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Differential ecophysiological responses to inorganic nitrogen sources (ammonium versus nitrate) and light levels in the seagrass *Zostera noltei*

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ABSTRACT: Seagrasses can use both ammonium (NH_4^+) and nitrate (NO_3^-) as inorganic nitrogen (N) sources. However, NO_3^- uptake and assimilation are energetically more expensive and tightly regulated than NH_4^+ uptake. The objective of this study was to test the complex interactive effects between different forms of N enrichment (NH_4^+ and NO_3^-) and light levels on the morphological and physiological traits in the intertidal seagrass *Zostera noltei*. Plants were cultured over 40 d under 2 levels of light (low and high) with 2 inorganic N concentrations supplied at the same dose, NO_3^- (25 µM) and NH_4^+ (25 µM), and a control, following a 2-factorial design. Results showed a differential response in *Z. noltei* depending on the inorganic N source and light dose. NH_4^+ enrichment negatively affected almost all morphometric and dynamic variables analyzed, both in isolation and combined with low light conditions. In contrast, NO_3^- enrichment had a positive effect on *Z. noltei* survival compared with the control treatment in terms of net growth rate and rhizomatic growth, mainly under high light conditions. Therefore, our study demonstrated that the effects promoted by nutrient enrichment largely depend on the source of N used. Light levels play a crucial role in this response by potentially shifting the effects from toxic (under low light) to beneficial (under high light) when NO_3^- is the main N source. Our findings highlight that N form in eutrophication events should be considered when evaluating the potential impacts of nutrient enrichment and light reduction on seagrass communities.

KEY WORDS: Seagrass · Nitrogen metabolism · Ammonium · Nitrate · Eutrophication · Toxicity · Dissolved inorganic nitrogen · Light intensity

1. INTRODUCTION

Seagrasses are coastal foundation species that are among the most productive coastal habitats. They provide a wide range of ecosystem services such as carbon (C) burial, amelioration of natural hazards and habitat and nursery functions (Nordlund et al. 2018). They sit between the land and the sea; therefore, the increase in human population density in coastal zones favors an increase in nutrient loads derived from watersheds and sewage and agricultural runoff, thereby driving eutrophication processes (Vitousek et al. 1997, Verhoeven et al. 2006). Eutrophication negatively affects seagrass ecosystems (Waycott et al. 2009) by both decreasing light levels and increasing the concentrations of dissolved inorganic nitrogen (DIN) (Touchette & Burkholder 2000). DIN usually reaches estuaries in the form of nitrate (NO_3^-) (Weller & Jordan 2020), with ammonium (NH_4^+) making up less than 10% of the DIN in these

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discharges (Ward et al. 2011). However, the load of NH_4^+ in coastal areas has increased worldwide in the last decade (Glibert et al. 2010, Malone & Newton 2020, Peñuelas & Sardans 2022).

Seagrasses typically exhibit higher uptake rates of NH_4^+ than NO_3^- (Lee & Dunton 1999, Dudley et al. 2001), primarily because NH_4^+ assimilation is energetically less costly (Turpin 1991). Previous studies have indicated that a moderate increase in NH_4^+ availability (<10 µM) stimulates seagrass growth and biomass when seagrasses grow under nutrient-limited conditions (e.g. Orth 1977, Alcoverro et al. 1997, Peralta et al. 2003, Invers et al. 2004). However, several studies have also demonstrated that high concentrations of NH_4^+ (~25 µM) can be toxic to some seagrass species in the presence of low light (LL) levels, phosphate deficiency, alkaline pH, high temperature and/or high salinity, among other factors (e.g. Burkholder et al. 1992, van Katwijk et al. 1997, Brun et al. 2002, 2008, van der Heide et al. 2008, Christianen et al. 2011, Villazán et al. 2013). The negative effects of high NH_4^+ concentrations on seagrasses have traditionally been explained by intracellular accumulation of NH_4^+, which can affect internal pH and enzyme kinetics, uncouple photosynthetic ATP production, increase respiration and decrease the uptake of other cations (e.g. Marschner 1995). In addition, continued uptake and assimilation of NH_4^+ can deplete C reserves and thus compete with other C-demanding or energy-consuming metabolic pathways. For example, carbohydrate reserves (mainly in the form of sucrose and starch) in the seagrass *Zostera noltei* have been reported to be crucial for avoiding NH_4^+ toxicity. If internal carbohydrate reserves (mainly sucrose) fall below a critical level, NH_4^+ can become toxic because NH_4^+ is not assimilated into amino acids as a result of the limited available C skeletons (Brun et al. 2002). In the case of NO_3^-, toxicity effects have rarely been described (but see Burkholder et al. 1992, 1994), possibly because of the tight interdependence between nitrogen (N) and C metabolism within plants, which require a continual supply of energy and C skeletons for NO_3^- assimilation and a partitioning of photosynthetic products among carbohydrate synthesis, amino acid synthesis and other plant functions (Huppe & Turpin 1994, Foyer et al. 2001, Stitt et al. 2002). Therefore, different effects have been recorded in seagrasses exposed to high levels of NO_3^-, such as decreases in their C reserves (Jiang et al. 2013), an increased rate of *Labyrinthula zosterae* infection in the presence of the herbicide Diuron (Hughes et al. 2018) or decreased shoot survival under diminished light (Burkholder 2000).

Beyond the likely direct toxic effects of nutrients on seagrasses, a common indirect phenomenon during eutrophication events is diminished light resulting from the proliferation of epiphytes and macroalgae in seagrass communities. Many studies have examined the responses of seagrasses to diminished light (Brun et al. 2008, Collier et al. 2009, Christianen et al. 2011, Serrano et al. 2011) and their subsequent recovery dynamics (Longstaff & Dennison 1999, Longstaff et al. 1999, Bité et al. 2007, Biber et al. 2009, Collier et al. 2009). During periods of depressed photosynthesis caused by light limitation, seagrasses mobilize stored non-structural carbohydrates (NSCs) to maintain metabolic processes (Alcoverro et al. 1999, Ralph et al. 2007). Shading-induced NSC depletion may modify the responses of seagrasses to other environmental stresses, such as high levels of N, because NSC reserves play an important role in determining seagrass growth (Ralph et al. 2007).

To our knowledge, a direct comparison of the 2 common sources of DIN (oxidized vs. reduced) applied at the same dose to a seagrass community is lacking in the literature. This study aimed to fill this research gap by exploring the complex interactive effects between DIN supply in different forms (NH_4^+ and NO_3^-), both alone and combined with light availability (2 contrasting light intensities), on the ecophysiological responses of the intertidal seagrass *Z. noltei*. We examined the effects of light levels and DIN sources on morphometric (i.e. above- [AG] and belowground [BG] biomass and leaf and root lengths [L_L and L_R]), dynamic (i.e. survival, net growth rate [NGR], shoot and internode appearance rates [SAR and IAR], rhizomatic growth rate [RGR]) and physiological traits (i.e. internal N, C and NSC reserves). The fast-growing *Z. noltei* was used as a model species because it is widely distributed along the coasts of the Atlantic Ocean (Green & Short 2003), in areas usually subjected to high nutrient levels that are exhibiting declining seagrass population trends (Short et al. 2011), necessitating protection and monitoring. Although the effects of nutrient enrichment and diminished light on this species have been investigated (e.g. Brun et al. 2002, 2008, Cabaço et al. 2013, Villazán et al. 2013, 2016), most studies have focused on NH_4^+ enrichment, while less attention has been paid to NO_3^- (e.g. uptake processes; Alexandre et al. 2011). We hypothesized that both forms of DIN would have negative effects on this species under LL conditions by increasing the demand on C reserves, whereas we expected to observe positive effects for both DIN forms in plants under high light (HL) conditions.

2. MATERIALS AND METHODS

2.1. Experimental setup

A 2-factorial experiment was conducted at an indoor mesocosm system at the Faculty of Marine and Environmental Sciences of the University of Cádiz in the spring (from March to April). Healthy appearing shoots with intact rhizomes of *Zosters noltei* were collected from an intertidal seagrass meadow at Santibáñez (Cádiz Bay Natural Park; 36.47° N, 6.25° W, Cádiz, Southern Spain), transported to the laboratory and kept in aerated seawater under saturating light (~231 µmol photons m^{-2} s^{-1}; Peralta et al. 2002) in a 16 h light:8 h dark cycle at 15°C for 2 d before the experiment. Apical shoots formed by 2 rhizome internodes with one apical shoot and one lateral shoot, joined to the associated roots, were selected as the experimental plant unit (EPU). Before transplantation, epiphytes were wiped away from shoots with a soft tissue paper.

The EPUs were allocated into 20 l experimental aquaria (n = 18) (Fig. 1), each filled with approximately 2–3 l of pre-washed sandy sediment that had been sieved (1 mm) to remove fauna and large particles and 15 l of sand-filtered seawater from the bay (salinity: approximately 35 psu). The natural seawater used in the aquaria contained low levels of NH_4^+ (0.7 ± 0.06 µM), NO_3^- (0.68 ± 0.12 µM) and phosphate (1.5 ± 0.28 µM; Fig. 1).

Aquaria were illuminated by lamps with cool fluorescent tubes (T5 High Output Blau Aquaristic aquarium color extreme fluorescent bulbs) in a 16 h light: 8 h dark cycle. The water temperature was kept constant at 17°C to achieve optimal growth (Nejrup & Pedersen 2008). Next, 36 EPUs were planted in each of the 18 aquaria (n = 648 EPUs), which were then provided with either 25 µM NH_4^+, 25 µM NO_3^- or no inorganic N (as a control) and exposed to 2 contrasting light levels, corresponding to sub-saturating (LL: 52 ± 5.01 mol photons m^{-2} s^{-1}) and saturating (HL: 262 ± 13.5 mol photons m^{-2} s^{-1}) light conditions for this spe-

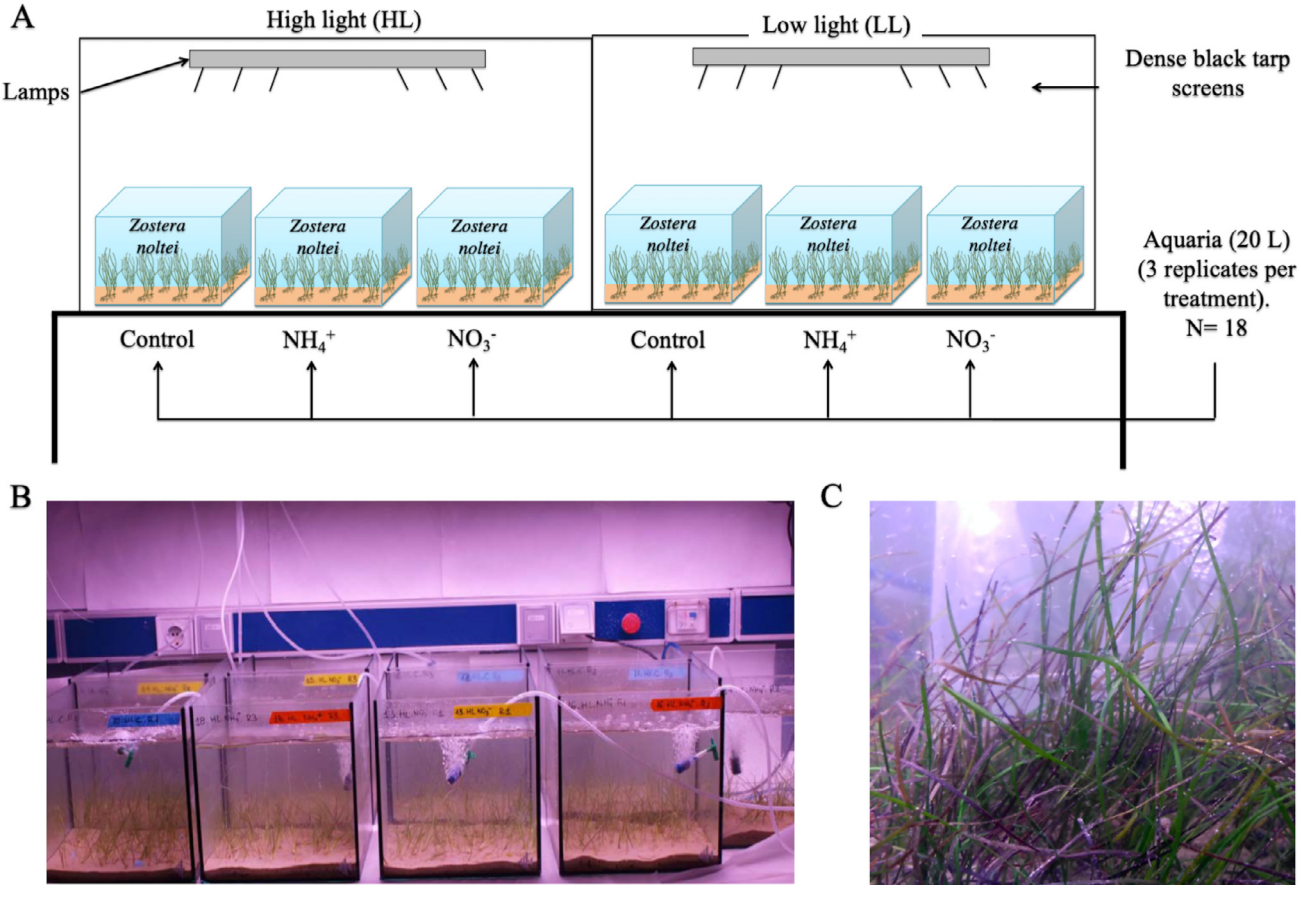

Fig. 1. (A) Simplified diagram of the experimental treatments. (B) Image of several experimental aquaria under high light conditions. (C) *Zostera noltei* in a control treatment

cies (Peralta et al. 2002). NH_4^+ and NO_3^- were added from a stock solution to each aquarium (25 µM treatments) as a daily pulse (375 µmol of both N forms). The NH_4^+ concentration was chosen because concentrations above 25 µM are known to be harmful to *Z. noltei* (Brun et al. 2002, 2008). The same concentration of 25 µM of NO_3^- was also selected so that the N load throughout the experiment was equal to the treatments with NH_4^+. Seawater samples were collected from each aquarium and filtered through Whatman GF/F filters (0.7 µm) before and 10 min after NH_4^+/NO_3^- addition and then were immediately frozen at −20°C for further analysis. Water sampling for analyses was repeated 3 times wk^{-1}, and physico-chemical parameters (i.e. light, temperature, salinity and pH) were monitored on Days 0, 2, 5 and 7 each week during the experiment (40 d). Water in all aquaria was renewed weekly (approximately every 6–7 d) to prevent any excessive accumulation of NH_4^+ or NO_3^-. During water renewal, the aquarium walls were cleaned with soft tissues to remove salt and epiphytes and floating (detached) seagrass leaves. Before and after water renewal, water samples were collected to calculate the nutrient accumulation rate throughout the incubation period. The mean net N uptake rates of NO_3^- and NH_4^+ (µmol N gWW^{-1} d^{-1}) (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m702p057_supp.pdf) were estimated on DIN supply treatments (i.e. LL + NH_4^+, LL + NO_3^-, HL + NH_4^+, HL + NO_3^-) among periods of seawater renewal over the course of the experiment (40 d), based on the total DIN added during the time interval (25 µM DIN multiplied by the number of times DIN was supplied, n), the aquarium volume (V = 15 l) and the amount of DIN before water renewal (DIN_{ren} concentration, µM multiplied by V), divided by the seagrass biomass in the aquarium and by the elapsed time (t) between water renewals. Moreover, aquaria positions were randomly interchanged at each renewal period (i.e. weekly) to minimize the effects of any slight differences in experimental conditions among the treatments (e.g. light or aeration). The analytical methods used to determine NH_4^+ and NO_3^- were essentially an adaptation of the spectrophotometric methods described by Hansen & Koroleff (1999) using a UNICAM UV-1700 PharmaSpec spectrophotometer. NH_4^+ was determined on the basis of the reaction of 120 µl of salicytate-catalyst (reagent A), and 200 µl of nitrate-hypochlorite as a catalyzer (mixing alkaline-citrate and Na-hypochlorite 10%), measured at 64 nm (Bower & Holm-Hansen 1980). NO_3^- measurements were based on the colorimetric measurement of nitrite formed after the reduction of NO_3^- by nitrate reductase. The NO_3^- produced was measured spectrophotometrically (540 nm) after the addition of 600 µl of vanadium chloride plus 150 µl of reagent mix (sulfanilamide acid and N-1-naphthylethylenediamine dihydrochloride) to 750 µl of samples (Schnetger & Lehners 2014). In both cases, standard curves were constructed according to the same procedures with known concentrations of NH_4^+ and NO_3.

2.2. Biological measurements

At the beginning of the experiment, morphometric measurements (L_L, number of leaves and AG and BG biomasses) were conducted on 10 EPUs randomly selected from the pool of collected plants. Before transplantation into aquaria, each EPU was weighed (initial wet weight, WW) and each rhizome was individually tagged with a label. Then each EPU was weighted (g WW), and the initial number of leaves per plant was recorded. At the end of the experiment, all surviving plants were carefully harvested and weighed (mg WW) to estimate the net growth production per EPU (mg WW EPU^{-1} d^{-1}) from the net change in individual plant weight during the experiment. At the end of the experiment, morphometric measurements were collected from all plants (L_L, L_R and internode abundance). In addition, each harvested EPU was split into leaves (AG) and rhizomes/roots (BG), freeze-dried and weighed to determine the AG:BG ratio. According to procedures described in Peralta et al. (2006) and de los Santos et al. (2010) (Table 1), morphometric information was used to calculate plant dynamic properties (survival, NGR, SAR and IAR, RGR), to estimate the growth of the plants over the duration of the experiment (40 d).

2.3. Physiological traits

The concentrations of NSCs (i.e. sucrose and starch) were measured in leaf and rhizome samples (n = 6) from each aquarium at the end of the experimental period. Samples were freeze-dried and ground before analysis. Total NSCs were measured according to Brun et al. (2002). Sugars (sucrose and starch) were first solubilized by 4 sequential extractions in 96% (v/v) ethanol at 80°C for 15 min. The ethanol extracts were evaporated under a stream of air at 40°C, and the residues were then dissolved in 10 ml of deionized water for analysis. Starch was extracted from the ethanol-insoluble residue by incubation for 24 h in 1 N NaOH. The sucrose and

Table 1. Morphometric and dynamic response variables of *Zostera noltei* measured in this study. DW: dry weight; WW: wet weight. Subscripts i and f are initial and final conditions (40 d), respectively; t = time; IA: internode abundance; EPU: experimental plant unit

Variable	Unit	Description
Morphometric variables		
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
Leaf length (L_L)	cm leaf EPU^{-1}	Mean values of shoots for all EPUs in each aquarium
Root length (L_R)	cm root EPU^{-1}	Mean values of roots for all EPUs in each aquarium
Dynamic variables		
Survival (S)	%	S = live EPUs / initial EPUs × 100
Net growth rate (NGR)	mg WW d^{-1} EPU^{-1}	NGR = (biomass$_f$ − biomass$_i$) / (t_f − t_i)
Shoot appearance rate (SAR)	no. shoots d^{-1} EPU^{-1}	SAR = (SA$_f$ / SA$_i$) / (t_f − t_i)
Internode appearance rate (IAR)	no. internodes d^{-1} EPU^{-1}	IAR = (IA$_f$ / IA$_i$) / (t_f − t_i)
Rhizomatic growth rate (RGR)	cm d^{-1}	RGR = (L_{Rf} − L_{Ri}) / (t_f − t_i)

starch content were determined spectrophotometrically with a resorcinol and anthrone assay with absorbances of 486 and 640 nm, respectively, with sucrose as the standard. NSC concentration was calculated as the sum of AG and BG sucrose and starch in each plant (Alcoverro et al. 1999). Total C and N content was determined in duplicate freeze-dried, ground samples of leaves and roots/rhizomes from each aquarium with a Perkin-Elmer 2400 elemental analyzer.

2.4. Statistical analyses

Before any statistical analysis, data were verified for normality (Shapiro-Wilk normality test) and homoscedasticity (Bartlett test for homogeneity of variance test). Repeated measures ANOVA was used to test whether light, salinity and pH at the end of each week varied over the course of the experiment and between treatments. We used 2-factorial permutational multivariate analysis of variance (PERMANOVA) to test the overall effects of N (control, NH_4^+ enrichment and NO_3^- enrichment) and light conditions (LL vs. HL) on morphometric and dynamic variables (i.e. AG:BG ratio, L_L, L_R, internode abundance, survival, NGR, SAR, IAR and rhizome growth rate). The multivariate approach was chosen because some of the measured response variables were likely to be correlated. To test the effects of the treatment factors on each response variable more specifically, after the multivariate analyses we performed univariate PERMANOVA (2- or 3-factorial), as suggested by Quinn & Keough (2002). A 2-way ANOVA was used for sucrose, starch and total N and C content in rhizomes and roots. When ANOVA assumptions were not satisfied (i.e. N and C in leaves), a non-parametric comparison (Kruskal-Wallis matched pairs test) was applied to assess statistically significant differences. When significant differences were found, the Tukey post hoc test was applied to compare both the levels and interaction factors. Data are presented as means ± SE. The significance level (α) was set at 0.05.

To evaluate the additive, synergistic or antagonistic effects of the significant interactions that arose under 40 d of stressors, we compared the observed responses to pairs of stressors with an additive null model (Darling & Côté 2008). We tested whether the effects of combined stress imposed by LL, NH_4^+ enrichment and NO_3^- enrichment were either additive or non-additive (i.e. synergistic or antagonistic) by using the relative response ratios (RR) for each variable in the following equation:

$$RR = (\text{stress treatment} - \text{non-stressed}) / \text{non-stressed} \quad (1)$$

where 'stress treatment' is the measured mean response for each stress treatment (i.e. LL, NH_4^+ or NO_3^- enrichment, and combinations of these treatments), and 'non-stressed' represents the control conditions (i.e. HL, N control). We used an additive null model as the expected additive response (Darling & Côté 2008):

$$RR_{Additive} = RR_{Stressor\ 1} + RR_{Stressor\ 2} \quad (2)$$

Error terms were calculated separately for each RR, and a bootstrap procedure was used to estimate the means and confidence intervals of each response

variable (Efron & Tibshirani 1986). Bootstrap means and confidence intervals were computed by resampling 1500 values among the original data for each parameter with the 'bootES' package v.1.2 in R (Gerlanc & Kirby 2016). Each set of drawn numbers was then combined to estimate relative responses with Eqs. (1) & (2).

We then compared the observed combined response and the expected additive response. If the observed combined response was less than the expected additive response, the effect was classified as antagonistic. Otherwise, if the observed combined response was greater than the expected additive response, the effect was classified as synergistic. If the observed combined response overlapped with the expected additive response, the effect was classified as additive. Statistical analyses were performed in R statistical software v.4.0.2 (R Core Team 2019).

3. RESULTS

3.1. Physico-chemical traits

The temperature in the seawater averaged 16.8 ± 0.06°C, pH averaged 8.04 ± 0.12 and salinity averaged 35.61 ± 0.17 across all treatment combinations and sampling days. Repeated measures ANOVA did not reveal any significant variations in these physico-chemical variables over time or across treatments (all $p > 0.39$). The NH_4^+ and NO_3^- concentrations in the water after enrichment (i.e. 10 min after 25 µM addition) differed considerably depending on the treatment (Table S1) and averaged 0 µM in treatments without N addition (data not shown). NH_4^+ and NO_3^- accumulated in the seawater, particularly under LL. The LL + NH_4^+ treatment, in comparison with the other treatments, showed a continual decrease in NH_4^+ uptake capacity (Fig. S1; ANOVA, $p < 0.001$). The net uptake rate represented a mean value of 15 ± 5% of the DIN added in the LL + NH_4^+ treatment, whereas the rest of the treatments averaged 65–75%, thus indicating accumulation of NH_4^+ in the LL + NH_4^+ treatment over time (Fig. S1).

3.2. Morphometric and dynamic traits

The multivariate response of all morphometric and dynamic variables was affected by both N forms (i.e. NH_4^+ and NO_3^-) and light conditions (LL vs. HL) (Fig. 2, Table S2). However, no significant differences were observed in the interaction between both factors (N and light), except for the maximum leaf length (L_{Lmax}).

L_{Lmax} was significantly affected by the interaction between N and light. Plants growing in LL were longer than those growing in HL under control and NO_3^- treatments (PERMANOVA, $F_{1,2} = 5.30$, $p = 0.002$ and $F_{1,2} = 3.27$, $p = 0.001$; Fig. 2B). In contrast, this pattern was reversed under NH_4^+ loading: L_{Lmax} was lower under the LL + NH_4^+ than HL + NH_4^+ treatment (PERMANOVA, $F_{1,2} = 4.33$, $p = 0.03$). Similar L_{Lmax} values were observed in HL for both N treatments. An inverse pattern was found in the maximum root length (L_{Rmax}); significant differences were detected in HL, with L_{Rmax} decreasing under NO_3^- and NH_4^+ enriched treatments compared with the control (PERMANOVA, $F_{1,2} = 2.56$, $p = 0.002$ and $F_{1,2} = 3.89$, $p = 0.001$; Fig. 2C). Survival increased significantly from 70% (control treatment) to 80% with NO_3^- enrichment in HL (PERMANOVA, $F_{1,2} = 3.03$, $p = 0.002$). However, it was approximately 60% lower in the LL + NH_4^+ than the LL + control treatment (PERMANOVA, $F_{1,2} = 0.79$, $p < 0.001$; Fig. 2D). This decrease was more pronounced in LL for the control vs. NH_4^+ enriched treatment, and survival reached values near 20% in the latter (Fig. 2D). Moreover, in LL, the addition of NH_4^+ resulted in a negative NGR (−1.50 mg WW d^{-1}) compared to those in the HL treatment (5 mg WW d^{-1}). NGR was significantly higher in HL under NO_3^- enrichment than in the control treatment (PERMANOVA, $F_{1,2} = 0.98$, $p = 0.029$; Fig. 2E). A similar pattern was found for the SAR (Fig. 2F). However, NH_4^+ affected the SAR negatively compared to NO_3^- and the control treatment, and the lowest values were observed in LL. The IAR was lower (no significant difference) under the NO_3^- and NH_4^+ treatments than the control treatment in LL. RGR was significantly higher under NO_3^- enrichment than in the control treatment (PERMANOVA, $F_{1,2} = 2.29$, $p = 0.04$) but was slightly lower with NH_4^+ treatment in HL (PERMANOVA, $F_{1,2} = 1.67$, $p = 0.022$; Fig. 2G,H).

3.3. Physiological traits

AG and BG N content was affected by both factors (N and light) and by their interactions. Foliar N was significantly higher under NH_4^+ load than under control and NO_3^- treatments (Kruskal-Wallis test, $\chi^2_{1,5} = 3.056$, $p = 0.031$ and $\chi^2_{1,5} = 1.089$, $p = 0.023$). The N content in rhizomes and roots differed between light treatments and was higher under LL, except in the NH_4^+ treatments, which showed an inverse pattern.

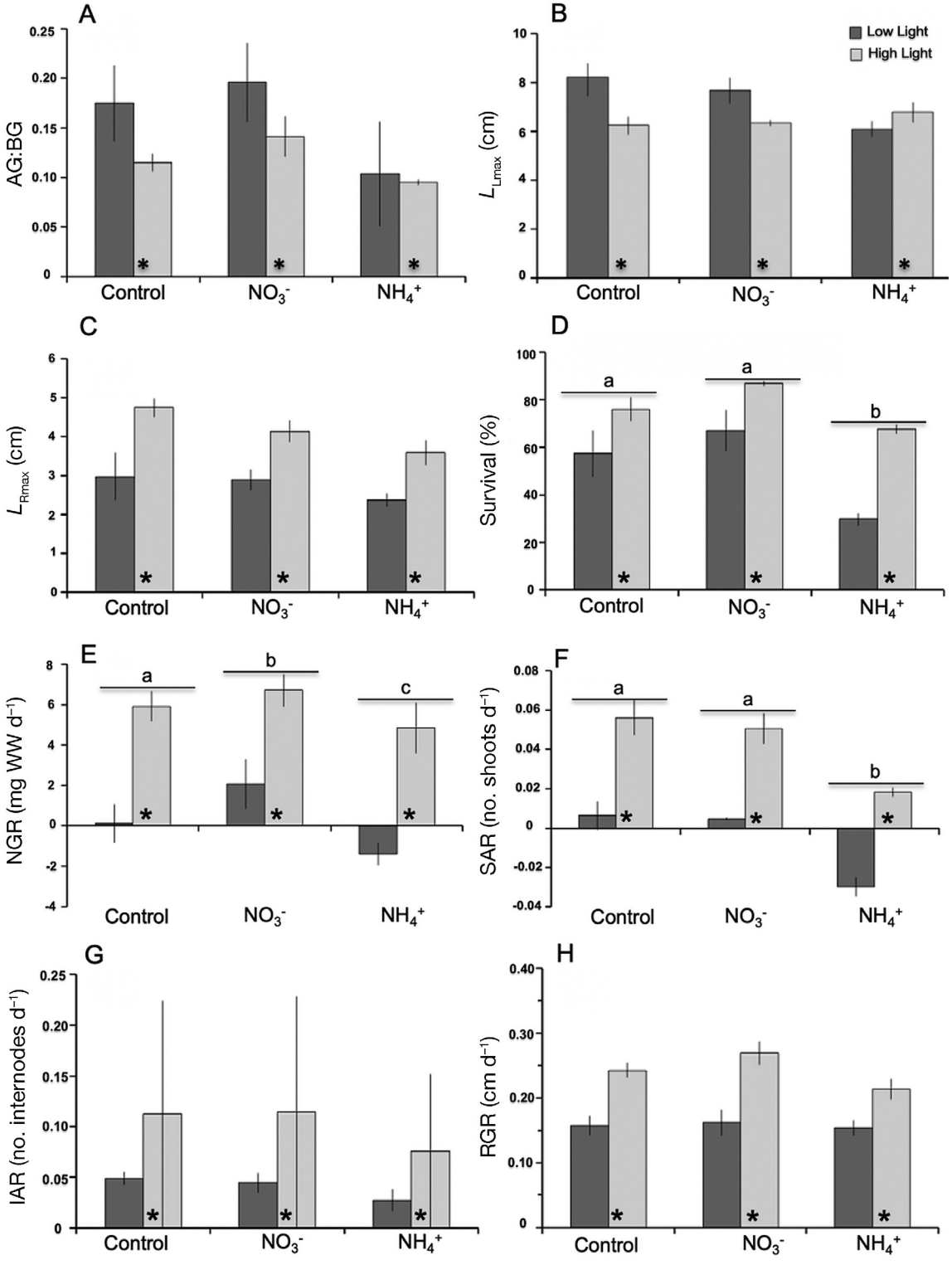

Fig. 2. (A–C) Morphometric and (D–H) dynamic responses of *Zostera noltei* to nitrogen and light treatments. (A) aboveground:belowground (AG:BG) ratio, (B) maximum leaf length (L_{Lmax}), (C) maximum root length (L_{Rmax})), (D) survival, (E) net growth rate (NGR), (F) shoot appearance rate (SAR), (G) internode appearance rate (IAR) and (H) rhizomatic growth rate (RGR). Dissolved inorganic nitrogen supply: 25 µM NH_4^+ or 25 µM NO_3^-; control: no inorganic N added. Horizontal lines with letters above indicate significant differences among nutrient enrichment treatments; asterisks inside bars show significant difference among light treatments. Data represents means ± SE (n = 3)

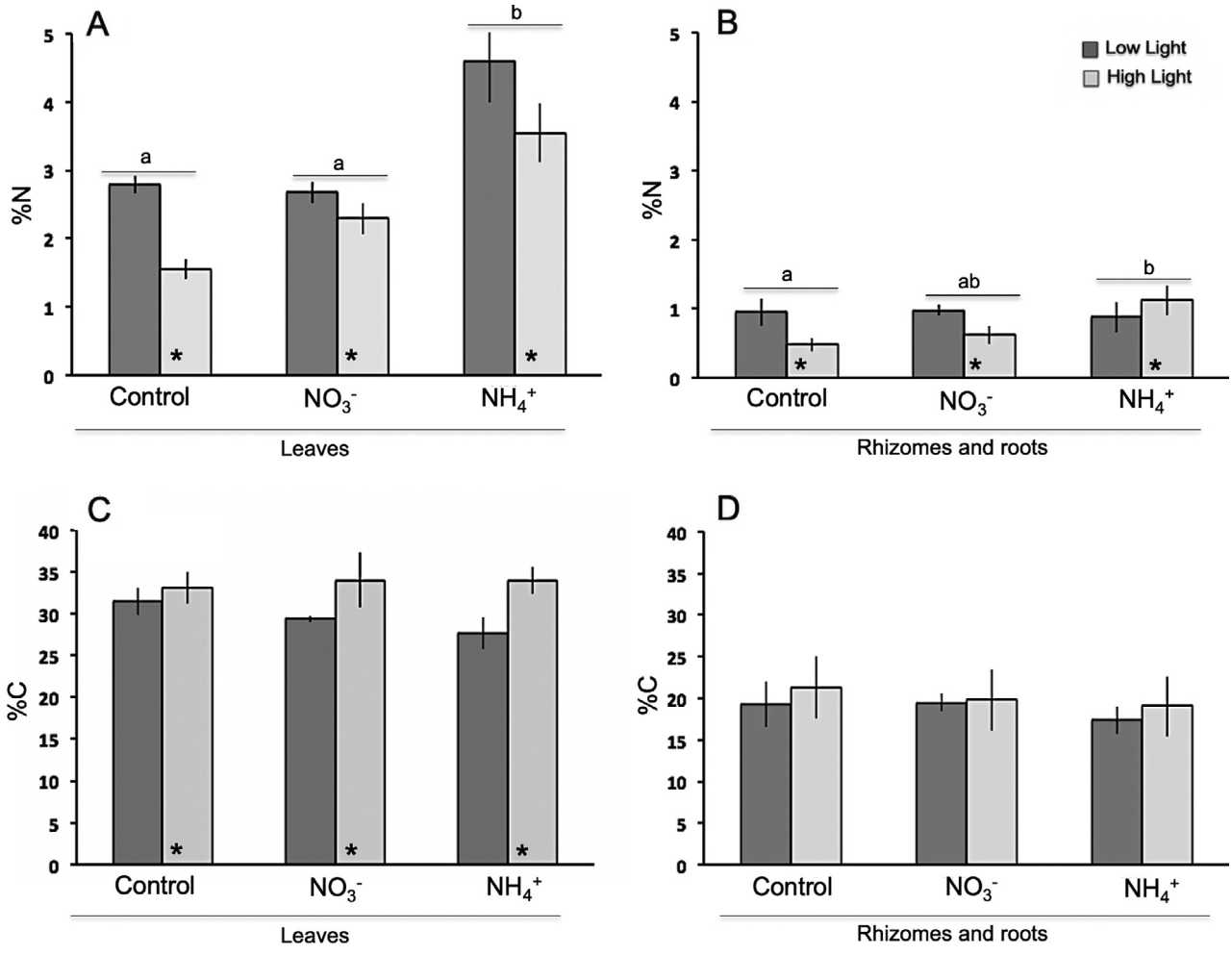

Fig. 3. (A,B) Nitrogen and (C,D) carbon content in (A,C) leaves and (B,D) rhizomes and roots of *Zostera noltei* under different nitrogen and light treatments. See Fig. 2 for further details

The highest N content in rhizomes and roots was found for the HL + NH_4^+ treatment compared with the control and NO_3^- treatments (2-way ANOVA in HL, $F_{1,2}$ = 11.16, p = 0.001 and $F_{1,2}$ = 9.43, p = 0.03; Fig. 3B, Table S3). The foliar C content was influenced by light treatments and was significantly lower in LL under control and nutrient-enrichment treatments (Fig. 3C). However, the C content in rhizomes and roots was not influenced by light or N supply (Fig. 3D).

The sucrose/starch concentrations responded negatively to NH_4^+ enrichment and LL (Fig. 4, Table S3) compared with the controls, but no differences were observed under NO_3^- enrichment (except for a starch increase in rhizomes/roots). Sucrose content (in leaves and rhizome/root parts) was substantially lower under NH_4^+ load and showed the largest decrease in LL, with a foliar sucrose content 40% lower than that in the LL + NO_3^- treatment (2-way ANOVA, $F_{1,2}$ = 1.04, p = 0.0012; Fig. 4A). The content of starch was lower in rhizomes and roots than leaves, and NH_4^+ enrichment resulted in a significantly lower overall starch content than did control and NO_3^- treatments. Plants cultivated under NO_3^- enrichment had significantly higher content of starch in rhizomes and roots than those under the control treatment (2-way ANOVA, $F_{1,2}$ = 0.89, p = 0.010; Fig. 4D) and NH_4^+ treatments (2-way ANOVA, $F_{1,2}$ = 1.45, p = 0.031; Fig. 4D) under HL conditions.

The combined effects of light and NH_4^+ enrichment on the morphometric and physiological responses did not generally differ from the expected additive effects, except in the case of the sucrose content of leaves (Table 2, Fig. 4A), for which the combined effect was higher than the expected additive effect. The combined effects of light and NO_3^- enrichment

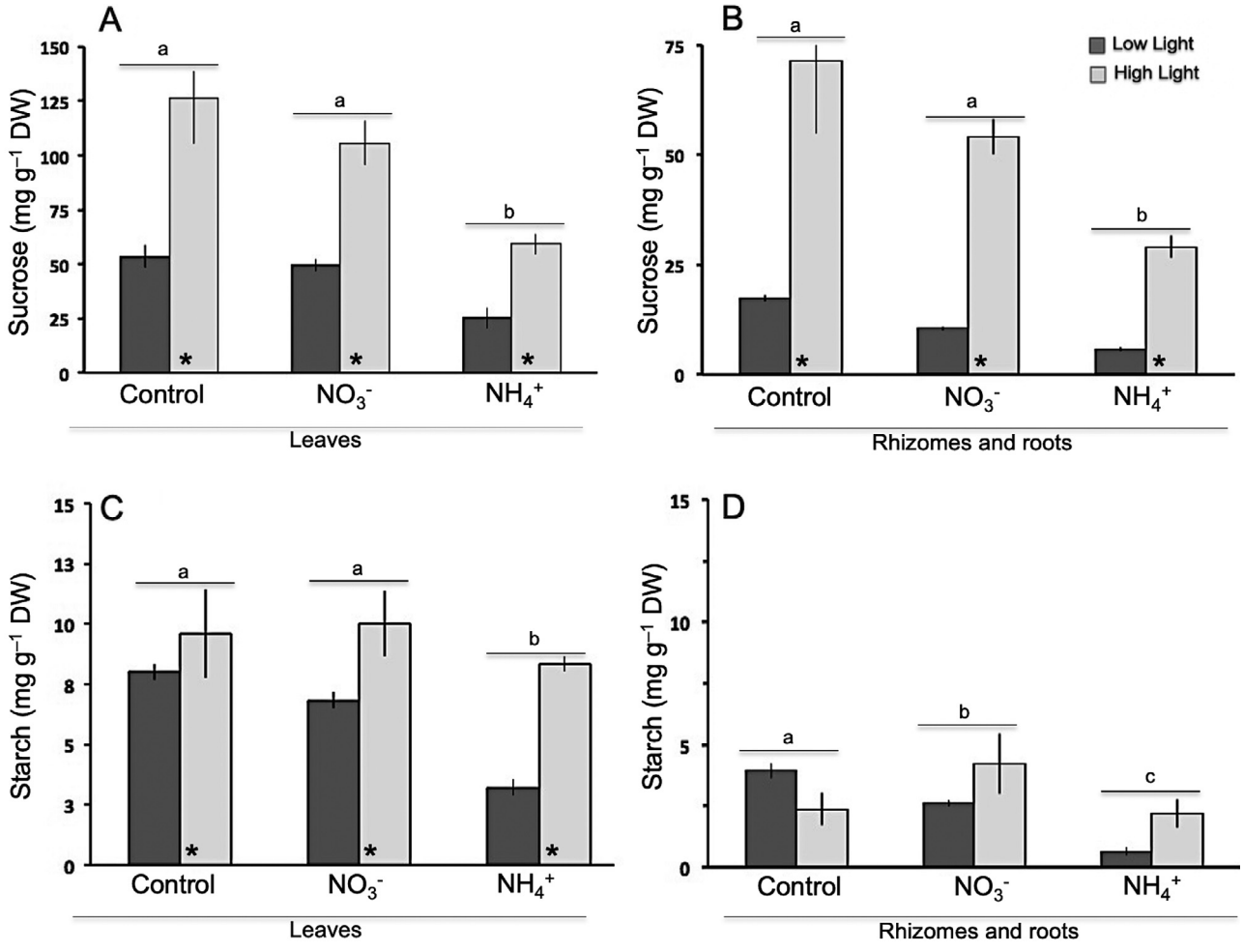

Fig. 4. (A,B) Sucrose and (C,D) starch concentrations in (A,C) leaves and (B,D) rhizomes and roots of *Zostera noltei* under different nitrogen and light treatments. See Fig. 2 for further details

at physiological levels were also additive, but the starch content in rhizome/root parts showed an antagonistic effect because the combined effect was lower than the expected additive effect (Table 2, Fig. 3D).

4. DISCUSSION

As expected, our experiments indicated that diminished light decreased biomass and negatively affected most dynamic parameters in *Zostera noltei*. The ecophysiological responses of *Z noltei* were conditioned by the nature of each DIN form supplied: negative effects were observed with NH_4^+ and neutral and/or slightly positive effects were observed with NO_3^-. In addition, light levels boosted these responses. Therefore, our initial assumption that NO_3^- might have a negative effect on this species was not supported. Instead, a dual behavior of NH_4^+ (i.e. as a nutrient and as a toxic element) was demonstrated and was found to be highly dependent on light levels. The large decrease in AG biomass and SAR observed under LL conditions indicated the high sensitivity of *Z. noltei*. Decreases in AG biomass and shoot density under limited light conditions have often been reported for *Z. noltei* and other seagrass species (Ralph et al. 2007); it has been described as a plasticity mechanism to maximize available understory light by decreasing self-shading in the population (Collier et al. 2012). Meanwhile, leaves were longer under LL than HL conditions. This morphological plasticity is also a well-described mechanism in seagrasses and land plants that substantially enhances light harvesting under LL conditions (Erftemeijer & Stapel 1999, Collier et al. 2007, Ralph et al. 2007,

Table 2. Relative response ratios (Eq. 1) of significant morphometric and physiological variables (see abbreviations in Table 1) in *Zostera noltei* plants when exposed to a single factor: low light (LL), high N (NO_3^- vs. NH_4^+) and when these single factors were combined. The expected additive response is the null model to which the combined response was tested. Values shown are adjusted bootstrap means and 95% confidence interval (in brackets). Add.: additive; Antag.: antagonistic; Synerg: synergistic

	LL alone	High NO_3^- alone	High NH_4^+ alone	Expected additive response (LL + NO_3^-)	Observed combined response (LL + NO_3^-)	Effect	Expected additive response (LL + NH_4^+)	Observed combined response (LL + NH_4^+)	Effect
Survival	−24.4% (−54, −5)	14.5% (5, 27)	−11% (−21, −1)	−9.9% (−49, 22)	−11.6% (−32, 9)	Add.	−35.4% (−74, −6)	−61% (−72, −49)	Add.
NGR	−97.9% (−127, −66)	13.5% (−17, 40)	−17.9% (−67, 15)	−84.4% (−144, −26)	−64.9% (−99, −24)	Add.	−115.8% (−194, −51)	−123.6% (−148, −101)	Add.
SAR	−88.7% (−119, −59)	−9.9% (−42, 24)	−67.3% (−101, −48)	−98.6% (−160, −36)	−91.7% (−119, −71)	Add.	−156% (−220, −107)	−153.4% (−184, −131)	Add.
Sucrose (leaves)	−57.6% (−88, −36)	−16.4% (−54, 19)	−53.1% (−88, −33)	−74% (−142, −17)	−60.8% (−54, 19)	Add.	−110.7% (−176, −69)	−80% (−54, 19)	Synerg.
Starch (leaves)	−16.6% (−67, 7)	4.4% (−48, 34)	−13.3% (−66, 11)	−12.2% (−115, 42)	−28.8% (−48, 34)	Add.	−29.8% (−133, 19)	−66.5% (−48, 34)	Add.
Sucrose (roots/rhizomes)	−75.6% (−110, −49)	−24.1% (−61, 10)	−59.3% (−94, −29)	−99.7% (−172, −40)	−85.3% (−121, −58)	Add.	−134.9% (−204, −78)	−91.9% (−128, −65)	Add.
Starch (roots/rhizomes)	66.2% (4, 116)	77.7% (−17, 193)	−7% (−81, 59)	143.8% (−14, 309)	9.4% (−140, −30)	Antag.	59.2% (−77, 175)	−72.3% (−140, −30)	Add.

Niinemets 2010, Poorter et al. 2019). Indeed, the RGR was also lower under LL conditions, in agreement with findings from previous experiments (Denninson & Alberte 1982, Bintz & Nixon 2001, Peralta et al. 2002). The diminished RGR and branching frequency observed in this work and in other studies (Bulthuis 1983, Abal et al. 1994, Gordon et al. 1994, Vermaat & Verhagen 1996, Krause-Jensen et al. 2000, Peralta et al. 2002) under LL conditions may also explain the observed decrease in SAR observed in our light treatments, as shoot appearance is mainly affected by the growth of the apical shoot in this species (Peralta et al. 2006). Regarding internal composition, the N content in leaves was higher than that in BG tissues (rhizomes and roots) regardless of light level, whereas the N content in both tissues decreased with increasing light levels. Similar responses have been observed in other studies with *Z. noltei* (Pérez-Lloréns & Niell 1993, Vermaat & Verhagen 1996, Peralta et al. 2002). This observation may be explained by dilution processes (Stocker 1980): when N utilization is faster than uptake, stored N resources are gradually diluted during growth. Meanwhile, lower C content in leaves under LL conditions is in concordance with the observed decrease in NSCs, because under such conditions, NSCs are mobilized to meet respiratory demands and balance C budgets (Kraemer & Alberte 1993, Zimmerman & Alberte 1996, Lee & Dunton 1997, Brun et al. 2003a, 2008).

Although the effects of DIN enrichment on seagrasses are well documented, potential differences in ecophysiological responses associated with DIN forms are frequently overlooked. In our study, a significantly higher NGR was observed under NO_3^- enrichment than in the control treatment, independent of light conditions, and survival and SAR of *Z. noltei* were not compromised in these treatments — a finding opposite from our hypothesis. This result indicates that under our control conditions, experimental plants were nutrient-limited, and even under LL conditions, plants benefit from having this surplus of N (i.e. NO_3^-). Moreover, NSCs under LL and HL were similar to those in control treatments, and significant differences were found only in BG starch, thus underscoring the positive effects of NO_3^- in our experimental design. However, opposite results have been found by Burkholder et al. (1992) and Burkholder et al. (1994) in *Z. marina*, in which NO_3^- appeared to damage the plants' meristems and led to leaf loss under pulsed daily additions (approximately 3.5, 7 or up to 10 µM NO_3^- d^{-1} for 14 wk). In studies with other species (e.g. *Thalassia hemprichii*), a neutral effect has been found only with use of NO_3^- (Jiang et al. 2013, Ow et al. 2016), but positive effects in growth and survival under NO_3^- enrichment have

often been observed (Orth 1977, Peralta et al. 2003, van Lent & Verschnure 1995). The lack of consistent results emphasizes the need for direct comparative studies on NO_3^- enrichment and calls attention to the presence of other factors causing stress to the plants, given that negative effects of NO_3^- enrichment are associated with the tight coupling between C and N metabolism and consequently with decreased C-skeleton availability to respond to such additional stress (Jiang et al. 2013, Hughes et al. 2018).

In contrast, NH_4^+ enrichment triggered negative effects independently of light conditions, but also boosted those found under LL conditions. Unlike NO_3^- enrichment, NH_4^+ enrichment led to lower growth, survival and SAR than did control and NO_3^- treatments under both light conditions. Moreover, a remarkable increase in internal N in leaves was observed under NH_4^+ enrichment, reaching concentrations greater than 4% under LL treatments. This value is high, given that the N-limitation threshold in seagrasses is approximately 1.2–1.3% of N in AG biomass (Duarte 1990). The uptake of NH_4^+ occurs primarily through passive and unregulated processes (Britto & Kronzucker 2002) and has a positive linear relationship with external concentrations (Pedersen et al. 1997, Alexandre et al. 2011), thus leading to high internal concentrations of NH_4^+ (Villazán et al. 2015). To limit these toxic effects, plants must assimilate this NH_4^+ into amino acids to prevent intracellular storage of NH_4^+ (Britto & Kronzucker 2002, Marschner 1995, Pedersen et al. 1997, van Katwijk et al. 1997, Villazán et al. 2015), thus increasing the N content in tissues. Similar responses have been observed in laboratory experiments in several species (Longstaff & Dennison 1999, Egea et al. 2018, Moreno-Marin et al. 2018) as well as in seasonal studies in *Z. noltei* beds (Pérez-Lloréns & Niell 1993, Vermaat & Verhagen 1996, Brun et al. 2003b). In contrast, the poor ability of *Z. noltei* to survive under very LL conditions may be explained by the restricted sucrose mobilization throughout the plant under LL levels and the small starch reservoir that this species must use to meet C demands (Brun et al. 2003a). Interestingly, LL and NH_4^+ enrichment affected the lengths of the leaves, following an opposite pattern from that observed in the other LL treatments because the leaves were shorter. This is a clear example of a tradeoff: to improve light harvesting efficiency under LL conditions, leaves must be longer, but longer leaves could increase passive NH_4^+ uptake and consequently exacerbate NH_4^+ toxicity, potentially compromising plant survival. This tradeoff may also partially explain why NH_4^+ accumulated in seawater during the experimental period, particularly in LL treatments, in agreement with the observed decrease in DIN uptake capacity under the LL + NH_4^+ treatment (Fig. S1). Our findings may indicate that NH_4^+ toxicity has a negative feedback effect under LL conditions because NH_4^+ toxicity is concentration-dependent (van Katwijk et al. 1997, Brun et al. 2002, van der Heide et al. 2008). Therefore, the lower the AG biomass (e.g. because of shoot mortality, shorter leaves, lower biomass, etc.), the lower the NH_4^+ uptake from the water, thus increasing the NH_4^+ concentration in the water and enhancing its toxicity in a continuous feedback mechanism.

Although the mechanistic processes underlying physiological responses to separate factors (e.g. light and nutrients) can be explored in indoor mesocosms and are well described here, the complexity in nature —where factors interact simultaneously and plants may have opposing responses (e.g. leaf length) to the factors present—makes the final response difficult to predict. Several meta-analyses have indicated important roles for synergistic and antagonistic effects in marine organisms (Crain et al. 2008, Jackson et al. 2016). Some studies on seagrasses have demonstrated that synergistic interactions occur when plants are exposed to a combination of stressors, such as light, salinity, temperature and eutrophication (Collier et al. 2011, Salo & Pedersen 2014, Ontoria et al. 2019). However, as shown by this study, a large fraction of the responses at the physiological level were additive, which is consistent with previous studies of combined multiple stressors on seagrasses (e.g. Egea et al. 2018, Moreno-Marin et al. 2018). Therefore, stressor responses appear to be highly plastic and context-dependent, and designing ecologically realistic experiments that consider the impact of local stressors (e.g. nutrient inputs) within the context of global stressors (e.g. climate change) will be particularly valuable (Gunderson et al. 2016). In this sense, our results highlight aspects that should be considered in setting up and performing experiments. First, the passive uptake of NH_4^+ (in contrast to NO_3^-) may affect nutrient enrichment, given that some effects were found to depend on the N source used (i.e. NH_4^+ vs. NO_3^-). Furthermore, factors such as hydrodynamics (e.g. narrow boundary layers; La Nafie et al. 2012), temperature (van Katwijk et al. 1997, Brun et al. 2002), pH (van der Heide et al. 2008, Egea et al. 2020), shoot density (van der Heide et al. 2008), salinity (Villazán et al. 2013) and phosphate presence (Brun et al. 2008), among others, can influence NH_4^+ effects. Moreover, other plant traits may affect the whole response of the plant to combined

stressors. In addition, most of these interrelationship pathways are bidirectional and also affect habitat complexity (van der Heide et al. 2008) and secondarily affect the whole seagrass community (e.g. herbivore and filter-feeder abundance; Jiménez-Ramos et al. 2017).

In summary, our study showed that the form of DIN supplied (reduced vs. oxidized) is of critical importance in seagrass ecosystems because different forms may have opposite ecological consequences. *Z. noltei* exhibited a positive response under NO_3^- enrichment independent of light conditions, but showed diminished growth, survival and NSCs with NH_4^+ enrichment, mainly under LL conditions. Although we found positive effects of NO_3^- enrichment, extrapolation of these results to *in situ* conditions must be performed with caution, as complex relationships in the ecosystem and other indirect effects (e.g. increasing photosynthetic growth, decreasing C reserves within the plant, enhancing the settling of organic matter into the sediment, etc.) may blunt this initially beneficial effect.

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