



Harnessing facilitation: Why successful re-introduction of *Stratiotes aloides* requires high densities under high nitrogen loading



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ABSTRACT

Dense mats of floating vegetation form complex structures that support high biodiversity in wetlands. Since the 1960s, however, high-density stands of *Stratiotes aloides* have shown strong declines throughout Europe and restoration efforts have often failed, both of which have been linked to high nitrogen (N) input rates. The low success rate of reintroduction is striking, since seemingly healthy, high-density stands are observed to grow under similar environmental conditions. We therefore hypothesise that N tolerance of *S. aloides* increases with density due to joint N uptake and detoxification. To test our hypothesis, we set up a controlled full factorial mesocosm experiment in which we manipulated N loading and *S. aloides* density, and investigated interacting effects on habitat biogeochemistry and plant performance. High-density *S. aloides* stands strongly lowered ammonium availability through shared uptake, even at extremely high N loads. Furthermore, dense stands strongly reduced water layer oxygen concentrations, which stimulated sediment phosphorus mobilisation, and increased carbon dioxide concentrations, which enhanced underwater photosynthesis. High-density stands thus not only detoxify ammonium, but also facilitate population growth through habitat manipulation. Combined, these mechanisms enabled *S. aloides* to remain viable at high loads of 800 kg N ha⁻¹ y⁻¹, whereas low-density stands already collapsed at 200 kg N ha⁻¹ y⁻¹. Overall, our results show that under similar environmental conditions, high-density stands can thrive, while restoration or natural recruitment by a low number of individuals may be impossible. We therefore conclude that acknowledging and harnessing intraspecific facilitation can be vital for successful conservation and restoration.

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1. Introduction

The floating aquatic macrophyte *Stratiotes aloides* characterises many meso- to eutrophic wetlands in Europe and Asia. This species is found mainly in sheltered bays of large lakes, backwater ponds, ditches and canals and shows a vigorous asexual reproduction, which often results in high-density, floating stands (Cook and Urmi-König, 1983). Due to their diverse structure, these floating *S. aloides* stands support high biodiversity (Sugier et al., 2010) and are essential for a number of associated endangered species such as the Green hawkler (Rantala et al., 2004; Suhonen et al., 2013) and the Black tern (Van der Winden et al., 2004). Healthy *S. aloides* plants show an alternating life cycle of submerged and emergent life stages, with plants becoming buoyant in spring, forming dense floating mats in summer and sinking again in autumn (Cook and Urmi-König, 1983; Roelofs, 1991). Since the 1960s, however, *S. aloides* populations have been in severe decline all over Europe (Mason and Bryant, 1975; Roelofs, 1991; Smolders et al., 2003; Zantout et al., 2011; Abeli et al., 2014). This has resulted in either local extinction, or the occurrence of (often submerged) remnant

populations. Although restoration efforts of wetlands in general, and *S. aloides* in particular, have increased over the last decades, the mostly small-scale and low-density reintroduction attempts have been largely unsuccessful (Gosling and Baker, 1980; Kurstjens et al., 2008; Termaat, 2010; Abeli et al., 2014).

Both the strong decrease in natural populations and the lack of successful reintroduction of *S. aloides* have been related to increased availability of ammonium (NH₄⁺) in the environment (Zantout et al., 2011; Abeli et al., 2014). Laboratory experiments have shown that growth and vitality of individually grown *S. aloides* plants are already severely hampered at NH₄⁺ water layer concentrations beyond 50 μmol l⁻¹ (Roelofs, 1991; Smolders et al., 1996), and in the field the species is hardly found when concentrations exceed 30 μmol NH₄⁺ l⁻¹ (Smolders and Roelofs, 1993). Therefore, one would expect *S. aloides* to be absent in areas with high N loads. Paradoxically, however, seemingly healthy, dense stands do still occur in agricultural systems, which are characterised by relatively high nitrogen (N)-loads of 100 to 320 kg ha⁻¹ y⁻¹ (Roelofs, 1991; Smolders et al., 2000; Saunders and Kalf, 2001; Geurts et al., 2010; Bakker, 2012).

In this study, we investigated the hypothesis that high-density stands of *S. aloides* buffer against NH₄⁺ toxicity by reducing ambient concentrations through shared uptake and manipulating habitat

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biogeochemistry. This would explain both the occurrence of high-density stands and the lack of restoration success in areas with high N loads. Intraspecific, density-dependent facilitation has been shown to alleviate environmental stress in a wide range of terrestrial, marine and freshwater ecosystems (Bertness and Callaway, 1994; Bruno et al., 2003; Le Bagousse-Pinguet et al., 2012; He et al., 2013). In addition, density-dependent detoxification of NH_4^+ has previously been reported for seagrasses (van der Heide et al., 2008; van der Heide et al., 2010), but it is yet unknown whether this also occurs in freshwater macrophytes.

To test our hypothesis, we set up a full factorial mesocosm experiment, in which we grew *S. aloides* at high or low plant density to determine density-related effects of three environmentally relevant N loads on plant growth and toxicity effects. The results of this experimental work will be especially important for future restoration efforts, as it may explain why low-density introductions of *S. aloides* and other species show low success rates under environmental stress such as high N loads.

2. Methods

2.1. Experimental set-up

S. aloides plants were collected in November 2012 from a peatland area in the Netherlands (52°44'17.02"N; 6°7'3.12"E) and transported to the greenhouse facility of Radboud University. In January 2013, 18 large mesocosms (700 L; \varnothing 90 cm) were filled with 100 l (15 cm) of organically rich soil, originating from a minerotrophic peatland in the Netherlands (52°18'32"N; 4°45'42"E) and a water layer of 500 l (75 cm) artificial surface water (deionised water, with the addition of 700 $\mu\text{mol l}^{-1}$ $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 75 $\mu\text{mol l}^{-1}$ KCl and 450 $\mu\text{mol l}^{-1}$ $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$). To create two densities of *S. aloides* stands, half of the basins received 3 plants (low-density, LD; 83.4 ± 2.3 g DW m^{-2} ; 5 plants m^{-2}) and the other half 10 plants (high-density, HD; 253.2 ± 1.0 g DW m^{-2} ; 16.7 plants m^{-2} ; comparable to high density in the field (personal observation)). To maintain a low density in the LD treatment, all offsets (young plants grown through vegetative reproduction) were removed. After 5 weeks of acclimatisation, both the HD and LD stands of *S. aloides* received artificial rainwater to which we added 0.2, 1.2 and 6.0 g l^{-1} NH_4Cl at a rate of 800 ml week^{-1} , resulting in three different N loadings of 40, 200 and 800 kg N ha^{-1} y^{-1} . These loadings were based on only atmospheric deposition (low), a combination of atmospheric deposition and agricultural run-off (medium; (Morris, 1991; Saunders and Kalff, 2001; Geurts et al., 2010; Bakker, 2012) and very high loads that occurred in Europe in the 1970s when *S. aloides* declined most severely (Saunders and Kalff, 2001). All combinations of density and N loading were performed in three replicates. During the experiment, water temperatures were similar for all mesocosms, ranging from 18.1 to 23.0 °C. Light was mostly natural, but an artificial light regime (Philips, Master SonT, 400 W) was implemented to prevent fluctuations below 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$. On average, PAR at the water level was 260 ± 15 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and 550 ± 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (mean \pm SEM) during the cold (January–April) and warm (May–August) season, respectively. To prevent algal growth affecting our treatments, we removed (filamentous) algal biomass bi-weekly. High algal biomass production was, however, only observed in basins after *S. aloides* started dying.

2.2. Chemical analyses

NH_4^+ concentrations in the water layer were checked colourimetrically every week using reagents (JBL, Neuhofen/Pfalz, Germany) and a field spectrophotometer (Spectroquant NOVA 60, MERCK, Schiphol-Rijk, the Netherlands) to roughly ascertain differences between NH_4^+ treatments. Every two weeks, samples were taken from the sediment pore water, using ceramic soil moisture cups (Eijkelkamp, Giesbeek, the Netherlands) fixed at –10 cm in

the sediment, and from the water layer. pH was measured with a standard Ag/AgCl electrode (Orion, Thermo Fisher Scientific, Waltham, MA, USA) combined with a pH meter (Tim840 titration manager; Radiometer Analytical, Lyon, France). Total inorganic carbon (TIC) was measured by injecting 0.2 ml of sample into a compartment with 1 ml phosphoric acid (0.4 M) attached to an infra-red gas analyser (IRGA; ABB Analytical, Frankfurt, Germany), after which concentrations of bicarbonate (HCO_3^-) and carbon dioxide (CO_2) were calculated based on the pH equilibrium.

Concentrations of phosphate (PO_4^{3-}), nitrate (NO_3^-) and NH_4^+ were measured colourimetrically on an Auto Analyser 3 system (Bran & Luebbe, Norderstedt, Germany) using ammonium molybdate (Henriksen, 1965), hydrazine sulphate (Kamphake et al., 1967) and salicylate (Grasshof and Johannse, 1972), respectively. The availability of ammonia (NH_3) was calculated using temperature, pH and NH_4^+ concentrations. Every two weeks, oxygen (O_2) concentrations were measured in all basins at three depths (5 cm, 20 cm and 40 cm below the water surface), using an optical oxygen probe (Hach LDO, Hach, Loveland, CO, USA).

2.3. Plant growth

Plants were emergent when they were introduced into the experimental basins, but most sank in the first month, after which their buoyancy was recorded during the experiment. Furthermore, the numbers of plants, offsets and roots were recorded monthly. After 8 months of treatment, all biomass was harvested and the number of leaves and roots, fresh weight (FW) and dry weight (DW; 48 h, 60 °C) was determined. Plants from the LD basins treated with 800 kg N ha^{-1} y^{-1} died prematurely and the remaining biomass was harvested after 4 months of treatment.

After 3 months of experimental treatments, photosynthetic quantum yield of dark-adapted leaves was determined using Pulse Amplitude Modulated (PAM) fluorescence (Junior PAM, Heinz Walz GmbH, Effeltrich, Germany), whereas chlorophyll content (a + b) was determined by extraction on a pooled sample of three leaves per basins according to Wintermans and De Mots (1965) and Lichtenthaler and Wellburn (1983) using a spectrophotometer (Lambda 25, UV/VIS Spectrometer, PerkinElmer Instruments, Waltham, MA, USA). Dried material was analysed for C and N content using an elemental analyser (Carlo Erba NA1500, Thermo Fisher Scientific, Waltham, MA, USA). Furthermore, 200 mg of dried soil was digested with 4 ml of HNO_3 (65%) and 1 ml of H_2O_2 (30%) using a microwave oven (MLS 1200 Mega, Milestone Inc., Sorisole, Italy), after which digestates were diluted and analysed for P and K using ICP spectrometry (ICP-OES iCAP 6000; Thermo Fisher Scientific, Waltham, MA, USA).

Amino acid contents were determined 21 days before the start of the experiment and after 62 and 139 days of treatment, by extraction from representative, pooled subsamples according to Van Dijk and Roelofs (1988), using norValine as internal standard. Freeze-dried extracts were dissolved in 0.01 N HCl and analysed by High Performance Liquid Chromatography (Varian 920-LC Analytical HPLC, Varian Inc., Palo Alto, CA, USA).

2.4. Statistical analyses

All data was checked for normality of residuals and homogeneity of variance using Shapiro Wilk's test and Levene's test for equality of variance, respectively. Non-normal or heteroscedastic data were log transformed to authorise use of parametric tests. Data on PO_4^{3-} , NH_4^+ , NH_3 , NO_3^- and amino acid contents were analysed over time, while data on O_2 concentrations were analysed over water depth using linear mixed models (LMM) combined with Bonferroni post hoc tests. Data on chemical composition of the water layer and pore water and final plant parameters were analysed by two-way ANOVA with Tukey post

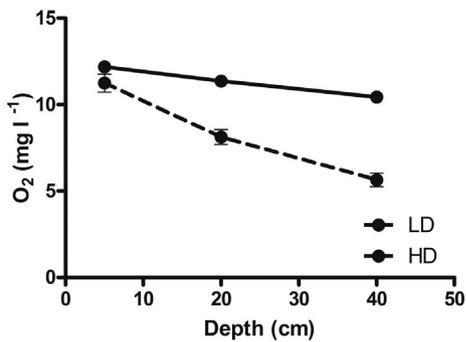


Fig. 1. Mean O_2 concentrations (\pm SEM) of the water layer at 5, 20 and 40 cm depth in high-density (HD) or low-density (LD) stands of *Stratiotes aloides*. HD stands of *S. aloides* lowered the O_2 concentration in the water layer, whereas O_2 availability remained constant underneath LD stands ($P < 0.001$).

hoc tests. All analyses were carried out with SPSS (v21, IBM Statistics, 2012).

3. Results

3.1. Density effects on biogeochemistry

HD stands of *S. aloides* reduced the O_2 concentration in the water layer by approximately 30% at 20 cm and 50% at 40 cm depth ($P < 0.001$), whereas in LD treatments, O_2 concentrations in the water layer did not differ between higher and lower depths (Fig. 1). In addition, HD stands of *S. aloides* had a higher availability of CO_2 (199.4 ± 19.2 vs. 29.8 ± 4.4 ; mean \pm SEM; $P < 0.001$) and lower pH (7.5 ± 0.1 vs. 8.0 ± 0.1 ; mean \pm SEM; $P < 0.001$) in the water layer than LD stands. There were no effects of N loading on O_2 and CO_2 concentrations.

NH_4^+ loading of $800 \text{ kg ha}^{-1} \text{ y}^{-1}$ led to accumulation of NH_4^+ ($P < 0.001$; Fig. 2), NO_3^- ($P < 0.001$; Fig. 2) and NH_3 ($P < 0.001$; Fig. 2) in the water layer. For LD, NH_4^+ ($P < 0.001$) and NH_3 ($P < 0.001$) concentrations were much higher (Fig. 2), although NH_3 concentrations showed high variation (since calculations are based on variable data from both pH (logarithmic scale) and NH_4^+ measurements). In addition,

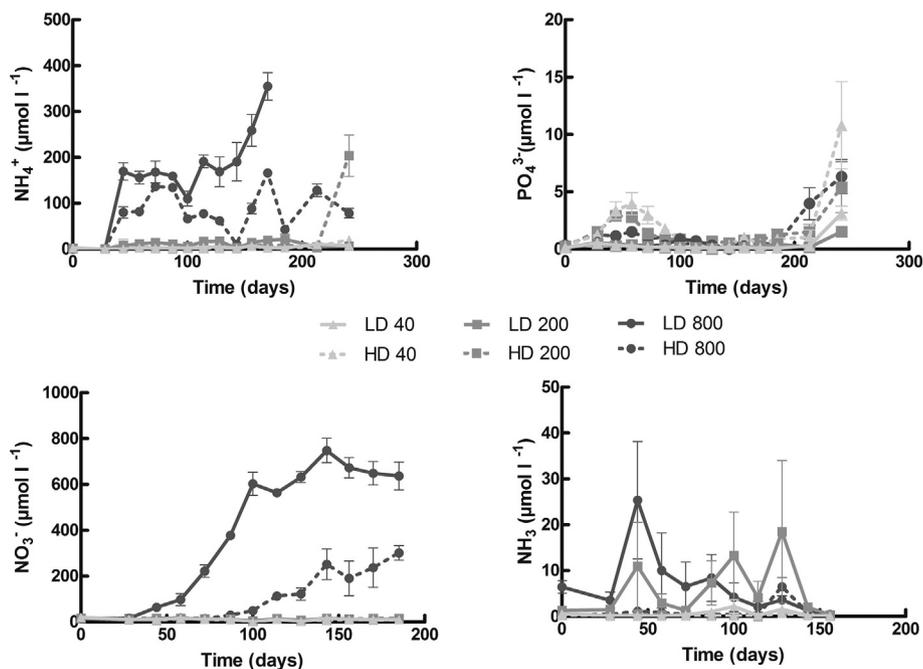


Fig. 2. Concentrations of NH_4^+ , NO_3^- , NH_3 and PO_4^{3-} in the water layer of high-density (HD) and low-density (LD) stands of *Stratiotes aloides*, receiving 40, 200 or $800 \text{ kg N ha}^{-1} \text{ y}^{-1}$. Concentrations of NH_4^+ , NO_3^- and NH_3 were higher in the water layer of LD stands ($P < 0.001$) and increased with increasing NH_4^+ loads ($P < 0.001$). PO_4^{3-} concentrations were higher in the water layer of HD stands.

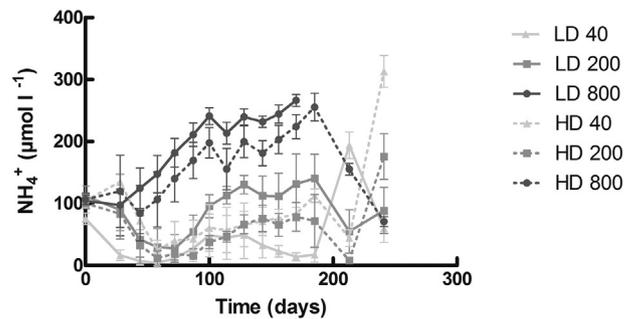


Fig. 3. NH_4^+ concentration in the porewater of basins with low-density (LD) and high-density (HD) stands of *Stratiotes aloides*, subjected to 40, 200 and $800 \text{ kg N ha}^{-1} \text{ y}^{-1}$. Porewater NH_4^+ concentrations were increased with increasing NH_4^+ loads ($P = 0.026$) and were higher for LD than HD stands ($P < 0.001$).

NO_3^- accumulation started earlier and reached up to two times higher concentrations compared to HD stands ($P < 0.001$). Porewater NH_4^+ concentrations were low at the start of the experiment, with values of $100 \pm 5 \text{ µmol l}^{-1}$. While these concentrations dropped to $50 \pm 14 \text{ µmol l}^{-1}$ and $89 \pm 21 \text{ µmol l}^{-1}$ under N loadings of 40 and $200 \text{ kg N ha}^{-1} \text{ y}^{-1}$, respectively, they increased to values of around $223.9 \pm 15.2 \text{ µmol l}^{-1}$ at $800 \text{ kg N ha}^{-1} \text{ y}^{-1}$ ($P < 0.001$; Fig. 3). HD stands showed lower NH_4^+ concentrations in the porewater than LD stands ($P = 0.026$; Fig. 3). Furthermore, HD stands showed higher net PO_4^{3-} mobilisation rates to the water layer, leading to higher PO_4^{3-} concentrations ($P < 0.001$) during the first two months (Fig. 2).

3.2. Plant growth

LD stands receiving $800 \text{ kg N ha}^{-1} \text{ y}^{-1}$ showed high mortality after 3 months of treatment, with no plants surviving after approximately 4 months (Table 1). Reduced vitality was observed in LD stands receiving NH_4^+ loadings of $200 \text{ kg N ha}^{-1} \text{ y}^{-1}$, which is illustrated by a lower final biomass compared to the lowest NH_4^+ loading of $40 \text{ kg N ha}^{-1} \text{ y}^{-1}$ (Table 1). Although no mortality was observed in HD stands, the final biomass of stands receiving $800 \text{ kg N ha}^{-1} \text{ y}^{-1}$ was lower than that of

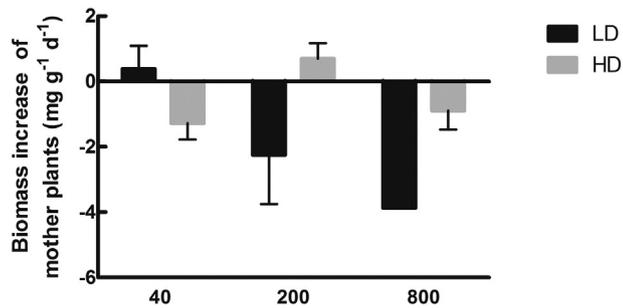


Fig. 4. Biomass increase (%) of mother plants of *Stratiotes aloides* grown in high-density (HD) or low-density (LD) stands at different NH_4^+ loadings. In general, growth was reduced most strongly in plants subjected to high NH_4^+ loading ($P = 0.046$) or grown at LD ($P = 0.011$). An interaction effect ($P = 0.006$) indicated that plants from HD stands had their optimal growth rate at NH_4^+ loadings of $200 \text{ kg N ha}^{-1} \text{ y}^{-1}$, whereas those from LD stands grew best at a loading of $40 \text{ kg N ha}^{-1} \text{ y}^{-1}$.

stands receiving only $200 \text{ kg N ha}^{-1} \text{ y}^{-1}$ ($P = 0.001$; Table 1). Effects of enhanced NH_4^+ loading were more profound for LD than for HD stands, indicating an interactive effect of density and NH_4^+ effects on final plant biomass ($P = 0.001$). This was also illustrated by a lower PAM for LD plants under high N load, but not for HD plants receiving similar loads ($P < 0.001$; Table 1).

Plant biomass production showed a density-dependent response to high N loads ($P = 0.006$). HD stands showed 20–30% decreases in mother plant biomass under either low ($40 \text{ kg ha}^{-1} \text{ y}^{-1}$) or high ($800 \text{ kg N ha}^{-1} \text{ y}^{-1}$) N loads, while under intermediate loads ($200 \text{ kg N ha}^{-1} \text{ y}^{-1}$) mother plant biomass increased by approximately 20% (Fig. 4). For LD stands, on the other hand, increasing N loads led to a strong decrease in plant biomass ($P = 0.046$), due to increased mortality of mother plants (Fig. 4). In general, LD stands showed lower biomass increases than plants from the HD stands ($P = 0.011$). LD stands subjected to $200 \text{ kg N ha}^{-1} \text{ y}^{-1}$ showed a large variation in biomass increase, since plants from two basins decreased by 93–98% in, whereas those from the third basin increased their biomass by 20%. High NH_4^+ loads not only lowered biomass, but also resulted in a lower number of roots ($P = 0.001$) with eventually no roots remaining in LD treatments receiving $800 \text{ kg N ha}^{-1} \text{ y}^{-1}$ (Table 1). During the experiment,

5.0 ± 2.1 , 14.0 ± 3.6 and 2.7 ± 0.3 offsets (including removed offsets) were produced by the LD stands receiving 40 , 200 and $800 \text{ kg N ha}^{-1} \text{ y}^{-1}$ respectively, while HD plants produced 0.3 ± 0.3 , 3.7 ± 1.8 and 3.0 ± 2.5 offsets. HD plants had, on average, higher F_V/F_M ratios ($P < 0.001$; Table 1). At both densities, plants subjected to NH_4^+ loadings of $800 \text{ kg N ha}^{-1} \text{ y}^{-1}$ contained lower concentrations of chlorophyll a and b ($P = 0.013$; Table 1).

3.3. N accumulation in plant tissue

Increasing the NH_4^+ load from 40 to 200 and $800 \text{ kg N ha}^{-1} \text{ y}^{-1}$ resulted in a 1.5 and 2.5 times higher N contents of the leaf tissue, respectively ($P < 0.001$; Table 1), although no differences in N contents were found between plants from LD and HD stands. HD stands showed 25% higher P content than LD stands ($P = 0.002$). N:P ratios increased with increasing N load ($P < 0.001$), with values more than doubled at N loads of $800 \text{ kg ha}^{-1} \text{ y}^{-1}$ compared with $40 \text{ kg ha}^{-1} \text{ y}^{-1}$. This effect was more profound in LD than in HD stands ($P = 0.038$).

Plants subjected to high NH_4^+ loads responded by producing high concentrations of N-rich free amino acids (FAA) (Table 2). LD stands produced more N-rich FAA than HD stands ($P = 0.004$), whereas both showed increased FAA production with increasing N load ($P < 0.001$). With increasing N load, the contribution of asparagine (Asn; C:N ratio 0.5) increased from 30 to 70% of total N-rich amino acids (Table 2). At high N loads, N-rich FAA made up $1.7 \pm 0.1\%$ of total plant N for HD plants, and $15.3 \pm 5.5\%$ for LD plants (data not shown).

4. Discussion

Here, we studied the density-dependent response of *S. aloides* to high availability of NH_4^+ . Our results clearly show that high-density (HD) stands facilitate their own growth and survival under elevated environmental N loading through joint uptake of NH_4^+ and alleviating adverse effects of high N loads. As a result, HD stands still thrived under extremely high NH_4^+ loads of $800 \text{ kg N ha}^{-1} \text{ y}^{-1}$, whereas plants growing at a lower density (LD) collapsed when receiving NH_4^+ loads of $200 \text{ kg N ha}^{-1} \text{ y}^{-1}$. Since the N content of plants grown at HD or LD showed no differences, the main reason for a higher tolerance of HD stands to increasing N loads lies in the fact that NH_4^+ is taken up by a

Table 1
Plant parameters of *Stratiotes aloides* plants grown at high or low density, with different loadings of NH_4^+ . Overall effects ($P < 0.05$) of density are indicated in bold (bold > not bold), whereas effects overall effects of NH_4^+ loads are indicated by different superscript letters (a, b, c). P values of density, NH_4^+ and interaction effects ($D * \text{NH}_4^+$) are given (ns is not significant). PAM stands for Pulse Amplitude Modulation, and represents a relative measure of the activity of Photosystem II.

	LD			HD			Density P	NH_4^+ P	$D * \text{NH}_4^+$ P
	$40 \text{ kg N ha}^{-1} \text{ y}^{-1}$	$200 \text{ kg N ha}^{-1} \text{ y}^{-1}$	$800 \text{ kg N ha}^{-1} \text{ y}^{-1}$	$40 \text{ kg N ha}^{-1} \text{ y}^{-1}$	$200 \text{ kg N ha}^{-1} \text{ y}^{-1}$	$800 \text{ kg N ha}^{-1} \text{ y}^{-1}$			
Final number of plants	3.0 ± 0.0	$2.7 \pm 0.3^*$	0.0 ± 0.0	9.7 ± 0.9	11.7 ± 1.7	11.7 ± 1.7			
Final biomass per basin (g DW)	29.6 ± 5.1^{ab}	$12.7 \pm 11.5^{b,*}$	0.3 ± 0.1^a	17.4 ± 2.7^{ab}	33.8 ± 1.1^b	21.6 ± 2.1^a	<0.001	0.001	0.001
Final number of roots per plant	18.33 ± 2.55^b	8.39 ± 4.20^a	0.00 ± 0.00^a	13.54 ± 3.33^b	15.43 ± 2.17^a	1.88 ± 0.57^a	ns	0.001	ns
Emergent after (days)	211 ± 0^b	$211^{a,*}$	Never ^a	165 ± 24^b	69 ± 39^a	30 ± 0^a			
Chlorophyll a + b ($\text{mg g}^{-1} \text{ DW}$) ^{**}	0.93 ± 0.07^b	0.78 ± 0.13^b	0.26 ± 0.12^a	0.85 ± 0.06^b	1.06 ± 0.04^b	0.71 ± 0.10^a	$0.074/\text{ns}$	0.013	ns
PAM ^{**}	0.72 ± 0.01	0.74 ± 0.01	0.61 ± 0.03	0.75 ± 0.00	0.74 ± 0.03	0.79 ± 0.01	<0.001	$0.084/\text{ns}$	<0.001
C ($\text{mmol kg}^{-1} \text{ DW}$) ^{**}	$26,780 \pm 848^a$	$28,580 \pm 63^b$	$30,036 \pm 425^c$	$28,848 \pm 620^a$	$29,930 \pm 288^b$	$31,382 \pm 341^c$	0.002	<0.001	ns
N ($\text{mmol kg}^{-1} \text{ DW}$) ^{**}	1373 ± 184^a	2365 ± 105^b	3808 ± 87^c	1651 ± 110^a	2205 ± 189^b	3622 ± 132^c	ns	<0.001	ns
P ($\text{mmol kg}^{-1} \text{ DW}$) ^{**}	110.0 ± 9.4	119.9 ± 13.4	179.6 ± 71.0	134.1 ± 0.8	156.5 ± 12.7	160.5 ± 14.4			
K ($\text{mmol kg}^{-1} \text{ DW}$) ^{**}	1103 ± 114	1042 ± 45	1139 ± 260	1140 ± 61	1135 ± 95	1114 ± 11			
C:N ratio g g^{-1} ^{***}	17.17 ± 1.73^c	10.40 ± 0.46^b	6.76 ± 0.06^a	15.09 ± 0.90^c	11.78 ± 0.87^b	7.45 ± 0.33^a	ns	<0.001	ns
C:P ratio g g^{-1} ^{**}	189.4 ± 41.5	244.4 ± 27.3	283.8 ± 42.8	215.2 ± 4.2	193.6 ± 15.0	199.3 ± 20.9			
N:P ratio g g^{-1} ^{***}	5.71 ± 0.82^a	9.21 ± 1.42^b	16.30 ± 2.65^b	5.56 ± 0.33^a	6.37 ± 0.21^b	10.38 ± 1.12^b	ns	0.025	ns

* Plants from two out of three basins in this treatment showed strong decreases in biomass and did not become emergent, whereas the third showed no reduced growth.

** Values are presented from measurements after 3 months of treatment. Since plants from the LD treatments receiving $800 \text{ kg N ha}^{-1} \text{ y}^{-1}$ were still alive at this stage, effects of density and N stress can be compared between all treatments.

Table 2

Concentrations of N-rich amino acids ($\mu\text{mol g}^{-1}$ DW) in leaves of *Stratiotes aloides* grown in high-density (HD) or low-density (LD) stands, subjected to different NH_4^+ loads. Measurements were carried out 21 days before and 62 and 139 days after start of the treatments. After 139 days of treatment, plants from LD stands subjected to $800 \text{ kg N ha}^{-1} \text{ y}^{-1}$ had died, indicated by †. The carbon to nitrogen ratio of each N-rich amino acids is given in the left column in brackets.

Amino Acid Name (abbreviation)	Date Days	LD			HD		
		40	200	800	40	200	800
Asparagine (Asn) (0.50)	–21	4.7 ± 4.1	2.9 ± 2.4	6.2 ± 2.8	9.8 ± 4.0	5.7 ± 3.8	1.8 ± 0.5
	62	1.1 ± 0.2	74.8 ± 29.0	266.7 ± 112.5	0.3 ± 0.1	9.3 ± 5.2	25.0 ± 1.3
	139	0.9 ± 0.8	40.5 ± 24.3	†	0.7 ± 0.1	9.7 ± 5.4	29.3 ± 7.4
Glutamic acid (Glu) (0.40)	–21	2.4 ± 0.8	1.9 ± 0.4	3.1 ± 0.9	3.1 ± 0.9	1.7 ± 0.6	2.1 ± 0.2
	62	2.1 ± 0.4	8.1 ± 2.4	6.8 ± 0.3	1.2 ± 0.3	1.4 ± 0.5	2.9 ± 1.6
	139	1.4 ± 0.6	3.2 ± 1.3	†	2.5 ± 0.6	2.2 ± 0.3	3.4 ± 0.3
Ethanolamine (Etam) (0.50)	–21	0.9 ± 0.2	1.2 ± 0.3	2.4 ± 0.4	4.7 ± 1.4	2.3 ± 0.9	2.4 ± 1.2
	62	3.4 ± 0.4	5.6 ± 1.2	12.1 ± 2.5	1.6 ± 0.2	1.8 ± 0.5	2.8 ± 0.1
	139	2.1 ± 0.9	4.7 ± 2.3	†	1.9 ± 1.5	2.9 ± 0.3	3.9 ± 0.5
Histidine (His) (0.50)	–21	0.3 ± 0.3	0.2 ± 0.2	0.4 ± 0.4	0.5 ± 0.3	0.6 ± 0.4	0.2 ± 0.0
	62	2.2 ± 1.9	4.0 ± 2.2	12.3 ± 1.2	0.0 ± 0.0	0.4 ± 0.4	2.9 ± 0.2
	139	0.1 ± 0.1	2.8 ± 1.3	†	0.4 ± 0.1	0.9 ± 0.6	3.6 ± 1.1
Arginine (Arg) (0.66)	–21	0.4 ± 0.3	0.0 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
	62	0.0 ± 0.0	4.0 ± 2.4	20.0 ± 3.3	0.0 ± 0.0	0.2 ± 0.1	1.5 ± 0.3
	139	0.0 ± 0.0	8.3 ± 1.3	†	0.4 ± 0.1	1.1 ± 0.6	7.3 ± 3.3
N in N-rich amino acids	–21	8.7 ± 5.5	6.2 ± 2.5	12.3 ± 4.1	18.2 ± 4.2	10.4 ± 5.6	6.6 ± 1.4
	62	8.8 ± 1.8	96.6 ± 37.1	317.9 ± 117.7	3.1 ± 0.4	13.0 ± 6.6	35.1 ± 2.5
	139	4.8 ± 0.5	60.0 ± 28.9	†	6.6 ± 1.8	17.0 ± 6.6	47.5 ± 9.2

higher number of plants. This shared uptake thus functions as a detoxification mechanism under high NH_4^+ stress through intraspecific facilitation, as shown for seagrasses (van der Heide et al., 2008; van der Heide et al., 2010) but formerly unknown for freshwater macrophytes.

4.1. N loading effects are density-dependent

Since NH_4^+ is taken up passively by plants (Marschner, 1995) and is toxic inside plant cells (Britto and Kronzucker, 2002), excess NH_4^+ is usually stored in N-rich free amino acids (Rabe, 1990). Build-up of N-rich free amino acids is considered indicative for physiological stress in *S. aloides* (Smolders et al., 2000). In our experiment, excess N was mainly (49–77%) stored as free asparagine, which reached concentrations of up to $267 \mu\text{mol g}^{-1}$ in LD stands receiving $800 \text{ kg N ha}^{-1} \text{ y}^{-1}$. This level is equal to concentrations reported for *S. aloides* growing under N rich conditions in the field (Smolders et al., 2000). While total N concentrations in shoots were similar for HD and LD stands, LD stands had significantly higher concentrations of free N-rich amino acids than HD, with 15% of total N present as free amino acids compared to only 1.7% in HD stands. In the field, *S. aloides* was found to contain 5.4–27.5% of total N as free amino acids under high N loading (Smolders et al., 2000). Although strong build-up of N-rich free amino acids in LD stands subjected to $800 \text{ kg N ha}^{-1} \text{ y}^{-1}$ shows that these plants try to detoxify NH_4^+ , the high mortality indicates that loads are too high for plants to cope with.

Apart from increased mortality, N loads $\geq 200 \text{ kg N ha}^{-1} \text{ y}^{-1}$ resulted in decreased biomass production in LD stands. Furthermore, even though all LD stands initially produced offsets, they would not be able to increase their density sufficiently to match HD stands in a field situation. Although LD plants subjected to $200 \text{ kg N ha}^{-1} \text{ y}^{-1}$ produced a high number of offsets (± 14), this production was most likely the result of our continued removal of offsets to maintain a LD in our mesocosm. Normally, plants would only produce 1.7 ± 0.2 offsets each (Harpenslager et al., 2015). Plants would then have had to invest in these offsets for approximately one year before they become independent adult plants. Furthermore, since young plants are more susceptible to high NH_4^+ loading (Smolders et al., 1996) and herbivory, it is highly unlikely that these plants would have reached maturity.

4.2. Habitat modification further reduces N stress

In HD stands, strong habitat modification provides additional benefits in terms of C and P availability. First of all, CO_2 accumulates in the

water layer underneath a dense plant cover, resulting in both a lower pH, which reduces build-up of highly toxic NH_3 , and a higher availability of CO_2 , which stimulates underwater photosynthesis. CO_2 concentrations below $200 \mu\text{mol l}^{-1}$, such as those observed in our LD stands, reduce underwater photosynthesis of *S. aloides* and inhibit their ability to become buoyant, due to a switch to less efficient, HCO_3^- -based photosynthesis (Cook and Urmi-König, 1983; Nielsen and Borum, 2008; Harpenslager et al., 2015). Due to the much higher CO_2 availability in HD stands, plants became buoyant 3 months earlier than those in LD stands. Secondly, after becoming emergent, HD stands completely closed off the water surface, thereby reducing atmospheric O_2 flux to the water layer similar to other excessively growing floating aquatic macrophytes (Caraco et al., 2006). Low O_2 concentrations enhance the mobilisation of phosphorus (P) from the sediment, by weakening the bonds of iron (Fe)–P complexes under anaerobic conditions (Smolders et al., 2006). Indeed, HD stands had both a higher P availability in the water layer and a higher P content in the plants than LD stands, which is highly beneficial since *S. aloides* is known to have a high P requirement (De Lyon and Roelofs, 1986; Smolders et al., 1996).

4.3. Implications for restoration and conservation

While the concept of facilitation is now generally accepted in ecological theory (Bruno et al., 2003; He et al., 2013)) and its importance has been shown in different stressful habitats, including eutrophic freshwater systems (Le Bagousse-Pinguet et al., 2012), intertidal systems (Bertness and Leonard, 1997), deserts (Padilla and Pugnaire, 2006) and tropical regions (Gomez-Aparicio, 2009), the implementation of these positive intra- and interspecific interactions in restoration and conservation biology remains limited (Padilla and Pugnaire, 2006; Gomez-Aparicio, 2009; Silliman et al., 2015). We here use self-facilitation of *S. aloides* under high N loading as an example to show that lack of intraspecific facilitation could add to the low success rate of low-density (LD) reintroduction efforts. Due to self-facilitating feedback mechanisms that are present in high-density (HD) stands, these plants not only improve their own growth, but also become more robust with respect to toxicity, such as a high input of N, but probably also to other environmental stressors that may limit growth of *S. aloides*, such as herbivory (Veen et al., 2013), CO_2 and PAR limitation (Harpenslager et al., 2015), algal growth and high concentrations of toxic compounds in the sediment such as H_2S (Roelofs, 1991).

While LD or HD stands of *S. aloides* showed no effects of NH_4^+ toxicity when receiving N loads of up to $40 \text{ kg N ha}^{-1} \text{ y}^{-1}$, loads of

200 kg N ha⁻¹ y⁻¹ or higher induced toxicity in LD stands, while HD stands still thrived. Combinations of atmospheric deposition of 15–40 kg N ha⁻¹ y⁻¹ (Morris, 1991), external loadings of 100 to 320 kg N ha⁻¹ y⁻¹ (Saunders and Kalff, 2001; Bakker, 2012) and internal N mobilisation rates of – 13 to 188 kg ha⁻¹ y⁻¹ (Geurts et al., 2010) illustrate that this load of 200 kg N ha⁻¹ y⁻¹ can be considered realistic in peatlands affected by agricultural activities in Western Europe. In other parts of the world, N loadings of surface waters can be even higher (Erisman et al., 2003; Bouwman et al., 2013). This implies that although introduction of low plant density (around 5 plants m⁻²) may still be successful under more pristine conditions, systems with a higher nutrient loading, such as those in agricultural areas, require introduction in high densities (approximately 15 plants m⁻²). Higher densities can also be created by building temporary enclosures from which plants can colonise the rest of the area. This will also prevent additional stress from herbivory (Veen et al., 2013). In systems receiving extremely high N loads of 800 kg ha⁻¹ y⁻¹, HD stands showed early warning signs of N toxicity (e.g. higher free N-rich amino acid content (Marschner, 1995; Britto and Kronzucker, 2002; Miller and Cramer, 2005), lower root: shoot ratio (Britto and Kronzucker, 2002; Tylova et al., 2008; Stevens et al., 2011) and a lower growth rate (Britto and Kronzucker, 2002; Stevens et al., 2011)). Despite the fact that these stands were still vital, such a system may eventually not be sustainable, and N loads should be severely reduced before attempting to restore *S. aloides* vegetation in such systems.

5. Conclusion

We here show that successful conservation and restoration of plant populations in freshwater systems may require the acknowledgement and harnessing of intraspecific facilitation. As the importance of this density-dependent facilitation increases with the level of environmental stress in the habitat, an assessment should be carried out beforehand, to prevent unsuccessful restoration or conservation attempts. Although low stress levels may not require harnessing of facilitation, this may well be a prerequisite at higher stress levels, while at very high stress levels measures will never be successful until habitat conditions have been improved.

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