., Roelofs, J.G.M., 1996. The effects of ammonium on growth, accumulation of free amino acids and nutritional status of young phosphorus deficient *Stratiotes aloides* plants. *Aquat. Bot.* 53, 85–96.
- Suzuki, M.S., Ovalle, A.R.C., Pereira, E.A., 1998. Effects of sand bar openings on some limnological variables in a hypertrophic tropical coastal lagoon of Brazil. *Hydrobiologia* 368, 111–122.
- Terrados, J., Duarte, C.M., Fortes, M.D., Borum, J., Agawin, N.S.R., Bach, S., Thampanya, U., Kamp-Nielsen, L., Kenworthy, W.J., Geertz-Hansen, O., Vermaat, J., 1998. Changes in community structure and biomass of seagrass communities along gradients of siltation in SE Asia. *Estuar. Coast. Shelf S.* 46, 757–768.
- Thursby, G.B., Harlin, M.M., 1982. Leaf-root interaction in the uptake of ammonia by *Zostera marina*. *Mar. Biol.* 72, 109–112.
- Tomassen, H.B.M., Smolders, A.J.P., Lamers, L.P.M., Roelofs, J.G.M., 2003. Stimulated growth of *Betula pubescens* and *Molinia caerulea* on ombrotrophic bogs: role of high levels of atmospheric nitrogen deposition. *J. Ecol.* 91, 357–370.
- Udy, J.W., Dennison, W.C., 1997. Physiological responses of seagrasses used to identify anthropogenic nutrient inputs. *Mar. Freshw. Res.* 48, 605–614.
- Uku, J., Beer, S., Bjork, M., 2005. Buffer sensitivity of photosynthetic carbon utilisation in eight tropical seagrasses. *Marine Biol.* 147, 1085–1090.
- Van der Heide, T., Smolders, A., Rijkens, B., van Nes, E.H., van Katwijk, M.M., Roelofs, J., 2008. Toxicity of reduced nitrogen in eelgrass (*Zostera marina*) is highly dependent on shoot density and pH. *Oecologia*. 158, 411–419.
- Van Dijk, H.F.G., Roelofs, J.G.M., 1988. Effects of excessive ammonium deposition on the nutritional-status and condition of pine needles. *Physiol. Plantarum* 73, 494–501.
- Van Katwijk, M.M., Vergeer, L.H.T., Schmitz, G.H.W., Roelofs, J.G.M., 1997. Ammonium toxicity in eelgrass *Zostera marina*. *Mar. Ecol. Prog. Ser.* 157, 159–173.
- Van Katwijk, M.M., Schmitz, G.H.W., Gasseling, A.P., van Avesaath, P.H., 1999. Effects of salinity and nutrient load and their interaction on *Zostera marina*. *Mar. Ecol. Prog. Ser.* 190, 155–165.
- Verheij, E., Erfteimeijer, P.L.A., 1993. Distribution of seagrasses and associated macroalgae in South Sulawesi, Indonesia. *Blumea* 38, 45–64.
- Verhoeven, K.J.F., Simonsen, K.L., McIntyre, L.M., 2005. Implementing false discovery rate control: increasing your power. *Oikos* 108, 643–647.
- Vermaat, J.E., Agawin, N.S.R., Duarte, C.M., Fortes, M.D., Marba, N., Uri, J.S., 1995. Meadow maintenance, growth and productivity of a mixed Philippine seagrass bed. *Mar. Ecol. Prog. Ser.* 124, 215–225.
- Walling, D.E., 2006. Human impact on land-ocean sediment transfer by the world's rivers. *Geomorphology* 79, 192–216.
- Williams, S.L., 2007. Introduced species in seagrass ecosystems: status and concerns. *J. Exp. Mar. Biol. Ecol.* 350, 89–110.
- Yuhi, M., 2008. Impact of anthropogenic modifications of a river basin on neighboring coasts: case study. *J. Waterw. Port. C: Div.* 134, 336–344.