Ammonium toxicity in eelgrass *Zostera marina*

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ABSTRACT: Seagrasses are declining all over the world, resulting in a substantial loss of biodiversity, coastal sediment stabilization and nursery areas of economically important fish. The seagrass decline has often been associated with increasing eutrophication of coastal areas. We tested possible toxic effects of high nitrogen concentrations in the water layer on the seagrass *Zostera marina* L., which is often the sole higher plant inhabiting coastal zones in the northern hemisphere. Plants grown in either mud or sand were subjected to various water ammonium and nitrate concentrations, whereby ammonium and nitrate supply were balanced (both 25 µM or 75 µM), or unbalanced (ammonium 125 µM and nitrate 25 µM, and vice versa). We used 2 temperatures, 15 and 20°C. Analyses were made after 2 and 5 wk of exposure. In an additional experiment, 9 µM ammonium and 3 µM nitrate were supplied. An ammonium concentration of 125 µM in the water layer was toxic for *Z. marina*: the plants became necrotic within 2 wk. After 5 wk, plants in all treatments except for the 9 µM treatment were either necrotic or had died. This suggests that toxicity occurs at ammonium concentrations as low as 25 µM. Nitrate treatment had no effect. Ammonium toxicity effects were more pronounced in plants grown on sand and at the higher temperature. It is argued that the ammonium toxicity effects on *Z. marina* are expected to be strongest in autumn when irradiance decreases, temperature is still high, and ambient ammonium concentrations rise.

KEY WORDS: *Zostera marina* · Seagrass · Ammonium toxicity · Eutrophication · Nitrogen · Ammonium · Nitrate

INTRODUCTION

Seagrasses are the only submerged vascular plants inhabiting shallow coastal seas. They contribute substantially to biodiversity and coastal sediment stabilization and provide nursery areas for economically important species (e.g. Heck et al. 1995). Like most seagrasses throughout the world, many eelgrass *Zostera marina* L. populations in coastal areas of the northern hemisphere are under great pressure, or have already disappeared. This may be due to eutrophication, which elevates nitrogen. *Z. marina* is adapted to low nitrogen concentrations (Borum et al. 1989, Hemminga et al. 1991, Pedersen & Borum 1992). Increased nitrogen levels often induce increased growth and biomass (Short 1983a, 1987, Williams & Ruckelshaus 1993, Bohrer et al. 1995, van Lent et al. 1995) and affect plant

morphology, tissue C/N ratios, density and reproductive strategy (Harlin & Thorne-Miller 1981, Short 1983a, 1987, van Lent et al. 1995). In some cases no significant growth response to nitrogen enrichment has been reported (Dennison et al. 1987, Murray et al. 1992). In other cases, however, decline in eelgrass was observed, not only as a consequence of shading due to increased algal growth (Neckles et al. 1993, Williams & Ruckelshaus 1993, Harlin 1995, Short et al. 1995, Taylor et al. 1995), but also as a direct effect of increased nitrogen in the form of nitrate (Burkholder et al. 1992, 1994). It is well known that high ammonium levels can be toxic to plants. Submerged aquatic plants may be particularly vulnerable to high water nitrogen concentrations because not only the roots, but also the leaves, are encompassed by water. Leaves, unlike roots, cannot regulate nitrogen uptake, e.g. through the reduction of root hairs (Short 1983a, Marschner 1995). Until recently, ammonium toxicity to submerged aquatic plants has been given little attention. It has

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been observed in a few freshwater aquatic plants (Glänzer 1974, Grube 1974, Agami et al. 1976, Glänzer et al. 1977, Roelofs 1991, Smolders et al. 1996); however, it has not, as yet, been reported for *Z. marina* or any other seagrass.

Increased external nitrogen concentrations may result in an increased tissue nitrogen content of *Zostera marina* (Harlin & Thorne-Miller 1981, Short 1987, Borum et al. 1989, Burkholder et al. 1992, 1994). This may cause a lowering of the concentration of phenolics, which increases the susceptibility to wasting disease (L. H. T. Vergeer unpubl. results, Buchsbaum et al. 1990). In this way, increased nitrogen concentrations may have an indirect adverse effect on plant vitality. However, Ravn et al. (1994) found a positive correlation between phenolic acid contents and tissue nitrogen content.

It is well known that, in many terrestrial and freshwater plant species, the presence of high ammonium concentrations can decrease the uptake of other cations like potassium, magnesium or calcium, or even lead to their exclusion (e.g. Pearson & Stewart 1993, Marschner 1995, Smolders et al. 1996). It is not known whether this also occurs in the marine environment with its high availability of potassium, sodium, magnesium and calcium.

Optimal growth for most plant species is usually obtained with a mixed supply of ammonium and nitrate (Marschner 1995); ammonium toxicity can be alleviated by nitrate supply (e.g. Hecht & Mohr 1990, Ikeda 1991, Feng & Barker 1992, Adriaanse & Human 1993).

Both roots and leaves of *Zostera marina* are capable of nitrogen absorption (Iizumi & Hattori 1982, Thursby & Harlin 1982, Short & McRoy 1984, Pedersen & Borum 1992, Hemminga et al. 1994). Ammonium is preferred above nitrate (Thursby & Harlin 1982, Short & McRoy 1984, Hemminga et al. 1994). *Z. marina* leaves have a greater affinity for ammonium than do the roots (Thursby & Harlin 1982, Short & McRoy 1984, Pedersen & Borum 1992, Hemminga et al. 1994). However, depending on the nutritional status of the sediment and overlying water, and presumably on the root:shoot ratio, root uptake is more important in some ecosystems (Iizumi & Hattori 1982, Short & McRoy 1984) and leaf uptake in others (Hemminga et al. 1994), or they may be equally important (Pedersen & Borum 1992).

In this study, the effect of nitrogen form on size, condition and a number of shoot tissue constituents of *Zostera marina* was examined through the supply of ammonium and nitrate in balanced and unbalanced ratios. It was hypothesized that excess ammonium and/or nitrate may be toxic to *Z. marina*, which would indicate why this seagrass is absent in eutrophicated areas. It would also indicate why perennial populations in northwest Europe are found in locations with low water nitrogen concentrations throughout the year, whereas annual populations are found in submersed habitats with higher nitrogen concentrations (in the Netherlands: autumn and winter concentrations rising above 15 µM ammonium and 50 µM nitrate, unpubl. data Dutch Ministry of Transport, Public Works and Water Management 1980–1990, van Lent & Verschuure 1994a). Due to contamination by ammonium and nitrate of the various synthetic sea salt mixes tested, low levels of ammonium and nitrate could not be applied to the plants.

MATERIALS AND METHODS

Zostera marina L. plants were subjected to water ammonium and nitrate concentrations of 9:3, 25:25, 25:50, 25:125, 75:75 and 125:25 µM, respectively. We used 2 sediment types, mud and sand, and 2 (growing season) temperatures, 15 and 20°C. The ammonium: nitrate treatment of 25:50 µM was applied at 15°C only. The ammonium:nitrate treatment of 9:3 µM was applied at 17°C and on mud only. Sampling was conducted after 2 and 5 wk. The 9:3 treatment was sampled after 3 and 6 wk.

Culture experiment. *Zostera marina* plants were collected and rinsed in the harbour-canal of Goes (SW Netherlands, 51° 32' N, 3° 50' E, 12 October 1992). The plants for the ammonium:nitrate 9:3 µM treatment were collected in the Wadden Sea (Eems and Terschelling, 53° 22' N, 5° 13' E, 11 August 1993). The plants were transported (at 8°C, corresponding to the temperature in Goese Sas) to the laboratory, and maintained overnight at 4°C. The following day, pairs of plants were placed in 75 ml jars filled with coarse sand or mud originating from dune sand or from a *Z. marina* habitat in Zandkreek, respectively (both in the Netherlands). A thin layer of sand was put over the mud to counter nutrient exchange with the overlying water. Twenty jars per container were placed in 18 containers filled with synthetic sea water (25.4‰ S, Wimex, of Wiegandt GmbH, Krefeld, Germany), and maintained under an 8 h dark:16 h light cycle corresponding to growing season conditions; light intensity just below the water is surface averaged 90 µE m^{-2} s^{-1}. The containers were placed in random sequence in a temperature controlled (15°C) water bath (Figs. 1 & 2). For technical details of the set-up, see Roelofs et al. (1984). The plants were allowed to acclimate for 3 wk. Macroalgae were removed, with caution taken not to damage the plants, from the second week onwards (*in situ*, water dynamics would have impeded macroalgal growth). On 5 November 1992 (9:3 treatment: 27 August 1993), the treatments started: NaNO$_3$ and NH$_4$Cl enriched synthetic sea water was pumped
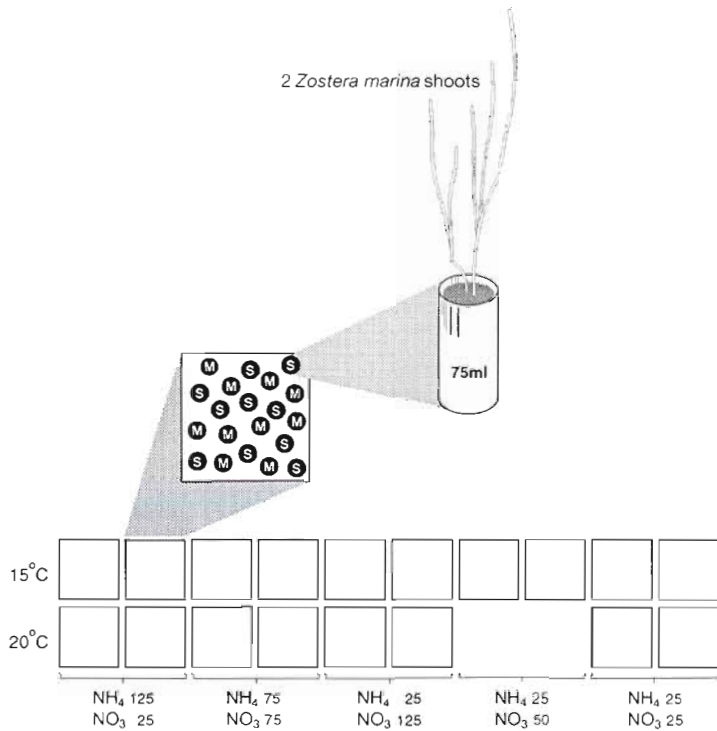
Fig. 1. Scheme of the experimental set-up. In the actual set-up, the containers were randomly placed. S: sand, M: mud

through the containers. [In making the stock solutions, it was calculated that the 25.4‰ S synthetic sea water (Wimex, 3 batches tested) already contained 10 µM ammonium and 25 µM nitrate on average. Sea salts of Reef Crystals (Aquarium Systems, Sarrbourg, France, 3 batches) were also tested, and appeared to contain even more ammonium and/or nitrate on average, which was also the case with Rila sea salts, tested by Rohrmann et al. (1992). Also, the variation was larger in Reef Crystals sea salt than in Wimex sea salt.] The ammonium:nitrate 9:3 µM treatment was applied using a self-prepared saltmix, derived from uncontaminated 'pro analysi' salts. Regrettably, it was too expensive and time-consuming to prepare this mix for all treatments. Each container was replenished once a day from its own stock container. The containers for the 20°C treatment were thermostatically heated. Water in the containers was gently aerated to ensure complete mixing.

Sampling. In Goese Sas, water samples were taken on 3 September and 12 October (date of collection). Water was sampled in the culture

Fig. 2. Photograph of the experimental set-up

experiment at the onset of the treatments, and after 2 and 5 wk. Three water samples were taken per occasion per container Alkalinity and pH were measured instantaneously in 1 sample, the 2 other samples were filtered, whereafter citric acid was added to one of them in order to prevent precipitation of metals (for ICP analysis, see below). The samples were stored at −20°C until further analysis.

Eight to 10 *Zostera marina* plants (5 jars) per sediment type were sampled from each container after 2 and 5 wk. The sediments were stored at 4°C. The sediments of the 5 jars were then mixed and water content was calculated from weight loss after drying 25 to 40 g of the wet sediment at 105°C over 48 h. Seventy to 80 g of fresh sediment were placed in a 500 ml polyethylene bottle with 200 ml of double-distilled water and shaken for 1 h. This mixture was centrifuged for 20 min at 11 000 rpm (r_{max} = 19 690 × g) and the supernatant stored at −20°C.

Plant analysis. The plants were sampled to measure length, width, wasting disease-like lesions, necrosis and dry weight. Leaf scores for wasting disease-like lesions and necrosis, based on the percentage of total leaf surface (Table 1), were estimated for each of the first 3 leaves separately. The leaf scores were averaged to calculate shoot values, whereby damage on young leaves was given a higher weight than damage on older leaves as the duration of their exposure to possible negative influences is less than that of older leaves (Giesen 1990). Distinction was made between discoloured leaf surface and infected leaf surface, the former being used to assess necrosis (including wasting disease-like lesions), the latter to assess only wasting disease-like lesions (examples in, among others, den Hartog et al. 1996). Three plants were dried at 70°C over 48 h to determine dry weight.

Chemical analysis. Two or 3 of the sampled plants were used to determine the concentration of phenolics in the shoots, using the method of Hagerman & Butler (1978), as described in Mole & Waterman (1987). The plants were freeze-dried for 2 d and

ground with the use of liquid nitrogen. Ten milligrams were extracted with 5 ml 80% ethanol for 10 min at 80°C. One millilitre of the extract was mixed with 2 ml sodium dodecyl sulfate (SDS) and 1 ml FeCl₃. The absorption was measured at 510 nm using a Schimadzu spectrophotometer (UV-120-01). Tannic acid (Sigma Chemical Company) was used as the standard.

Chlorophyll was extracted from leaf segments of 3 to 5 cm length which were taken from 3 cm below the top of the first fully grown leaf. The pieces of 2 shoots were taken together. The pieces were blotted dry and weighed. Chlorophyll *a* (chl *a*) was measured spectrophotometrically after extraction in 80% ethanol. Ca 2 ml extractant per 10 mg (fresh weight) of leaf material was used. The acidification method was used to correct for phaeophytin (Moed & Hallegraeff 1978). Calculations of chl *a* concentrations were performed according to Roijackers (1981).

Two or 3 shoots were used to analyze various chemical constituents of the shoots. The shoots were digested with sulfuric acid and hydrogen peroxide: 50 mg of oven-dried (48 h, 70°C) and ground shoot tissue were dissolved in 5 ml concentrated H_2SO_4, incubated at room temperature for 24 h, heated to 150°C and digested by slowly adding 2 ml 30% H_2O_2. The volume of the digest was brought to 100 ml with double-distilled water.

In the water samples, sediment extracts and shoot digests, total P, Mg, Ca, Fe, Mn, total S (only in sediment extracts), and Zn (only in shoot digests) were measured with an Inductively Coupled Plasma spectrophotometer (ICP), type IL Plasma 200. Ammonium, nitrate and chloride were measured colorimetrically with a Technicon AAII system according to Kempers & Zweers (1986), Grasshoff et al. (1983), and O'Brien (1962), respectively. In the shoot digestion process, nitrogen is partly converted to ammonium, and partly to nitrate; therefore, for shoots, total N is presented, i.e. ammonium+nitrate contents. K^+ and Na^+ were determined by flame photometry. pH of the water samples was measured with a pH electrode; alkalinity was estimated by titration with 0.01 M HCl down to pH 4.2.

Statistical analysis. Water and sediment parameters were normally distributed, as were length, width, biomass, phenolic contents and chl *a* of the shoots. Necrosis, wasting disease-like lesions and water content, being (based on) percentages, were normally distributed after arcsine transformation. The remaining chemical parameters of the shoots were lognormally distributed. The back-transformed means were used as a central measure. As a measure of variance, standard error of the mean was used. For lognormal parameters, this was calculated according to Mood et al. (1974).

Table 1. *Zostera marina*. Leaf scores for necrosis or wasting disease-like lesions, based on the percentage of damaged leaf surface, from which shoot values were calculated (based on Giesen 1990). All shoot values, including those for the 9:3 treatment, were pooled for each container

| | Damaged leaf surface (%) | | | | | |
	0	1	2–5	6–10	11–30	>30
First leaf	0	0.2	0.4	0.6	0.8	1.0
Second leaf	0	0.1	0.2	0.4	0.6	0.8
Third leaf	0	0	0.1	0.2	0.4	0.6
Only the first 3 leaves were included in the calculation						

Analysis of variance was used in this split-plot experiment (sediment type being a subplot factor, the containers being the experimental unit), whereby N-treatment, temperature and their interaction were tested with the following error-term: N-treat-ment × temperature × replicate + N-treatment × replicate + temperature × replicate, and sediment type and the interactions of sediment type with the other parameters were tested against the residual error (Steel & Torrie 1980, Freund & Littell 1985). For comparison of means, Tukey's test was used. The ANOVA and Tukey's test were carried out using the Statistical Analysis System, procedure GLM (SAS 1989).

The results were ordinated using Redundance Analysis (RDA, which is the canonical form of Principal Components Analysis, PCA) to detect and illustrate the correlations between the various shoot response parameters and the effects of the treatments on them. RDA extracts ordination axes on the basis of the shoot response variables, like in PCA, but with the constriction that they should be a linear combination of the explanatory variables, i.e. the treatments (Jongman et al. 1995). We chose scaling 2 (covariance biplot), because we were also interested in the correlations of the shoots responses, rather than on the 'distances' between the containers, which would require scaling 1 (ter Braak 1994). The ordination was carried out with help of the program CANOCO (ter Braak 1987).

RESULTS

Water

The measured chemical properties of the water were not influenced by the treatments, except pH and alkalinity, and, of course, ammonium and nitrate (Table 2). At 20°C, pH was lower than at 15°C, whereas alkalinity was higher. In the high ammonium treatment, pH tended to be lower than in the other treatments.

Ammonium concentrations in containers were lower than the concentrations applied (Fig. 3): application of 125, 75, 25 and 9 µM ammonium resulted in an actual concentration in the water of circa 70, 20, 10 and 3 µM, respectively. With pH values of the water being 8.5 on average (Table 2), associated ammonia must have been formed in appreciable quantities and its loss into the air could account for some of the missing

nitrogen. Also, large quantities were probably absorbed by the plants. This is supported by our finding that on the last sampling date, when the majority of plants in the highest ammonium concentration had died, the ammonium concentration was higher than on the previous dates (Fig. 3). The nitrate concentrations in the water corresponded with the concentrations applied, indicating that no large losses of nitrate occurred (Fig. 3). The nitrate concentrations measured on the first 2 sampling dates were found to be higher than 100 µM, but unfortunately no discrete analyses were made.

At the collection site, the ammonium concentration was 16 µM on the day of collection; nitrate was not measured. One month earlier, ammonium concentration was 15 µM and nitrate 0.8 µM.
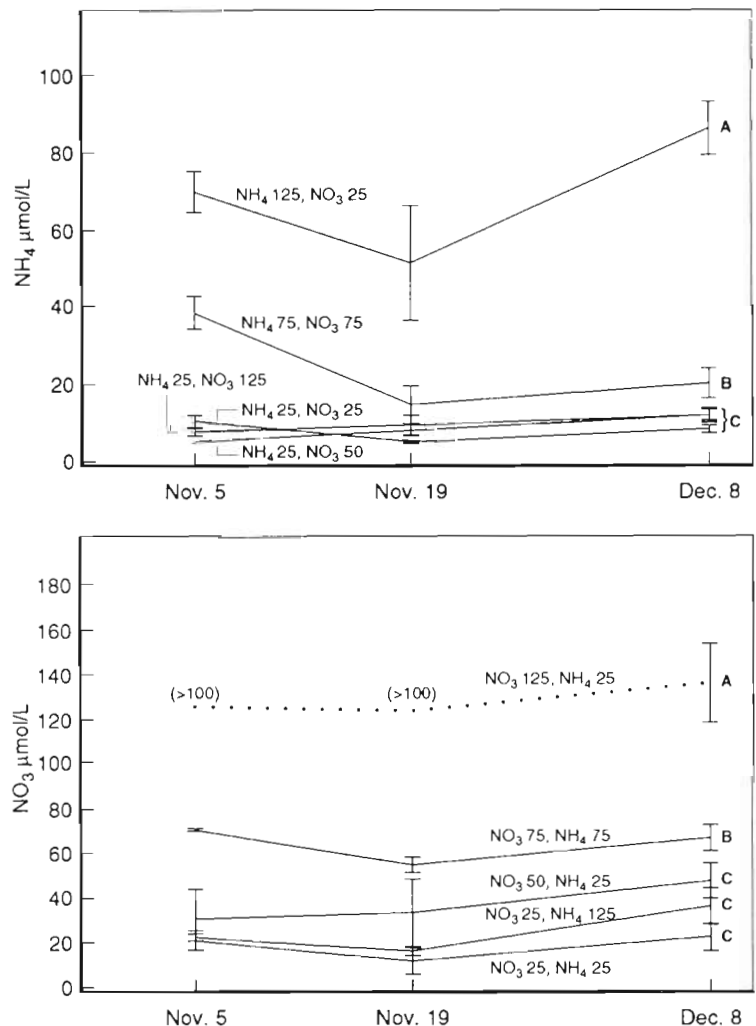

Fig. 3. Ammonium and nitrate in the water at the onset of the treatment and after 2 and 5 wk. N-treatments are presented in µM. Means and SEM are plotted. Different letters denote significant differences at the 5% level according to Tukey's test

Table 2. pH and chemical composition of the water and the effects of N-treatment and temperature and their interactive effect (ANOVA). Values are means of sampling results at the onset of the treatment and after 2 and 5 wk; data pooled due to sampling date having no effect. nd: not detectable. •$0.01 \leq p \leq 0.05$, ••$0.001 \leq p < 0.01$, all other effects were non-significant

	Mean	SEM	Significance		
			N-treat.	Temp.	N-treat. × Temp.
pH	8.5	0.0	••		••
Alkalinity (meq l^{-1})	2.03	0.04			•
Cl (mM)	402	6			
S (mM)	17.7	0.3			
P (mM)	nd	nd			
Na (mM)	334	4			
Mg (mM)	33.1	1.0			
K (mM)	7.5	0.1			
Ca (mM)	5.45	0.17			
Fe (μM)	0.35	0.04			
Mn (μM)	0.30	0.02			

Sediments

Sediment nutrient values at 2 and 5 wk are shown in Table 3. Nitrogen and sulfate were found in lesser quantities in sandy sediment than in muddy sediment. Phosphorus and manganese were higher in the sandy sediment, probably due to its terrestrial origin. Over the time course of the experiment, magnesium, calcium and sulfate concentrations rose considerably, indicating sulfide oxidation. Nitrate could not be measured due to the high sulfur concentration.

Zostera marina

The number of shoots increased over time in the 9 μM ammonium treatment (Fig 4). In the other treatments, the number of shoots had decreased by ca 50% after the acclimation period and the first 2 wk of treatment. After 5 wk, a further decrease was observed in the higher ammonium treatments, especially those at 20°C, in which the majority of plants died. No substantial change in shoot numbers occurred in the other treatments (Figs. 5 & 6).

High ammonium concentration in the water layer was toxic for *Zostera marina*. The plants became necrotic within 2 wk, especially those at 20°C and on sand (Fig. 5). Necrosis was typified by brown-black discolouration. After 5 wk, plants of all treatments were necrotic, except for those in the 9 μM ammonium treatment (Figs. 4 & 6). The plants were generally smaller (shorter, leaves narrowed, weight lost) after 5 wk as compared to the first sampling date. This was more severely the case in the 125 μM ammonium 15°C
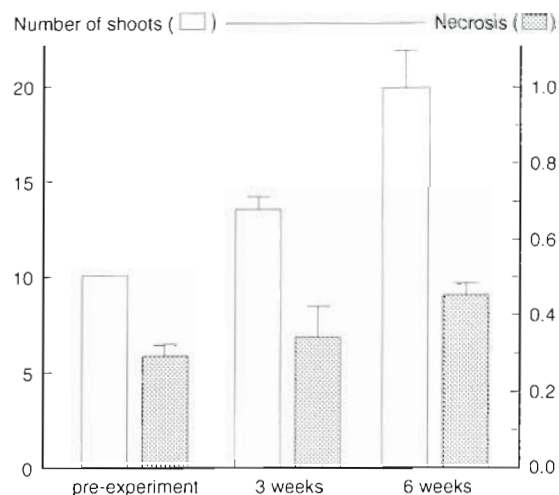

Fig. 4. Number of shoots and necrosis of *Zostera marina* in the 9 μM ammonium:3 μM nitrate treatment

treatment and the 75 μM ammonium 20°C treatment (Tables 4 to 7). In the lower ammonium treatments, on the other hand, temperature effect on plant size seemed to be the reverse, i.e. the plants were somewhat larger at 20°C (Table 6). Hence, the ammonium and temperature treatments showed an interactive effect on plant length (Table 7).

After 2 wk, the nitrogen treatments had influenced the shoot tissue nitrogen, potassium, phenolics, zinc and the ratios of shoot tissue nitrogen to potassium, magnesium and phosphorus (Tables 4 & 5, Figs. 5 & 6). These nitrogen treatment effects can be attributed to the ammonium treatment, rather than the nitrate treatment (Fig. 7, Tukey's Comparison of Means). The nitrogen content in the leaves amounted to 3.5% of the dry weight at the highest ammonium treatment (Table 4). After 5 wk, most ions and nutrient concentrations measured in the shoot tissue were highly variable, which may be the result of tissue damage and/or physiologi-

Table 3. Average water-extractable nutrient concentrations (μmol kg^{-1} DW) in the sediments measured after 2 and 5 wk. Values are pooled exclusive of the 9:3 treatment

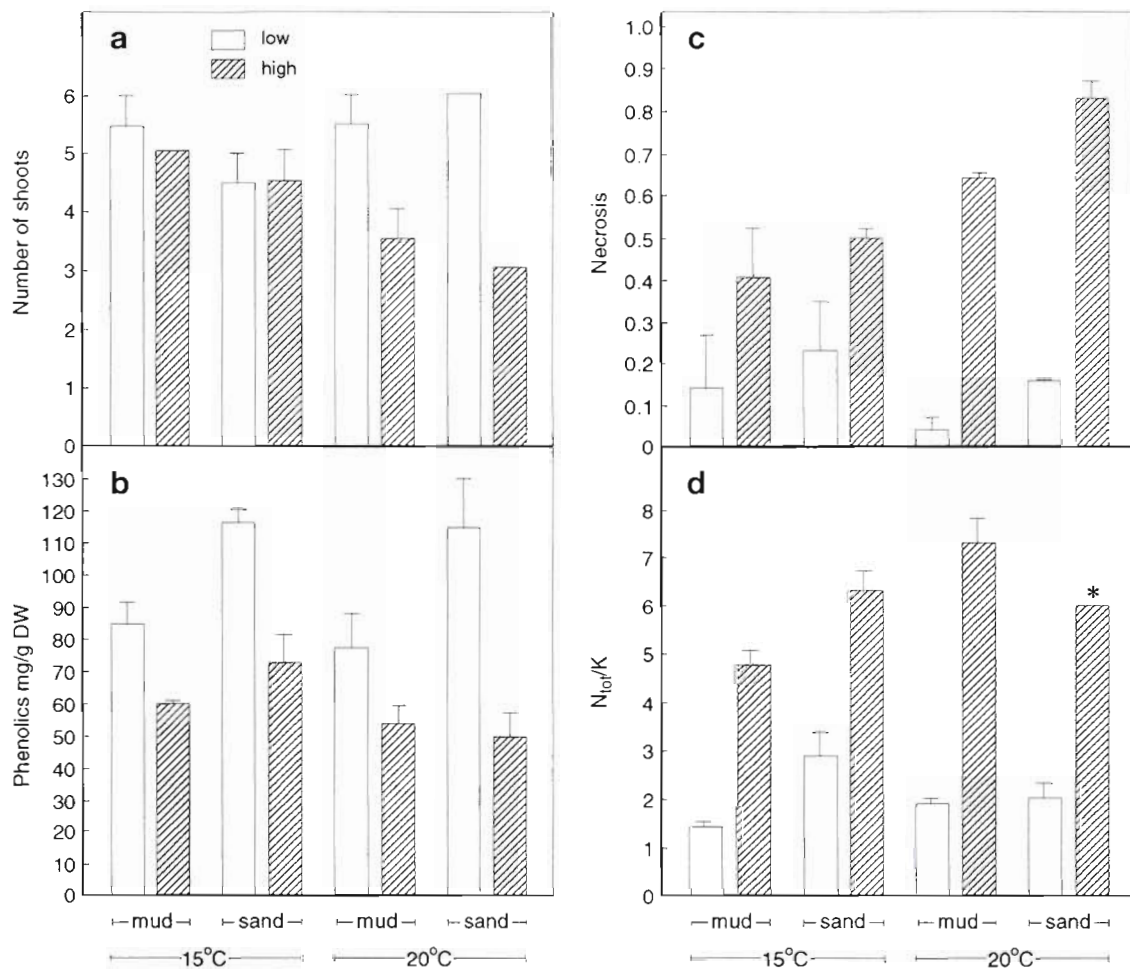|	2 wk		5 wk	
	Mud	Sand	Mud	Sand
NH$_4$	101	37	213	70
K	164	119	239	161
Ca	1060	940	1370	1310
Mg	3400	3500	5100	5500
Fe	2.0	1.6	2.8	3.3
Mn	0.06	20.5	0.08	13.9
P$_{tot}$	3.2	5.9	2.8	3.5
SO$_4$	2600	1900	3500	3300
Water content %	22	21	23	22

Fig. 5. Effect of 'high' (125 μM) and 'low' (25 μM) ammonium and of sediment type on (a) number of shoots, (b) phenolic content, (c) necrosis, and (d) N_{tot}:K ratio of *Zostera marina* after 2 wk of treatment. NO_3 supply for both treatments was 25 μmol l^{-1}. Index of necrosis: see 'Materials and methods'. *Only 1 replicate

cal breakdown. The effects that occurred after 5 wk were therefore less pronounced than the effects after 2 wk (Table 7). However, clear effects of ammonium treatments after 5 wk were still found with regard to plant number and size (smaller in high ammonium treatments), shoot tissue phenolics (lower in high ammonium treatments), and nitrogen and nitrogen: potassium ratios (higher in high ammonium treatments) (Tables 6 & 7, Fig. 6).

Phenolic content was inversely correlated with shoot tissue nitrogen content, nitrogen ratios and necrosis (Fig. 7, p ≤ 0.001). However, no correlation with wasting disease was found. Necrosis was further correlated with shoot tissue nitrogen content and the nitrogen ratios, and inversely correlated with number of shoots (Fig. 7, p ≤ 0.001).

In treatments with sandy sediment, plants had fewer shoots, were more necrotic and had higher shoot tissue magnesium, zinc, phosphorus, and phenolic contents

and lower shoot tissue potassium concentrations than plants in the muddy sediment (Tables 4 & 5, Figs. 5 & 7).

DISCUSSION

High ammonium concentration in the water layer was toxic for *Zostera marina*: the plants became necrotic within 2 wk. After 5 wk, plant size was much reduced, and a number of plants had died. At this time, plants in all treatments were necrotic, except for those in the 9 μM ammonium treatment (applied in a separate experiment), implying that the 25 μM ammonium treatment was also toxic.

Toxic effects of ammonium on *Zostera marina* have not been previously recorded. However, many indications of toxic effects can be found in the literature. For example, growth inhibition by high ammonium concentrations is suggested by the results of Dennison et
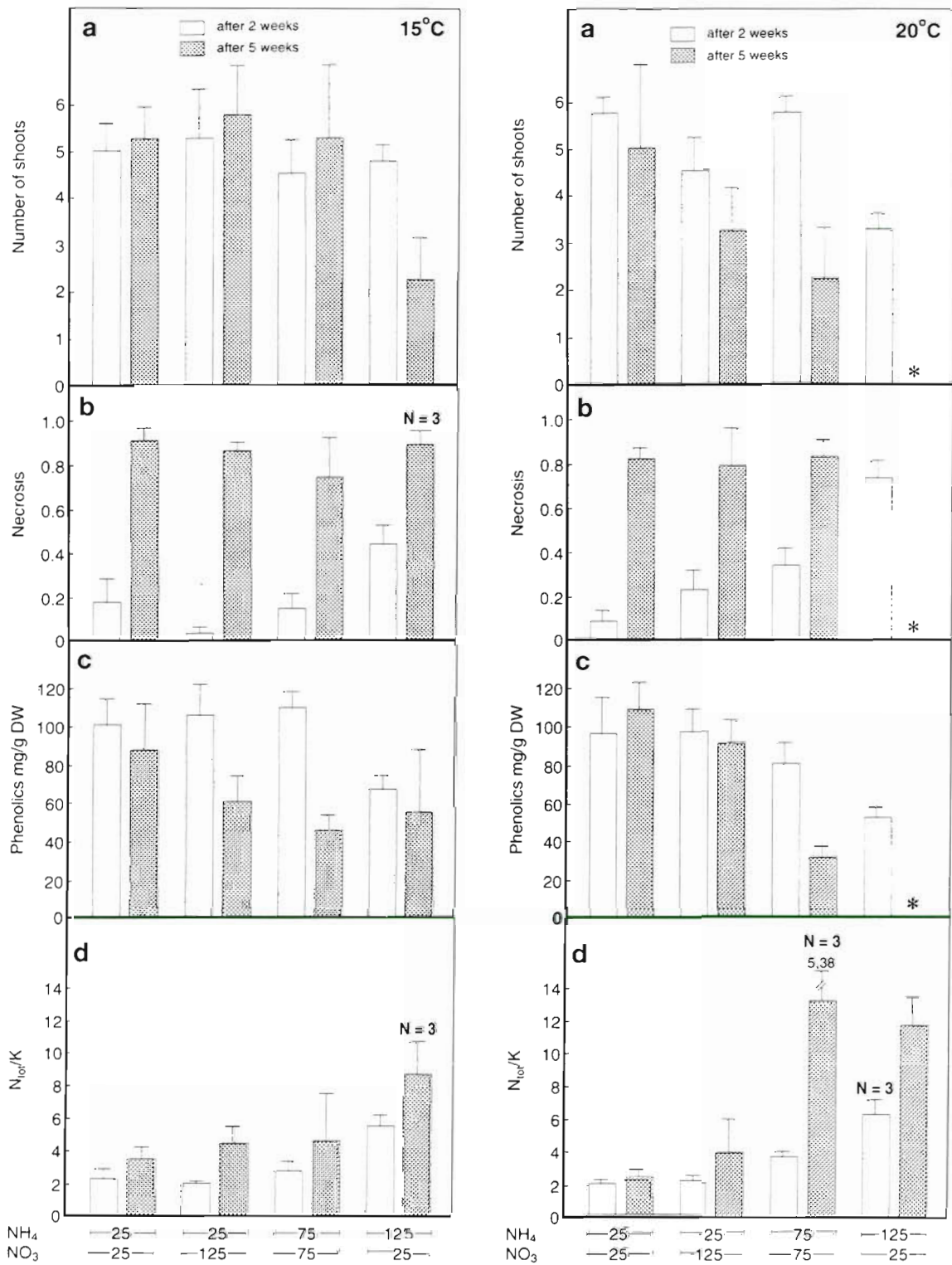
Fig. 6. Effect of N-treatments after 2 and 5 wk on (a) number of shoots, (b) necrosis, (c) phenolic content, and (d) N_{tot}:K ratio of *Zostera marina* at 2 temperatures. NH_4 and NO_3 treatments are in µM. Averages (back-transformed) of the 2 sediment types are presented. *Not measured due to the limited number of plants that survived

Table 4. *Zostera marina*. Characteristics and chemical composition of seagrass shoots under different N-treatments ($NH_4:NO_3$, $\mu mol\ l^{-1}$) after 2 wk. Means or back-tranformed means (for arcsine- and log-transformed parameters) are presented. The index for necrosis was calculated from % brown colouration of leaf surface, including infected leaf surface, and the index for wasting disease was calculated only from % infected leaf surface ('Materials and methods'). nm: not measured (insufficient plant material)

| | 25:25 | | | | 25:50 | | 25:125 | | | | 75:75 | | | | 125:25 | | | |
| | 15°C | | 20°C | | 15°C | | 15°C | | 20°C | | 15°C | | 20°C | | 15°C | | 20°C | |
	Mud	Sand	Mud	Sand	Mud	Sand	Mud	Sand	Mud	Sand	Mud	Sand	Mud	Sand	Mud	Sand	Mud	Sand
No. of shoots	5.5	4.5	5.5	6.0	6.5	4.5	6.5	4.0	5.0	4.0	5.0	4.0	5.5	6.0	5.0	4.5	3.5	3.0
Length (cm)	44	40	38	34	39	34	36	42	40	36	43	37	42	32	38	43	21	29
Width (mm)	2.3	2.5	2.1	2.3	2.3	1.8	2.0	2.6	2.4	2.4	2.5	2.3	2.4	2.0	2.0	2.4	1.5	1.9
Biomass (mg)	79	56	65	55	69	54	56	43	72	66	76	69	85	45	57	68	23	45
Necrosis	0.14	0.23	0.04	0.15	0.03	0.07	0.03	0.04	0.24	0.22	0.13	0.16	0.30	0.36	0.40	0.50	0.64	0.83
Wasting disease	0.01	0.03	0.02	0.04	0.03	0.07	0.03	0.04	0.16	0.05	0.07	0.06	0.08	0.04	0 01	0.01	0.02	0.00
Water content (%)	84	85	82	82	85	79	83	87	83	85	87	84	85	86	86	87	89	81
Phenolics (mg g^{-1} DW)	85	117	78	115	113	138	88	123	103	91	111	107	79	81	60	73	54	49
Chl a (μg g^{-1} FW)	43	74	28	24	46	27	38	27	67	25	87	50	35	39	52	64	nm	39
N_{tot} (μmol g^{-1} DW)	1080	1720	1390	1200	1280	1250	1570	1600	1310	1560	1960	2370	2240	2430	2920	2880	2830	2740
P_{tot} (μmol g^{-1} DW)	53	85	50	80	64	84	60	86	61	99	53	75	58	82	56	103	45	62
Na (μmol g^{-1} DW)	1190	960	1140	1050	1150	1160	1220	1140	1330	1320	1140	980	1210	1200	1170	1230	1640	1250
K (μmol g^{-1} DW)	690	600	740	610	780	680	810	780	720	660	880	720	670	610	610	460	450	460
Mg (μmol g^{-1} DW)	213	256	181	251	173	260	194	235	193	258	192	218	212	277	209	343	226	336
Ca (μmol g^{-1} DW)	230	242	207	180	139	186	125	140	206	261	125	126	131	227	166	187	191	222
Fe (μmol g^{-1} DW)	2.7	1.2	1.5	1.0	1.4	1.5	1.2	0.5	1.8	1.4	2.0	0.6	2.2	1.3	1.9	2.9	6.1	1.4
Mn (μmol g^{-1} DW)	2.6	4.1	4.1	4.4	2.7	5.0	3.5	2.8	2.3	2.7	3.7	4.0	2.7	5.7	3.3	4.8	1.4	3.6
Zn (μmol^{-1}g DW)	0.7	1.1	0.8	1.3	0.9	1.2	1.3	1.5	1.2	1.8	1.9	2.3	1.5	3.5	0.7	3.0	0.5	3.4
N_{tot}:K	1.6	2.9	1.9	2.0	1.6	1.8	1.9	2.0	1.8	2.4	2.2	3.3	3.4	4.0	4.8	6.3	6.3	6.0
N_{tot}:Mg	5.1	6.7	7.7	4.8	7.4	4.8	8.1	6.8	6.8	6.0	10.2	10.8	10.5	8.8	14.0	8.4	12.5	8.2
N_{tot}:P_{tot}	20	20	28	15	20	15	26	19	21	16	37	32	39	30	52	28	63	44

al. (1987). Also, eelgrass plants used in an experiment by Borum et al. (1989) may have suffered from ammonium toxicity, as it was reported that all older leaves 'showed signs of progressing senescence' after 3 wk of exposure to a water ammonium concentration of 50 µM (and additional pulses of 90 µM).

The brown-black discolouration of the *Zostera marina* leaves that we observed has also been reported for *Callitriche* spp., *Potamogeton* spp., *Elodea canadensis*, aquatic *Ranunculus* spp. and *Nymphaea caerulea* as a symptom of ammonium toxicity (Glänzer 1974, Grube 1974, Agami et al. 1976, Glänzer et al. 1977). Additionally, these investigators reported brown discolouration of the chloroplasts, which are the sites of ammonia assimilation in leaves (Marschner 1995), with necrosis starting at the leaf tips, and being more severe on older leaves than on younger ones. We also observed the latter symptom with *Z. marina*.

Table 5. p-values of the ANOVA model for the effect of N-treatment, sediment type, temperature and interactions (see Table 3) on the characteristics and chemical composition of *Zostera marina* plants after 2 wk. •$0.01 \le p \le 0.05$, ••$0.001 \le p < 0.01$, •••$p < 0.001$, all other effects were non-significant. There were no significant effects on width, biomass, wasting disease, chl a, Ca and Fe

	N-treat.	Sedim.	Temp.	N-treat. × Sedim.	N-treat. × Temp.	Sedim. × Temp.	N-treat. × Sedim. × Temp.
No. of shoots		••				•	
Length			•				
Necrosis	••	•	•				
Water content		•		••			•
Phenolic conc.	•••	••	•			•	•
N_{tot}	•••					•	•
P_{tot}		•••					
Na		•	•				
K	••	••	•				
Mg		•••					
Mn		•					
Zn	•	••					
N_{tot}/K	•••	•		•		••	••
N_{tot}/Mg	•	••		•			
N_{tot}/P_{tot}	••	•••		•			

Table 6. *Zostera marina*. Characteristics and chemical composition of seagrass shoots under different N-treatments after 5 wk. Means or back-transformed means (for arcsine- and log-transformed parameters) are presented

	25:25				25:50		25:125				75:75				125:25			
	15°C		20°C		15°C		15°C		20°C		15°C		20°C		15°C		20°C	
	Mud	Sand	Mud	Sand	Mud	Sand	Mud	Sand	Mud	Sand	Mud	Sand	Mud	Sand	Mud	Sand	Mud	Sand
No. of shoots	5.5	5.0	7.0	3.0	7.0	6.5	6.5	5.0	3.5	3.0	7.0	3.5	3.5	1.0	2.0	2.5	0[a]	0[a]
Length (cm)	35	28	36	35	33	26	38	28	43	29	33	33	19	12	14	14	–	–
Width (mm)	2.4	2.0	2.4	2.6	2.0	2.1	2.4	2.0	2.6	2.1	2.0	2.5	2.3	2.3	1.8	1.9	–	–
Biomass (mg)	46	31	54	58	50	42	38	32	58	29	49	51	22	25	15	27	–	–
Necrosis	0.88	0.93	0.77	0.87	0.83	0.85	0.81	0.90	0.87	0.66	0.53	0.90	0.79	0.85	0.94	0.80	–	–
Wasting disease	0.06	0.04	0.06	0.09	0.04	0.01	0.02	0.01	0.31	0.16	0.07	0.16	0.00	0.00	0.00	0.00	–	–
Water content (%)	86	86	85	82	84	84	87	87	88	88	87	87	88	90	91	88	–	–
Phenolics (mg g^{-1} DW)	89	86	117	100	88	114	58	62	79	103	47	43	36	27	19	90	–	–
N_{tot} (µmol g^{-1} DW)	1600	1520	1630	1510	1200	1900	1840	2450	690	1600	2050	1060	1970	1520	2480	2520	2000	870
Na (µmol g^{-1} DW)	1300	1500	1600	1200	1090	940	930	960	1370	1210	820	1320	1620	1760	1230	1100	1850	1040
K (µmol g^{-1} DW)	560	380	740	760	620	590	480	510	430	320	390	460	220	90	250	250	170	80
Mg (µmol g^{-1} DW)	458		223		99		117		390		417		388		292		466	205
N_{tot}:K	2.8	4.0	2.2	2.0	1.9	3.2	3.8	4.8	1.6	4.9	5.2	2.3	9.0	17.7	9.9	10.3	12.1	11.1
N_{tot}:Mg	3.5		7.3		12.1		15.7		1.8		4.9		5.1		8.5		4.3	4.2

[a]Only fragments of shoots were available

Generally, plants do not regulate nitrogen uptake as there is no feedback inhibition mechanism (Rabe 1990). *Zostera marina* roots appear to have a saturating ammonium level in the sediment, but at higher concentrations uptake begins again (Iizumi & Hattori 1982). This was also found to be the case in the roots of terrestrial plants, where a Michaelis-Menten type uptake system operates at low concentrations, and a linear system operates at higher ammonium concentrations (for review see Kronzucker et al. 1996). The leaves of terrestrial plants also take up ammonium (for reviews see Pearson & Stewart 1993, Fangmeier et al. 1994, Marschner 1995). The leaves of *Z. marina* rapidly take up ammonium, mostly in direct proportion to the ammonium concentration in the water column (Iizumi & Hattori 1982, Thursby & Harlin 1982, Short & McRoy 1984, Asmus 1986). However, low concentrations were applied in these studies: maxima of 20, 45, 12 and
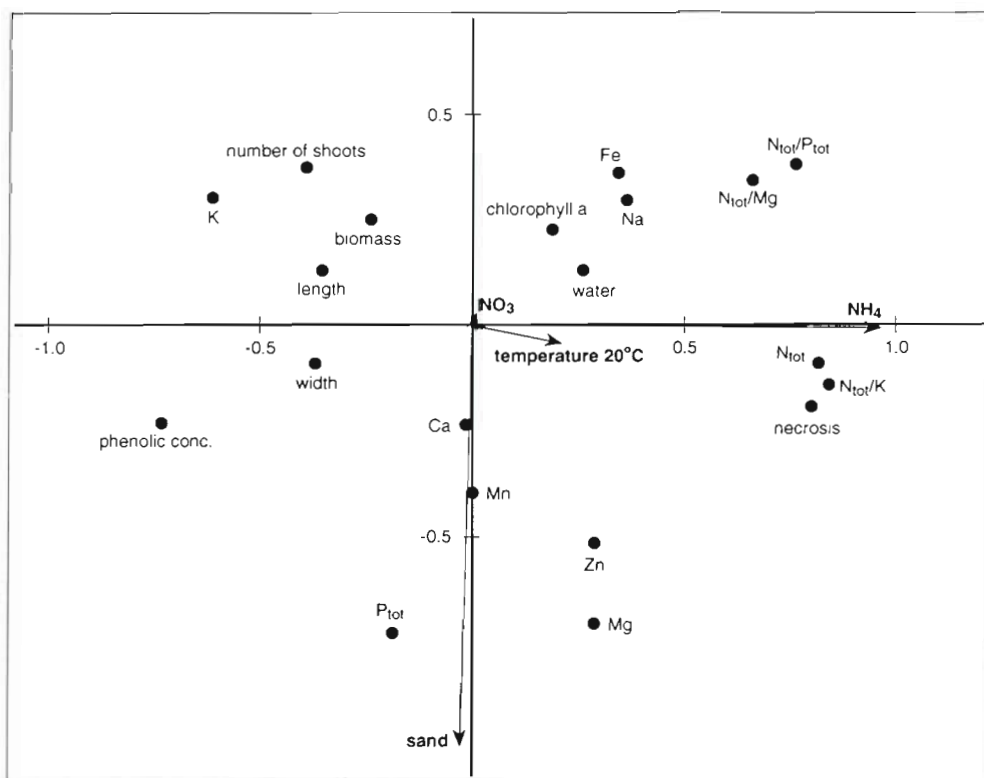

Fig. 7. Ordination diagram based on a redundancy analysis (RDA) displaying the effect of combined treatments of NO_3 (25, 50, 75 and 125 µM) and NH_4 (25, 75 and 125 µM), sediment type (sand vs mud) and temperature (15°C vs 20°C) on various plant parameters

Table 7. p-values of the ANOVA model for the effect of N-treatment, sediment type, temperature and interactions (see Table 5) on characteristics and chemical composition of *Zostera marina* plants after 5 wk. •$0.01 \leq p \leq 0.05$, ••$0.001 \leq p < 0.01$, all other effects were non-significant. There were no significant effects on width, biomass, necrosis, water content, Mg and N_{tot}:Mg (Mg measurements were mostly missing for sandy sediment: tested was the effect of N-treatment, temperature and N-treatment × temperature on muddy sediment)

	N-treat.	Sedim.	Temp.	N-treat. × Sedim.	N-treat. × Temp.	Sedim. × Temp.	N-treat. × Sedim. × Temp.
No. of shoots	••	••	••				
Length	••	•			••		
Wasting disease					•		
Phenolic conc.	•	•		•			
N_{tot}			•				
Na						•	
K	••		•				
N_{tot}:K	•	••	•		•		••

10 µM, respectively. Our experiment shows increasing shoot tissue nitrogen concentration in *Z. marina* at increasing ambient ammonium levels, indicating non-saturating uptake to at least 125 µM ammonium (this was not found for increasing ambient nitrate concentrations). Ammonium uptake by the tropical seagrass *Thalassia hemprichii* showed a gradually saturating uptake curve in a study in which maximal supply was 140 µM ammonium (Stapel et al. 1996). In *Cerastophyllum demersum* (an aquatic plant without roots), no saturation occurs at concentrations as high as 2.4 mM, and biphasic uptake, as described for roots, seems to occur (Toetz 1973). This was also found to be the case for *Lemna gibba* (Ullrich et al. 1984).

The physiological mechanism of ammonium toxicity is thought to lie in the ability of ammonia to uncouple photosynthetic electron transport, which may lead to necrosis through inhibition of photosynthesis. A secondary toxic effect may result from an inability to buffer the protons released from ammonium assimilation, affecting the function of many enzymes and membrane processes (e.g. Pearson & Stewart 1993, Marschner 1995).

The ammonium continuously entering the cells must be rapidly assimilated to prevent physiological damage (e.g. Magalhães & Huber 1989, Marschner 1995). This requires carbon skeletons, which may become limiting (e.g. Givan 1979, Magalhães & Huber 1989, Flaig & Mohr 1991, Marschner 1995). Carbon skeletons are provided by photosynthesis, the rate of which is dependent on illumination. Low light availability has been shown to decrease carbohydrate stores in *Zostera marina* (Kraemer & Alberte 1995, Zimmerman et al. 1995a). Also, glutamine synthetase, which is a key enzyme in ammonium assimilation (e.g. Miflin & Lea 1976, Givan 1979, Magalhães & Huber 1989), is stimulated upon illumination (Pregnall et al. 1987, Marschner 1995). As a result, low light availability will aggravate the effect of ammonium toxicity. In our experiment, illumination was presumed to be saturating as plants were collected at the end of autumn; they are able to adapt to low irradiance by decreasing their light saturation level (Zimmerman et al. 1995a). However, when light saturation is at a low level, the total amount of carbon fixed is lower than that fixed at a high saturation level. We would therefore expect that ammonium toxicity effects will be less severe in spring and summer.

Another aggravating factor is the relatively high temperature in the experiment (15 and 20°C), which would have caused enhanced metabolic activity, i.e. ammonium uptake (e.g. Lycklama 1963, Toetz 1973, Wang et al. 1993), and to a lesser extent carbon-consuming respiration (Drew 1979, Marsh et al. 1986, Zimmerman et al. 1989). It is not surprising, therefore, that we find a stronger toxic effect at 20°C than at 15°C.

Why are eelgrass leaves so susceptible to ammonium in the water layer when, in contrast, the roots are exposed to relatively high concentrations [usually between 10 and 300 µM ammonium (Harlin & Thorne-Miller 1981, Iizumi et al. 1982, Kenworthy et al. 1982, Dennison et al. 1987, Williams & Ruckelshaus 1993, Hemminga et al. 1994, van Lent & Verschuure 1994a) and occasionally even above 1000 µM ammonium (Short 1983b, Pedersen & Borum 1993)]? Firstly, leaves take up ammonium at a higher rate than roots (Thursby & Harlin 1982, Short & McRoy 1984, Pedersen & Borum 1992, Hemminga et al. 1994). Secondly, the pH of the water varies, but is circa 8.2 on average (Stumm & Morgan 1981), whereas the pH in typical marine sediments is around 7.5 (Berner 1980). A higher pH may result in an increased uptake rate of ammonium, as was found for *Ceratophyllum demersum* and *Lemna gibba* (Toetz 1973, Ullrich et al. 1984), probably owing to an increase in the proportion of the molecular

species (NH₃) (Marschner 1995). It should be noted that, in most studies of terrestrial plants, no such relationship between pH and uptake rate could be established (for review see Wang et al. 1993). Thirdly, eelgrass is known to aerate its rhizosphere to a small extent, thereby, through nitrification, very locally decreasing the ammonium concentration (Iizumi et al. 1980, Smith et al. 1984).

Phenolic content was inversely correlated with shoot tissue nitrogen concentration, which is in agreement with results of Buchsbaum et al. (1990) and L. H. T. Vergeer (unpubl. results). Contrary to their findings, however, no correlation with wasting disease was found. Necrosis, on the other hand, did show a significant correlation with phenolic content. However, the rapidity of plant deterioration argues against any causality: the plants were probably injured before any effect of decreased phenolic content had time to manifest in the plants.

The shoot tissue nitrogen content in *Zostera marina* leaves increased to 3.5% of the dry weight at the highest ammonium treatment (125 µM). Possibly, at these values free ammonium begins to accumulate and protein breakdown increases. In natural habitats, nitrogen content of *Z. marina* lies between 1 and 3% nitrogen during the growing season (Thayer et al. 1977, Harlin & Thorne-Miller 1981, Kenworthy & Thayer 1984, Short 1987, Pellikaan & Nienhuis 1988, Pedersen & Borum 1993, van Lent & Verschuure 1994a). Shoot tissue nitrogen:potassium ratio increased at high ammonium treatments and was highly correlated with necrosis, as is often observed in terrestrial plants (e.g. Marschner 1995) as well as in the aquatic *Stratiotes aloides* (Smolders et al. 1996). Interestingly, the increase of the shoot tissue nitrogen: potassium ratio at high ammonium treatments resulted not only from an increase in shoot tissue nitrogen concentration, but also from a significant (p < 0.01) decrease in shoot tissue potassium concentration, despite the high availability of potassium in the surrounding sea water. Shoot tissue magnesium concentrations were not influenced by the nitrogen treatments, but showed a remarkable correlation with necrosis and sediment type. Generally, the altered ion composition in the plants probably does not cause necrosis, but merely accompanies it.

Unlike the studies of Burkholder et al. (1992, 1994), no effects of nitrate on plant vitality or any other plant parameter were found in this study. Nitrate uptake by roots, and probably also by leaves, is inhibited by the presence of ammonium in the water (Iizumi & Hattori 1982, Zimmerman et al. 1987). Possibly the 25 µM ammonium present in the nitrate treatments inhibited nitrate uptake, and so suppressed any nitrate effects.

Compared to muddy sediment, plants in sandy sediment showed higher mortality, were more necrotic, and had higher shoot tissue magnesium, zinc, phosphorus, and phenolic contents and lower shoot tissue potassium concentrations. The tissue phosphorus and potassium effects corresponded to the differences in sediment properties. Zinc concentration was not measured in the sediment. The magnesium concentration was equal in both sediment types, leaving the strongly increased shoot tissue magnesium concentration on sand an interesting but unexplained phenomenon.

We did not expect that sandy sediment would have a negative influence on plant vitality under ammonium stress, because the lower ammonium concentration and higher phosphate concentration in sand were thought to give a better nutrient balance. Also, the expectedly more aerobic condition of sand was thought to be advantageous for the plants, as less energy would be needed for aeration of the root/ rhizomes. A possible explanation may be that photosynthesis is carbon limited. Eelgrass is known to increase photosynthesis with an extra supply of carbon dioxide (Madsen et al. 1993, Zimmerman et al. 1995b), which demonstrates that carbon limitation is not an uncommon phenomenon in *Zostera marina*. When carbon is limiting, muddy sediment may favour the plant in 2 ways: Firstly, muddy, organic sediment contains higher inorganic carbon levels than sandy sediment (e.g. Valiela 1984). Inorganic carbon can be taken up by the roots of *Z. marina* and transported to the leaves through the lacunal gas spaces (Wetzel & Penhale 1979, Penhale & Thayer 1980, Penhale & Wetzel 1983), thereby providing an extra carbon source, analogous with the isoetid plants (e.g. Søndergaard & Sand-Jensen 1979, Roelofs et al. 1984, Madsen et al. 1993). Secondly, plants grown on the anaerobic muddy sediment generally possess larger lacunal gas spaces (Penhale & Wetzel 1983) and, therefore, a larger capacity for carbon dioxide storage and recycling of respirative-CO_2 than do plants grown on sand. The importance of C-skeletons for the handling of excess ammonia was outlined above.

CONCLUSIONS AND ECOLOGICAL IMPLICATIONS

Ammonium was shown to be toxic to *Zostera marina* at an applied concentration of 125 µM and probably also at 25 µM ammonium. At 9 µM ammonium, no toxic effects were found. The effect was stronger at a higher temperature (20°C as compared to 15°C) and on sand (as compared to mud). It was argued that the toxicity will increase with lower irradiance. Toxic effects of ammonium will therefore be felt primarily in

autumn. In eutrophicated areas, this coincides with rising ambient nutrient concentrations due to decreased algal growth. It is expected that this will culminate in intensification of the normal end-of-season decline of Z. marina populations.

Earlier ammonium increase may cause a premature die-off, as was observed year after year in the small annual Zostera marina population of the western Wadden Sea (C. H. R. Hermus pers. comm., van Katwijk pers. obs.). At this location, ammonium usually increases in August/September, in contrast to other eelgrass habitats (e.g. eastern Wadden Sea and southwestern Netherlands), where ammonium increase begins in October (Helder 1974, Asmus 1986, van Lent & Verschuure 1994a, unpubl. data 1980–1990 from the Dutch Ministry of Transport, Public Works and Water Management).

High ammonium concentrations may prevent an annual population from adopting a perennial reproduction strategy. For example, in the Netherlands, 2 non-tidal brackish water lakes, Lake Veere (eutrophic, average ammonium concentration in summer below 5 µM, but in autumn increasing towards 23 µM) and Lake Grevelingen (oligotrophic, average ammonium concentration during autumn increasing from 3 µM to 11 µM), are inhabited by an annual and perennial eelgrass population, respectively [van Lent & Verschuure 1994b, ammonium data (unpubl.): 1980–1990 from the Dutch Ministry of Transport, Public Works and Water Management]. Formerly, both lakes were estuaries inhabited by an annual eelgrass population (Beeftink 1965, C. den Hartog pers. comm.). In Lake Grevingen, the population developed a perennial strategy after its habitat became submerged. In Lake Veere this may have been prevented by recurrent ammonium intoxication in autumn.

Generally, ammonium toxicity may be one of the underlying causes of the disappearance of many Zostera marina populations in eutrophicated coastal seas throughout the northern hemisphere. It may prevent eelgrass recovery and hamper revegetation efforts, especially at greater depth and in the case of perennial plants. The importance of sufficient carbon reserves for transplantation success has already been pointed out by Zimmerman et al. (1995a). In eutrophicated areas, this will become even more important due to (and may be partly explained by) the ammonium susceptibility of Z. marina as shown in this study. As seagrass species are closely related (Larkum & den Hartog 1989), it is likely that other seagrasses are also susceptible to ammonium toxicity. It would therefore be worthwhile to estimate ammonium toxicity in other seagrasses so as to gain a better understanding of global seagrass decline.

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