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Differential responses of two wetland graminoids to high ammonium at different pH values

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ABSTRACT

Enhanced soil ammonium (NH_4^+) concentrations in wetlands often lead to graminoid dominance, but species composition is highly variable. Although NH_4^+ is readily taken up as a nutrient, several wetland species are known to be sensitive to high NH_4^+ concentrations or even suffer toxicity, particularly at low soil pH. More knowledge about differential graminoid responses to high NH_4^+ availability in relation to soil pH can help to better understand vegetation changes. The responses of two wetland graminoids, *Juncus acutiflorus* and *Carex disticha*, to high (2 mmol·l^{-1}) *versus* control (20 μmol·l^{-1}) NH_4^+ concentrations were tested in a controlled hydroponic set up, at two pH values (4 and 6). A high NH_4^+ concentration did not change total biomass for these species at either pH, but increased C allocation to shoots and increased P uptake, leading to K and Ca limitation, depending on pH treatment. More than 50% of N taken up by *C. disticha* was invested in N-rich amino acids with decreasing C:N ratio, but only 10% for *J. acutiflorus*. Although both species appeared to be well adapted to high NH_4^+ loadings in the short term, *C. disticha* showed higher classic detoxifying responses that are early warning indicators for decreased tolerance in the long term. In general, the efficient aboveground biomass allocation, P uptake and N detoxification explain the competitive strength of wetland graminoids at the expense of overall biodiversity at high NH_4^+ loading. In addition, differential responses to enhanced NH_4^+ affect interspecific competition among graminoids and lead to a shift in vegetation composition.

INTRODUCTION

Increased nitrogen (N) availability is known lead to biodiversity loss and degradation of ecosystem functions, often related to enhanced dominance of N-limited graminoids (Bobbink *et al.* 1998). For several decades, N availability in wetlands has been greatly enhanced by the increased input of the mobile N-form nitrate (NO_3^-) leaching from surrounding agricultural land (Britto & Kronzucker 2002; Miller & Cramer 2004; Sutton *et al.* 2011) and forests (Dise & Wright 1995) through enhanced atmospheric N deposition (Bobbink *et al.* 1998) and increased decomposition of organic soil with concomitant N mineralisation (Lamers *et al.* 2001; Geurts *et al.* 2010). In wetland soils, NO_3^- is generally only present near the soil surface during relatively wet or waterlogged conditions. Instead, the more immobile NH_4^+ is the dominant N form under anaerobic conditions due to limited oxygen availability, leading to low NH_4^+ oxidation rates, increased NO_3^- losses through denitrification and dissimilatory NO_3^- reduction to NH_4^+ (DNRA; Burgin & Hamilton 2008). A much increased N input may therefore lead to significantly increased NH_4^+ concentrations in such wetland systems (Britto & Kronzucker 2002; Miller & Cramer 2004), affecting competition among species and leading to vegetation changes.

Although NH_4^+ is readily taken up as a nutrient, various species from dry, moist and wetland ecosystems appear to be sensitive to high NH_4^+ concentrations, and this N form can even become toxic (Van Katwijk *et al.* 1997; Britto & Kronzucker 2002; Stevens *et al.* 2011). Several typical sensitivity symptoms have regularly been found when plants were exposed to high NH_4^+ concentrations, including suppressed growth (or even mortality), lower photosynthesis, a decrease in root–shoot ratio, decreased uptake of cations and increased uptake of anions, and increased metabolic costs due to assimilation of free amino acids (Britto & Kronzucker 2002; Tylová *et al.* 2008; Van der Heide *et al.* 2008; Christianen *et al.* 2011; Stevens *et al.* 2011; Fritz *et al.* 2014). However, tolerance to NH_4^+ appears to differ widely among species and even among plants belonging to the same species (Cruz *et al.* 2011).

It is often assumed that plants naturally growing in NH_4^+-rich, acidic systems are more tolerant to high NH_4^+ concentrations, whereas acid-sensitive species from more buffered systems are more sensitive to NH_4^+ (De Graaf *et al.* 1998; Britto *et al.* 2001; Britto & Kronzucker 2002; Van den Berg *et al.*

2005). Additionally, NH_4^+ toxicity is often found to be related to a low pH of the rhizosphere, while in more buffered conditions in the rhizosphere, toxicity symptoms are often minimised or even absent (Findenegg 1987; Lucassen *et al.* 2002; Van den Berg *et al.* 2005). Therefore, wetland species adapted to waterlogged, buffered conditions may be expected to cope well with strongly increased NH_4^+ levels in soil pore water (concentrations in the millimolar range) as long as the pH is in the neutral range. However, due to differences in their growth responses and tolerances, species (including graminoids) are expected to show differential responses.

In the present study, we therefore tested the responses and tolerances to greatly enhanced NH_4^+ availability for two graminoid species that often dominate vegetation in wet grasslands and marshes, sharp-flowered rush (*Juncus acutiflorus*) and brown sedge (*Carex disticha*). First, we tested whether these wetland species indeed prefer uptake of NH_4^+ rather than NO_3^-. Second, their responses and tolerances to strongly increased NH_4^+ concentrations were tested under neutral and acidic conditions, by quantifying several factors that control NH_4^+ tolerance of wetland graminoids. As both species naturally occur on buffered soils, we expected high NH_4^+ loadings to become toxic only at low pH, while at neutral pH a fertilising effect was expected. *J. acutiflorus* is often found in more nutrient-rich conditions than *C. disticha*, and increased dominance of *J. acutiflorus* at the expense of smaller sedges such as *C. disticha* has been regularly observed in wet meadows affected by increased N input (personal observations). As dominance of graminoids often affects biodiversity in wetlands, more knowledge of differential responses to highly increased NH_4^+ availability may help to understand interspecific competition among graminoids and related changes in vegetation composition.

MATERIAL AND METHODS

Test species

Two very common graminoid species for Europe, sharp-flowered rush (*J. acutiflorus*) and brown sedge (*C. disticha*), which usually occur on moist or wet soils in grasslands, sedge fens and dune valleys were used. The soils in these habitats are generally buffered at pH 5–6, and can be characterised as mesotrophic to moderately eutrophic. In the Netherlands, the soils of well-developed vegetation, in which both species are present but not dominant, contain NH_4^+ concentrations ranging from 10 to 20 $\mu mol \cdot l^{-1}$ (unpublished field data). Both species are known to have special adaptations, such as root aerenchyma and radial oxygen loss (ROL) from the roots, to function well in waterlogged conditions, although the spatial ROL pattern differs between the species (Lamers *et al.* 2013).

Plant collection

Before the start of the experiments, sods containing *C. disticha* and *J. acutiflorus* were collected from a wet meadow in a nature reserve near Nijmegen, the Netherlands (De Bruuk, 51°46′ N, 5°53′ E) in mid-June 2009 (N sensitivity experiment) and 2011 (N uptake experiment). Ramets of both species were carefully taken from the sods. The roots of equal-sized individuals (10–15 cm shoot length for both species) were cut off and the remaining shoots were put into an aerated $^1/_8$ Hoagland nutrient solution (Hoagland & Arnon 1950) for 4 weeks, in order to develop new roots to a length of approximately 2 cm, after which the experiments described below were initiated.

Nitrogen uptake experiment

The preferential N form (NO_3^- or NH_4^+) for both plant species was explored in an N uptake experiment. For this experiment, a hydroponic system was used with 1.5 l opaque plastic containers. After an acclimatisation period of 7 days in aerated $^1/_8$ Hoagland nutrient solution at either pH 4 or pH 6 (containing both NO_3^- and NH_4^+), plants were provided with the following nutrient solution: 100 $\mu mol \cdot l^{-1}$ Ca^{2+}, 100 $\mu mol \cdot l^{-1}$ Mg^{2+}, 200 $\mu mol \cdot l^{-1}$ K^+, 100 $\mu mol \cdot l^{-1}$ SO_4^2-, 200 $\mu mol \cdot l^{-1}$ PO_4^{3-}, 0.8 $\mu mol \cdot l^{-1}$ Mn^{2+}, 0.8 $\mu mol \cdot l^{-1}$ H_3BO_3, 0.7 $\mu mol \cdot l^{-1}$ Zn^{2+}, 0.27 $\mu mol \cdot l^{-1}$ Fe (applied as Fe-EDTA), 0.2 $\mu mol \cdot l^{-1}$ Cu^{2+}, 0.008 $\mu mol \cdot l^{-1}$ Mo (De Graaf *et al.* 1998), with both NO_3^- (20 $\mu mol \cdot l^{-1}$) and NH_4^+ (20 $\mu mol \cdot l^{-1}$) at either pH 4 or pH 6 (n = 5). A permanent air-flow through the nutrient solution prevented anaerobic conditions, and 1-cyanguanidine (1% molar concentration of the N concentration) was added to the nutrient solution to inhibit nitrification (Van den Berg *et al.* 2005). The experiment was performed in a climate-controlled room with a 16:8 h day:night regime, at an average temperature of 20 °C during the day and 17 °C at night, a relative humidity of 50–70% and photosynthetically active radiation (PAR) of 300–400 µmol photons $m^{-2} \cdot s^{-1}$. Three individuals of the same species were placed in each plant container, using polystyrene trays for flotation. After 21 days, either $Na^{15}NO_3$ or $^{15}NH_4Cl$ (1% molar concentration of total N) was added to determine uptake rates of both N forms by the different species. After 2 h of labelling, plants were harvested and uptake rates calculated from the accumulation of enriched N in this period.

Nitrogen sensitivity experiment

After an acclimatisation period of 3 weeks in aerated $^1/_8$ Hoagland nutrient solution, plants of both species were exposed to four different treatments in a full factorial designed hydroponic system, differing in N concentration and pH (n = 4). Two individuals of the same species were placed in each plant container using polystyrene trays for flotation and to prevent algal growth. A permanent air-flow through the nutrient solution prevented anaerobic conditions. In total, 32 plant containers were used. The experiment was performed in a climate-controlled room with similar conditions as described above, and lasted for 8 weeks. Two different nutrient solutions were used as treatments: 20 $\mu mol \cdot l^{-1}$ NH_4^+ and 2 $mmol \cdot l^{-1}$ NH_4^+ at either pH 4 or pH 6. Concentrations of micronutrients were similar to those in the former experiment (see above) for all four solutions. The pH of the nutrient solution was set using 1.2 $mol \cdot l^{-1}$ HCl and 1 $mol \cdot l^{-1}$ NaOH, pH was checked daily and adjusted when required. The nutrient solution in each container was continuously refreshed by pumping at a rate of 1.5 $l \cdot day^{-1}$ from a separate 25-l opaque storage tank, filled with weekly-refreshed nutrient solution. In order to prevent nitrification, 1-cyanguanidine (1% molar concentration of NH_4^+) was added to all storage tanks (Van den Berg *et al.* 2005). During the experiment, growth rates of roots and shoots were monitored by measuring their maximum length. After 8 weeks, all plants were harvested.

Chemical analysis

For both experiments, fresh weight of all root and shoot material was determined directly after harvesting, and for the N sensitivity experiment roots were scanned to determine total root length using WinRHIZO (Regent Instruments, Quebec, Canada). In addition, the number of leaves was counted for each individual plant. In the N sensitivity experiment, a fresh subsample (1 g) of all shoots was directly frozen at −80 °C for amino acid analysis. Free amino acids were extracted using ethanol according to Van Dijk & Roelofs (1988). Twenty amino acids were quantified by measuring fluorescence after pre-column derivation with 9-fluorenylmethyl-chloroformate (FMOC-Cl) and measured with Norvaline as internal standard using HPLC (Model 920-LC; Varian Liquid Chromatography, Palo Alto, CA, USA). Another fresh subsample of the shoot (2 cm^2) was used to determine chlorophyll concentrations directly after harvest, using ethanol extraction (Wintermans & De Mots 1965) and a spectrophotometer (Model UV-1205; Shimadzu, Tokyo, Japan).

Root and shoot material was dried for 24 h at 70 °C to determine dry weight (biomass). Total concentrations of macro- and micronutrients in roots and shoots were measured by digesting 200 mg dried, homogenised material in sealed Teflon vessels in an Ethos D microwave lab station Terminal 20 (Milestone Pharmatech, New Brunswick, NJ, USA) with 4 ml nitric acid (65% v/v) and 1 ml hydrogen peroxide (30% v/v). This material was then analysed for total concentrations of P, Ca, K, Mg, Fe and S using Inductively Coupled Plasma emission spectrophotometry (ICP, Iris Intrepid II; Thermo Fisher Scientific, Waltham, MA, USA). In order to measure C and N concentrations, 4 mg dried, homogenised root and shoot material were transferred to tin cups and combusted in a CNS elemental analyser (Model EA NA1500; Carlo Erba Instruments, Milan, Italy). For the N uptake experiment, isotopic N composition in the dry plant tissue was determined using an elemental analyser (Model EA 1110; Thermo Fisher Scientific) coupled to an Isotopic Ratio Mass Spectrometer (IRMS, Model Finnigan Delta Plus; Thermo Fisher Scientific). Total uptake rates were calculated from enrichment of ^{15}N in plant tissue, after correction of the ^{15}N background due to natural abundance and the dilution factor of the added amount of ^{15}N.

Statistical analysis

Data of sub-replicates from the two or three plants growing in one container were pooled by calculating average values for each container, leaving four or five replicates per treatment (sensitivity experiment and uptake experiment, respectively). Data are expressed as mean ± SEM. If necessary, data were transformed logarithmically to meet the criteria of ANOVA testing and all data were analysed using the SPSS 16.0 package (SPSS, Chicago, IL, USA). A one-way ANOVA was carried out to test for possible differences in chemical composition, root:shoot ratio and biomass, using treatment types as class variables. A two-way ANOVA was used to test for interactions between pH and N treatments.

RESULTS

Uptake of NH_4^+ *versus* NO_3^-

Both *C. disticha* and *J. acutiflorus* were able to take up NH_4^+ as well as NO_3^- at both pH 4 and pH 6 when the two N forms were available (Fig. 1). Due to high variation in root biomass, there were no significant differences between the uptake of NH_4^+ or NO_3^- when expressed per gram root dry weight (data not shown). Expressed per gram total biomass of the plants, however, both species showed higher uptake rates for NH_4^+ than for NO_3^- ($P < 0.05$; Fig. 1). The rates for NH_4^+ uptake were two times (pH 4) and three times (pH 6) those for NO_3^- in *C. disticha*, and only 1.5 times in *J. acutiflorus*. In this experiment, pH neither affected N uptake nor N allocation (results not shown) in the two species.

Effects of low *versus* high NH_4^+ on biomass production

The initial total plant biomass was 0.7 ± 0.3 g FW (0.12 ± 0.05 g DW) for *C. disticha* and 1.2 ± 0.6 g FW (0.11 ± 0.05 g DW) for *J. acutiflorus*, without differences between treatments (data not shown). None of the treatments in the N sensitivity experiment led to mortality of individual plants. For both species, increased NH_4^+ concentrations did not enhance total plant biomass (Fig. 2). For *C. disticha,* shoot biomass significantly ($P < 0.001$) increased at 2 mmol·l^{-1} NH_4^+, while root biomass decreased ($P < 0.001$). Shoot biomass showed a higher increase at pH 6 than at pH 4 ($P < 0.03$). As a result, root:shoot ratios of *C. disticha* were lower ($P < 0.001$) at 2 mmol·l^{-1} NH_4^+, and lower at pH 6 ($P < 0.01$). For *J. acutiflorus*, an interaction ($P < 0.03$) between pH and high NH_4^+ was found in shoot biomass. This led to an increase of shoot biomass in the high NH_4^+ treatment compared to the low NH_4^+ treatment at pH 4, while at pH 6 no significant differences were found. Root biomass was lower ($P < 0.001$) at high NH_4^+ concentrations, both at pH 4 and pH 6. As a result, an interaction ($P = 0.05$) between high NH_4^+ and pH was found for the root:shoot ratio, which was mostly caused by a decrease of root biomass (pH 4 and pH 6), and to a lesser extent by the increase in shoot biomass (pH 4). Moreover, for *J. acutiflorus*, lower total biomass at pH 6 than at pH 4 was reached ($P < 0.05$),

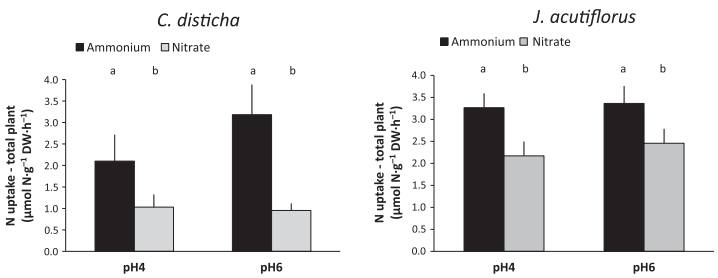

Fig. 1. Uptake rates for either NO_3^- or NH_4^+ (μmol N g^{-1} total DW h^{-1}; ±SEM) at two pH values for *C. disticha* and *J. acutiflorus*, when both N forms were available at 20 μmol·l^{-1}. Significant differences between the treatments are indicated by different letters.

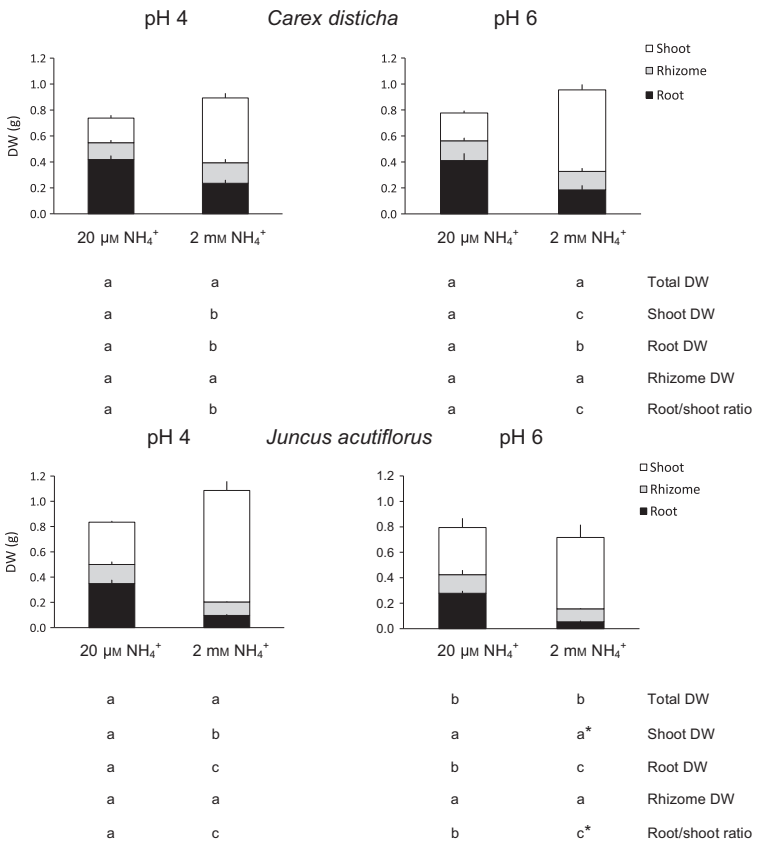

Fig. 2. Total biomass (g DW; ±SEM) of *C. disticha* and *J. acutiflorus* after growing on 20 μmol·l^{-1} NH_4^+ or 2 mmol·l^{-1} NH_4^+, at either pH 4 or pH 6, divided into shoot biomass, rhizome biomass and root biomass. Significant differences are indicated by different letters, *indicates a significant interaction between pH and N effects.

regardless of the N treatment. No differences in rhizome biomass were found for either species.

Macro- and micro-nutrient content in plant tissue

As a result of the high NH_4^+ treatment, a significant increase in N concentration ($P < 0.001$) was found in shoot and root tissue of both species at both pH values, without interaction with pH (Table 1). Consequently, the C:N ratio in both root and shoot tissue was significantly reduced in the 2 mmol·l^{-1} NH_4^+ treatment from 50 to 10 and from 22 to 9 in the root and shoot, respectively (data not shown). The P concentration showed an increase ($P < 0.01$) in shoot tissue of both species at high NH_4^+, and also in root tissue for *J. acutiflorus* ($P < 0.001$). As a result of the relatively higher increase in N than in P concentration, N:P ratios still doubled at the high NH_4^+ treatment in shoot and root tissue of both species ($P < 0.001$ and $P < 0.05$, respectively). The K concentration, in contrast, was lower ($P < 0.001$) in shoots of *C. disticha* treated with high NH_4^+. In root biomass of *C. disticha*, interacting effects ($P < 0.05$) were found, indicating lower K concentrations at high NH_4^+ and high pH. Consequently, N:K ratios in roots of *C. disticha* showed an interaction effect ($P < 0.05$) between NH_4^+ and pH, and an increase in shoots only with high NH_4^+ ($P < 0.001$). For *J. acutiflorus*, pH and NH_4^+ showed an interaction ($P < 0.0015$) in shoot tissue, with the lowest K concentrations at high NH_4^+ and low pH. In root tissue of *J. acutiflorus,* K concentrations were also lower ($P < 0.001$) at high NH_4^+, regardless of pH. This led to increased N:K ratios ($P < 0.001$) in roots of *J. acutiflorus*, with an interaction effect ($P = 0.05$) between increased NH_4^+ and low pH.

The Fe concentrations in shoot and root tissue of *C. disticha* showed no differences between treatments (Table 1). Also *J. acutiflorus* showed no differences in Fe concentrations in roots. Although an interaction ($P < 0.05$) between N and pH treatments was found for shoots of this species, no clear pattern could be observed. In shoots of both species, lower concentrations of Ca were found at high NH_4^+ ($P < 0.001$) and at low pH ($P < 0.01$). In root tissue, lower ($P < 0.001$) Ca concentrations were found only for *C. disticha* at low pH, regardless of the N treatment. The Mg concentrations in root tissue of both species were also reduced ($P < 0.001$) at high NH_4^+ and at a low pH ($P < 0.001$). In shoot tissue, lower ($P < 0.05$) Mg concentrations were only found at a low pH, but no differences between NH_4^+ treatments were found. In the high NH_4^+ treatment, sulphur (S) concentrations increased ($P < 0.005$) in root tissue at both pH values, while for shoot tissue this was only found at pH 6 ($P < 0.05$). When the dilution effect due to an increase in shoot biomass at high NH_4^+ was considered, calculations might show whether certain cations became limited for this treatment during the experiment (Timmer & Stone 1978; De Graaf *et al.* 1998). According to these calculations, Ca became limited at pH 4 for both species, and K became limited only for *J. acutiflorus* at both pH values.

Nitrogen allocation to free amino acids

Both species responded to enhanced NH_4^+ concentrations by significantly ($P < 0.001$) increasing their total free amino acid concentrations in shoot tissue (Table 2). The strong correlation ($R^2 = 0.87$) between the total free amino acid concentration and the total N concentration in shoot tissue (Fig. 3) highlights

Table 1. Concentrations of total C, N, P, K, Fe, Ca, Mg, S, N:P ratio and N:K ratio (±SEM) in shoots and roots of *C. disticha* and *J. acutiflorus* grown on 20 μmol·l^{-1} NH_4^+ or 2 mmol·l^{-1} NH_4^+ at either pH 4 or pH 6.

		Shoot							Root						
		NH_4 20 μM		NH_4 2 mM		*P* value			NH_4 20 μM		NH_4 2 mM		*P* value		
		pH 4	pH 6	pH 4	pH 6	N	pH	N*pH	pH 4	pH 6	pH 4	pH 6	N	pH	N*pH
Carex disticha															
C	mmol·g^{-1} DW	35 ± 1.1	35 ± 0.4	36 ± 1.2	38 ± 1.1	n.s.	n.s.	n.s.	38 ± 0.9	36 ± 0.7	38 ± 1.2	37 ± 0.4	n.s.	n.s.	n.s.
N	mmol·g^{-1} DW	1.5 ± 0.1	1.4 ± 0.1	3.4 ± 0.1	3.7 ± 0.1	0.00	n.s.	n.s.	0.6 ± 0.0	0.7 ± 0.1	3.6 ± 0.2	3.2 ± 0.1	0.00	n.s.	n.s.
P	μmol·g^{-1} DW	113 ± 5	128 ± 7	144 ± 9	141 ± 9	0.01	n.s.	n.s.	61 ± 3	86 ± 9	93 ± 14	93 ± 11	n.s.	n.s.	n.s.
K	μmol·g^{-1} DW	551 ± 17	547 ± 12	389 ± 68	309 ± 24	0.00	n.s.	n.s.	470 ± 28	345 ± 17	96 ± 13	56 ± 7	0.00	0.00	0.04
Fe	μmol·g^{-1} DW	1.2 ± 0.2	0.9 ± 0.1	1.1 ± 0.2	1.1 ± 0.1	n.s.	n.s.	n.s.	5.9 ± 2.2	4.7 ± 0.9	3.2 ± 1.0	5.5 ± 1.1	n.s.	n.s.	n.s.
Ca	μmol·g^{-1} DW	203 ± 8	279 ± 31	96 ± 13	130 ± 11	0.00	0.01	n.s.	34 ± 1	47 ± 4	32 ± 3	59 ± 5	n.s.	0.00	n.s.
Mg	μmol·g^{-1} DW	101 ± 6	138 ± 18	101 ± 9	174 ± 12	n.s.	0.00	n.s.	61 ± 2	107 ± 5	27 ± 3	42 ± 2	0.00	0.00	0.00
S	μmol·g^{-1} DW	66 ± 5	61 ± 5	67 ± 6	91 ± 8	0.02	n.s.	0.03	49 ± 4	41 ± 4	64 ± 4	70 ± 8	0.00	n.s.	n.s.
N:P ratio	g·g^{-1}	6.0 ± 0.3	5.0 ± 0.5	10.8 ± 0.9	12.2 ± 0.5	0.00	n.s.	n.s.	4.6 ± 0.2	3.8 ± 0.4	18.6 ± 1.9	16.9 ± 2.0	0.00	n.s.	n.s.
N:K ratio	g·g^{-1}	1.0 ± 0.1	0.9 ± 0.1	3.4 ± 0.5	4.4 ± 0.3	0.00	n.s.	n.s.	0.5 ± 0.0	0.7 ± 0.1	14.2 ± 1.8	23.8 ± 2.3	0.00	0.00	0.01
Juncus acutiflorus															
C	mmol·g^{-1} DW	33 ± 0.7	32 ± 0.5	34 ± 1.1	33 ± 0.9	n.s.	n.s.	n.s.	36 ± 0.7	37 ± 1.1	36 ± 0.6	33 ± 2.2	n.s.	n.s.	n.s.
N	mmol·g^{-1} DW	1.6 ± 0.1	1.3 ± 0.1	3.0 ± 0.1	2.9 ± 0.1	0.00	n.s.	n.s.	0.4 ± 0.0	0.8 ± 0.1	1.9 ± 0.2	1.8 ± 0.2	0.00	n.s.	n.s.
P	μmol·g^{-1} DW	143 ± 6	119 ± 12	159 ± 8	175 ± 15	0.01	n.s.	n.s.	72 ± 4	66 ± 5	129 ± 6	163 ± 19	0.00	n.s.	n.s.
K	μmol·g^{-1} DW	947 ± 6	854 ± 51	239 ± 44	423 ± 44	0.00	n.s.	0.01	269 ± 16	226 ± 13	103 ± 15	117 ± 27	0.00	n.s.	n.s.
Fe	μmol·g^{-1} DW	1.5 ± 0.2	1.0 ± 0.1	1.2 ± 0.1	1.4 ± 0.2	n.s.	n.s.	0.03	19 ± 7	20 ± 9	54 ± 17	33 ± 15	n.s.	n.s.	n.s.
Ca	μmol·g^{-1} DW	91 ± 7	148 ± 17	40 ± 6	83 ± 10	0.00	0.00	n.s.	68 ± 2	89 ± 2	71 ± 5	81 ± 14	n.s.	n.s.	n.s.
Mg	μmol·g^{-1} DW	160 ± 5	159 ± 11	113 ± 11	182 ± 21	n.s.	0.02	0.01	45 ± 2	80 ± 3	25 ± 2	43 ± 2	0.00	0.00	n.s.
S	μmol·g^{-1} DW	104 ± 2	117 ± 8	109 ± 7	145 ± 15	n.s.	0.02	n.s.	42 ± 2	45 ± 2	81 ± 6	86 ± 10	0.00	n.s.	n.s.
N:P ratio	g·g^{-1}	5.1 ± 0.4	4.8 ± 0.1	8.8 ± 0.3	9.1 ± 2.2	0.00	n.s.	n.s.	2.6 ± 0.2	5.4 ± 0.1	7.1 ± 0.5	6.5 ± 1.6	0.00	n.s.	0.02
N:K ratio	g·g^{-1}	0.6 ± 0.0	0.5 ± 0.0	5.2 ± 0.8	3.0 ± 0.6	0.00	0.03	0.05	0.6 ± 0.1	1.3 ± 0.1	8.6 ± 1.4	7.7 ± 1.8	0.00	n.s.	n.s.

the investment of both species into N-rich amino acids to store available N. The strongest response was shown by *C. disticha*, with 73–75% of the total amino acid concentration, consisting of N-rich amino acids at the high NH_4^+ treatment. The dominant N-rich free amino acid was asparagine, which accounted for 71–73% of the total free amino acid concentration. At the same time, other free amino acids, such as glutamine and aspartic acid, showed a decrease ($P < 0.001$) in the shoot tissue of *C. disticha*. Although *J. acutiflorus* showed a similar response to enhanced NH_4^+, this was much less extreme than in *C. disticha*. *J. acutiflorus* also showed an increase ($P < 0.001$) in total free amino acid concentration (Table 2). However, only 29–41% of the total free amino acid concentration was invested in N-rich amino acids ($P < 0.01$), mostly of asparagine (14–28%) and glutamine (11–12%). The total N concentration and the free amino acid concentration in *J. acutiflorus* increased less than in *C. disticha* (Fig. 3). Most of the total N is located in the shoot tissue of both species (Fig. 4), showing strong and significant interactions ($P < 0.01$) between high NH_4^+ and pH. However, *C. disticha* distributed more N ($P < 0.001$) towards the roots in response to high NH_4^+, while this response was absent for *J. acutiflorus*.

DISCUSSION

Nutrient and C allocation

The use of NO_3^- is an energy-demanding process for plants, because it has to be reduced to NH_4^+ before assimilation (Marschner 1995; Miller & Cramer 2004). Because NH_4^+ uptake is energetically more favourable, and both species often occur in wet, relatively NH_4^+-rich environments, a preference for NH_4^+ was to be expected. We indeed found that particularly *C. disticha*, and to a lesser extent also *J. acutiflorus*, preferentially took up NH_4^+. Although NH_4^+ uptake was higher, NO_3^- was also taken up readily, especially by *J. acutiflorus*. In anoxic wetland soils, NH_4^+ is the most common N species present, but radial oxygen loss enhances oxidation of the rhizosphere (Visser *et al.* 2000; Colmer 2003), leading to enhanced nitrification around root surfaces (Engelaar *et al.* 1995; Armstrong & Armstrong 2001). With this rhizosphere modifying strategy, wetland plants might facilitate the uptake of either NO_3^- or NH_4^+ (Chang *et al.* 2010; Konnerup & Brix 2010). Interestingly, *J. acutiflorus*, which showed the highest affinity for NO_3^-, also has the highest radial oxygen loss (Lamers *et al.* 2013). In addition, high affinities for NO_3^- in addition to NH_4^+ are also beneficial for both species when occurring in riparian wetlands.

Surprisingly, total biomass of both species was not affected at the high NH_4^+ treatment (2 mmol·l^{-1}), while total biomass of other wetland species, such as *Acorus calamus* (Vojtísková *et al.* 2004) and *Glyceria maxima* (Tylová *et al.* 2008), decreased as a result of sensitivity to high NH_4^+ loadings (respectively, 14.8 and 3.7 mmol·l^{-1}). In our experiments, there was neither a negative (toxic) nor a positive (nutrient) effect. Instead, *J. acutiflorus* and *C. disticha* only showed modified biomass allocation, in the form of a decreased root:shoot ratio, as result of high NH_4^+. This ability to change only biomass allocation, without decreasing total biomass, suggests high NH_4^+ tolerance. Such a tolerance to high NH_4^+ loadings in wetland graminoids was so far only found for *Phragmites australis* (Tylová *et al.* 2008; Engloner 2009). Given the low N:P ratios in the aboveground

Table 2. Free amino acid (AA) concentrations (μmol N g^{-1} DW ± SEM) in shoots of *C. disticha* and *J. acutiflorus*, grown on 20 μmol·l^{-1} NH_4^+ or 2 mmol·l^{-1} NH_4^+ at either pH 4 or pH 6.

		Carex disticha							*Juncus acutiflorus*						
		NH_4 20 μM		NH_4 2 mM		*P* value			NH_4 20 μM		NH_4 2 mM		*P* value		
		pH 4	pH 6	pH 4	pH 6	N	pH	N*pH	pH 4	pH 6	pH 4	pH 6	N	pH	N*pH
Total AAs	μmol N g^{-1} DW	51 ± 8	53 ± 7	680 ± 81	497 ± 70	0.00	n.s.	n.s.	84 ± 9	64 ± 5	164 ± 19	217 ± 21	0.00	n.s.	0.03
N-rich AAs	μmol N g^{-1} DW	2.9 ± 1.0	7.1 ± 3.1	497 ± 74	371 ± 66	0.00	n.s.	n.s.	8.2 ± 1.1	6.5 ± 1.2	49 ± 9	91 ± 11	0.00	n.s.	0.01
Asparagine	μmol N g^{-1} DW	0.8 ± 0.4	4.0 ± 3.1	487 ± 73	365 ± 65	0.00	n.s.	n.s.	1.0 ± 0.3	0.8 ± 0.4	23 ± 5	61 ± 5	0.00	0.03	0.01
Aspartic acid	μmol N g^{-1} DW	3.3 ± 0.3	3.3 ± 0.5	0.5 ± 0.5	1.2 ± 0.7	0.00	n.s.	n.s.	5.1 ± 0.5	3.9 ± 0.2	9.3 ± 1.2	8.3 ± 1.5	0.00	n.s.	n.s.
Glutamine	μmol N g^{-1} DW	0.9 ± 0.3	1.2 ± 0.3	0 ± 0	0 ± 0	0.00	n.s.	n.s.	2.5 ± 0.8	1.7 ± 0.4	21 ± 5	24 ± 12	0.00	n.s.	n.s.
Glutamic acid	μmol N g^{-1} DW	12.6 ± 0.8	11.8 ± 1.8	11.1 ± 1.5	9.4 ± 1.2	n.s.	n.s.	n.s.	12.9 ± 1.3	12.1 ± 0.7	20 ± 4	23 ± 2	0.00	n.s.	n.s.
Arginine	μmol N g^{-1} DW	0.2 ± 0.1	0.3 ± 0.1	6.2 ± 1.0	3.2 ± 1.3	0.00	n.s.	0.05	0.6 ± 0.2	0.3 ± 0.0	1.0 ± 0.4	1.7 ± 1.1	n.s.	n.s.	n.s.
Alanine	μmol N g^{-1} DW	4.5 ± 0.5	4.1 ± 0.6	18.4 ± 2.9	14.4 ± 1.8	n.s.	n.s.	n.s.	4.8 ± 0.6	2.9 ± 0.1	10.0 ± 0.8	12.2 ± 3.2	0.01	n.s.	n.s.
Serine	μmol N g^{-1} DW	6.0 ± 1.3	5.8 ± 1.3	83.3 ± 7.5	46.4 ± 8.9	0.00	n.s.	n.s.	5.2 ± 0.6	4.0 ± 0.6	11.9 ± 2.4	14.1 ± 1.8	0.00	n.s.	n.s.

biomass (<14 $g \cdot g^{-1}$) that we found for both species, it seems likely that growth was N-limited in the low NH_4^+ treatments (Wassen *et al.* 1995; Koerselman & Meuleman 1996; Verhoeven *et al.* 1996; Olde Venterink *et al.* 2002; Güsewell 2004, 2005). However, at the high NH_4^+ treatments, this seems very unlikely. In addition, as higher NH_4^+ availability increased P uptake, and N:P ratios remained below this critical value, P limitation was even more unlikely. However, high NH_4^+ levels decreased K concentrations in the shoot, leading to increased N:K ratios. The fact that these ratios were >2.1 indicate that growth may have become (co-)limited by K at high NH_4^+ (Olde Venterink *et al.* 2002), as confirmed for *J. acutiflorus* using the method of Timmer & Stone (1978) and De Graaf *et al.* (1998). These results are in agreement with Tylová *et al.* (2008), who also found induced K shortage in the wetland species *G. maxima* as a result of high NH_4^+ levels. Although total biomass was not affected, *J. acutiflorus* had lower shoot biomass, as might be expected with high NH_4^+ at pH 6. Although it is difficult to explain this response from our results, previous research suggests that cation deficiency might indeed be responsible for reduced growth of *J. acutiflorus* (Smolders *et al.* 1997).

Effects of pH

A low pH was expected to have a negative effect on cation uptake at increasing NH_4^+ levels (Findenegg 1987; Lucassen *et al.* 2002; Van den Berg *et al.* 2005). A higher proton concentration impairs the uptake of NH_4^+ because it is accompanied by the release of protons to the rhizosphere (Miller & Cramer 2004). Lucassen *et al.* (2002) found that the wetland species *Cirsium dissectum* suffered from severe growth reduction and even mortality at high NH_4^+ and pH 4, but not at pH 6, due to internal acidification of the roots. In contrast, neither growth nor NH_4^+ uptake rates of both tested graminoids was significantly affected by low pH in our study. It has been suggested that the uptake of NH_4^+ is most favourable when co-transported with anions, since the assimilation of these anions could enhance rhizosphere alkalinisation (Britto & Kronzucker 2002). Although increasing S concentrations with increasing NH_4^+ concentration were found earlier for *Gentiana pneumonanthe*, *Calluna vulgaris*, *Deschampsia flexuosa* (Van den Berg *et al.* 2005) and *Ricinus communis* (Van Beusichem *et al.* 1988), we found this effect mainly for root tissue. Uptake of other anions, such as bicarbonate (HCO_3^-), could have caused a similar effect. Since we used a nitrification inhibitor, NO_3^- concentrations are unlikely to have induced this response.

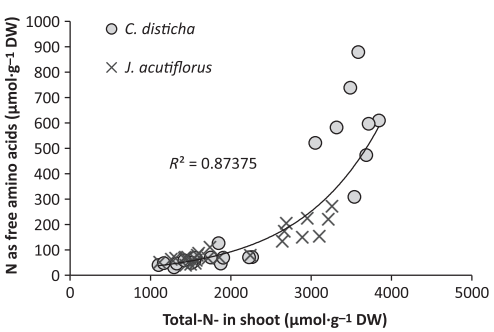

Fig. 3. Correlation between total N concentrations (µmol N g^{-1} DW) in shoots of *C. disticha* and *J. acutiflorus*, and their N concentrations as free amino acids (µmol N g^{-1} DW).

Nutrient or toxin?

Biomass and resource allocation reflects the plasticity of plants to adapt to new situations, and to be able to compete with other fast-growing species under more nutrient-rich conditions. Both species were able to increase their competitive strength with respect to light acquisition, at a lower root biomass, showing high C efficiency. Investing less C, while additional N is demanded for chlorophyll synthesis during biomass allocation to the shoot (Van Dijk & Roelofs 1988), seems adaptively beneficial with highly increased NH_4^+ levels. This result shows that N eutrophication does not have to lead to higher total biomass production of plants *per se* in order to increase their competitive strength. It also suggests that the positive aboveground response that is generally found as a result of eutrophication, without considering belowground responses, may reflect changed biomass allocation rather than growth stimulation; especially if other nutrients such as K may have become (co-)limited as a result of enhanced uptake of NH_4^+. On the other hand, such changes in plant morphology and physiology as a result of increased NH_4^+ might still negatively

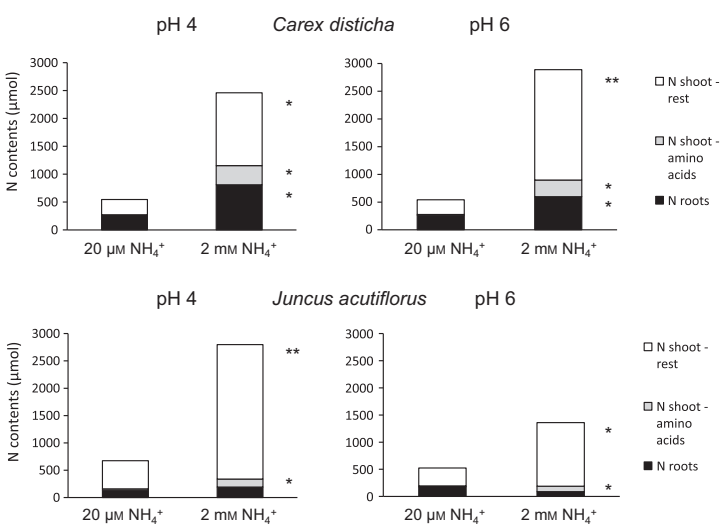

Fig. 4. Total-N content (µmol) in plants of *C. disticha* or *J. acutiflorus*, in roots, in free amino acids in the shoot and in the rest of the shoot, after growing on low (20 µmol·l^{-1}) or high (2 mmol·l^{-1}) NH_4^+ concentrations at either pH 4 or pH 6. *Indicates a significant increase due to the N treatment, **indicates a significant increase due to an interacting effect of the N and pH treatment.

affect plant fitness in the long term. The observed low root: shoot ratio in combination with impaired uptake per unit root biomass may reduce the uptake rates of other essential nutrients, and make plants more vulnerable to desiccation (Marschner 1995; De Graaf *et al.* 1998). In addition, N enrichment of plant tissue may enhance herbivory (Mattson 1980). Although a reduced root:shoot ratio is recognised as a symptom of NH_4^+ toxicity (Britto & Kronzucker 2002; Stevens *et al.* 2011), its occurrence alone may not certify actual toxicity. However, deficient plant Ca and K concentrations found for both graminoids in response to enhanced NH_4^+ uptake (Van Beusichem *et al.* 1988; Britto & Kronzucker 2002; Tylová *et al.* 2008; Stevens *et al.* 2011), and strongly increased concentrations of free (N-rich) amino acids in their shoot tissue (Marschner 1995; Smolders *et al.* 2000; Britto & Kronzucker 2002; Miller & Cramer 2004; Stevens *et al.* 2011), do indicate that both species suffer from N overload. NH_4^+ is taken up through passive diffusion, but it also has to be assimilated immediately because of its toxicity when accumulating in plant tissue. Consequently, uptake of NH_4^+ directly results in a high demand for C skeletons during assimilation (Miller & Cramer 2004).

Differential responses

High accumulation of free N-rich amino acids can be a good indicator for N saturation in plants (Van Dijk & Roelofs 1988; Rabe 1990; Näsholm *et al.* 1994; Smolders *et al.* 2000; Tomassen *et al.* 2003). In our study, *C. disticha* stored much more total N (up to 50%) in the form of free amino acids in aboveground biomass than *J. acutiflorus* (10%). Smolders *et al.* (1996) found extremely high accumulation of total N (82–97%) as free amino acids, mostly as asparagine, in P-deficient *Stratiotes aloides* subjected to high NH_4^+ levels. In our study, *C. disticha* also predominantly invested in the amino acid asparagine, which is known to be a storage compound and the major transport compound of N from the root to the leaves (Miflin & Lea 1977; Lam *et al.* 1996; Lea *et al.* 2007); its amide group can be used in the assimilation of proteins, contributing to different metabolic pathways (Miflin & Lea 1977). Asparagine can readily accumulate at high rates when plants are growing in a N-rich medium, especially when another mineral ion, probably K or Ca in our study, becomes limited for growth (Rabe 1990; Lea *et al.* 2007). In contrast, *J. acutiflorus* did accumulate a wider variety of free amino acids in addition to asparagine, including glutamine, glutamic acid, alanine and serine. Glutamine synthesis is the initial step from which other amino acids that are used in the metabolism of developing plant parts are synthesised (Miflin & Lea 1977). This might indicate that although free amino acid concentrations are increasing, *J. acutiflorus* still seems to be coping relatively well with increased NH_4^+ uptake, while *C. disticha* appears to be more sensitive. As total biomass was not enhanced, however, *J. acutiflorus* might additionally have accumulated non-amino acid N compounds that were not measured in our study. Cruz *et al.* (2011) suggested that plant tolerance to NH_4^+ might differ among species. Our results indeed showed differential responses between specific wetland-adapted graminoids. Tylová *et al.* (2008) suggested that different responses between wetland species upon high NH_4^+ could be related to their rooting strategy in hypoxic soils. The deeper rooting strategy and higher ROL (Lamers *et al.* 2013) might enhance NH_4^+ tolerance of *J. acutiflorus*, although this was not studied here. Our results imply that in addition to increased growth rates, differential tolerances to high NH_4^+ concentrations among graminoids might lead to changes in composition; in our case higher competitive strength of *J. acutiflorus* and lower competitive strength of *C. disticha* in the long term.

CONCLUSIONS

The wetland graminoids *J. acutiflorus* and *C. disticha* appeared to be well adapted to a highly increased NH_4^+ concentration, and showed high tolerance in the short term. They both showed high C efficiency, allocating biomass towards aboveground plant tissue without any change in total biomass. This efficient C allocation response enhances the competitive strength of graminoids upon N enrichment, which will lead to changes in plant composition at the expense of wetland biodiversity. In addition, both species showed several well-known response mechanisms in order to detoxify high NH_4^+ loadings, including decreased root: shoot ratios, increased allocation of N into free amino acids and indications of cation (K, Ca) deficiency. However, differential responses were found, as *C. disticha* showed a higher classic detoxifying responses than *J. acutiflorus*, which are early warning indicators for decreased tolerance to high NH_4^+ loadings in the long term. The differential responses of graminoid species will also affect their interspecific competition when NH_4^+ availability increases. Even though high tolerance was observed in the short term, it can be expected that the plasticity and adaptive ability with respect to high NH_4^+ loadings might decrease the competitive strength of *C. disticha*, and presumably also of *J. acutiflorus*, in the long term.

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