



High ammonium availability amplifies the adverse effect of low salinity on eelgrass *Zostera marina*

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ABSTRACT: Climate change intensifies the frequency and intensity of rainfall events, which increases the discharge of freshwater and nutrients to coastal areas. This may lower salinity and increase nutrient availability and, thus, affect estuarine eelgrass populations. We studied the interactive effect of increasing NH_4^+ levels and low salinity on estuarine eelgrass *Zostera marina*, grown in microcosm at various combinations of NH_4^+ enrichment (0, 10 and 25 μM) and salinity (5, 12.5 and 20). Increasing NH_4^+ had a positive effect on eelgrass performance as long as salinity was kept at ambient level (20). N enrichment was followed by an increase in pigments, photosynthesis and various growth variables and a decrease in stored carbon concentrations (sucrose and starch). Low salinity had an overall negative effect on plant fitness; pigment concentration, photosynthesis and growth were reduced while mortality increased. Exposure to low salinity was also followed by a decrease in sucrose, suggesting that it was used as an osmolyte and/or that photosynthesis could not cover energy requirements needed for osmoregulation or repairing processes. Concomitant exposure to high NH_4^+ and low salinity turned the positive effect of NH_4^+ into a strong, negative synergistic effect. Several growth-related variables were affected significantly and mortality increased substantially. We suggest that this simultaneous exposure intensified competition for energy and C skeletons affecting other metabolic processes (e.g. growth, repair processes) negatively. Our results suggest that climate change driven alterations in precipitation and NH_4^+ loading might seriously impact estuarine eelgrass communities.

KEY WORDS: Carbon reserves · Dissolved inorganic nitrogen · Eutrophication · Hyposalinity · Osmoregulation · Seagrass

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INTRODUCTION

Eelgrass *Zostera marina* L. is the dominant seagrass species in the Northern Hemisphere. Eelgrass is especially common in estuaries (Short et al. 2007) where it occurs at salinities ranging from 5 to 35 (den Hartog 1970). Most estuaries are influenced by substantial freshwater inputs (from precipitation, riverine run-off and point sources), which also carry large

amounts of particulate and dissolved nutrients from the catchment area (Howarth et al. 1996). Ongoing climate changes have several potential consequences (e.g. rising temperatures, more frequent heat wave events, sea-level rise, higher frequency of severe storms) that may affect life in the sea. The frequency and intensity of precipitation events are predicted to increase in NW Europe and NE America (Meier et al. 2012, IPCC 2013), which is likely to

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increase the discharge of nutrient- and particle-rich freshwater to estuarine systems and, thus, potentially alter both the salinity and nutrient loads in these habitats.

Both increased nutrient loading and altered salinity regimes may affect seagrass fitness negatively and, thus, contribute to the loss of seagrass populations (Zieman 1975, Rudnick et al. 2005, Kahn & Durako, 2006, Jiang et al. 2013). High nutrient loading causes accumulation of drift macroalgae, epiphytes and phytoplankton, which may impair light conditions for seagrasses (Sand-Jensen & Borum 1991, McGlathery 2001, Brun et al. 2003, Lyons et al. 2012). Nutrient enrichment may further intensify the flux of organic matter to the sediment, thereby enhancing the risk of anoxia and sulphide toxicity (Holmer & Bondgaard 2001, Borum et al. 2005, Pérez et al. 2007, Olivé et al. 2009). While relatively small increases in the availability of dissolved inorganic nitrogen (DIN) (NH_4^+ and NH_3^-) may stimulate growth of seagrasses in pristine, oligotrophic environments (e.g. Short 1987, Powell et al. 1989, Short et al. 1990, Perez et al. 1994, Alcoverro et al. 1997), higher concentrations of NH_4^+ and NH_3^- can be toxic to seagrasses (e.g. Burkholder et al. 1992, 1994, van Katwijk et al. 1997). High availability of dissolved DIN may lead to accumulation of NH_4^+ within plant cells, which can affect intracellular pH, enzyme kinetics, photosynthetic ATP production and uptake of other ions (Marschner 1995, Britto & Kronzucher 2002). High availability of DIN may further lead to an imbalance in the C economy in seagrasses because intracellular accumulation of NH_4^+ stimulates N assimilation (i.e. the synthesis of amino acids and proteins), which increases the need for energy and C skeletons and, thus, intensifies the competition with other metabolic processes for these resources (Brun et al. 2002, 2008, Villazán et al. 2013b).

Exposure to non-lethal, but sub-optimal salinity alters the ionic composition in cells, disturbs membrane integrity, affects enzyme kinetics and may thus lead to lower photosynthetic rates, growth and reproduction, and eventually increase mortality in seagrasses (Irlandi et al. 2002, Kahn & Durako 2006, Chollett et al. 2007, Nejrup & Pedersen 2008, Salo & Pedersen 2014, Salo et al. 2014, Sandoval-Gil et al. 2014). Exposure to hypo- or hypersaline conditions requires osmoregulation to sustain cell turgor and a favorable ionic composition within cells. Exposure to hypo- or hypersaline conditions is therefore followed by elevated energetic expenditures related to uptake and export of ions, synthesis or breakdown of organic osmolytes and repair processes (Hellebusi 1976, Karsten 2012).

The individual effects of low salinity and NH_4^+ enrichment on seagrasses are generally well studied, whereas studies on the combined effects of multiple stressors on seagrasses are relatively rare (but see Kahn & Durako 2006, Jiang et al. 2013, Villazán et al. 2013b, Salo & Pedersen 2014). Marine organisms are typically exposed to more than one environmental stressor at a time and organisms are generally expected to be more sensitive to a given stressor when simultaneously affected by another (e.g. Myers 1995, Paine et al. 1998). The combined effect of 2 or more stressors may be additive or non-additive, while non-additive effects may be synergistic or antagonistic; some studies indicate that the effects of multiple stressors on seagrasses can be synergistic (e.g. La Nafie et al. 2012, Salo & Pedersen 2014). The possible presence of non-additive effects of multiple stressors makes it difficult to predict consequences of ongoing climate changes on seagrasses because these changes are expected to alter several environmental and potentially stressful factors at the same time (e.g. temperature, light conditions, salinity, nutrient availability). If the combined effect of multiple stressors is synergistic then the predictions based on the impact of single stressors (i.e. assuming additive effects) will underestimate the total effect of changes in these stressors (Darling & Côté 2008).

Estuarine seagrasses are exposed to short- and long-term variations in both salinity and NH_4^+ concentration, and ongoing climate change is expected to increase fluctuations in these variables, which will likely alter the level of stress (strength and duration) imposed on these organisms. It is therefore valuable to study potential interactive effects between environmental factors that may stress eelgrass. We hypothesize that stress imposed by low salinity and high NH_4^+ availability will lead to an increased competition for energy and carbon within plants and we expect these to have either additive or synergistic impacts on plant fitness. We tested therefore if low salinity enhanced the expected adverse effect of NH_4^+ enrichment in eelgrass in a factorial experiment with 3 levels of NH_4^+ availability (target concentrations: 0, 10 and 25 μM) and 3 levels of salinity (5, 12.5 and 20) using a range of physiological response variables (e.g. photosynthesis, pigment concentrations, growth, survival, nutrient uptake rates) that are indicators of plant fitness. In addition, we measured intracellular concentrations of total N, NH_4^+ , Na^+ , K^+ , Cl^- and non-structural carbohydrates to investigate the underlying mechanisms for the observed physiological responses.

MATERIALS AND METHODS

Experimental setup

A 2-factorial aquaria experiment was conducted during October and November 2012 to test how NH_4^+ availability and salinity affected eelgrass response. Eelgrass shoots were collected from Isefjorden (55° 42' 44" N, 11° 47' 35" E), Denmark, at a depth of 0.5 to 1 m at the beginning of October 2012. Water temperature at the sampling site was ca. 16°C and salinity was ca. 20. Healthy looking shoots with intact rhizomes and roots were transferred to the laboratory where they were kept for 2 to 3 d in aerated seawater under sub-saturating light (ca. 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) at 15°C. Shoots were standardized to have 3 to 4 (visible) leaves and 3 rhizome internodes (by removing older leaves and rhizome internodes) prior to the experiment. 27 aquaria (volume = 20 l) were filled with 2 to 3 l of sediment from the Isefjord and 15 l of water with salinity of 20 mixed from seawater (salinity 30) and tap water.

Twelve haphazardly chosen eelgrass shoots were planted in each of the 27 aquaria, which were then subjected to a factorial design with 3 target concentrations of NH_4^+ (0, 10 and 25 μM ; treatments hereafter called 'control', +N and +NN, respectively) and 3 levels of salinity (5, 12.5 and 20), with 3 replicate aquaria in each treatment combination. Target levels of salinity in the aquaria were obtained by lowering the salinity by ca. 3 units every second day. The first pulse of NH_4^+ was added 3 d after the target levels of salinity were reached in all the aquaria.

The water used in the experiment contained low levels of NH_4^+ (ca. 0.5 μM). Additional NH_4^+ was added to the aquaria (in the +N and +NN treatments) from an NH_4Cl stock-solution every day. Concentrations of NH_4^+ were selected to correspond to those in a previous study on NH_4^+ effects on eelgrass (Villazán et al. 2013b). The daily loading of NH_4^+ to each aquaria corresponded to 0 μmol in the control treatment, 150 $\mu\text{mol d}^{-1}$ in the +N treatment and 375 $\mu\text{mol d}^{-1}$ in the +NN treatment, respectively. Concentrations of NH_4^+ were measured twice weekly in all aquaria; water samples were collected before and after nutrient additions and analyzed in accordance with Bower & Holm-Hansen (1980).

The concentration of NH_4^+ in water before adding a new pulse of NH_4^+ was close to zero in most treatments (Table 1), although accumulation of NH_4^+ was observed in aquaria from the low salinity (5) to the high N (25 μM) treatment (Table 1). The experiment lasted for 4 wk after the first NH_4^+ addition.

Dissolved inorganic carbon (DIC) concentrations in the water were measured twice (2nd and 4th week) in all aquaria by Gran titration. DIC concentrations averaged $3.37 \pm 0.06 \text{ mM}$ (mean \pm SE), across 3 replicate aquaria and over 2 sampling dates in each of the 9 treatment combinations, which is high enough to saturate DIC uptake and photosynthesis in eelgrass (Sand-Jensen & Gordon 1984). Light intensity above the aquaria was close to the saturating level for growth (ca. 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$; Olesen & Sand-Jensen 1993) and was provided by halogen lamps (12 V, 35 W) in a 16:8 h light:dark cycle. Water temperature was kept constant at 15°C to obtain optimal growth conditions (Nejrup & Pedersen 2008). The water was aerated to ensure mixing and about half the volume in each aquarium was changed weekly to reduce the risk of C, phosphorus and/or micronutrient limitation and to prevent growth of epiphytes.

Physiological and morphological responses

Maximum net photosynthetic rate (P_{max}) and dark respiration was measured as O_2 production and consumption under saturating light and in darkness, respectively. At the conclusion of the experiment (during the last week) 2 randomly chosen shoots from each aquarium were incubated in an 800 ml gas-tight, transparent chamber equipped with a circulation pump (AquaBee, 300 l h^{-1}) to ensure

Table 1. Average ammonium concentrations (μM) in aquaria over the course of the experiment to study the interactive effect of increasing NH_4^+ levels and low salinity on estuarine eelgrass *Zostera marina*. Target concentrations of NH_4^+ were 0 μM (control), 10 μM (+N) and 25 μM (+NN). Samples for these measurements were taken before and after adding new ammonium in each treatment combination. NH_4^+ values are shown as mean \pm 1 SE across 3 replicate aquaria and over 16 sampling events in each treatment

Sampling time	Salinity	NH_4^+ (μM)		
		Control	+N	+NN
Prior to adding NH_4^+	20	0.35 \pm 0.12	0.39 \pm 0.20	2.58 \pm 1.22
	12.5	0.43 \pm 0.13	0.70 \pm 0.29	4.84 \pm 1.41
	5	0.09 \pm 0.07	1.29 \pm 0.44	15.81 \pm 3.20
After adding NH_4^+	20	0.35 \pm 0.12	9.95 \pm 0.48	25.90 \pm 1.45
	12.5	0.24 \pm 0.22	10.82 \pm 0.30	29.10 \pm 1.68
	5	0.10 \pm 0.07	12.52 \pm 0.92	41.62 \pm 4.09

water movement within the chamber. The chamber was filled with seawater (salinity corresponding to that in the respective aquaria). To prevent O₂ saturation in the chambers during incubations, the O₂ level of the water was reduced to ca. 70% of air saturation by bubbling with gaseous N₂. The chamber was finally submerged into a water bath at constant temperature (15°C) and equipped with a Clark-type O₂ microelectrode (OX-500, Unisense) connected to a pico-amperimeter (Picoammeter PA2000, Unisense) and to a data logger (ADC-16, Pico Technology). A lamp with 8 halogen spots (OSRAM Decostar 51; 12 V, 35 W) illuminated the set-up (375 μmol photons m⁻² s⁻¹) for the P_{\max} measurements. The O₂ concentrations were recorded every minute and rates of O₂ release or consumption (mg O₂ g⁻¹ dry weight [DW] h⁻¹) were calculated from periods with a constant change in O₂ concentration over a minimum of 15 min.

Chlorophyll fluorescence was measured using PAM fluorometry (Hansatech Instruments). Maximum quantum yield (F_v/F_m) of photosystem II was measured on 3 randomly selected and dark-adapted (15 min) plants from each aquarium.

Fresh biomass of each plant was measured at the start and at the end of the experiment. Net production (NP; g fresh weight [FW] plant⁻¹ d⁻¹) was estimated from net changes in individual plant weights (FW) over the course of the experiment. Leaf elongation rate (LER) was measured using the plastochrone interval technique, as described by Sand-Jensen (1975). Appearance of new internodes per shoot (IAR) was recorded, and mortality rates were determined from the final number of dead plants in each aquarium at the end of the experiment. Leaf necrosis was estimated as the proportion of dark brown or black colored leaf tissue in the first 3 leaves of each shoot in accordance with van Katwijk et al. (1997).

Nutrient uptake rates

Uptake rates of PO₄³⁻ and NH₄⁺ were measured on whole eelgrass shoots with attached roots and rhizomes using the perturbation method (Pedersen 1994) during the last week of the experiment. Two randomly selected plants from each aquarium were introduced into a glass chamber containing 1 l of seawater with the same salinity as in the respective treatment. Chambers were kept at 15°C and the water in each chamber was bubbled with air to ensure mixing and to reduce boundary layer effects.

Nutrients (NH₄⁺ or PO₄³⁻) were added to each chamber from stock solutions (NH₄Cl or KH₂PO₄, respectively) to obtain initial concentrations of 25 μM of NH₄⁺ or 8 μM of PO₄³⁻. Triplicate water samples were taken after 0 and 120 min, respectively. Incubation time was selected according to the existing literature on seagrass nutrient uptake, where 120 min is often used to avoid nutrient depletion in the culture medium (Touchette & Burkholder 2000, Villazán et al. 2013a).

Concentrations of NH₄⁺ and PO₄³⁻ were analyzed immediately in accordance with Bower & Holm-Hansen (1980) and Murphy & Riley (1962), respectively. At the end of each uptake measurement, plants were weighed (FW) and returned to their aquaria. Nutrient uptake rates (V , μmol g⁻¹ FW h⁻¹) were estimated from changes in nutrient concentration (S) over the course of the incubation using Eq. (1), where S_0 and vol_0 are the initial nutrient concentration and water volume, S_f and vol_f are the final nutrient concentration and water volume, respectively, and t is the time elapsed between sampling events.

$$V = \frac{(S_0 \times vol_0) - (S_f \times vol_f)}{(t \times FW)} \quad (1)$$

Biochemical responses

Biochemical responses were analyzed at the end of the experiment. Chlorophyll_{a+b} concentrations were determined on duplicate leaf samples from each aquarium. Samples were freeze-dried to constant weight (i.e. DW), ground and extracted overnight in 96% ethanol. The extractant was homogenized and filtered before determining the chlorophyll concentrations spectrophotometrically at 665 and 649 nm (Wintermans & DeMotts 1965). Total N content was determined on duplicate freeze-dried and ground samples of leaves from each aquarium using a Carlo-Erba NA-1500 CNS analyzer.

Intracellular concentrations of inorganic ions (NH₄⁺, Na⁺, K⁺ and Cl⁻) were determined in leaf samples from one randomly selected plant from each aquarium. Leaves were cleaned for epiphytes and salts using MilliQ water, dried to constant weight at 60°C and ground. The plant material was dissolved in 15 ml of 3.5 mM HNO₃, stirred for 30 min and subsequently centrifuged at 2000 × g for 5 min. The supernatant was passed through a 0.45 μm filter and concentrations of NH₄⁺, Na⁺, K⁺

and Cl^- were finally analyzed using an ion chromatograph (Metrohm 850 ProfIC AnCat-MCS) with chemical suppression and conductimetric detection (Marín-Guirao et al. 2013).

Concentrations of sucrose and starch were measured on triplicate, freeze-dried and ground leaf and root/rhizome samples from each aquarium. Total non-structural carbohydrates (TNC) were measured following Brun et al. (2002). Sugars (sucrose and hexoses) were first solubilized by 4 sequential extractions in 96% (v/v) ethanol at 80°C for 15 min. Ethanol extracts were evaporated and residues were dissolved in deionized water for analysis. Starch was extracted from the ethanol-insoluble residues by incubation in 1 N NaOH for 24 h. Sucrose and starch contents of extracts were determined spectrophotometrically using resorcinol and anthrone assays at 486 and 640 nm, respectively, with sucrose as a standard.

Statistical analysis

The experiment was originally designed as a 2-factorial experiment with categorical factors salinity and NH_4^+ treatments. However, we were unable to keep NH_4^+ concentrations constant (across levels of salinity within each category of NH_4^+) so we decided to treat NH_4^+ treatment as a continuous (i.e. covariate) variable. We used therefore permutational ANCOVA with one continuous variable (NH_4^+ concentration) and 1 (salinity) or 2 (salinity and plant part) categorical variables to analyze the data. Analyses were conducted with the PERMANOVA package in PRIMER+ v. 6 (Anderson et al. 2008). Categorical factors were considered fixed and all response data and the covariate (i.e. average NH_4^+ concentration in each aquarium) were $\log(x+1)$ transformed prior to analysis to increase linearity between the response and NH_4^+ concentration. Resemblance matrixes were based on Euclidean distances and the analyses were conducted using Type I SS with 9999 permutations under a reduced model (Anderson et al. 2008). MDS plots and PERMDISP were used to inspect the dispersion of the data. All tests were carried out using $\alpha = 0.05$.

Permutational multivariate ANCOVA was first used to test the overall effect of NH_4^+ enrichment and salinity on all physiological and morphological variables, i.e. P_{\max} , respiration, F_v/F_m , leaf elongation, number of new internodes, number of standing leaves, net production, necrosis, mortality and nutrient (PO_4^{3-} and NH_4^+) uptake rates. Data were nor-

malized prior to analysis to minimize scale differences among response variables. The multivariate approach was chosen because many of the measured response variables were likely inter-correlated. The effect of treatment factors on each individual response variable was subsequently analyzed using univariate 1-factor ANCOVA as recommended by Quinn & Keough (2002). For sucrose and starch, we used univariate 2-factor ANCOVA including plant parts (i.e. leaves and roots/rhizomes) as a second categorical factor.

A significant interaction in the ANCOVA analyses mentioned above indicates a non-additive (i.e. synergistic or antagonistic) effect and we tested therefore whether combined stress imposed by low salinity and high ammonium was additive or non-additive. We did so for the physiological and morphological response variables in Table 2 (below) by estimating the relative response ratio (RR) for each variable as:

$$\text{RR} = \frac{\text{Stress treatment} - \text{Non-stressed}}{\text{Non-stressed}} \quad (2)$$

where 'Stress treatment' and 'Non-stressed' are the measured mean responses in the stress treatments (i.e. low salinity, high N and the combination of low salinity and high N, respectively) and the non-stressed situation (i.e. high salinity and low N), respectively. We used an additive null model as the combined stressor expectation (Darling & Côté 2008):

$$\text{RR}_{\text{Additive}} = \text{RR}_{\text{Stressor 1}} + \text{RR}_{\text{Stressor 2}} \quad (3)$$

The relative responses were calculated as combinations of individual variables, each measured with their own error and the bootstrap procedure (Efron & Tibshirani 1986) was therefore used to estimate means and confidence intervals of each response variable. Bootstrap means and confidence intervals were computed for each response variable by first resampling 1500 values among the original data for each parameter. Each set of drawn numbers was then combined to estimate relative responses using Eqs. (2) & (3). The 1500 resulting estimates were then organized into 500 data sets, each with $n = 3$ as in the original data and means were estimated for each. The 500 new mean values were finally used to estimate bootstrap means and 95% confidence intervals for each response variable. Confidence intervals were estimated from the 2.5 and 97.5 percentiles of the 500 means obtained by permutation (Meyer et al. 1986).

RESULTS

Physiological and morphological variables

The multivariate response of all physiological and morphological variables was affected by the interaction between NH_4^+ and salinity (Table 2). Enrichment with NH_4^+ had an effect on the multivariate response at salinity 20, but not at 12.5 and 5. High NH_4^+ loading altered most of the individual response variables negatively at salinity 5, whereas there was no clear or even a positive effect of NH_4^+ enrichment at higher salinities.

P_{max} was affected by the interaction between NH_4^+ and salinity (Fig. 1A, Table 2). P_{max} was unaffected by NH_4^+ at salinity 5 and 12.5, but increased from 1.63 to 5.26 $\text{mg O}_2 \text{g}^{-1} \text{DW h}^{-1}$ with increasing NH_4^+ concentration at salinity 20. Subsequent analysis showed that the combined effect of both stressors was synergistic (Table 3). Dark respiration rate (R_D) was influenced by salinity, but not by NH_4^+ (Table 2, Fig. 1A), being significantly higher at salinity 5 than at 12.5 and 20. F_v/F_m was only affected by salinity (Table 2), being lower at salinity 5 than at 12.5 and 20 (Fig. 1B).

The interaction between NH_4^+ and salinity affected leaf elongation rate (Table 2), which was reduced by ca. 50% with increasing NH_4^+ at low salinity, but increased with increasing NH_4^+ -loading at salinities 12.5 and 20 (Fig. 1C). The combined effect of low salinity and high NH_4^+ was synergistic (Table 3). Internode appearance rate (IAR) was only affected significantly by salinity (Fig. 1D, Table 2), being lower at salinity 5 than at 12.5 and 20. The final number of leaves per shoot was affected by the interaction between NH_4^+ and salinity (Fig. 1E, Table 2) and the combined effect was synergistic (Table 3): plants had less leaves per shoot at salinity 5 and the number decreased with increasing NH_4^+ load. No effect of NH_4^+ was evident at salinity 12.5 and 20. Net production was affected by the interaction between NH_4^+ and salinity (Fig. 1F, Table 2), being low and decreasing with increasing NH_4^+ (from 8 $\text{g FW plant}^{-1} \text{d}^{-1}$ in the control to ca. 2 $\text{g FW plant}^{-1} \text{d}^{-1}$ in the +NN treatment) at low salinity, but increasing slightly with increasing NH_4^+ -load at salinities 12.5 and 20 (Fig. 1F). The combined effect of low salinity and high NH_4^+ was synergistic (Table 3). Necrosis was affected by salinity, being more widespread at salinity 5 than at 12.5 and 20. Necrosis was not affected by NH_4^+ , nor by the interaction between NH_4^+ and salinity (Fig. 1G, Table 2). Mortality was influenced by both NH_4^+ and salinity (Fig. 1H, Table 2) being close to 0 at salinity 20 across all NH_4^+ -treatments, but increasing with NH_4^+ en-

Table 2. Results of multivariate and univariate permutational ANCOVA analyses examining the effects of salinity (S) (categorical variable) and ammonium concentration (N) (continuous variable) on physiological and morphological responses of eelgrass *Zostera marina*. P_{max} : photosynthetic rate; F_v/F_m : maximum quantum yield; LER: leaf elongation rate; IAR: internode appearance rate; NP: net production. **Bold** p-values indicate significant results ($\alpha = 0.05$)

Variable, factors	df	MS	Pseudo-F	p
Multivariate response				
N	1	38.62	9.71	<0.001
S	2	67.65	17.01	<0.001
N × S	2	14.28	3.59	<0.001
Residual	21	3.98		
Univariate responses				
P_{max}				
N	1	9.58×10^{-2}	14.67	0.002
S	2	4.98×10^{-2}	7.63	0.003
N × S	2	6.17×10^{-2}	9.45	0.001
Residual	21	6.53×10^{-3}		
Respiration rate				
N	1	4.84×10^{-4}	0.05	0.835
S	2	5.62×10^{-2}	5.50	0.012
N × S	2	5.29×10^{-3}	0.52	0.603
Residual	21	1.02×10^{-2}		
F_v/F_m				
N	1	1.00×10^{-4}	0.43	0.488
S	2	1.49×10^{-3}	7.33	0.005
N × S	2	7.23×10^{-5}	0.36	0.704
Residual	21	2.03×10^{-4}		
LER				
N	1	1.10×10^{-2}	11.71	0.003
S	2	0.27	187.70	<0.001
N × S	2	3.29×10^{-2}	35.11	<0.001
Residual	21	9.37×10^{-4}		
IAR				
N	1	3.94×10^{-5}	0.79	0.391
S	2	7.60×10^{-4}	15.17	<0.001
N × S	2	1.54×10^{-4}	3.06	0.066
Residual	21	5.01×10^{-5}		
No. of leaves				
N	1	1.63×10^{-3}	1.73	0.198
S	2	5.55×10^{-2}	58.99	<0.001
N × S	2	7.94×10^{-3}	8.44	0.001
Residual	21	9.41×10^{-4}		
NP				
N	1	2.83×10^{-6}	2.52	0.127
S	2	1.02×10^{-4}	91.01	<0.001
N × S	2	2.30×10^{-5}	20.44	<0.001
Residual	21	1.12×10^{-6}		
Necrosis				
N	1	7.80×10^{-2}	2.16	0.160
S	2	1.54	42.67	<0.000
N × S	2	6.93×10^{-2}	1.92	0.166
Residual	21	3.60×10^{-2}		
Mortality				
N	1	2.20	15.09	<0.001
S	2	1.18	8.10	0.002
N × S	2	0.26	1.80	0.192
Residual	21	0.15		
NH_4^+ uptake rate				
N	1	0.25	44.20	<0.001
S	2	5.30×10^{-2}	9.19	0.001
N × S	2	6.63×10^{-3}	1.15	0.330
Residual	21	5.77×10^{-3}		
PO_4^{3-} uptake rate				
N	1	7.66×10^{-2}	29.09	<0.001
S	2	1.26×10^{-2}	4.79	0.019
N × S	2	1.60×10^{-2}	6.09	0.010
Residual	21	2.63×10^{-3}		

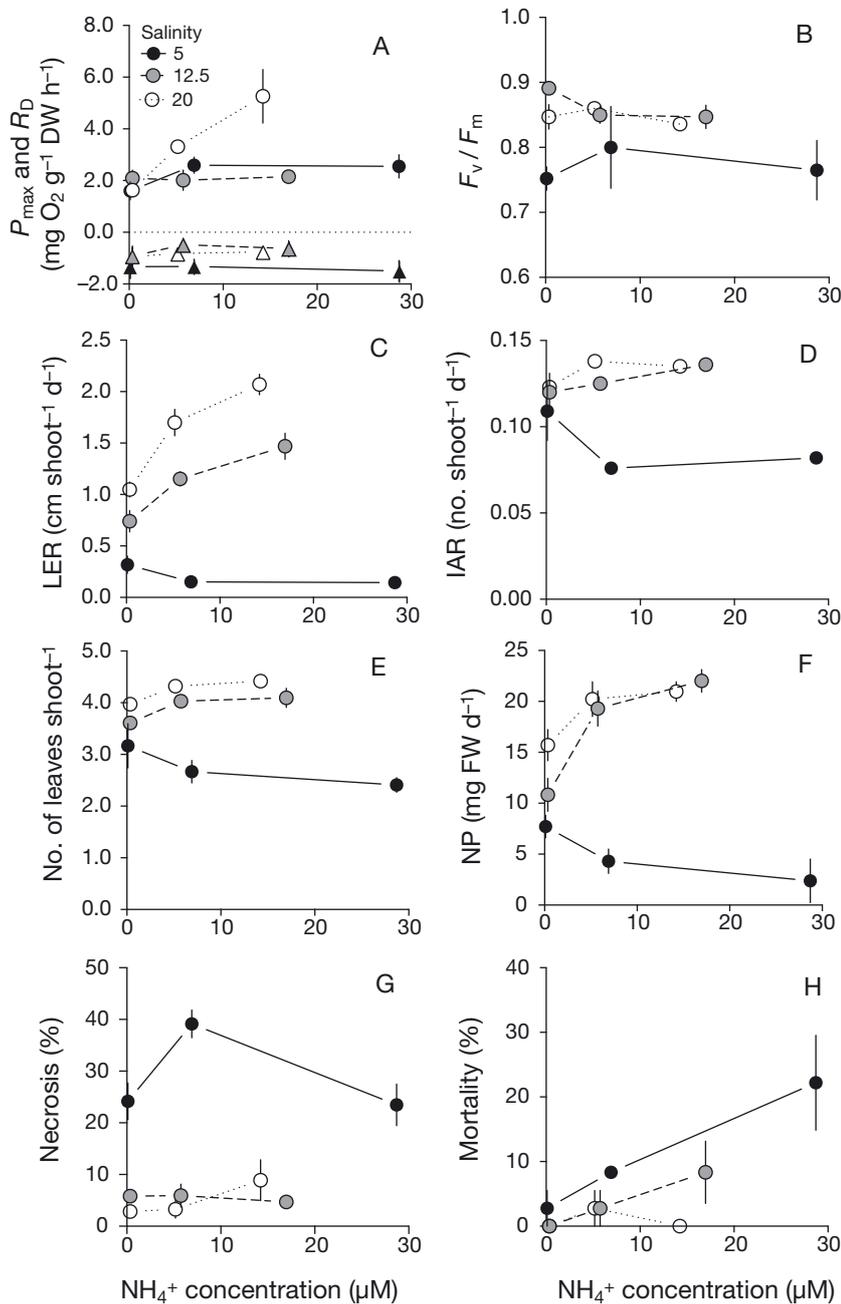


Fig. 1. *Zostera marina*. Physiological and morphological variables of eelgrass plants exposed to 3 levels of salinity (5, 12.5 and 20) and 3 levels of NH_4^+ : (A) maximum photosynthetic rate (P_{max} , circles) and dark respiration rate (R_D , triangles); (B) maximum quantum yield (F_v/F_m); (C) leaf elongation rate (LER); (D) internode appearance rate (IAR); (E) number of leaves per shoot; (F) net production (NP); (G) degree of necrosis; (H) mortality. Values are means (across 3 replicate aquaria) $\pm 1 \text{ SE}$

enrichment at salinities 12.5 and 5. Subsequent analysis suggested that the combined effect of low salinity and high NH_4^+ was synergistic (Table 3) although this was not supported by a significant interaction in the ANCOVA analysis (Table 2).

Nutrient uptake rates

Uptake rates of NH_4^+ were affected by both factors (NH_4^+ and salinity), but not by their interaction (Table 2, Fig. 2A). Low salinity had a negative effect on NH_4^+ uptake rate, which also decreased with increasing levels of the NH_4^+ treatments, being ca. 50% lower in the +NN treatment than in the control. In contrast, PO_4^{3-} uptake rates (Fig. 2B) decreased with decreasing salinity and increased with NH_4^+ enrichment, except at low salinity where uptake rate remained unaffected by NH_4^+ level, which resulted in a significant interaction between salinity and NH_4^+ treatment (Table 2). The effect of low salinity and high NH_4^+ was synergistic according to the results in Table 3.

Biochemical responses

Chlorophyll_{a+b} content was affected by both the NH_4^+ and the salinity treatments, but not by their interaction (Fig. 3A, Table 4). Low salinity led to lower levels of chlorophyll_{a+b} while increasing NH_4^+ levels were followed by an overall increase in chlorophyll_{a+b} content.

Total N content was only affected by the NH_4^+ treatment (Fig. 3B, Table 4) and increased with NH_4^+ loading. The concentration of intracellular NH_4^+ (Fig. 3C) followed the same pattern: it was significantly affected by NH_4^+ , but not by salinity and nor by the interaction between NH_4^+ and salinity (Table 4).

The interaction between NH_4^+ and salinity had a significant effect on the concentrations of Na^+ , K^+ and Cl^- (Fig. 4, Table 4). The concentration of Na^+ (Fig. 4A) and K^+ (Fig. 4B) increased with increasing NH_4^+ loading at salinity 20, were largely unaffected by NH_4^+ at salinity 12.5 and decreased slightly with increasing NH_4^+ -loading at salinity 5. The concentration of Cl^- (Fig. 4C) increased with increasing NH_4^+ at salinity 12.5 and 20, respectively, but was unaffected by

Table 3. *Zostera marina*. Relative response ratios (Eq. 2) of growth-related response variables (Table 2) in eelgrass plants when exposed to low salinity alone, to high N availability alone and to the combination of low salinity and high N availability. The estimated additive effect is the null model to which the combined response is tested. Values shown are adjusted bootstrap means and 95% confidence interval (in brackets). For abbreviations see Table 1 legend

Variable	Low salinity alone	High NH ₄ ⁺ alone	Expected additive response	Observed combined response	Effect
P_{\max}	+26% (-8, 62)	+217% (111, 347)	+243% (128, 385)	+25% (-1, 52)	Neg. synergistic
Respiration	+11% (-104, 144)	-49% (-107, 40)	+38% (-186, 156)	+14% (-77, 123)	Additive
F_v/F_m	-11% (-16, -6)	-1% (-5, 3)	-12% (-20, -4)	-9% (-20, -1)	Additive
LER	-69% (-79, -56)	+95% (70, 121)	+26% (-6, 54)	-86% (-90, -82)	Neg. synergistic
IAR	-13% (-36, 8)	+9% (0, 19)	-3% (-28, 19)	-36% (-50, -20)	Additive
NP	-52% (-66, -38)	+31% (9, 56)	-21% (-51, 13)	-80% (-94, -68)	Neg. synergistic
No. of leaves	-20% (-34, -7)	+11% (7, 15)	-9% (-25, 8)	-36% (-41, -30)	Neg. synergistic
Necrosis	+622% (229, 1117)	+430% (-66, 478)	+782% (253, 1433)	+830% (317, 1324)	Additive
Survival	-3% (-7, 0)	0% (0, 0)	-3% (-7, 0)	-15% (-21, -9)	Neg. synergistic
NH ₄ ⁺ uptake rate	-51 (-65, -31)	-64 (-83, -37)	-115 (-146, -74)	-80 (-88, -71)	Additive
PO ₄ ³⁻ uptake rate	+33% (-44, 127)	+430% (297, 544)	+464% (289, 644)	+91% (8, 202)	Neg. synergistic

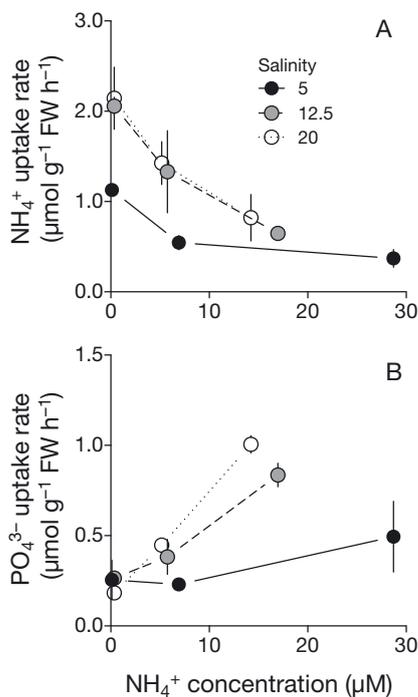


Fig. 2. *Zostera marina*. (A) NH₄⁺ and (B) PO₄³⁻ uptake rates measured during the last week of the experiment on eelgrass plants exposed to 3 levels of salinity and 3 levels of NH₄⁺. Values are means (across 3 replicate aquaria) ± 1 SE

NH₄⁺ treatment at salinity 5. The K⁺:Na⁺-ratio (molar) was modified by both main factors (Table 4). The ratio decreased with increasing salinity and increased with NH₄⁺ enrichment at salinities 12.5 and 20, but remained largely similar at salinity 5 (Fig. 4D).

Sucrose concentrations were affected by NH₄⁺ treatment and salinity and by the NH₄⁺ × plant part interaction (Fig. 5A,B, Table 5). The sucrose content decreased with decreasing salinity and increasing N loading. The reduction in sucrose concentration with increasing NH₄⁺ was larger in leaves than in roots/rhizomes. The starch content was one order of magnitude lower than the sucrose content (Fig. 5C,D). Starch content was affected significantly by all main factors (Table 5), being lower in roots/rhizomes than in leaves and decreasing with decreasing salinity. The starch content decreased generally with increasing NH₄⁺-load.

DISCUSSION

Eelgrass is common and often abundant in estuarine habitats where it is exposed to natural and/or anthropogenic driven variations in salinity and

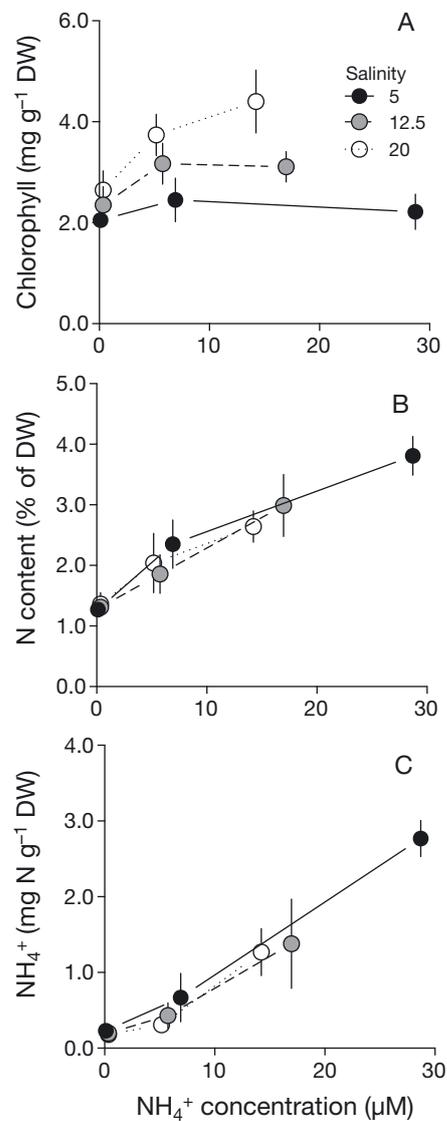


Fig. 3. *Zostera marina*. Chlorophyll content and N pools (total N and intracellular ammonium N) in leaves of eelgrass plants exposed to 3 levels of salinity and 3 levels of NH_4^+ : (A) chlorophyll_{a+b} content; (B) total N content; (C) intracellular NH_4^+ content. Values are means (across 3 replicate aquaria) ± 1 SE

nutrient availability. Our results showed that low salinity had a strong negative effect on eelgrass performance while increasing NH_4^+ availability mostly had a positive effect on eelgrass variables related to growth and survival. Interestingly, the combined effect of low salinity and high NH_4^+ availability turned the positive impact of high NH_4^+ availability into a strong negative and, in most cases, synergistic effect as indicated from the significant interaction effects between salinity and NH_4^+ concentration.

Table 4. Results of multivariate and univariate permutational ANCOVA analyses examining the effects of salinity (S) (categorical variable) and ammonium concentration (N) (continuous variable) on chlorophyll content, total N content, ammonium content, the content of different ions (Na^+ , K^+ , Cl^-) and the $\text{K}^+:\text{Na}^+$ molar ratio in leaves of eel grass plants. **Bold** p-values indicate significant results ($\alpha = 0.05$)

Variable, factors	df	MS	Pseudo-F	p
Univariate responses				
Chlorophyll content				
N	1	2.96×10^{-2}	5.98	0.024
S	2	5.27×10^{-2}	10.63	<0.001
N \times S	2	1.18×10^{-2}	2.38	0.114
Residual	21	4.94×10^{-3}		
N pools				
Total N content				
N	1	0.72	108.71	<0.001
S	2	2.05×10^{-2}	3.11	0.065
N \times S	2	8.84×10^{-4}	0.13	0.879
Residual	21	6.60×10^{-3}		
Ammonium content				
N	1	0.55	42.05	<0.001
S	2	2.16×10^{-2}	1.64	0.221
N \times S	2	6.07×10^{-3}	0.46	0.641
Residual	21	1.31×10^{-2}		
Ions				
Na^+				
N	1	3.04×10^{-3}	1.76	0.195
S	2	0.14	83.06	<0.001
N \times S	2	4.52×10^{-2}	26.13	<0.001
Residual	21	1.73×10^{-3}		
K^+				
N	1	4.15×10^{-2}	13.40	0.002
S	2	1.71×10^{-2}	5.51	0.014
N \times S	2	6.94×10^{-2}	22.39	<0.001
Residual	21	3.10×10^{-3}		
Cl^-				
N	1	0.15	19.45	<0.001
S	2	0.16	21.45	<0.001
N \times S	2	0.11	14.69	<0.001
Residual	21	7.63×10^{-3}		
$\text{K}^+:\text{Na}^+$				
N	1	8.17×10^{-3}	5.44	0.030
S	2	5.62×10^{-2}	37.43	<0.001
N \times S	2	3.48×10^{-3}	2.31	0.124
Residual	21	1.50×10^{-3}		

Eelgrass was only marginally affected by intermediate salinity (12.5 compared to 20) while exposure to low salinity (5) had a negative effect on most growth-related variables. Hyposaline conditions led to reduced growth (lower leaf elongation, internode appearance and net production), shoots with fewer and more necrotic leaves, reduced nutrient uptake capacity and elevated mortality. Most of these responses were expected and largely similar to those reported from other studies on low salinity stress in eelgrass (e.g. Nejrup & Pedersen 2008, Salo & Peder-

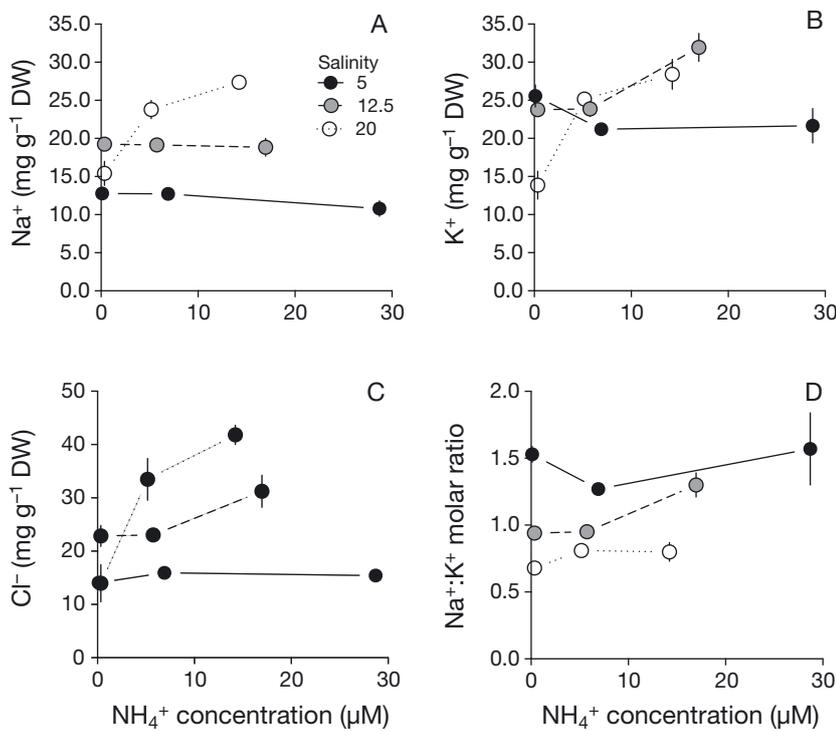


Fig. 4. *Zostera marina*. Intracellular concentrations of (A) Na^+ , (B) K^+ and (C) Cl^- ; and (D) $\text{K}^+:\text{Na}^+$ molar ratio in leaves of eelgrass plants exposed to 3 levels of salinity and 3 levels of NH_4^+ . Values are means (across 3 replicate aquaria) ± 1 SE

Table 5. Results of multivariate and univariate permutational ANCOVAs examining the effects of salinity (S) (categorical variable), plant tissue (Ti) (i.e. leaves or roots/rhizomes; categorical variable) and ammonium concentration (N) (continuous variable) on sucrose and starch concentrations. **Bold** p-values indicate significant results ($\alpha = 0.05$)

Variable, factors	df	MS	Pseudo-F	p
Sucrose				
N	1	1.54	128.33	<0.001
S	2	0.50	41.71	<0.001
Ti	1	4.41×10^{-2}	3.69	0.062
N \times S	2	6.28×10^{-2}	0.52	0.605
N \times T	1	0.15	12.64	<0.001
S \times Ti	2	1.10×10^{-2}	0.92	0.410
S \times N \times Ti	2	1.28×10^{-2}	1.07	0.361
Residual	42	1.20×10^{-2}		
Starch				
N	1	0.30	37.85	<0.001
S	2	0.13	16.07	<0.001
Ti	1	0.21	27.26	<0.001
N \times S	2	6.99×10^{-5}	8.90×10^{-3}	0.991
N \times T	1	1.80×10^{-2}	2.29	0.134
S \times Ti	2	1.04×10^{-2}	1.33	0.283
S \times N \times Ti	2	1.55×10^{-2}	1.98	0.151
Residual	42	7.86×10^{-3}		

sen 2014, Salo et al. 2014). The negative effect of low salinity on growth, nutrient uptake and survival may have been caused by cell damages due to inappropriate osmoregulation (e.g. cell rupture, changes in ion composition and pH, disturbance of inner membranes), reduced photosynthetic capacity and/or by increasing metabolic costs related to osmoregulation. The photosynthetic apparatus is often considered one of the most sensitive components in plant cells (Maxwell & Johnson 2000). The marked decrease in pigment concentration (20 to 50%) in low salinity plants seemed to support this expectation at first, but gross photosynthetic rates (i.e. P_{max}) remained largely unaffected by salinity unless plants were simultaneously exposed to high levels of NH_4^+ . Also, F_v/F_m was only marginally (<10%) reduced at low salinity and values remained within those considered typical for unstressed plants (i.e. 0.8; Björkman & Demmig 1987, Johnson et al. 1993). In other words, reduced photosynthetic efficiency could not

explain the negative effect that low salinity had on growth and survival.

Low salinity had a negative effect on the uptake capacity of NH_4^+ and PO_4^{3-} and the relative effect of low salinity remained largely the same across all levels of the N-treatment (i.e. no interaction between salinity and NH_4^+). The correlation between net uptake of NH_4^+ and PO_4^{3-} and salinity may have been caused by the active export of ions during osmoregulation and/or by increased competition for energy that could affect active nutrient uptake across the plasma membrane (in case of PO_4^{3-}) and/or the assimilation of NH_4^+ and PO_4^{3-} (McGlathery et al. 1996, Touchette 2007, Garrote-Moreno et al. 2015). Hyposaline conditions caused a net loss of Na^+ , K^+ and Cl^- from eelgrass and lead to a marked increase in $\text{K}^+:\text{Na}^+$ ratios, indicating unbalanced, disturbed ionic conditions in the plants (Kirst 1990, Touchette 2007, Choi et al. 2010). Osmoregulation is related to increased metabolic costs and respiration almost doubled with decreasing salinity (independently of N-treatment). At the same time C reserves in low salinity plants were reduced by ca. 50% over the course of the experiment. The concomitant increase in respiration and elevated mobilization of stored C

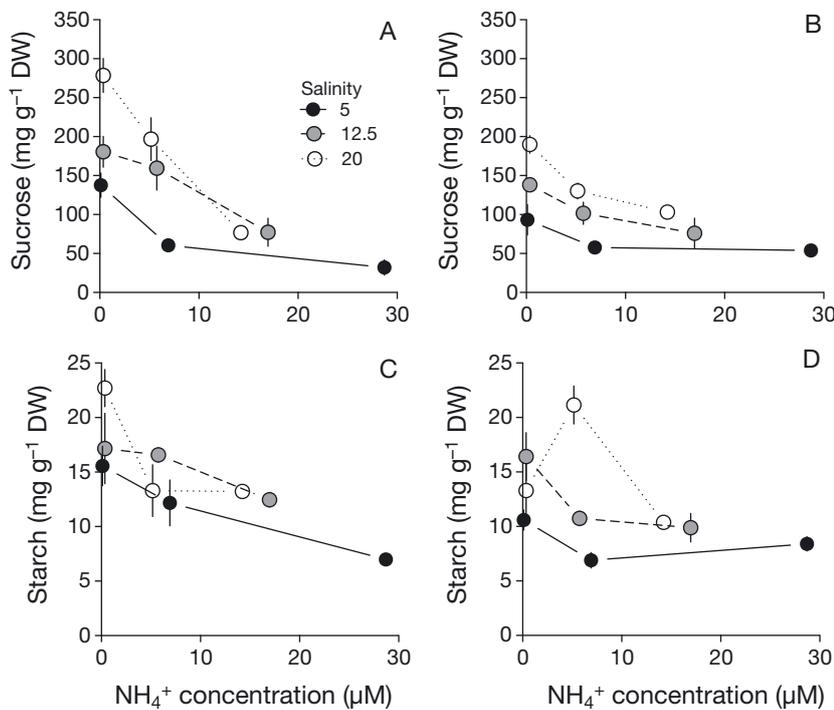


Fig. 5. *Zostera marina*. Sucrose concentration in (A) leaves, and (B) roots/rhizomes, and starch content in (C) aboveground (i.e. leaves) and (D) belowground (i.e. roots/rhizomes) tissues in eelgrass plants exposed to 3 levels of salinity and 3 levels of NH_4^+ . Values are means (across replicate aquaria) ± 1 SE

suggests that exposure to low salinity was related to a substantial increase in the cost of osmoregulation and/or the repair of damages and that photosynthesis was unable to cover these elevated energy requirements.

Exposure to elevated levels of nitrogen and, especially, to NH_4^+ can be both beneficial and disadvantageous to seagrasses depending on the nutrient status of the plants. Positive effects (e.g. increased photosynthesis and growth) are often seen in nutrient-poor environments where plants may be N limited (Touquette & Burkholder 2000). A number of studies have, however, shown that even relatively low concentrations of NH_4^+ can have an adverse effect on eelgrass (van Katwijk et al. 1997, Villazán et al. 2013b). These effects can either be due to direct toxic effects of NH_4^+ , or be due to increased energy requirements driven by elevated assimilation of NH_4^+ into amino acids and proteins (Brun et al. 2002, 2008, Villazán et al. 2013b). N assimilation requires energy and carbon skeletons, which must be provided by photosynthesis or mobilized from C reserves within the plant (Marschner 1995). Villazán et al. (2013b) demonstrated that NH_4^+ enrichment has a positive effect on eelgrass fitness as long as light conditions are kept

sufficient, but that exposure to similar NH_4^+ levels under low light conditions results in exhaustion of internal C stores and reduced plant performance. This relationship indicates that N assimilation competes with other metabolic process for C and energy and that long-term exposure to elevated NH_4^+ levels may affect the C balance in seagrasses. Thus, the simultaneous exposure to high NH_4^+ availability and an additional stressor is therefore likely to lead to a negative effect of NH_4^+ on plant performance as has been previously demonstrated in *Zostera noltei* (Brun et al. 2008, La Nafie et al. 2012).

Exposure of eelgrass to high levels of NH_4^+ at ambient salinity (20) had an overall positive effect on plant performance. Photosynthesis, growth and P uptake rate were stimulated while N uptake rate was reduced and mortality remained unaffected by N treatment as long as salinity was kept high. The positive effect of NH_4^+ enrichment may have been caused by low DIN concentrations in the water

used for our control treatment (Table 1), which may have left plants from this treatment slightly N limited. The total N content in plants from the control treatment was close to the limiting level for seagrasses (1.2 to 1.3% N of DW; Duarte 1990) by the end of the experiment and N enrichment may therefore have released plants from N limitation in the +N and +NN treatments. The positive effect of NH_4^+ enrichment on growth was, however, also followed by a significant accumulation of intracellular NH_4^+ , which may be the major reason why N uptake rates were reduced at low salinity. Nutrient uptake rates are controlled by the external concentration of nutrients, but also by the nutrient quota within the plant and high internal concentrations of NH_4^+ may exert a negative feedback on N uptake rates in algae and seagrasses (e.g. Pedersen 1994, McGlathery et al. 1996, Pedersen et al. 1997). The uptake of PO_4^{3-} was, in contrast, stimulated by NH_4^+ enrichment. Increasing the N quota in N-enriched plants would typically lead to higher protein and enzyme levels (e.g. Villazán et al. 2013b), which could stimulate active uptake and assimilation of PO_4^{3-} .

The positive effect of N enrichment on eelgrass performance was followed by a 50 to 60% reduction

in the content of sucrose and starch. This reduction indicates that photosynthesis, although stimulated by N enrichment, was unable to cover the energy and C demand related to increasing N assimilation and, thus, that competition for C and energy would have intensified and potentially have become harmful to the plants if they had been exposed to these conditions beyond the experimental period of 4 wk.

The positive effect of high N availability that we observed at high salinity was completely reversed when plants were exposed to low salinity. Only few studies have assessed the potential interaction effect between high NH_4^+ availability and low or high salinity in seagrasses, but Kahn & Durako (2006) showed that the combination of high NH_4^+ availability and low salinity impairs *in situ* growth of *Thalassia testudinum* while van Katwijk et al. (1999) found that exposure of eelgrass to hypersaline conditions (salinity 30 as opposed to 23) leads to increasingly negative effects of high nutrient availability on eelgrass.

The combined effects of high NH_4^+ availability and low salinity on eelgrass performance were largely the same as the individual effects of low salinity, i.e. reduced growth, reduced shoot size, more necrotic leaves, lower nutrient uptake rates and elevated mortality, but the responses were in most cases much stronger than when exposed to low salinity alone, and also stronger than the algebraic sum of the individual response to low salinity and high NH_4^+ availability, respectively (Table 3). A combined response of 2 stressors that is larger than the sum of the individual responses is a strong indication of a synergistic effect. All growth-related response variables in Table 3, except for R_D , F_v/F_m , IAR, necrosis and N uptake rate, had combined responses that were substantially larger than the sum of the 2 individual responses, underlining that simultaneous exposure to low salinity and high NH_4^+ availability had an overall negative synergistic effect on eelgrass.

The underlying mechanisms behind an interaction between NH_4^+ enrichment and low or high salinity are not well understood. We suggest that the interaction effects are caused by a combination of the physical and chemical cell damages imposed by low salinity and high internal NH_4^+ concentrations, respectively, combined with an enhanced competition between NH_4^+ assimilation and osmoregulatory processes for energy and carbon skeletons. Any physical and/or chemical damages require energy for repair processes and low salinity may thus increase the demand for energy needed for osmoregulation while at the same time it may lower C fixation through photosynthesis. High NH_4^+ availability increases the

need for energy to support assimilation of NH_4^+ . These energy-consuming processes will likely divert energy away from other important metabolic processes (repair, growth etc.), which may impair the carbon balance within plants and consequently reduce plant growth and ultimately increase mortality. Thus, any factor that increases the demand for energy and interacts with low salinity and/or NH_4^+ enrichment, e.g. high temperature (Salo & Pedersen 2014), low irradiance (Villazán et al. 2013b, Jiang et al. 2013) or hydrodynamics (La Nafie et al. 2012), may amplify the negative effects of these stressors.

We examined the physiological responses of eelgrass to combinations of salinity and NH_4^+ enrichment, and tested these interactions at steady state levels of stress for 4 wk. The amount of stress is equal to the product of its intensity and duration (Schulze et al. 2005) and short-term exposure to stressful levels of salinity and NH_4^+ should thus lead to less severe effects. Future studies should evaluate the response to unfavorable levels of salinity and NH_4^+ as a function of exposure time and, also, include studies on the potential recovery after exposure to stressful conditions (i.e. resilience) to fully evaluate the consequences of changes in salinity and NH_4^+ at several time scales.

Eelgrass habitats that experience low salinity and relatively high concentrations of NH_4^+ are common in inner parts of estuaries, in shallow sheltered bays and/or close to river mouths. Most climate models predict an increased frequency and intensity of rainfall along the northern Atlantic coast of Europe as a result of ongoing climate changes (IPCC 2013). More intense and frequent precipitation events will increase the seaward transport of freshwater and dissolved nutrients from catchments and especially those dominated by agricultural areas (Meier et al. 2012). Since low salinity combined with high levels of NH_4^+ has negative additive or synergistic effects on estuarine seagrasses, seagrass populations are at risk of experiencing an increased level of stress in the future. The occurrence of synergistic responses to multiple stressors should therefore be taken into account in the future management of coastal areas.

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