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Elevated ammonium concentrations and low light form a dangerous synergy for eelgrass *Zostera marina*

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ABSTRACT: We studied the effect of ecologically relevant ammonium concentrations and light on several morphological and physiological properties, nitrogen metabolism and carbon reserves of eelgrass *Zostera marina* L. Eelgrass was grown under mesocosm conditions at 3 levels of ammonium enrichment (target concentrations of 0, 10 and 25 µM) and 2 levels of light (low and high light). High ammonium supply combined with low light had a negative effect on several morphological and physiological response parameters, while no such effects were found when ammonium was supplied under high light. N enrichment caused an increase in the content of total N, intracellular ammonium, free amino acids and residual N in the plants and this response was more pronounced under low-light conditions than under high light. The soluble proteins content decreased, in contrast with external ammonium enrichment. The accumulation of free amino acids and residual N in NH_4^+-enriched plants was followed by a substantial drop in carbohydrate reserves (sucrose and starch), which was larger in plants grown under low-light conditions. Our results indicate that N enrichment increases the demand for C skeletons and energy, and that photosynthesis cannot supply enough C and energy to cover that demand under low-light conditions. Eelgrass plants exposed to reduced light conditions, for example close to their depth limit or when covered by drift macroalgae, may thus be especially susceptible to enhanced ammonium concentrations. Our study demonstrates that ammonium toxicity may explain why eelgrass and other seagrasses deteriorate under nutrient-rich, low-light conditions.

KEY WORDS: Dissolved inorganic nitrogen · Light · Nitrogen metabolism · Carbon reserves · Seagrass · Eutrophication

INTRODUCTION

Seagrasses are the dominant benthic primary producers in many coastal areas and they provide many ecologically and economically important services to marine ecosystems (Costanza et al. 1997, Duarte 2000, Waycott et al. 2009). Seagrass ecosystems have declined worldwide over the last 4 to 5 decades (Orth et al. 2006, Waycott et al. 2009, Short et al. 2011) as a consequence of increasing anthropogenic nutrient loading and subsequent eutrophication (Short et al. 1995, Short & Wyllie-Echevarria 1996, Burkholder et al. 2007). High nutrient availability affects seagrasses in several ways. The major effects are indirectly caused by the proliferation of phytoplankton, epiphytic microalgae and fast-growing drifting macroalgae promoting light attenuation (Sand-Jensen & Borum 1991, Hernández et al. 1997, Valiela et al. 1997, Hauxwell et al. 2001, McGlathery 2001, Bryars et al. 2011, Lyons et al. 2012) or increasing the sedi-

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ment organic matter load, which may reduce oxygen levels and increase the risk of anoxia (Greve et al. 2003) and sulfide intrusion into the plants (Holmer & Bondgaard 2001, Borum et al. 2005, Pérez et al. 2007, Olivé et al. 2009). Furthermore, there may be a direct effect of high nutrient availability on seagrasses since exposure to high concentrations of NH_4^+ can be toxic to higher plants (e.g. Marschener 1995, Britto & Kronzucker 2002, Brun et al. 2002, 2008).

A moderate increase in the availability of inorganic nitrogen (<10 µM) may stimulate growth and biomass of seagrasses when these are growing under nutrient-limited conditions (e.g. Orth 1977, Alcoverro et al. 1997, Peralta et al. 2003, Invers et al. 2004). However, some studies have shown little or no effect of nutrient enrichment (e.g. Harlin & Thorne-Miller 1981, Dennison et al. 1987, Murray et al. 1992, Pedersen & Borum 1993, Pedersen 1995, Lee & Dunton 2000), most likely because these studies were carried out in areas with relatively high ambient availability of nutrients where the plants under study were nutrient replete. A growing body of evidence suggests that enrichment by inorganic nitrogen (N), especially NH_4^+, can have an adverse effect on seagrasses by reducing photosynthesis, growth and survival (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).

Adverse effects of high NH_4^+ concentrations on seagrasses and other higher plants have traditionally been explained by internal accumulation of NH_4^+, which may affect internal pH and enzyme kinetics, uncouple the production of ATP during photosynthesis, increase respiration and reduce the uptake of other cations (e.g. Marschener 1995). Other studies indicate that high NH_4^+ concentrations may cause enhanced ethylene synthesis, increased energy consumption related to active efflux of NH_4^+, and reduced photo-protection (Britto et al. 2001, Britto & Kronzucker 2002). The negative effect of high NH_4^+ availability on plants may also be related to an imbalance in the carbon (C) economy of the plants since accumulation of internal NH_4^+ stimulates the synthesis of amino acids in plants (Marschener 1995). This synthesis requires C skeletons and energy, which must be provided directly from photosynthesis or be mobilized from C reserves within the plant. Continuous uptake and assimilation of NH_4^+ can therefore drain the C reserves and, thus, compete with other C-demanding or energy-consuming metabolic processes.

The aims of this study were to test whether elevated, but ecologically relevant, levels of NH_4^+ affect eelgrass fitness and to study the underlying mechanisms behind this toxicity in terms of N metabolism and the possible consequences for the C reserves in the plant. We cultivated *Zostera marina* plants under 3 different NH_4^+ concentrations (0, 10 and 25 µM) at 2 different light levels (low and high) for 5 wk. We hypothesized that increasing concentrations of NH_4^+ in the growth media would cause increasingly negative effects in *Z. marina*, because C reserves may be drained in order to support the assimilation of NH_4^+. We expected that low light would enforce and high light alleviate the potential negative effects of NH_4^+.

MATERIALS AND METHODS

A 2-factorial culture experiment was conducted from October to November 2011 (ca. 5 wk) to test how NH_4^+concentrations and light levels affected eelgrass *Zostera marina*. Individual shoots of *Z. marina* were collected from Isefjorden, Denmark, at a depth of 1–2 m in late September 2011. Healthy looking shoots with intact rhizomes (6–9 internodes) were transferred to the laboratory where they were held in aerated water from the sampling site under sub-saturating light (ca. 30 µmol photons m^{-2} s^{-1}) in a 16 h light:8 h dark cycle at 15°C until used in the experiment (ca. 1 wk). Shoots were first 'standardized' to have 4 (visible) leaves and 4 rhizome internodes (by removing older leaves and internodes) before being used in the experiment. Each of 18 aquaria (volume = 20 l) was filled with ca. 2–3 l of sediment from the sampling site and 15 l of filtered water from the North Sea. The salinity of the seawater was adjusted to 20‰ by dilution with tap water and the temperature was kept constant at 15°C to obtain optimal growth conditions for the plants (Nejrup & Pedersen 2008). The water in the aquaria was aerated to ensure mixing and changed weekly to avoid nutrient limitation and excessive growth of phytoplankton. Light above the aquaria was provided by lamps with halogen spots (12 V, 35 W) in a 16 h light:8 h dark cycle.

Fourteen eelgrass plants were planted in each of the 18 aquaria, which were then subjected to 3 target concentrations of NH_4^+ (0, 10 and 25 µM; treatments called C, +N and +NN, respectively) and 2 levels of light (26 ± 3 and 70 ± 9 µmol photons m^{-2} s^{-1} PAR; treatments called LL and HL, respectively) with 3 replicate aquaria within each treatment combination. The light intensity provided in the LL treatment was low, but above the light compensation point (I_C) of *Zostera marina*, while that provided in the HL treat-

ment was close to saturating levels (I_K) (Marsh et al. 1986, Olesen & Sand-Jensen 1993).

The water added to the aquaria contained low levels of ammonium (ca. 1 µM) and nitrate (2–3 µM), and ammonium was added to the aquaria (in the +N and +NN treatments) from a NH_4Cl stock solution every day to keep the concentrations as close to the target concentrations as possible. The NH_4^+ addition corresponded to 150 µmol aquaria^{-1} d^{-1} in the +N treatment and 375 µmol aquaria^{-1} d^{-1} in the +NN treatment. The concentration of ammonium was monitored twice weekly in all aquaria. Water samples were collected just before and right after addition of ammonium. The concentrations before adding new ammonium averaged 0.8 ± 0.2 µM in the control treatment, 0.7 ± 0.2 µM in the +N treatment, and 1.2 ± 0.2 µM in the +NN treatment (mean ± SD across 3 replicate aquaria and over 10 sampling dates in each treatment). The concentration of ammonium just after adding ammonium averaged 0.8 ± 0.2 µM in the control treatment, 11.2 ± 0.3 µM in the +N treatment, and 24.7 ± 0.4 µM in the +NN treatment. All water in the aquaria was changed once weekly to prevent accumulation of ammonium (especially in the +NN treatment) and to reduce the risk of limitation by phosphorus or micronutrients.

Physiological and morphological responses

Prior to transplantation into the aquaria, each plant was weighed (initial fresh weight biomass) and marked for measuring leaf elongation rate. At the end of the experiment, all surviving plants were harvested and each plant was weighed (fresh weight, FW) and the number of leaves per shoot was counted. Net production (g FW plant^{-1} d^{-1}) was estimated from the net change in individual plant weights over the course of the experiment while the production of new leaves (plastochrone interval) and leaf elongation rate was measured using the leaf-marking technique (Sand-Jensen 1975). The appearance of new side-shoots per original shoot was recorded. Survival rate was estimated from the number of surviving plants in each aquarium at the end of the experiment. Leaf necrosis was quantified as the area with brown–black discolouration of the 3 youngest leaves on each shoot.

Maximum net photosynthetic rate (P_{max}) and dark respiration were measured as O_2 production or consumption under saturating light conditions (ca. 150 µmol photons m^{-2} s^{-1} PAR) or in darkness. Four randomly chosen eelgrass shoots were collected from each aquarium at the end of the experiment and incubated in a 800 ml gas-tight, transparent chamber equipped with a circulation pump (AquaBee, 300 l h^{-1}) used to ensure circulation within the chamber. Two shoots were fixed in each chamber, which was filled with natural seawater without ammonium enrichment (salinity 20‰) having an O_2 concentration corresponding to ca. 70% of air saturation to prevent supersaturation of O_2 in the chamber during incubations. The chamber was finally submerged into a water bath with constant temperature (15°C). The chamber was equipped with a Clark-type O_2 microelectrode (OX-500, Unisense) that was connected to a pico-amperemeter (Picoammeter PA2000, Unisense) and a Pico Technology ADC-16 data logger. A lamp with 8 halogen spots (OSRAM Decostar 51; 12 V, 35 W) illuminated the set-up. The water bath held 2 replicate chambers at a time. The O_2 concentrations were recorded every minute throughout the incubations and rates of O_2 release or uptake were calculated from periods with constant changes in O_2 concentration over a minimum of 10–15 min.

Biochemical responses

Total C and N

Total C and N content were determined on duplicate freeze-dried, ground samples of leaves and roots/rhizomes from each aquarium using a Carlo-Erba NA-1500 CHNS analyzer.

Intracellular inorganic N

Intracellular concentrations of NH_4^+ and NO_3^- were measured on duplicate leaf and rhizome samples from each aquarium. Samples were rinsed in deionized water and ca. 0.5 g (FW) was ground in 20 ml of boiling deionized water (Dortch et al. 1984). Samples were sonicated for 10 min and then centrifuged for 20 min at 5000 × *g*. The concentration of NH_4^+ and NO_3^- was finally measured in the supernatant according to Bower & Holm-Hansen (1980) and Grasshoff et al. (1983).

Free amino acids

Intracellular concentrations of free amino acids (FAA) were measured on duplicate leaf and rhizome samples from each aquarium. Leaves or rhizome

internodes were cut from the plants and wiped with a piece of cloth to remove attached epiphytes and debris. Samples were transferred to a 20 ml glass vial with 10 µl 96% ethanol for extraction. The extract was then transferred to a 1.5 ml HPLC vial with 70 µl 10 mM borate buffer at pH 8.8. Primary and secondary amines in the sample were derivatized with 20 µl 10 mM 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (Liu et al. 1995) using a AccQ Tag kit (Waters Corp.). The derivatives were heated to 55°C for 10 min to degrade a tyrosine side product that interferes with the chromatographic separation of amino acids. The derivatives were separated on a Waters Alliance 2695 separation module with a 3.9°–150 mm Nova-Pak C-18 column. The solvents used for the separation were (1) 98.9 mM sodium acetate and 6.34 mM triethylenamine at pH 5.70, (2) 98.9 mM sodium acetate and 6.34 mM triethylenamine at pH 6.80, (3) acetonitrile, and (4) water. The separated amino acid derivatives were quantified by fluorescence (250 nm excitation and 395 nm emission) using a Waters 474 scanning fluorescence detector. The detection limit of the method was about 1 pmol of each amino acid. The amount of N bound in FAA was finally estimated using the specific C:N ratio of each of the identified amino acids.

Soluble proteins

The content of soluble proteins was determined on duplicate leaf and rhizome samples from each aquarium using a modification of the Bradford method (Jones et al. 1989). Fresh plant material (ca. 0.1 and 0.5 g for leaf and rhizome samples, respectively) was ground and transferred to a centrifuge tube with 1 ml 0.1 M NaOH (pH 12.8). The mixture was shaken on a vortex mixer and then sonicated for 1–2 min. Samples were left to extract for 30–60 min at room temperature before shaking once again. Samples were centrifuged for 5 min at 5000 × *g* and the supernatant was subsequently transferred to a test tube. Aliquots (0.1 ml) of each sample were mixed with 5 ml of Bradford reagent and soluble polyvinylpyrollidone (concentration: 3 mg PVP ml^{-1} reagent). The absorbance was read using a spectrophotometer at 595 nm after 5 and within 10 min after addition of the reagent. Blanks (aliquots of 0.1 M NaOH) and standards (0.1 ml aliquots of bovine serum albumin dissolved in 0.1 M NaOH) were treated as the samples. The amount of N bound in soluble proteins was finally estimated assuming an average C:N ratio of 6.1:1.

Chlorophyll-bound N

Chlorophyll *a* + *b* concentrations were determined on duplicate leaf samples from each aquarium using the method of Wintermans & De Mots (1965). Samples were freeze-dried, ground and extracted overnight in 96% ethanol. The extract was filtered and the chlorophyll concentrations were determined spectrophotometrically at wavelengths of 649, 665 and 750 nm. The amount of chlorophyll-bound N was estimated assuming that N constituted 6.23% of the molar weight of chlorophyll *a* (Stryer 1981).

Residual N

The amount of N not accounted for by the aforementioned analyses was termed residual N. This pool was likely made up by a mixture of structural proteins, cyclic amino acids and other low molecular weight N compounds, and was estimated as the total amount of N minus the N bound in intracellular NH_4^+, NO_3^-, chlorophyll, FAA and soluble proteins.

Sucrose and starch

The concentrations of sucrose and starch were measured on duplicate leaf and rhizome samples from each aquarium. Samples were freeze-dried and ground prior to analysis. Total non-structural carbohydrates 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. 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 keeping it for 24 h in 1 N NaOH. The sucrose and starch content of the extracts was determined spectrophotometrically using a resorcinol and anthrone assay with an absorbance of 486 and 640 nm, respectively, with sucrose as a standard.

Statistical treatment

We used 2-factorial (for physiological and morphological response variables) or 3-factorial (for biochemical response variables) permutational MANOVA (PERMANOVA) to test for effects of the treatments (NH_4^+ enrichment, light level and plant part, i.e.

leaves and roots/rhizomes) and their interactions. All treatment factors were considered fixed. The multivariate approach was chosen because all response variables were obtained from plants originating from the same experimental unit (aquarium) and because many of the response variables were likely intercorrelated. Data were normalized to minimize scale differences among response variables before analysis and PERMANOVA was executed using Type III sum of squares on geometric (Euclidean) distances and unrestricted permutation of raw data (Anderson et al. 2008).

Univariate permutational ANOVA (2- or 3-factorial) was subsequently used to test the effect of the treatment factors and their interactions on each response variable separately as suggested by Quinn & Keough (2002). 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 an α-level of 0.05.

RESULTS

Physiological and morphological properties

The composite response of all physiological and morphological parameters was affected by the interaction between NH_4^+ addition and light (PERMANOVA, p = 0.007; Table 1). Enrichment with NH_4^+ affected the composite response variable negatively at low light, but not at high light.

High NH_4^+ levels affected most of the individual response variables negatively under low-light conditions, whereas no clear or even positive effects of NH_4^+ were recorded under high-light conditions. Maximum photosynthetic and respiration rates (Fig. 1A) were not affected significantly by NH_4^+, light or their interaction (p > 0.05, Table 1), although P_{max} in plants cultivated in low light tended to decrease with increasing NH_4^+ loading and the opposite trend was recorded in plants cultivated in high light.

Net production (i.e. net changes in plant biomass) was significantly affected by the interaction between NH_4^+ and light (Fig. 1B, Table 1): NH_4^+ enrichment caused a marked reduction in net production at low light, decreasing from ca. 15 mg FW shoot^{-1} d^{-1} in the control to almost −10 mg FW shoot^{-1} d^{-1} under the highest NH_4^+ loading. NH_4^+ enrichment had, in contrast, no effect on net production under high-light conditions (mean across N levels was ca. 22 mg FW shoot^{-1} d^{-1}).

Table 1. Statistical results of the MANOVA (composite response) and ANOVA (individual responses) analyses examining the effect of light level and ammonium supply on various morphological and physiological properties of *Zostera marina*

Variable, factors	df	MS	Pseudo-*F*	p
MANOVA				
Ammonium supply (N)	2	11.79	2.30	0.029
Light (L)	1	38.88	7.60	0.002
L × N	2	14.56	2.84	0.007
ANOVA				
Photosynthetic rate (P_{max})				
Ammonium supply (N)	2	0.217	0.22	0.817
Light (L)	1	0.005	0.01	0.823
L × N	2	2.235	0.23	0.139
Respiration rate (*R*)				
Ammonium supply (N)	2	0.560	0.51	0.604
Light (L)	1	0.051	0.05	0.832
L × N	2	1.380	1.27	0.310
Net production (NP)				
Ammonium supply (N)	2	1.363	7.20	0.008
Light (L)	1	9.077	47.90	0.001
L × N	2	1.461	7.71	0.005
Leaf elongation rate (LER)				
Ammonium supply (N)	2	2.77	7.04	0.010
Light (L)	1	6.69	17.01	0.001
L × N	2	0.024	0.06	0.940
Plastochrone interval (PI)				
Ammonium supply (N)	2	0.658	2.47	0.132
Light (L)	1	11.921	44.74	0.001
L × N	2	0.282	1.06	0.388
Side-shoot appearance rate				
Ammonium supply (N)	2	0.068	0.09	0.935
Light (L)	1	0.551	0.70	0.432
L × N	2	3.402	4.29	0.031
Leaf abundance				
Ammonium supply (N)	2	2.401	15.04	0.001
Light (L)	1	6.865	43.02	0.001
L × N	2	1.709	10.71	0.001
Necrosis				
Ammonium supply (N)	2	3.285	26.71	0.001
Light (L)	1	3.671	29.85	0.001
L × N	2	2.641	21.48	0.001

Leaf elongation rate (Fig. 1C) was affected by both NH_4^+ loading and light, but not by their interaction (Table 1). Leaf elongation decreased from 2.6 to 2.1 cm shoot^{-1} d^{-1} with increasing NH_4^+ concentration at low light, but increased from 2.6 to 3.1 cm shoot^{-1} d^{-1} with increasing NH_4^+ concentration at high light. The plastochrone interval (Fig. 1D) was only affected significantly by light (Table 1), being 25–30% higher in plants cultivated under low light than in those exposed to high light. The plastochrone interval tended to increase with increasing NH_4^+ addition in plants held at low light.

The production of side-shoots (Fig. 1E) was affected by the interaction between NH_4^+ and light

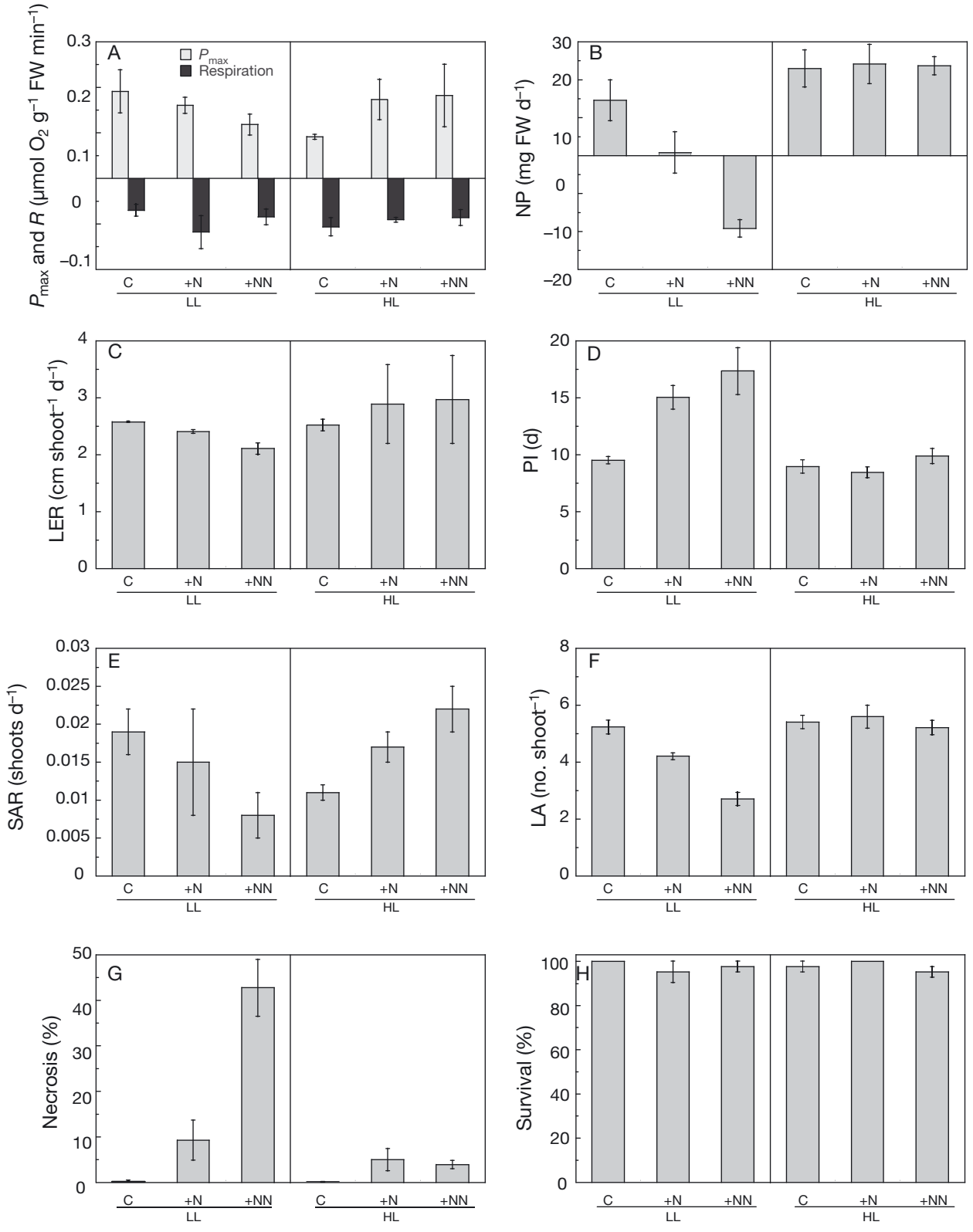

Fig. 1. *Zostera marina*. Dynamics and physiological features of plants under each ammonium and light treatment (means ± SE across 3 replicate aquaria): (A) maximum photosynthetic (P_{max}) and respiration rate (R), (B) leaf elongation rate (LER), (C) plastochrone interval (PI), (D) shoot appearance rate (SAR), (E) leaf abundance (LA), (F) net production (NP), (G) degree of necrosis, and (H) survival rate (SR). C, + N, +NN: 0, 10, 25 µM ammonium concentration, respectively; LL: low light; HL: high light

(Table 1). New side-shoots were produced at a rate of 0.018 shoot^{-1} d^{-1} without NH_4^+ enrichment in low light, but this rate was reduced to 0.007 shoot^{-1} d^{-1} in the high NH_4^+ treatment. In contrast, enrichment with NH_4^+stimulated the production of new shoots (from 0.012 to 0.022 shoot^{-1} d^{-1}) in high light. Leaf abundance (Fig. 1F) was significantly affected by the interaction between NH_4^+ and light (Table 1): the number of leaves per shoot was reduced from 5.2 in the control to ca. 2.5 at high NH_4^+ addition in low light.

The degree of necrosis (Fig. 1G) was affected by the interaction between NH_4^+ and light (Table 1). In low light, necrosis increased from ca. 0% in the control treatment to more than 40% in the +NN treatment. A similar pattern occurred under high light, although at much lower levels (max. ca. 5%). Survival (Fig. 1H) was unaffected by all treatment factors and remained close to 100% in all the treatment combinations.

N pools

The composite response of all N-related response parameters was affected by light and by the NH_4^+ × tissue interaction (PERMANOVA, p = 0.002 and p = 0.006, respectively; Table 2). The effect of NH_4^+ enrichment was stronger in leaves (all 3 treatment levels different from each other, $p < 0.05$) than in the roots/rhizomes (C treatment only different from the +NN treatment, p = 0.007). Total N and most of the N species within the plants (i.e. intracellular inorganic N, FAA and residual N) increased substantially with NH_4^+ enrichment, although the content of soluble proteins showed the opposite pattern. All N species were typically more abundant in plants grown under low light than under high light, and levels were also higher in leaves than in the roots/rhizomes.

Total N (Fig. 2A) was significantly affected by plant part and the interaction between NH_4^+ and light (Table 2). Total N was 2-fold higher in leaves than in the roots/rhizomes, and increased about 30–50% with NH_4^+ addition, being ca. 2.5% of DW in the +NN treatment. The relative increase in total N with NH_4^+ enrichment was larger in plants cultivated under high light than under low light.

Intracellular NH_4^+ (Fig. 2B) constituted less than 1‰ of total N, but was affected significantly by NH_4^+ enrichment, light and plant part, but not by any of the interactions (Table 2). Intracellular NH_4^+ increased substantially with NH_4^+ enrichment and levels were

Table 2. Statistical results of the MANOVA (composite response) and ANOVA (individual responses) analyses examining the effect of light level, ammonium supply and plant tissue on various N pools (total N, ammonium-N, nitrate-N, free amino acid-N, soluble protein-N, chlorophyll-bound N and residual N) in *Zostera marina*

Variable, factors	df	MS	Pseudo-*F*	p
MANOVA				
Ammonium supply (N)	2	27.431	11.63	0.001
Light (L)	1	18.181	7.71	0.002
Tissue (Ti)	1	86.517	36.69	0.001
N × L	2	1.961	0.83	0.509
N × Ti	2	8.809	3.74	0.006
L × Ti	1	1.933	0.82	0.441
N × L × Ti	2	5.367	1.14	0.319
ANOVA				
Total N content				
Ammonium supply (N)	2	4.946	66.10	0.001
Light (L)	1	1.315	17.58	0.001
Tissue (Ti)	1	19.450	259.96	0.001
N × L	2	0.263	3.51	0.049
N × Ti	2	0.572	7.65	0.003
L × Ti	1	0.084	1.13	0.311
N × L × Ti	2	0.397	5.31	0.018
Ammonium content				
Ammonium supply (N)	2	4.563	9.11	0.003
Light (L)	1	2.992	5.98	0.026
Tissue (Ti)	1	8.279	16.53	0.001
N × L	2	0.053	0.11	0.888
N × Ti	2	1.081	2.16	0.138
L × Ti	1	0.287	0.57	0.471
L × N × Ti	2	0.016	0.03	0.969
Nitrate content				
Ammonium supply (N)	2	1.032	1.00	0.375
Light (L)	1	4.559	4.42	0.059
Tissue (Ti)	1	1.140	1.10	0.329
N × L	2	0.107	0.10	0.895
N × Ti	2	0.090	0.09	0.925
L × Ti	1	0.099	0.10	0.755
L × N × Ti	2	0.983	0.95	0.424
Free amino acids				
Ammonium supply (N)	2	6.834	49.78	0.001
Light (L)	1	0.830	6.05	0.024
Tissue (Ti)	1	8.789	64.02	0.001
N × L	2	0.684	4.98	0.017
N × T	2	3.034	22.10	0.001
L × Ti	1	0.367	2.68	0.107
L × N × Ti	2	0.307	2.24	0.141
Soluble proteins				
Ammonium supply (N)	2	3.238	8.39	0.002
Light (L)	1	4.448	11.52	0.002
Tissue (Ti)	1	8.315	21.54	0.001
N × L	2	0.045	0.12	0.883
N × T	2	2.792	7.24	0.006
L × Ti	1	0.276	0.72	0.362
L × N × Ti	2	0.274	0.71	0.502
Chorophyll *a* + *b*				
Ammonium supply (N)	2	0.434	2.41	0.132
Light (L)	1	1.416	7.85	0.009
N × L	2	0.277	1.54	0.250
Residual N				
Ammonium supply (N)	2	6.601	48.14	0.001
Light (L)	1	3.329	24.28	0.001
Tissue (Ti)	1	10.545	76.91	0.001
N × L	2	0.669	4.88	0.012
N × T	2	1.024	7.47	0.003
L × Ti	1	0.112	0.82	0.391
L × N × Ti	2	0.568	4.14	0.024

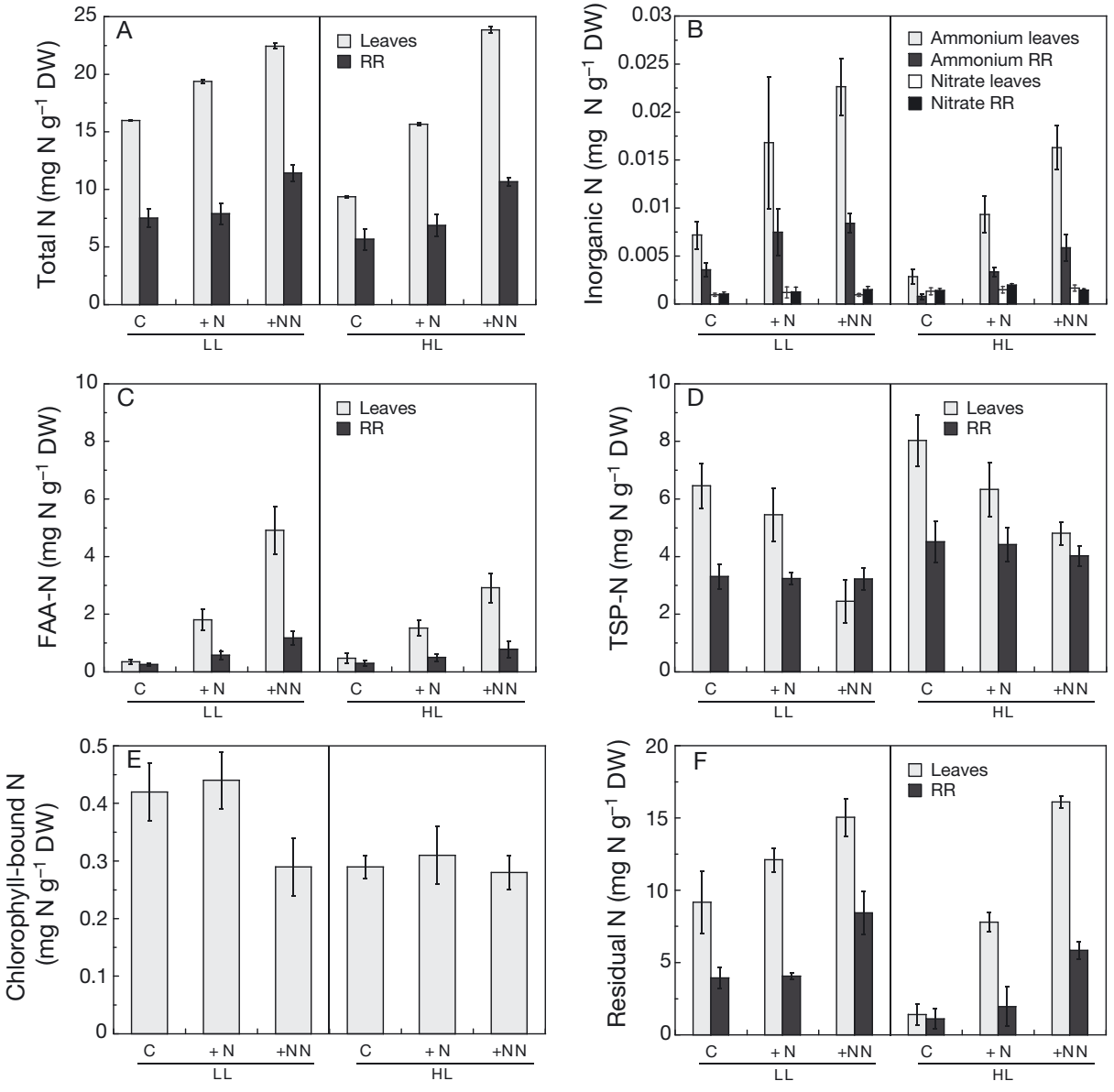

Fig. 2. *Zostera marina*. Nitrogen pools in aboveground (leaves) and belowground (root/rhizomes, RR) tissues under each ammonium and light treatments (means ± SE across 3 replicate aquaria): (A) total nitrogen content, (B) intracellular nitrogen content (ammonium and nitrate), (C) free amino acid nitrogen (FAA-N), (D) total soluble protein nitrogen (TSP-N), (E) chlorophyll-bound N, and (F) residual nitrogen. C, + N, +NN: 0, 10, 25 µM ammonium concentration, respectively; LL: low light; HL: high light

higher in plants grown in low light than in high light. Leaves contained always more NH_4^+ than the roots/rhizomes.

Intracellular NO_3^- made up less than 1‰ of total N (Fig. 2B) and was only affected by light ($p = 0.046$); levels were higher in plants cultivated under high light.

Nitrogen bound in free amino acids (FAA-N) made up between 4 and 12% of total N depending on treatment (Fig. 2C). FAA-N was affected by the interactions between NH_4^+ and light and NH_4^+ and plant part (Table 2). FAA-N increased more with NH_4^+ enrichment in the leaves than in the roots/rhizomes and more in low light than in high light.

The amount of N bound in soluble proteins (TSP-N) made up 25–60% of total N (Fig. 2D) and was affected significantly by light and by the interaction between NH_4^+ and plant part (Table 2), but responded quite different than the other N species. TSP-N in leaves decreased markedly with NH_4^+ enrichment, being 30–60% lower in plants from the +NN treatment than in those from the control treatment. TSP-N in the roots/rhizomes was relatively unaffected by

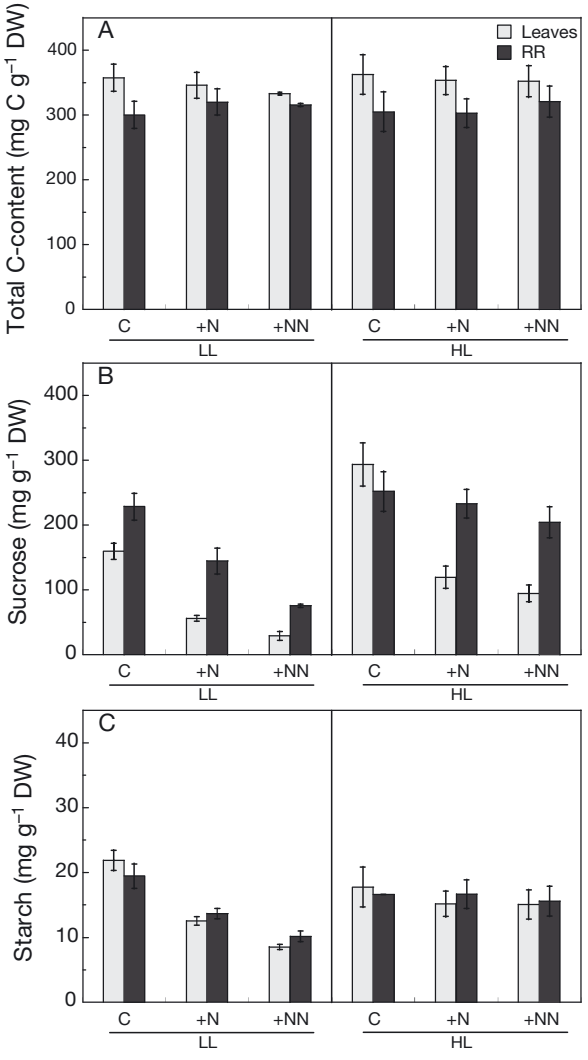

Fig. 3. *Zostera marina*. (A) Carbon content, (B) sucrose and (C) starch concentration in aboveground (leaves) and belowground (roots/rhizomes, RR) tissues under low light (LL) or high light (HL) and different ammonium supply (C, +N, +NN: 0, 10, 25 µM, respectively) as treatments. Data are means ± SE across 3 replicate aquaria

NH_4^+ treatment. TSP-N was higher in plants grown in high than in low light.

The amount of N bound in chlorophyll *a* + *b* (Chl-N) made up 1–2% of total N in the leaves (Fig. 2E). Chl-N was only affected by the light (Table 2), being ca. 30% higher in plants grown in low light than in high light.

The amount of residual N compounds made up 30–63% of total N depending on treatment and plant part (Fig. 2F). Residual N was affected by the highest order interaction (i.e. NH_4^+ × light × plant part); the amount increased with NH_4^+ enrichment, but more so in the roots/rhizomes than in the leaves and more so in high light than in low light.

Table 3. Statistical results of the MANOVA (composite response) and ANOVA (individual responses) analyses examining the effect of light level, ammonium supply and plant tissue on various carbon pools (total carbon, sucrose and starch) in *Zostera marina*

Variable, factors	df	MS	*F*	p
MANOVA				
Ammonium supply (N)	2	11.922	8.75	0.001
Light (L)	1	9.551	7.01	0.001
Tissue (Ti)	1	26.527	19.46	0.001
N × L	2	2.901	2.13	0.079
N × T	2	2.045	1.52	0.203
L × Ti	1	0.415	0.30	0.790
L × N × Ti	2	0.996	0.73	0.615
ANOVA				
Total carbon				
Ammonium supply (N)	2	0.030	0.08	0.916
Light (L)	1	0.320	0.83	0.370
Tissue (Ti)	1	21.725	56.21	0.001
N × L	2	0.438	1.13	0.349
N × T	2	1.013	2.62	0.090
L × Ti	1	0.387	1.00	0.308
L × N × Ti	2	0.165	0.43	0.655
Sucrose				
Ammonium supply (N)	2	7.231	41.18	0.001
Light (L)	1	8.274	47.12	0.001
Tissue (Ti)	1	4.796	27.31	0.001
N × L	2	0.060	0.34	0.736
N × T	2	0.783	4.46	0.014
L × Ti	1	0.020	0.11	0.721
L × N × Ti	2	0.774	4.41	0.026
Starch				
Ammonium supply (N)	2	4.661	5.82	0.007
Light (L)	1	0.957	1.19	0.304
Tissue (Ti)	1	0.007	0.01	0.939
N × L	2	2.403	3.00	0.073
N × T	2	0.279	0.35	0.719
L × Ti	1	0.008	0.01	0.916
L × N × Ti	2	0.057	0.07	0.920

C pools

The composite response of all C-related response parameters was affected by all main factors, i.e. N treatment, light and plant part (all $p < 0.001$; Table 3), but not by any of the interactions. Total C content (Fig. 3A) averaged 350.6 ± 10.4 and 309.7 ± 8.8 mg C g^{-1} DW in leaves and roots/rhizomes, respectively, and was only affected significantly by plant part (Table 3).

The concentration of sucrose (Fig. 3B) was affected by the highest order (NH_4^+ × light × plant part) interaction (Table 3). Sucrose decreased substantially with NH_4^+ enrichment and the decrease was largest in low-light plants where the content in leaves de-

Table 4. *Zostera marina*. C:N, sucrose-C:total C and sucrose-C:FAA-N ratios under each ammonium and light treatment in aboveground (leaves) and belowground (roots/rhizomes, RR) tissues (mean ± SE). C, + N, +NN: 0, 10, 25 µM, respectively; LL: low light; HL: high light

— Treatment —		C:N (molar ratio)		Sucrose-C:Total C (%)		Sucrose-C:FAA-N (mg C mg^{-1} N)	
Light	NH_4^+	Leaves	RR	Leaves	RR	Leaves	RR
LL	C	26.6 ± 2.7	46.6 ± 1.0	18.8 ± 1.4	32.0 ± 1.5	202.0 ± 37	370.3 ± 37.9
	+N	20.8 ± 0.3	47.3 ± 1.1	6.8 ± 0.7	18.9 ± 0.9	14.0 ± 2.6	122.8 ± 37.3
	+NN	17.4 ± 1.0	28.6 ± 3.9	3.7 ± 0.8	10.8 ± 0.6	2.9 ± 1.1	29.2 ± 2.9
HL	C	45.4 ± 2.4	62.6 ± 2.7	34.0 ± 3.4	33.1 ± 5.5	282.7 ± 68.1	448.6 ± 229.9
	+N	26.4 ± 1.3	52.1 ± 3.1	14.2 ± 2.0	29.6 ± 1.8	34.2 ± 7.1	205.3 ± 59.7
	+NN	17.3 ± 0.6	36.0 ± 4.0	11.4 ± 1.7	27.3 ± 4.1	13.6 ± 0.3	135.5 ± 37.3

creased to ca. 16% of that in plants from the control treatment. The decrease in sucrose content with NH_4^+ enrichment was more pronounced in leaves than in the roots/rhizomes.

The starch content was always one order of magnitude lower than that of sucrose (Fig. 3C). Starch was only affected significantly by NH_4^+ treatment (Table 3). The content of starch was rather similar in leaves and roots/rhizomes and NH_4^+ enrichment caused a significant drop in starch in both plant parts. Plants cultivated under high light had similar contents of starch across NH_4^+ treatments.

Ratios of C:N, sucrose-C:total C and sucrose-C:FAA-N were typically higher in the root/rhizomes than in leaves (Table 4). The C:N ratio mainly reflected variations in total N and declined with NH_4^+ enrichment. The sucrose-C:total C ratio mainly reflected changes in the sucrose content and was strongly influenced by NH_4^+ enrichment and light, reaching its lowest values in the +NN treatment under low light. The sucrose-C:FAA-N ratio was affected by NH_4^+ enrichment and light, being lowest at high NH_4^+-enrichment combined with low light.

DISCUSSION

Our study demonstrated that relatively high, but ecologically relevant, concentrations of NH_4^+ (i.e. in the range of 0–10 and 0–25 µM) in the water had significant negative effects on the composite and on several individual physiological responses that represented plant fitness. Exposure to 10 and 25 µM NH_4^+ for 5 wk lead to leaf necrosis, and slowed down the leaf growth rate, the production of side-shoots, the leaf abundance and the net growth rate, but did not affect photosynthesis, respiration, plastochrone interval or survival. The adverse effects of NH_4^+ were intensified when plants were cultured under relatively low light.

Toxic effects of high NH_4^+ concentrations are well studied among terrestrial plants, including crop plants (Britto & Kronzucker 2002). High water concentrations of NH_4^+ can stimulate leaf necrosis and reduce the photosynthetic performance, leaf elongation rate, shoot size, biomass and survival in several seagrass species (e.g. van Katwijk et al. 1997, Brun et al. 2002, 2008, van der Heide et al. 2008). The negative responses reported in these studies show a great deal of variability depending on the experimental set-up (i.e. applied N concentrations, pulsed versus constant enrichment, duration) and seagrass species involved. Most of these studies have, however, exposed plants to rather high concentrations of inorganic N, e.g. 100–200 µM NH_4^+ (van Katwijk et al. 1997, Brun et al. 2002, van der Heide et al. 2008, Christianen et al. 2011). Dissolved inorganic N concentrations undergo considerable seasonal variations in eutrophic estuaries, but rarely exceed 100–150 µM. A review on nutrient concentrations in 33 Danish estuaries (all considered eutrophic) revealed that the average (across estuaries) concentration of inorganic N ranges from ca. 100 µM in winter (October to March) to a few µM in summer and that the bulk of this nitrogen is in the form of NO_3^-, whereas NH_4^+ typically makes up less than 10–20% of the total inorganic N (Conley et al. 2000). Only 2 studies have so far investigated the effect of lower and more ecologically relevant NH_4^+ concentrations. Brun et al. (2002) found that leaf-elongation, plastochrone interval and net plant growth in *Zostera noltii* were affected negatively when exposed to a constant concentration of 16 µM NH_4^+, while Brun et al. (2008) reported that ca. 15 µM NH_4^+ had a negative effect on net shoot growth and photosynthetic performance (F_v/F_m) in *Z. noltii*. Brun et al. (2008) further documented that the adverse effect of elevated NH_4^+ was correlated to a reduction in sucrose within the plants and that the negative effects of NH_4^+ were alleviated by high light. These results indicate that the adverse effect of NH_4^+ may be related to increased

competition for C skeletons between NH_4^+ assimilation and other metabolic processes (Brun et al. 2008). Uptake of NH_4^+ by seagrasses depends on the external concentration in the medium (Thursby & Harlin 1982, Rubio et al. 2007, Villazán et al. 2013) and may be passive at high concentrations where low-affinity systems tend to operate (Britto & Kronzucker 2002). In order to avoid intracellular accumulation of toxic levels of ammonium, this compound is quickly assimilated into amino acids, which are used for the synthesis of proteins or stored if the assimilation of inorganic N exceeds the requirements needed for growth (e.g. Marschener 1995).

Five weeks of NH_4^+ enrichment led to a doubling of total N in the plants. All investigated N pools (with the exception of NO_3^- and soluble proteins) increased in response to NH_4^+ enrichment. Intracellular NH_4^+ increased almost 4-fold, but made up less than 1‰ of total N in all treatments, suggesting rapid assimilation or an active efflux of NH_4^+ (Britto & Kronzucker 2002). Rapid assimilation seems most feasible since the amount of FAA increased almost 7-fold in the +NN treatment relative to that in the control treatment. The amount of N bound in the residual N pool, i.e. aromatic and structural amino acids, structural proteins and other N compounds not accounted for in the chemical analyses, increased by a factor of 3. The pools of FAA-N and residual N were both rather large, making up 12.5% and 62.5% of total N in the +NN treatment, respectively. The large size and substantial increase of these N-pools during N-enrichment indicate that these N-compounds constitute the major storage compounds in eelgrass. Rapid assimilation and synthesis of amino acids and other N compounds were able to keep intracellular concentrations of NH_4^+ low in our plants despite a relatively high external concentration in the medium.

Soluble proteins decreased by almost 50% with increasing N enrichment, which was somewhat unexpected given the increase in total FAA and total N. Similar patterns have been observed in terrestrial plants exposed to high NH_4^+ concentrations and it has been suggested that high NH_4^+ availability either causes a higher turnover rate of proteins, or that energy and C skeletons are diverted from protein synthesis to NH_4^+ assimilation (e.g. Dominguez-Valdivia et al. 2008). This would explain why the concentration of soluble proteins was inversely related to the concentration of FAA. It would also explain why concentrations of soluble proteins were higher while concentrations of FAA were lower in high-light plants where more C and energy derived from photosynthesis were available.

Sustained synthesis and storage of amino acids may constitute a problem for seagrasses under low-light conditions since these processes require C skeletons and energy, both of which must be provided from photosynthesis or through mobilization of C reserves. Amino acids have C:N ratios ranging from 6:1 to 5:3, which means that 6 to 1.7 mol C are required for each mol N assimilated. Extended periods with high DIN availability and low light may therefore lead to competition between N assimilation and other metabolic processes for C and energy.

Ammonium enrichment caused the concentration of sucrose in the leaves to drop 68 and 84% (in high and low light, respectively) over the course of the experiment, whereas the concentrations in the roots/rhizomes decreased by 19 and 67%. The starch concentration in the leaves was also reduced, although less than sucrose (15 and 61% for high- and low-light plants, respectively). Because enrichment with NH_4^+ did not affect net photosynthesis and respiration significantly, the drop in sucrose and starch cannot be explained by a lower net gain of inorganic C in plants enriched with NH_4^+. We suggest that the depletion in sucrose and starch resulted from mobilization of C reserves to cover the demands related to enhanced assimilation of NH_4^+. A simple mass balance shows that this is indeed possible. The net uptake of NH_4^+-N over 35 d in the +N and high-light treatment amounted to ca. 250 µmol N plant^{-1} (taking growth and changes in total N into account). If all that NH_4^+-N was assimilated it would correspond to a C requirement of ca. 625 µmol C plant^{-1} assuming that glutamine (having a C:N ratio of 5:2) was the major amino acid being synthesized. Using the observed rates for photosynthesis and respiration (Fig. 1A), net photosynthesis should yield ca. 622 µmol C plant^{-1} over 35 d (using a 16 h light:8 h dark cycle), while mobilization of the sucrose and starch could provide 112 µmol C plant^{-1}. Photosynthesis and mobilization of C could thus cover the C demand needed for assimilation of the acquired N. A similar estimate for plants in the +NN, high-light treatment shows that photosynthesis and mobilization together could provide ca. 1070 of the 1088 µmol C plant^{-1} needed for assimilation of the acquired N.

We were unable to carry out the same sort of estimate for plants grown under low light and N enrichment due to the large amount of biomass lost by these plants over the course of the experiment. However, these plants were exposed to a light level close to their compensation irradiance and nearly all the C needed for N assimilation must therefore have been provided from mobilization of sucrose

and starch. A larger importance of sucrose and starch mobilization in low-light plants is indicated from the larger drop in both these compounds compared with the high-light plants. Thus, all the metabolic and catabolic processes in plants grown under low light and elevated NH_4^+ concentrations may have undergone tougher competition for C skeletons and energy, which may have affected growth and fitness of the plants. This hypothesis is supported by studies where addition of α-ketoglutarate (i.e. C skeletons) to N-enriched plants can stimulate N assimilation and the synthesis of amino acids (e.g. Magalhaes et al. 1992).

We found that that high, but ecologically relevant, concentrations of NH_4^+ can have an adverse effect on *Zostera marina*, especially under low-light conditions. Several measures for growth, but not survival, were affected negatively by the combination of elevated NH_4^+ concentrations and low light. Our experiment lasted only for 5 wk, but the sucrose reserves were almost completely depleted in low-light plants by the end of the experiment. We suggest that continued exposure to these conditions would have reduced survival substantially. The most vulnerable plants will therefore be those living in deeper waters close to their depth limit or those shaded by phytoplankton, epiphytes or drifting macroalgae. Light attenuation in the water column is the main predictor of eelgrass depth limits, but studies on the relationship between Secchi depth, light attenuation and seagrass depth limits often tend to overestimate predicted depth limits in eutrophic areas with a high turbidity (Duarte et al. 2007). Krause-Jensen et al. (2011) showed that sediment characteristics such as a high content of organic matter, total N, total P and hydrogen sulphide could partly explain why observed depth limits of eelgrass were lower than predicted in Danish coastal waters. Elevated concentrations of NH_4^+ near the bottom may also explain why the depth limits are lower than predicted from the light environment alone. Although the concentrations of NH_4^+ in the water column are typically low (<2 µM) during summer, little is known about the concentrations in the bottom water close to the sediment. Fast decomposition of sediment organic matter and anoxia may stimulate the release of sediment NH_4^+ into the water during summer. Conley et al. (2007) showed that the net flux of NH_4^+ from sediment to the bottom water could reach ca. 300 µmol m^{-2} h^{-1} during mid-summer in shallow Skive Fjord (Denmark). This efflux caused the concentration of NH_4^+ in the bottom water to increase from <5 µM to 50–100 µM for 1 mo, while no increase was detected in the surface waters. The NH_4^+ concentration in the bottom water surrounding eelgrass plants may thus be significantly higher than indicated from water samples taken further up in the water column, and they may reach concentrations at which the performance of eelgrass is affected.

Coastal eutrophication is often followed by accumulation of drifting macroalgae that may cover entire seagrass meadows (e.g. Rasmussen et al. 2013). Mass accumulation of macroalgae in seagrass meadows typically occurs in summer and may impair light availability, but it also may cause an increase in the concentrations of NH_4^+ within and below the mat. Field studies by Bierzychudek et al. (1993) and Hauxwell et al. (2001) demonstrated that the NH_4^+ concentration increased from a few µM in the water above the algal mats to more than 100 µM at the bottom of mats with a thickness of 20–30 cm. Similar results have been obtained in laboratory experiments using mats of the green alga *Chaetomorpha linum* (e.g. Krause Jensen et al. 1999, McGlathery et al. 1997). These studies show that seagrasses can be exposed to conditions of low light and very high NH_4^+ concentrations in summer when more optimal conditions (i.e. high insolation and low NH_4^+ concentration) otherwise are expected. Whether algal mats may cause a serious impact on the seagrasses may to a large extent depend on the duration of the algal cover.

In summary, high, but ecologically relevant NH_4^+ concentrations had a negative effect on eelgrass performance. Net photosynthesis was not affected by NH_4^+ enrichment, but other measures of growth were affected negatively by elevated NH_4^+ concentrations. The negative effects were much more apparent in plants cultivated under low light than under high light and the adverse effects were correlated to a substantial decrease in sucrose and starch reserves. The negative effect of elevated NH_4^+ concentrations on eelgrass thus seems to be related to an imbalance in the C economy of the plant.

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