

Flow velocity and light level drive non-linear response of seagrass *Zostera noltei* to ammonium enrichment

Beatriz Villazán^{1,*}, Fernando G. Brun¹, Vanessa González-Ortiz¹,
Francisco Moreno-Marín¹, Tjeerd J. Bouma², Juan J. Vergara¹

¹Departamento de Biología (Área de Ecología), Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz,
Campus de Excelencia Internacional del Mar (CEIMAR), Puerto Real 11510 Cádiz, Spain

²Netherlands Institute for Sea Research (NIOZ), PO Box 140, 4400 AC Yerseke, The Netherlands

ABSTRACT: We investigated the interactive effects of light (low and high light doses) and flow velocity (low, medium and high levels) under NH_4^+ enriched conditions on dynamic and morphological variables of *Zostera noltei* plants in a 5 wk flume experiment. Our results showed a non-linear response of *Z. noltei* in this factorial design, with the strongest negative effect of NH_4^+ enrichment recorded at intermediate flow velocities for almost all the dynamic response variables (i.e. survival, net production, shoot appearance rate). This negative effect of NH_4^+ enrichment was intensified under low light conditions, where net production was only positive in plants growing at high flow velocity. This positive effect of flow velocity was ascribed to the more horizontal position of the leaves, which allows for higher levels of light capture than under lower flow velocities. However, enhancing current velocity may increase NH_4^+ uptake rates until they reach adverse levels, which can potentially trigger NH_4^+ toxicity. This negative effect of flow velocity seems to be neutralized by the higher light capture at high flow, resulting in intermediate current velocities being more harmful for plants. In summary, our results highlight the importance of studying the complexity of interactions among multiple stressors that frequently co-occur in nature to improve our ability to forecast the response of seagrass populations to possible interaction effects in future global change scenarios.

KEY WORDS: Ammonium toxicity · Flume tank · Hydrodynamics · Light reduction · Multiple stressors · Seagrass · *Zostera noltei*

— Resale or republication not permitted without written consent of the publisher —

INTRODUCTION

Seagrasses are clonal marine plants that inhabit intertidal and subtidal areas, extending from tropical to temperate latitudes (den Hartog & Kuo 2006). These species constitute one of the most productive coastal ecosystems (Mann 2000) and have significant ecological, social and economic values (Costanza et al. 1997). Although many factors have been acknowledged to contribute to the decline of seagrass ecosystems (Waycott et al. 2009), nutrient enrichment has

been considered as one of the most important drivers (Burkholder et al. 2007).

Nutrients are required for photosynthetic organisms, and their availability usually stimulates growth and biomass production in seagrasses (e.g. Orth 1977, Alcoverro et al. 1997, Brun et al. 2006). However, high concentration of nutrients under some specific circumstances (e.g. low light levels, low water renewal time in the basin) may result in adverse effects on seagrasses due to algal overgrowth. Large algae accumulations may cause light

*Corresponding author: beatriz.villazan@uca.es

reduction (Burkholder et al. 2007) and enhance flux of organic matter into the sediment. This raises the risk of sediment anoxia (Greve et al. 2003) and promotes sulphide intrusion into plants (Borum et al. 2005, Pérez et al. 2007), which may reduce photosynthesis, growth and survival in seagrasses. In addition, if N is mainly available in its reduced form of ammonium (NH_4^+), seagrasses are also vulnerable to direct toxic effects (van Katwijk et al. 1997, Brun et al. 2008, Villazán et al. 2013b). Adverse effects of high NH_4^+ concentrations have been traditionally explained by internal accumulation of NH_4^+ (Marschner 1995), which may affect internal pH and enzyme kinetics, uncouple the photosynthetic production of ATP, increase respiration rates and reduce the uptake of some ions (e.g. potassium, magnesium, calcium or phosphate) (van Katwijk et al. 1997, Villazán et al. 2013a). To prevent internal NH_4^+ accumulation and to minimize its harmful effects, plants must rapidly assimilate NH_4^+ into amino acids and other N-organic compounds. This generates strong internal demands on ATP, phosphorous and organic C skeletons (Brun et al. 2002, 2008, Villazán et al. 2013b), and these organic C demands must be supplied either from photosynthesis or mobilized from internal C reserves. Therefore, any factor that increases the uptake rates of NH_4^+ and/or reduces photosynthetic rates or internal C reserves (i.e. less C and energy available for the plant) may enhance the level of NH_4^+ toxicity in plants.

In addition, phosphate is required in the NH_4^+ assimilation process as a part of the metabolic energy transfer molecule (i.e. ATP) and as a major component of organic compounds (Marschner 1995). Thus, when C turnover and ATP consumption increase as a result of NH_4^+ assimilation, an enhancement of phosphate demands would be expected (Brun et al. 2002, Villazán et al. 2015). Consequently, phosphate may play a key role in reducing NH_4^+ toxicity, as previously suggested for the seagrass *Zostera noltii* (Brun et al. 2002, 2008); no evidence of toxic effects of high phosphate availability has been found in previous studies.

Release of NH_4^+ into coastal areas is increasing and will continue to rise in the near future (Glibert et al. 2010), potentially affecting seagrass populations. In addition, hydrodynamic conditions in coastal areas are changing worldwide, due to anthropogenic engineering activities that change tidal flows (Kennish 2001) and the effects of climate change, which are expected to increase the frequency and intensity of storms and wave stress (Young et al. 2011). Previous studies have described a consistent relationship

between hydrodynamic conditions, NH_4^+ uptake and photosynthetic rates in seagrasses due to the influence of hydrodynamics on the mass transfer of resources to and from seagrass beds (Koch et al. 2006, Morris et al. 2008, 2013). Increased current velocity or waves may enhance NH_4^+ uptake rates of seagrasses by decreasing the diffusive boundary layer (DBL) thickness in leaves (Koch et al. 2006). At the same time, photosynthetic rates may be reduced by enhanced sediment resuspension (i.e. decreasing light levels; Koch 2001), or increased as a consequence of the higher carbon uptake rates due to the reduction of the DBL (Koch et al. 2006) and of the higher light levels captured by leaves due to their reconfiguration at high flow velocities (i.e. a more horizontal arrangement; Zimmerman 2003, de los Santos et al. 2010). In shallow areas, different environmental factors (e.g. NH_4^+ concentration, hydrodynamics, light levels) may drive either additive, synergistic or opposing effects on seagrass ecophysiology. Thus, the outcome of the interaction among NH_4^+ enrichment, light and hydrodynamic conditions is far from simple to predict, and their interactions may lead to non-linear responses in seagrasses under natural conditions, where multiple factors may act at the same time. In this study, we aimed to test the complex interactive effects between hydrodynamic conditions (3 different flow velocities) and light levels (2 contrasting light intensities) on the dynamic and morphometric responses of the seagrass *Z. noltii* growing under a continuous supply of NH_4^+ .

MATERIALS AND METHODS

Zostera noltii plants were collected in June 2010 from an intertidal bed at Los Toruños salt marsh (Cádiz Bay Natural Park; 36° 30' N, 6° 10' W, Cádiz, southern Spain). Plants were rinsed in seawater and sent to the Netherlands Institute for Sea Research (NIOZ) laboratory. Upon arrival less than 48 h after collection, experimental plant units (EPUs) formed by 2 rhizome internodes, one apical shoot, one lateral shoot and the associated roots were selected. Prior to transplantation, epiphytes were wiped with soft tissue paper. Before the experiment, the EPUs were kept for 5 d in a tank filled with aerated natural seawater from the Oosterschelde estuary, southwest Netherlands (salinity 30; nutrient concentrations 0.27 $\mu\text{mol } \text{NH}_4^+ \text{ l}^{-1}$, 1.59 $\mu\text{mol } \text{NO}_3^- \text{ l}^{-1}$, 0.22 $\mu\text{mol } \text{PO}_4^{3-} \text{ l}^{-1}$) under saturating light conditions (~18 mol photons $\text{m}^{-2} \text{ d}^{-1}$) in a 16 h light: 8 h dark cycle at 20°C.

Twelve flume tanks (Peralta et. al 2006) were used to expose *Z. noltei* plants to 3 contrasting current velocities: low velocity (LV) = 0.01 m s⁻¹, medium velocity (MV) = 0.10 m s⁻¹, and high velocity (HV) = 0.35 m s⁻¹. Furthermore plants were exposed to 2 light levels: low light (LL) = 47.3 µmol photons m⁻² s⁻¹, and high light (HL) = 220.7 µmol photons m⁻² s⁻¹. These light levels are equivalent, respectively, to daily doses of 3.1 ± 0.1 and 14.3 ± 0.1 mol photons m⁻² with an 18:6 photoperiod, which correspond to sub-saturating and saturating light conditions for this species (Peralta et al. 2002). The experiment lasted for 36 d from June to August; 4 replicates, represented by flume tanks, within each velocity treatment were used.

Velocity treatments were applied using individual water pumps for each flume and controlled with an ADV Doppler Nortek at 25 Hz (see Peralta et al. 2006 for further information). These velocity treatments were selected according to the range observed on tidal flats bordering intertidal vegetation and the velocity range registered in *Z. noltei* beds in Cádiz Bay. This observed range varied from low values of 0.02 m s⁻¹ to exceptionally high flow velocity values of 0.25 m s⁻¹ (Lara et al. 2012, Morris et al. 2013). Moreover, temperature, velocity and light levels were selected according to previous experiments performed with this species under comparable experimental conditions, in order to facilitate comparisons (Peralta et al. 2006, de los Santos et al. 2010).

Each flume tank was filled with natural seawater from the Oosterschelde estuary. Water was renewed twice per week to avoid excessive growth of phytoplankton. Photoperiod was set at an 18 h light: 6 h dark cycle. Temperature was kept constant at 20°C using independent cooling units for each flume to maintain optimal growth conditions for the plants (Peralta et al. 2002).

Five haphazardly selected EPUs were transplanted into a pot (12 × 12 × 25 cm) filled with a homogenous mixture of clay, sand and gravel. Subsequently, 4 pots were randomly positioned within each flume tank (i.e. a total of 20 EPUs per flume tank) and kept during a 1 wk acclimation period under saturating light conditions and at the same temperature and seawater conditions as those used for the experimental setup. During the experiment, 2 pots per flume tank were kept at HL while the other 2 pots were shaded with a neutral light-quality screen to apply the LL treatment, and selected flow velocities (LV, MV and HV) were applied to the tanks. Seawater was enriched with NH₄⁺ (50 µmol l⁻¹) and phosphate (5 µmol l⁻¹) in each flume from an NH₄Cl and KH₂PO₄

stock solution every 2 d. The pH was measured twice per week, and the mean recorded value was 8.2 (n = 8). At this pH level, less than 10 % of the total N is in the form of ammonia (NH₃) (Collos & Harrison 2014). Therefore, this study will use NH₄⁺ throughout, but this will be used to refer to both reduced forms (NH₄⁺ and NH₃).

The selected concentration of NH₄⁺ (i.e. 50 µmol l⁻¹) has been demonstrated to promote chronic toxicity in previous experiments with *Z. noltei* plants (Brun et al. 2002, 2008). It also has ecological relevance since this level can be found in nature when seagrass meadows grow in eutrophic waters or near wastewater discharge points (Burkholder et al. 2007, Cabaço et al. 2008). Phosphate was added simultaneously to NH₄⁺ in the flumes to maintain nutrient stoichiometry. In addition, previous studies have suggested that any process limiting phosphate availability may increase seagrass vulnerability to NH₄⁺ toxicity (Brun et al. 2002, 2008). In the present study, we used a high NH₄⁺ load in all the treatments, rather than using NH₄⁺ presence/absence as an additional factor. This choice was due to the restricted number of flumes available: 12 in total with 4 per velocity treatment. Using a full factorial design with 3 factors would require at least 18 flumes to perform only 3 independent replicates. In addition, using the same experimental conditions that have been used in some previous experiments from our research group (Peralta et al. 2006, de los Santos et al. 2010) allowed us to compare our results, generated by adding an NH₄⁺ supply, with their control treatments (i.e. without nutrient supply).

Water samples were collected twice, and nutrients were measured using a San Plus segmented flow Skalar Autoanalyser® model 8805. The average nutrient concentrations measured just after nutrient supply was added, for the whole experimental period in all the flumes, was 53.6 ± 0.8 µmol NH₄⁺ l⁻¹ and 5.0 ± 0.1 µmol PO₄³⁻ l⁻¹ (n = 80). Average remaining nutrient concentrations before nutrient addition in all the flumes was 6.4 ± 0.9 NH₄⁺ µmol l⁻¹ and 0.6 ± 0.1 PO₄³⁻ µmol l⁻¹ (n = 80).

Biological measurements

At the beginning of the experiment, morphometric measurements (leaf width, leaf length, number of leaves and above- and belowground biomass) were conducted on 10 EPUs haphazardly selected from the pool of collected plants. Prior to transplantation into pots, each EPU was weighed (initial fresh weight,

Table 1. Morphometric and dynamic response variables of *Zostera noltei* measured or calculated in this study. D_1 and D_2 : main rhizome diameters; subscripts i and f: initial and final conditions, respectively; M_i : meristems activated initially; M_f : meristems activated during experimental period; EPU: experimental plant unit

Variable	Unit	Description
Morphometric		
Aboveground biomass (AG)	g DW plant ⁻¹	Dry biomass of leaves
Belowground biomass (BG)	g DW plant ⁻¹	Dry biomass of rhizomes and roots
AG:BG ratio	dimensionless	$AG:BG = \frac{AG}{BG}$
Foliar necrosis	(%)	Mean values for all the shoots in an EPU
Leaf abundance (LA)	no. of leaves EPU ⁻¹	Mean values for all the shoots in an EPU
Leaf length (LL)	cm leaf EPU ⁻¹	Mean values for all the shoots in an EPU
Leaf width (LW)	mm leaf EPU ⁻¹	Mean values for all the shoots in an EPU
Leaf thickness (LTh)	mm leaf EPU ⁻¹	Mean values for all the shoots in an EPU
Leaf cross section (L_{sec})	mm ² leaf EPU ⁻¹	$L_{sec} = \frac{LW \cdot LTh}{2 \cdot 2}$
Internode abundance (IA)	no. of internodes EPU ⁻¹	Mean values in an EPU
Internode length (IL)	cm internode EPU ⁻¹	Mean values in an EPU
Internode cross section (I_{sec})	mm ² internode EPU ⁻¹	$I_{sec} = \frac{D_1 \cdot D_2}{2 \cdot 2} \cdot \pi$
Root length (RL)	cm root EPU ⁻¹	Mean values for all the shoots in an EPU
Dynamics		
Survival (SR)	(%)	$SR = \frac{\text{Live EPUs}}{\text{Initial EPUs}} \cdot 100$
Net growth rate (NGR)	g FW d ⁻¹ EPU ⁻¹	$NGR = \frac{\text{Biomass}_f - \text{Biomass}_i}{t_f - t_i}$
Shoot appearance rate (SAR)	no. of shoots d ⁻¹ EPU ⁻¹	$SAR = \frac{\sum M_f}{\sum M_i} \cdot \frac{1}{(t_f - t_i)}$
Internode appearance rate (IAR)	no. of internodes d ⁻¹ EPU ⁻¹	$IAR = \frac{IA_f - IA_i}{t_f - t_i}$

FW) and each rhizome was individually tagged with a label. At the end of the experiment, all surviving plants were carefully harvested and weighed again (FW) to estimate net growth production per plant (g FW plant⁻¹ d⁻¹) from the net change in individual plant weight during the experiment. At the end of the experiment, morphometric measurements were taken from all the plants (leaf abundance, leaf length, leaf cross section, internode cross section and internode length and root length). Furthermore, each harvested EPU was split into leaves (aboveground, AG), and rhizomes/roots (belowground, BG), freeze-dried and weighed to calculate the AG:BG ratio. Following procedures in Peralta et al. (2006) and de los Santos et al. (2010) (Table 1), morphometric information was used to calculate plant dynamic properties (net production, shoot and internode appearance rate), which estimate growth of the plants relative to the time span of the experiment. Leaf necrosis was quantified as the area with brown/black discolouration of the leaves on each shoot in relation to the total leaf surface (%).

Total carbon and nitrogen content

Total C and N content in tissues was determined using 4 ground, freeze-dried samples of leaves and rhizomes/roots from each treatment using a Carlo-Erba NA-1500 CHNS analyzer.

Estimation of phosphate and ammonium uptake rates

Foliar NH₄⁺ and PO₄³⁻ uptake rates (V) (μmol g⁻¹ dry weight [DW] h⁻¹) were calculated for each flume tank during the experiment. These calculations included plants growing at LL and HL within the same flume. Foliar uptake rates at each velocity treatment were estimated from changes in nutrient concentration (S) (μmol l⁻¹) in each flume, which was measured twice per week during the experiment. Uptake rates were computed as follows:

$$V = \frac{(S_i - Vol_i) - (S_f - Vol_f)}{t \times B} \quad (1)$$

where S_i and S_f are nutrient concentrations ($\mu\text{mol l}^{-1}$) at the beginning (initial, i) and end (final, f) of each sampling interval, t is the time elapsed between 2 successive samplings (h), B is the estimated foliar DW (g), and Vol is the volume (l) of each flume. NH_4^+ and PO_4^{3-} uptake rates ($n = 10$, twice a week for 5 wk) were plotted against time (36 d). Plotted uptake rate for each day is calculated as the mean \pm SE value of each velocity treatment ($n = 4$ flumes per velocity regime). DW at each sampling interval was estimated by assuming linear growth fitted between the initial and the final foliar DW of each flume tank. Foliar DW was calculated according to the ratio of total FW to foliar FW (1.56 ± 0.10 , $n = 10$) and FW was converted in DW according to the ratio of 4:1 g FW g^{-1} DW) ($n = 10$).

Statistical analysis.

We used a nested permutational MANOVA (PERMANOVA) to test the overall effects of the treatments (velocity and light levels) for the dynamic and morphometric plant response variables (i.e. survival, net growth rate, shoot appearance and internode appearance rates, necrosis, AG:BG biomass ratio, leaf abundance, leaf length, leaf cross section, internode cross section, internode length and root length). The experimental design included 1 random factor (tank) nested within velocity and 2 fixed factors, i.e. velocity (3 levels: LV, MV and HV) and light (2 levels: LL and HL). The multivariate approach was chosen because plant variables were measured in plants coming from the same experimental unit (tank) and because many of the plant variables are likely to be inter-correlated (Quinn & Keough 2002). Data were normalized (means were subtracted and the resulting difference was divided by standard deviation) to minimize scale differences among dynamic and morphometric variables before analysis. PERMANOVA was executed using Type III sum of squares on geometric (Euclidean) distances and unrestricted permutation of raw data (Anderson et al. 2008).

Univariate nested PERMANOVA (3 or 4 factorial) was subsequently used to test the effect of the factors on each variable separately as suggested by Quinn & Keough (2002). All factors (velocity and light) were considered fixed except for the nested factor (tank), which was assumed to be a random factor. For total C and N content, type of tissue was included as a fixed factor (2 levels; leaves and rhizomes/roots). These tests were also conducted using Type III sum of

squares on geometric (Euclidean) distances and unrestricted permutation of raw data.

All tests (permutational MANOVA and ANOVA) were carried out using a significance level of $\alpha = 0.05$. We used the estimation of p-values obtained by Monte Carlo sampling (Anderson et al. 2008). To test the interactions among factors, we used pairwise comparisons when significant interaction terms were detected (Anderson et al. 2008). PERMANOVA tests were performed using the software PRIMER v6.1.13 and PERMANOVA+ v1.0.3 statistical package.

Nutrient uptake rates at each velocity ($n = 4$) were plotted against experimental time. The data were fitted by least-squared regression analysis. To test differences in NH_4^+ and PO_4^{3-} uptake rates, a 1-way ANOVA test was used to check differences among slopes in each velocity treatment. When significant differences were found, a post-hoc Tukey test was performed (Zar 1984). Homoscedasticity and normality of the data were checked before conducting ANOVA tests, and NH_4^+ and phosphate uptake rates were log transformed due to detection of heteroscedasticity. Data are shown as mean \pm standard error (SE). Significance level was set at a probability of 5% ($\alpha = 0.05$).

RESULTS

Dynamic and morphometric response variables

The multivariate response of all dynamic and morphometric response variables was affected by velocity and light treatments separately (MANOVA, $p < 0.001$, Table 2), but no significant interactions were detected ($p = 0.284$, Table 2). In addition, the nested design showed that the flume tanks had no significant effects within velocity treatments ($p = 0.893$, Table 2). Low light negatively affected all response variables, regardless of the velocity regime (Table 2). In addition, the negative effect of NH_4^+ was stronger at medium than at low or high velocity regimes (Table 2, Figs. 1 & 2).

Survival rate, net production and shoot appearance rate were significantly affected by velocity and light but not by their interaction (Table 2). However, internode appearance rate was affected by light and by the interaction between light and velocity but not by velocity conditions alone (Table 2).

Under HL conditions, survival decreased from ~75% both at HV and LV to ~60% at MV (Fig. 1A). Under LL conditions, survival declined in comparison to HL in all the velocity treatments (Fig. 1A). The net

Table 2. Statistical results of the MANOVA and ANOVA analyses examining the effects of light, velocity and flume tank on dynamic and morphometric response variables of *Zostera noltei*. Significant results ($p < 0.05$) are given in **bold**

	df	MS	F	p
Composite response (MANOVA):				
Velocity	2,9	27.73	3.28	<0.001
Light	1,9	54.29	0.81	<0.001
Tank	9	8.36	0.81	0.893
Light × Velocity	2	12.21	1.18	0.284
Individual responses (ANOVA):				
Survival rate (SR)				
Velocity	2,9	3.21	5.71	0.015
Light	1,9	6.11	8.59	0.021
Tank	9	0.55	0.56	0.831
Light × Velocity	2	0.42	0.59	0.564
Net growth rate (NGR)				
Velocity	2,9	3.66	4.40	0.043
Light	1,9	8.95	5.88	0.038
Tank	9	0.83	1.00	0.449
Light × Velocity	2	0.76	0.50	0.619
Shoot appearance rate (SAR)				
Velocity	2,9	3.32	4.49	0.039
Light	1,9	7.82	28.19	<0.001
Tank	9	0.73	0.75	0.665
Light × Velocity	2	0.78	2.96	0.086
Internode appearance rate (IAR)				
Velocity	2,9	1.49	1.88	0.203
Light	1,9	13.49	49.04	<0.001
Tank	9	0.78	0.84	0.577
Light × Velocity	2	1.26	4.78	0.028
Necrosis				
Velocity	2,9	2.45	6.14	0.015
Light	1,9	1.08	1.19	0.288
Tank	9	0.37	0.37	0.941
Light × Velocity	2	2.87	3.46	0.073
Aboveground:belowground biomass (AG:BG)				
Velocity	2,9	7.37	4.23	0.045
Light	1,9	3.30	8.22	0.017
Tank	9	0.78	0.89	0.538
Light × Velocity	2	1.28	1.43	0.294
Leaf abundance (LA)				
Velocity	2,9	7.31	15.20	0.001
Light	1,9	10.45	32.27	<0.001
Tank	9	0.46	0.54	0.841
Light × Velocity	2	1.38	4.39	0.038
Leaf length (LL)				
Velocity	2,9	0.23	0.43	0.669
Light	1,9	0.63	0.35	0.577
Tank	9	0.52	0.55	0.832
Light × Velocity	2	1.57	0.87	0.445
Leaf cross section (L_{sec})				
Velocity	2,9	1.99	2.80	0.106
Light	1,9	1.69	0.99	0.342
Tank	9	0.70	0.77	0.642
Light × Velocity	2	1.15	0.67	0.533
Internode cross section (I_{sec})				
Velocity	2,9	0.90	0.81	0.475
Light	1,9	1.79	4.42	0.057
Tank	9	1.11	1.06	0.392
Light × Velocity	2	0.19	0.83	0.831

Table 2. (continued)

	df	MS	F	p
Internode length (IL)				
Velocity	2,9	2.13	2.37	0.142
Light	1,9	3.60×10^{-4}	3.45×10^{-4}	0.985
Tank	9	0.89	0.90	0.518
Light × Velocity	2	0.77	0.74	0.501
Root length (RL)				
Velocity	2,9	0.96	0.82	0.469
Light	1,9	1.02	0.77	0.406
Tank	9	1.18	1.23	0.292
Light × Velocity	2	0.32	0.24	0.789

growth rate (NGR) of plants under LL was only positive at HV, whereas negative values were recorded in EPUs grown at LV and MV. At HL levels, net production reached its minimum at MV, while higher and similar values were recorded at LV and HV (Fig. 1B). The shoot appearance rate was negatively affected under LL conditions in all the velocity regimes, reaching the lowest values at MV, while higher and similar values were recorded at LV and HV treatments (Fig. 1C). Under HL levels, the highest value of shoot appearance rate was found at HV (Fig. 1C). Internode appearance rate was affected negatively by LL in all the velocity treatments. The reduction was stronger under HV, decreasing from 0.05 to 0.02 internodes d⁻¹ EPU⁻¹. However, internode appearance rate was unaffected by velocity both under LL and under HL levels (Fig. 1D).

In contrast to the dynamic response variables, no significant differences were detected in most of the morphometric response variables. Only necrosis, AG:BG ratio and leaf abundance responded significantly to the treatments (Table 2). Necrosis was significantly affected by velocity conditions (Table 2), reaching the highest value at MV under both LL and HL conditions (70 and 60 % respectively; Fig. 2A). Necrosis was unaffected by light treatments in all the velocity regimes. The AG:BG ratio was affected significantly by light and velocity treatments but not by their interaction (Table 2) and was higher in plants cultivated under HL in all the velocity treatments (Fig. 2B). The lowest value was recorded at MV, with higher values under HL than LL (Fig. 2B). Leaf abundance was affected by velocity, light and their interaction (Table 2). The number of leaves per EPU was reduced from ~5 in LV and HV to 3 in MV at HL levels (Fig. 2C). The effect of LL was stronger in LV and HV, decreasing from ~5 to ~3 in LV and HV, while similar values were found in MV under LL and HL conditions (Fig. 2C). However, the rest of the mor-

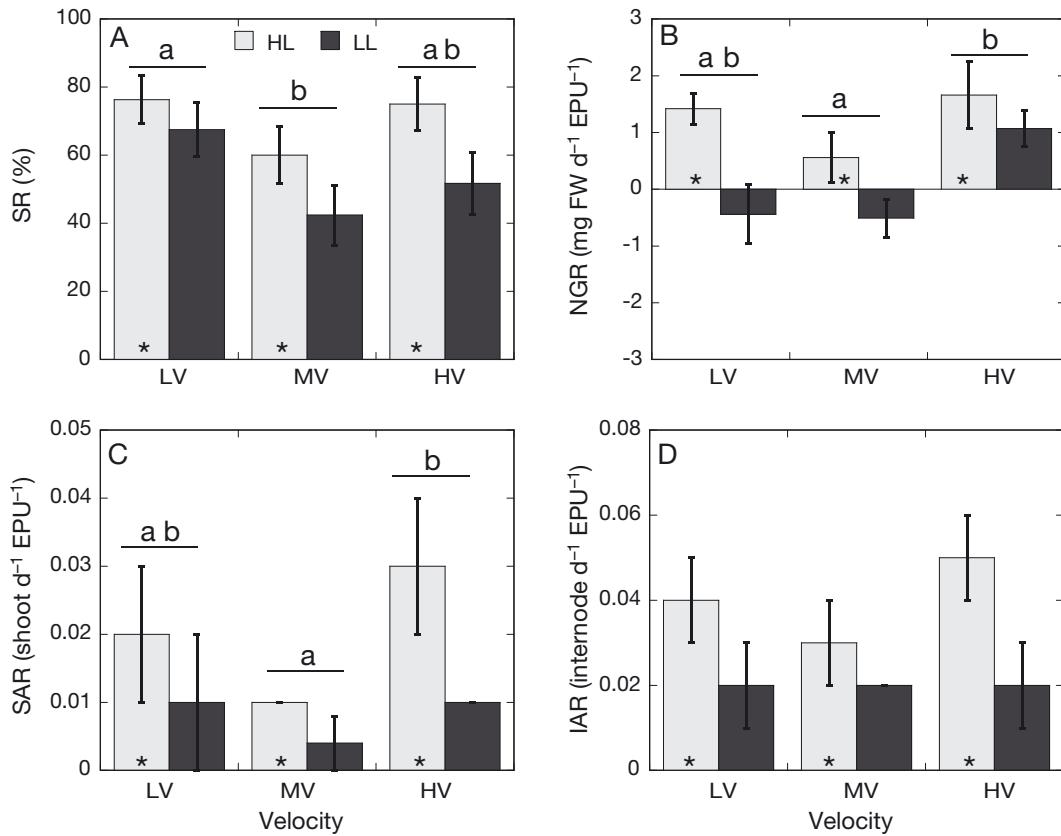


Fig. 1. Dynamic response variables of *Zostera noltei* under low (LV), medium (MV) and high (HV) velocity treatments and high (HL) and low (LL) light treatments with constant NH_4^+ addition ($50 \mu\text{mol l}^{-1}$): (A) survival rate (SR), (B) net growth rate (NGR), (C) shoot appearance rate (SAR), and (D) internode appearance rate (IAR). EPU: experimental plant unit. Grey and black bars show HL and LL treatments, respectively. Lines with letters above indicate significant differences among velocity treatments; asterisks inside bars show significant differences among light treatments. Data are means $\pm 1 \text{ SE}$

photometric response variables (i.e. leaf length, leaf and internode cross-section, internode and root length) were unaffected by the treatments (Table 2, Fig. 2D–H).

Total carbon and nitrogen content

Total C and N contents were higher in leaves than in the rhizomes/roots in all the assayed treatments. Leaf C content did not show any significant difference among light and velocity treatments, with a mean value ~27 % DW (Table 3, Fig. 3A). In the rhizomes/roots, total C content showed a slight increase with current velocity (Fig. 3A). Under LL levels, the lowest value of C content in leaves was recorded at MV (19.5 %), while leaves of EPUs cultivated at LV and HV had similar values (21.4 and 22.0 %, respectively). On the other hand, total N content was unaffected by light and velocity treatments but was different between plant tissues, i.e. AG versus BG

(Table 3). Total N content was lower in rhizomes/roots than in leaves with a mean value of ~2 % in leaves and 1 % in rhizomes/roots. A slight decrease of total N was found in rhizomes/roots in plants growing under MV at both light levels (Fig. 3B).

Ammonium and phosphate uptake rates

The uptake rates of both nutrients increased with incubation time and increasing velocity treatment (ANOVA, $p < 0.001$ for NH_4^+ and PO_4^{3-} uptake rates; Fig. 4). Uptake rates of NH_4^+ were approximately 1 order of magnitude higher than for PO_4^{3-} under all velocity conditions. In comparison to LV, the slopes of the PO_4^{3-} uptake in MV and HV were almost 4 and 16 times higher, respectively. With respect to NH_4^+ , the uptake rate was ~2-fold higher in MV and ~4-fold higher in HV compared with LV.

The net uptake ratio of $\text{NH}_4^+:\text{PO}_4^{3-}$ was unaffected by velocity conditions ($11.17 \pm 0.44 \mu\text{mol NH}_4^+ \mu\text{mol}^{-1}$

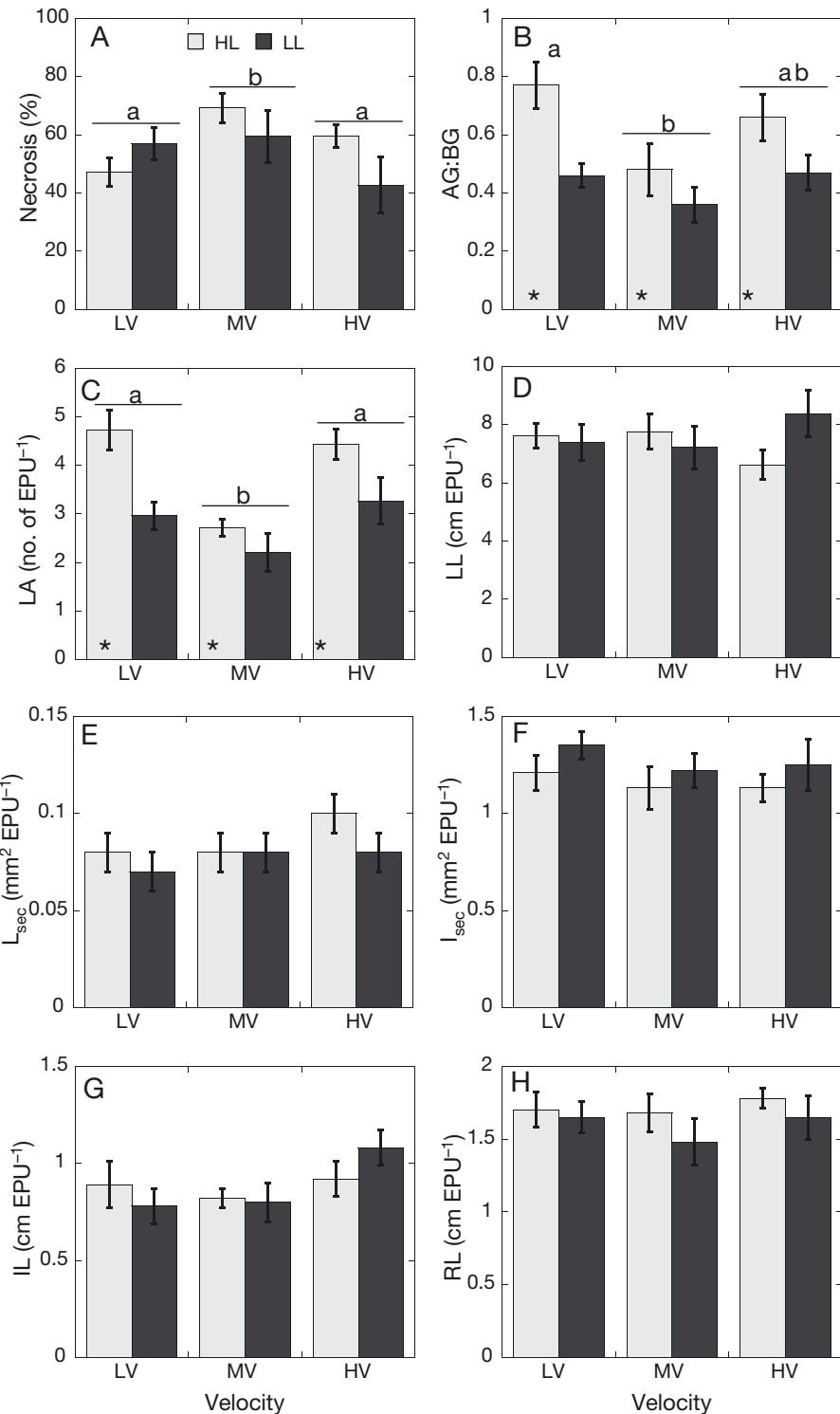


Fig. 2. Morphometric response variables of *Zostera noltei* under different velocity and light treatments with a constant NH_4^+ addition ($50 \mu\text{mol l}^{-1}$): (A) necrosis, (B) aboveground:belowground biomass ratio (AG:BG), (C) leaf abundance (LA), (D) leaf length (LL), (E) leaf cross section (L_{sec}), (F) internode cross section (I_{sec}), (G) internode length (IL), and (H) root length (RL). For explanation of other abbreviations and asterisks, see Fig. 1 legend. Data are means $\pm 1 \text{ SE}$

PO_4^{3-}). This value was similar to the ratio of concentrations supplied throughout the experiment ($10 \mu\text{mol NH}_4^+ \mu\text{mol}^{-1} \text{PO}_4^{3-}$).

DISCUSSION

The effects of combined multiple stressors are often assumed to be accumulative (Halpern et al. 2007); however, our study found a non-linear response in *Zostera noltei* plants when they were subjected to high NH_4^+ concentration under different flow velocities and light levels. Interestingly, more negative effects on *Z. noltei* performance were observed when plants were cultivated under moderate current velocity and low light levels. The individual effects of current velocity, light levels and NH_4^+ enrichment have been well studied (Peralta et al. 2006, Brun et al. 2008, de los Santos et al. 2010, Morris et al. 2013), whereas studies regarding the combined effects of these stressors on seagrasses are lacking.

Although this experimental design lacked a control treatment (i.e. without nutrient supply) to examine the likely negative effect of NH_4^+ supply, we can compare our results with previous experiments developed under similar experimental conditions using the same devices (Peralta et al. 2006, de los Santos et al. 2010). By doing this, the net plant growth rate measured using our experimental design (i.e. with NH_4^+ addition; mean value $\sim 1.2 \text{ mg FW EPU}^{-1} \text{ d}^{-1}$), pooled over velocity or light levels, was approximately 60% lower than that recorded in control treatments from these previous experiments (i.e. without NH_4^+ ; supply; average net production $\sim 3 \text{ mg FW EPU}^{-1} \text{ d}^{-1}$; Peralta et al. 2006, de los Santos et al. 2010). Therefore, it seems that, as previously demonstrated in this species, the chronic NH_4^+ concentration supplied in our

Table 3. Statistical results of the ANOVA tests examining the effects of light, velocity, flume tank and plant tissue on total C and N content in plants of *Zostera noltei*. Significant results ($p < 0.05$) are given in **bold**

	df	MS	F	p
Total C				
Velocity	2,9	0.30	0.34	0.731
Light	1,9	1.44	2.64	0.133
Tissue	1,9	20.01	116.22	<0.001
Tank	9	0.89	1.39	0.206
Light × Velocity	2	0.49	0.89	0.448
Velocity × Tissue	2	12.72	74.67	<0.001
Light × Tissue	2	5.95	28.18	<0.001
Light × Velocity × Tissue	2	12.35	58.95	<0.001
Total N				
Velocity	2,9	1.26	1.49	0.275
Light	1,9	0.77	1.99	0.191
Tissue	1,9	33.78	64.93	<0.001
Tank	9	0.85	1.66	0.104
Light × Velocity	2	0.29	0.76	0.489
Velocity × Tissue	2	9.11	17.51	0.001
Light × Tissue	2	17.46	34.32	0.001
Light × Velocity × Tissue	2	11.75	23.10	<0.001

experiment had a negative effect on plant performance (Brun et al. 2002, 2008).

NH_4^+ is a paradoxical nutrient. On one hand, it is an essential nutrient that plays a crucial role in plant metabolism (Marschner 1995), but on the other hand, elevated levels of NH_4^+ also may trigger toxicity phenomena in seagrasses (e.g. van Katwijk et al. 1997, Brun et al. 2002, 2008, Christianen et al. 2011, Villazán et al. 2013b, 2015), land plants (Britto & Kronzucker 2002) and micro- and macroalgae (Peckol & Rivers 1995, Collos & Harrison 2014). Several studies have shown that exposure to high, but ecologically relevant, NH_4^+ concentrations can be both beneficial and detrimental to seagrasses (Touche & Burkholder 2000, Burkholder et al. 2007). Positive effects such as increased photosynthesis and growth are often seen in nutrient-poor environments, where plants may be N-limited (Touche & Burkholder 2000, Brun et al. 2006). However, a number of studies have shown that even relatively low concentrations of NH_4^+ can have an adverse effect on seagrasses (e.g. van Katwijk et al. 1997, Brun et al. 2002, 2008, Villazán et al. 2013b, 2015).

Adverse effects of NH_4^+ are usually related to intracellular accumulation, since high intracellular concentration of NH_4^+ may produce toxic effects in plants by affecting intracellular pH, enzyme kinetics, photosynthetic ATP production and uptake of other

ions (Marschner 1995, Britto & Kronzucker 2002). This direct relationship between external and intracellular NH_4^+ concentrations was found in both *Posidonia oceanica* and *Zostera marina* plants cultivated under high NH_4^+ concentrations (Invers et al. 2004, Villazán et al. 2013b, 2015). Therefore, any process increasing NH_4^+ uptake and/or its intracellular accumulation should make seagrasses more prone to NH_4^+ toxicity.

The response of seagrasses to NH_4^+ enrichment differ when NH_4^+ is assayed as a single stressor compared to when different factors interact at the same time, altering the NH_4^+ uptake and/or N assimilation processes (van Katwijk et al. 1997, Brun et al. 2002, 2008, van der Heide et al. 2008, Christianen et al. 2011, Villazán et al. 2013b, 2015). Several factors may lead to higher NH_4^+ uptake rates and, hence, to the accumulation of NH_4^+ inside cells, thereby increasing the toxic effect of NH_4^+ on seagrasses. For

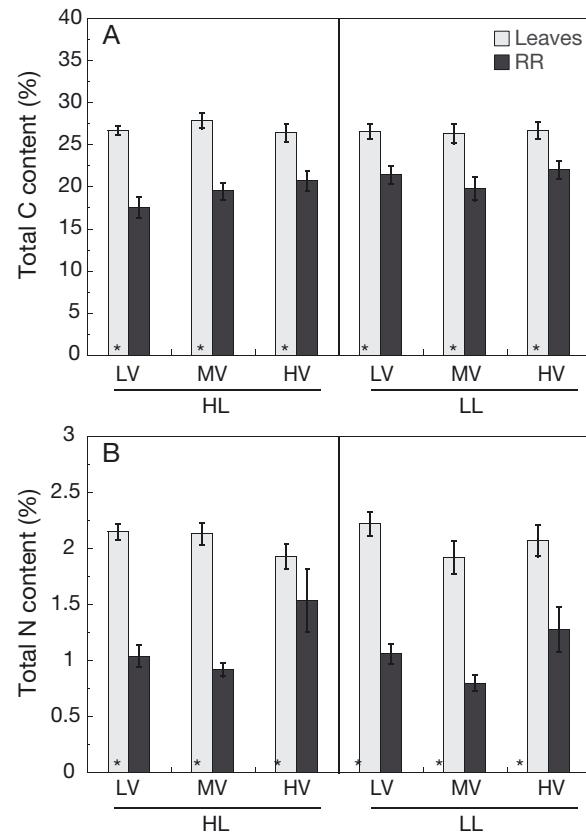


Fig. 3. (A) Total C and (B) N content in aboveground (AG) and belowground (BG) tissues of *Zostera noltei* under different velocity regimes and light treatments. Grey and black bars show AG (Leaves) and BG tissues (roots and rhizomes, RR), respectively. Asterisks inside bars indicate significant differences between AG and BG tissues among classes of velocity and light treatments. For explanation of other abbreviations, see Fig. 1 legend. Data are means \pm 1 SE

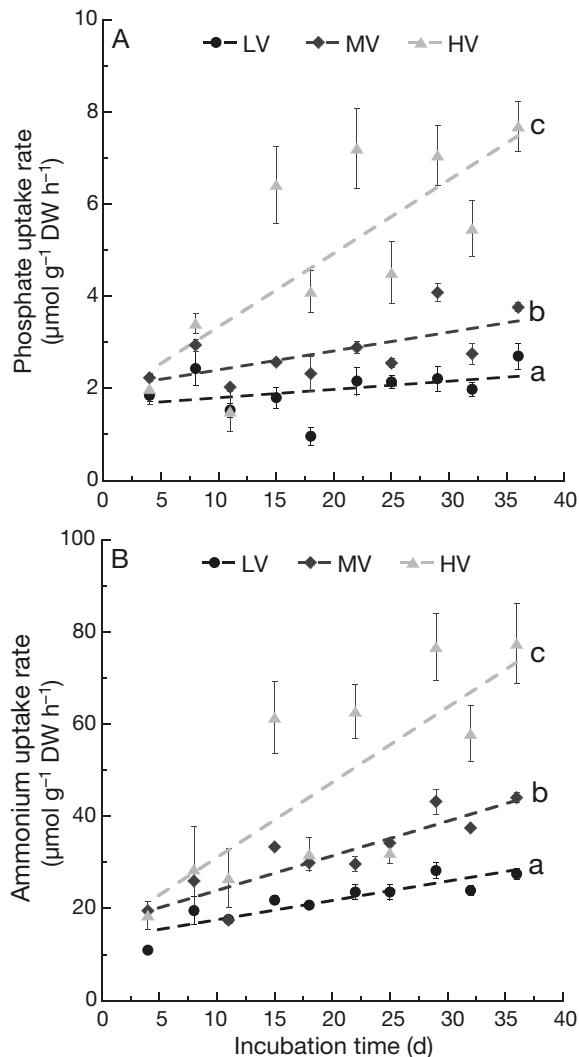


Fig. 4. (A) Foliar phosphate and (B) ammonium uptake rates under low (LV), medium (MV) and high (HV) velocity treatments versus incubation time (36 d). Letters indicate significant differences among velocity treatments (ANOVA, $\alpha = 0.05$). Data are presented as means $\pm 1 \text{ SE}$ ($n = 4$ replicates)

instance, higher availability of NH_4^+ in seawater (e.g. Short & McRoy 1984, Alexandre et al. 2011, Villazán et al. 2013a), seagrass meadow density (van der Heide et al. 2008) and hydrodynamic conditions (La Nafie et al. 2012) are factors that have been found to drive either synergistic or opposing effects in response to NH_4^+ loading in seagrass populations.

Increase in flow velocity decreases the thickness of the leaf boundary layer and enhances NH_4^+ uptake rates in seagrasses, since NH_4^+ uptake seems to be limited at velocities $< 0.3 \text{ m s}^{-1}$ (Cornelisen & Thomas 2004, Morris et al. 2008, 2013). However, previous studies combining flow velocity and NH_4^+ were focused on how flow velocity affects NH_4^+ uptake

rates in different seagrass species and only one study explored, to a limited extent, whether this higher NH_4^+ uptake scaled up to the physiological level of the whole plant (i.e. growth, production, mortality; La Nafie et al. 2012).

The expected increase in NH_4^+ uptake with flow is consistent with our results, since higher NH_4^+ uptake rates were measured with increasing water velocity (Fig. 4B). Consequently, the toxic effect of NH_4^+ derived from the accumulation of this ion in seagrass tissues is expected to be aggravated by increasing current velocity. However, a non-linear response with enhancing flow velocity was recorded, with a stronger toxic effect at medium than at high velocity. This result highlights the importance of analyzing the complete set of trade-offs caused by the alteration of some important factor, such as flow velocity. In this case, a higher flow velocity enhanced NH_4^+ uptake but, at the same time, improved light capture because leaves were positioned more horizontally at high velocities (Zimmerman 2003, de los Santos et al. 2010). As a result, plants may increase the photosynthetic gain of C skeletons and energy (i.e. non-structural carbohydrates and ATP) when grown under HV conditions; this promotes assimilation of NH_4^+ into amino acids, preventing high intracellular concentrations of this nutrient and, hence, improve growth conditions. Moreover, increased growth and dilution of intracellular NH_4^+ under HV conditions could be a possible mechanism behind reduced susceptibility to NH_4^+ toxicity at high flows.

However, both total C and N content remained almost unaltered among the different treatments. This lack of difference in total C content was also found in previous studies where NH_4^+ was used (Brun et al. 2002, 2008, Villazán et al. 2013b), and results were explained on the basis of the low intra- and interspecific variability of the C content in seagrasses (Duarte 1990). However, total N content in tissues increased when N was supplied in these same studies (Invers et al. 2004, Brun et al. 2008, Villazán et al. 2013b, 2015), which is in contrast with our findings in this study. Toxic NH_4^+ effects in seagrasses may lead to losses of seagrass biomass (van Katwijk et al. 1997, Brun et al. 2002, 2008, Villazán et al. 2013b, 2015), decreasing leaf-loss time and also termination of root and rhizome production (Brun et al. 2002). A significant fraction of the N may be lost directly to the sediments as dead roots and rhizomes, and the rest is lost as leaves or leaf bundles (Pedersen & Borum 1993, Hemminga et al. 1999). Therefore, the lower net production linked to the possible higher loss of biomass enriched in N under the medium

velocity treatment could explain why total N did not increase.

Phosphate uptake rates in our experimental design followed a positive relationship with flow velocity, with 4-fold higher uptake rates recorded at HV than at MV. The availability of phosphate is an essential factor in the response of seagrasses to NH_4^+ enrichment because phosphorus (P) plays an important role during NH_4^+ assimilation. P is an important element for metabolic energy transfer (Stitt 1997), and its limitation can depress photosynthesis since it affects the rate of synthesis and regeneration of substrates in the Calvin-Benson cycle (Dietz & Foyer 1986, Woodrow & Berry 1988). Thus, when C turnover increases as a result of N assimilation, the presence of P can ameliorate the negative effect of NH_4^+ as has been demonstrated in *Z. noltii* plants (Brun et al. 2002, 2008). Therefore, the higher phosphate uptake rates recorded in those plants subjected to HV conditions could also contribute to alleviate the toxic effects expected under this treatment. Moreover, the uptake of phosphate increased over time, which could be explained by the increase in the N quota in N-enriched plants. This would typically lead to higher protein and enzyme levels (e.g. Villazán et al. 2013b, 2015) because inorganic phosphate plays an important role in the assimilation process as a part of the metabolic energy transfer molecule (i.e. ATP; Brun et al. 2008, Villazán et al. 2015). Since phosphate uptake follows an active and regulated mechanism depending on plant requirements (Touchette & Burkholder 2000), the higher intracellular NH_4^+ levels would require higher levels of P to maintain the stoichiometric ratios between these 2 limiting nutrients. The same pattern was found for NH_4^+ uptake rates, which also increased over time. The growth of other organisms could contribute to the uptake of nutrients during the course of the experiment. Recent experiments from our research group have demonstrated that after 2 to 3 wk of culturing seagrasses in natural sediments with high levels of NH_4^+ , a large fraction of the NH_4^+ added to the water column was removed by microphytobenthos (F. Moreno-Marín et al. unpubl., B. Villazán et al. unpubl.). Therefore, the growth of microphytobenthos in the sediments of the miniflume tanks can help to explain the recorded increase in nutrient uptake over time in the flumes.

Organisms are generally expected to be more sensitive to a stress factor when simultaneously affected by another stress (Myers 1995). The possible presence of non-additive effects of multiple stressors, as demonstrated in this study, makes predicting the consequences of ongoing climate changes on sea-

grasses more complicated because these changes are expected to alter several environmental and potentially stressful factors at the same time (e.g. temperature, light, hydrodynamics, nutrient availability), which may interact in various ways. Therefore, the study of potential interactive effects among environmental factors that may cause stress in seagrasses is necessary to estimate and to manage the biological consequences of anthropogenic impacts on coastal ecosystems.

Acknowledgements. This study was supported by the national projects CTM2008-00012 (iMacHydro) and CTM2011-24482 (SEA-LIVE). B.V. holds a Formación de Personal Investigador (FPI) grant from the Ministerio de Economía y Competitividad. We thank B. Sinke and J. van Soelen for their technical assistance and Y. Argote Sebio for his valuable help with morphometric measurements. This is CEIMAR Journal publication no. 123.

LITERATURE CITED

- Alcoverro T, Romero J, Duarte CM, López NI (1997) Spatial and temporal variations in nutrient limitation of seagrass *Posidonia oceanica* growth in the NW Mediterranean. Mar Ecol Prog Ser 146:155–161
- Alexandre A, Silva J, Bouma TJ, Santos R (2011) Inorganic nitrogen uptake kinetics and whole-plant nitrogen budget in the seagrass *Zostera noltii*. J Exp Mar Biol Ecol 401:7–12
- Anderson MJ, Gorley RN, Clarke KR (2008) PERMANOVA+ for PRIMER. Guide to software and statistical methods. PRIMER-E, Plymouth
- Borum J, Pedersen O, Greve TM, Frankovich TA, Zieman JC, Fourqurean JW, Madden CJ (2005) The potential role of plant oxygen and sulphide dynamics in die-off events of the tropical seagrass, *Thalassia testudinum*. J Ecol 93:148–158
- Britto DT, Kronzucker HJ (2002) NH_4^+ toxicity in higher plants: a critical review. J Plant Physiol 159:567–584
- Brun FG, Hernández I, Vergara JJ, Peralta G, Pérez-Lloréns JL (2002) Assessing the toxicity of ammonium pulses to the survival and growth of *Zostera noltii*. Mar Ecol Prog Ser 225:177–187
- Brun FG, Pérez-Pastor A, Hernández I, Vergara JJ, Pérez-Lloréns JL (2006) Shoot organization in the seagrass *Zostera noltii*: implications for space occupation and plant architecture. Helgol Mar Res 60:59–69
- Brun FG, Olivé I, Malta E, Vergara JJ, Hernández I, Pérez-Lloréns JL (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, Tomasko D, Touchette B (2007) Seagrasses and eutrophication. J Exp Mar Biol Ecol 67:61–77
- Cabaço S, Machas R, Vieira V, Santos R (2008) Impacts of urban wastewater discharge on seagrass meadows (*Zostera noltii*). Estuar Coast Shelf Sci 78:1–13
- Christianen MJA, van der Heide T, Bouma TJ, Roelofs JGM, van Katwijk MM, Lamers LPM (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
- Collos Y, Harrison PJ (2014) Acclimation and toxicity of high ammonium concentrations to unicellular algae. *Mar Pollut Bull* 80:8–23
- Cornelisen CD, Thomas FIM (2004) Ammonium and nitrate uptake by leaves of the seagrass *Thalassia testudinum*: impact of hydrodynamic regime and epiphyte cover on uptake rate. *Mar Syst* 49:177–194
- Costanza R, d'Arge R, de Groot R, Farber S, and others (1997) The value of the world's ecosystem services and natural capital. *Nature* 387:253–260
- de los Santos CB, Brun FG, Bouma TJ, Vergara JJ, Pérez-Lloréns JL (2010) Acclimation of seagrass *Zostera noltii* to co-occurring hydrodynamyc and light stresses. *Mar Ecol Prog Ser* 398:127–135
- den Hartog C, Kuo J (2006) Taxonomy and biogeography of seagrasses. In: Larkum AWD, Orth LM, Duarte CM (eds) *Seagrasses: biology, ecology and conservation*. Springer, Dordrecht, p 1–23
- Dietz KJ, Foyer C (1986) The relationship between phosphate status and photosynthesis in leaves: reversibility of the effects of phosphate deficiency on photosynthesis. *Planta* 167:376–381
- Duarte CM (1990) Seagrass nutrient content. *Mar Ecol Prog Ser* 67:201–207
- Glibert PM, Allen JI, Bouwman AF, Brown CW, Flynn KJ, Lewitus AJ, Madden CJ (2010) Modeling of HABs and eutrophication: status, advances, challenges. *J Mar Syst* 83:262–275
- Greve TM, Borum J, Pedersen O (2003) Meristematic oxygen variability in eelgrass (*Zostera marina*). *Limnol Oceanogr* 48:210–216
- Halpern BS, Selkoe K, Micheli F, Kappel C (2007) Evaluating and ranking the vulnerability of global marine ecosystems to anthropogenic threats. *Conserv Biol* 21: 1301–1315
- Hemminga MA, Marbà N, Stapel J (1999) Leaf nutrient resorption, leaf lifespan and the retention of nutrients in seagrass systems. *Aquat Bot* 65:141–158
- Invers O, Kraemer GP, Pérez M, Romero J (2004) Effects of nitrogen metabolism and carbon reserves in the temperate seagrass *Posidonia oceanica*. *J Exp Mar Biol Ecol* 303: 97–114
- Kennish MJ (2001) Coastal salt marsh systems in the US: a review of anthropogenic impacts. *J Coast Res* 17: 731–748
- Koch EM (2001) Beyond light: physical, geological, and geochemical parameters as possible submersed aquatic vegetation habitat requirements. *Estuaries* 24:1–17
- Koch EW, Ackerman JD, Verduin JJ, van Keulen M (2006) Fluid dynamics in seagrass ecology – from molecules to ecosystems In: Larkum AWD, Orth RJ, Duarte CM (eds) *Seagrasses: biology, ecology and conservation*. Springer, Dordrecht, p 193–225
- La Nafie YA, de los Santos CB, Brun FG, van Katwijk MM, Bouma TJ (2012) Waves and high nutrient loads jointly decrease survival and separately affect morphological and biomechanical properties in the seagrass *Zostera noltii*. *Limnol Oceanogr* 57:1664–1672
- Lara M, Peralta G, Alondo JJ, Morris EP, González-Ortiz V, Rueda-Márquez JJ, Pérez-Lloréns JL (2012) Effects of intertidal seagrass habitat fragmentation on turbulent diffusion and retention time of solutes. *Mar Pollut Bull* 64:2471–2479
- Mann KH (2000) *Ecology of coastal waters with implications for management*, 2nd edn. Blackwell Science, Oxford
- Marschner H (1995) *The mineral nutrition of higher plants*, 2nd edn. Academic Press, London
- Morris EP, Peralta G, Brun FG, van Duren L, Bouma TJ, Pérez-Lloréns JL (2008) Interaction between hydrodynamics and seagrass canopy structure: spatially explicit effects on ammonium uptake rates. *Limnol Oceanogr* 53: 1531–1539
- Morris EP, Peralta G, Van Engeland T, Bouma TJ and others (2013) The role of hydrodynamics in structuring *in situ* ammonium uptake within a submerged macrophyte community. *Limnol Oceanogr Fluids Environ* 3:210–224
- Myers N (1995) Environmental unknowns. *Science* 269: 358–360
- Orth RJ (1977) Effect of nutrient enrichment on growth of the eelgrass, *Zostera marina*, in the Chesapeake Bay, Virginia, USA. *Mar Biol* 44:187–194
- Peckol P, Rivers JS (1995) Physiological responses of the opportunistic macroalgae *Cladophora vagabunda* (L.) van den Hoek and *Gracilaria tikvahiae* (MacLachlan) to environmental disturbances associated with eutrophication. *J Exp Mar Biol Ecol* 23:122–127
- Pedersen MF, Borum J (1993) An annual nitrogen budget for a seagrass *Zostera marina* population. *Mar Ecol Prog Ser* 101:169–177
- Peralta G, Pérez-Lloréns JL, Hernández I, Vergara JJ (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
- Peralta G, Brun FG, Pérez-Lloréns JL, Bouma TJ (2006) Direct effects of current velocity on growth, morphometry and architecture of seagrasses: a case study on *Zostera noltii*. *Mar Ecol Prog Ser* 327:135–142
- Pérez M, Invers O, Ruiz JM, Frederiksen MS, Holmer M (2007) Physiological responses of the seagrass *Posidonia oceanica* to elevated organic matter content in sediments: an experimental assessment. *J Exp Mar Biol Ecol* 344:149–160
- Quinn G, Keough M (2002) *Experimental design and data analysis for biologists*. Cambridge University Press, Cambridge
- Short FT, McRoy CP (1984) Nitrogen uptake by leaves and roots of the seagrass *Zostera marina* L. *Bot Mar* 27: 547–555
- Stitt M (1997) The flux of carbon between the chloroplast and the cytoplasm. In: Dennis DT, Turpin DH, Lefebvre DD, Layzell DB (eds) *Plant metabolism*. Longman Scientific & Technical, Singapore, p 382–400
- Touchette BW, Burkholder J (2000) Review of nitrogen and phosphorus metabolism in seagrasses. *J Exp Mar Biol Ecol* 250:133–167
- van der Heide T, Smolders A, Rijkens B, van Nes EH, van Katwijk MM, Roelofs J (2008) Toxicity of reduced nitrogen in eelgrass (*Zostera marina*) is highly dependent on shoot density and pH. *Oecologia* 158:411–419
- van Katwijk MM, Vergeer LHT, Schmitz GH, Roelofs JGM (1997) Ammonium toxicity in eelgrass *Zostera marina*. *Mar Ecol Prog Ser* 157:159–173
- Villazán B, Brun FG, Jiménez-Ramos R, Pérez-Loréns JL, Vergara JJ (2013a) Interaction between ammonium and phosphate uptake rates in the seagrass *Zostera noltii*. *Mar Ecol Prog Ser* 488:133–143
- Villazán B, Pedersen MF, Brun FG, Vergara JJ (2013b) Elevated ammonium concentrations and low light form a

- dangerous synergy for eelgrass *Zostera marina*. Mar Ecol Prog Ser 493:141–154
- Villazán B, Salo T, Brun FG, Vergara JJ, Pedersen MF (2015) High ammonium availability amplifies the adverse effect of low salinity on eelgrass *Zostera marina*. Mar Ecol Prog Ser 536:149–162
- Waycott M, Duarte CM, Carruthers TJB, Orth RJ, and others (2009) Accelerating loss of seagrasses across the globe threatens coastal ecosystems. Proc Natl Acad Sci USA 106:12377–12381
- Woodrow IE, Berry JA (1988) Enzymatic regulation of photosynthetic CO₂ fixation in C3 plants. Annu Rev Plant Physiol Plant Mol Biol 39:533–594
- Young IR, Zieger S, Babanin AV (2011) Global trends in wind speed and wave height. Science 332:451–455
- Zar JH (1984) Biostatistical analysis, 2nd edn. Prentice-Hall, Englewood Cliffs, NJ
- Zimmerman RC (2003) A biooptical model of irradiance distribution and photosynthesis in seagrass canopies. Limnol Oceanogr 48:568–585

Editorial responsibility: Christine Paetzold,
Oldendorf/Luhe, Germany

Submitted: October 13, 2014; *Accepted:* January 26, 2016
Proofs received from author(s): February 25, 2016