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Combined effects of water column nitrate enrichment, sediment type and irradiance on growth and foliar nutrient concentrations of *Potamogeton alpinus*

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SUMMARY

1. High water column NO_3^- concentrations, low light availability and anoxic, muddy sediments are hypothesised to be key factors hampering growth of rooted submerged plants in shallow, eutrophic fresh water systems. In this study, the relative roles and interacting effects of these potential stressors on survival, growth, allocation of biomass and foliar nutrient concentrations of *Potamogeton alpinus* were determined in a mesocosm experiment using contrasting values of each factor (500 versus 0 μmol L^{-1} NO_3^-; low irradiance, corresponding to the eutrophic environment, versus ambient irradiance; and muddy versus sandy sediment).
2. Low irradiance, high NO_3^- and sandy sediment led to reduced growth. In a muddy sediment, plants had lower root : shoot ratios than in a sandy sediment.
3. Growth at high NO_3^- and on the sandy sediment resulted in lower foliar N and C concentrations than in the contrasting treatments. The C : N ratio was higher at high NO_3^- and on the sandy sediment. Foliar P was higher on the muddy than on the sandy sediment but was not affected by irradiance or NO_3^-. The N : P ratio was lowest at high NO_3^- on the sandy sediment.
4. Total foliar free amino acid concentration was lowest on sand, low irradiance and high NO_3^-. Total free amino acid concentration and growth were not correlated.
5. Turbidity and ortho-PO_4^{3-} concentration of the water layer were lower at high water column NO_3^- indicating that the growth reduction was not associated with increased algal growth but that physiological mechanisms were involved.
6. We conclude that high water column NO_3^- concentrations can significantly reduce the growth of ammonium preferring rooted submerged species such as *P. alpinus*, particularly on sediments with a relatively low nutrient availability. Further experiments are needed to assess potential negative effects on other species and to further elucidate the underlying physiological mechanisms.

Keywords: eutrophication, free amino acids, nitrate inhibition, nitrogen enrichment, submerged macrophyte

Introduction

The excessive use of manure and synthetic fertilisers has resulted in leaching of nitrate (NO_3^-) from agricultural land, greatly increasing NO_3^- concentrations of groundwater and surface waters in many parts of Europe (Iversen, Grant & Nielsen, 1998). As a

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consequence, many shallow freshwater systems suffer from high NO_3^- loading (100–1000 μmol L^{-1}) of the water column [International Lake Environment Committee (ILEC), 2004]. Sheltered parts of these systems are additionally faced with high sedimentation rates, resulting in the accumulation of unstable sediments containing large amounts of easily degradable organic matter (Labadz, Butcher & Sinnott, 2002). As most of these sediments are anoxic, ammonium (NH_4^+) is the dominant N-ion in the interstitial water, contrasting with the overlying water where nitrate (NO_3^-) usually exists in much higher concentrations (Wetzel, 2001). Rooted submerged macrophytes, capable of taking up NH_4^+ and NO_3^- through both roots and shoots (Nichols & Keeney, 1976; Best & Mantai, 1978), are thus potentially faced with excessive N-assimilation rates in systems where N-concentrations in both sediment and water column are high.

Especially in poorly aerated water, elevated water column NH_4^+ concentrations can reduce the growth and vitality of submerged species, such as *Stratiotes aloides* L. (Smolders *et al.*, 1996). Moreover, growth reduction of *Ruppia drepanensis* Tineo occurred after enhancing pore water NH_4^+ levels (Santamaria, Dias & Hootsmans, 1994). It has been suggested that growth inhibition is related to the luxurious uptake and subsequent incorporation of NH_4^+ in N-rich free amino acids, the assimilation of which requires energy and carbon that cannot then be used for growth (Smolders *et al.*, 1996; Marschner, 1998).

The effects of high water column NO_3^- concentrations on the growth of rooted submerged species are not yet well understood. Indirectly, NO_3^- may influence the growth of these species. Since it is an energetically more favourable electron acceptor than iron (Fe), high NO_3^- concentrations in the upper sediment layers may result in decreased ortho-phosphate (PO_4^{3-}) pore water concentrations by maintaining iron in an oxidative state (Ripl, 1978; Smolders *et al.*, 1997; Lucassen *et al.*, 2004). Consequently, PO_4^{3-} will be better bound in the sediment and less PO_4^{3-} is expected to be available for the growth of rooted submerged plants when compared with NO_3^--poor waters. For phosphorus (P)-limited plants this may negatively affect growth.

Direct effects of high water column NO_3^- concentrations on the growth of rooted submerged species are known from a marine example. Unrelated to light reduction from phytoplankton and epiphytic algae on leaf tissues, relatively low NO_3^- enrichment of the water column directly reduced the growth of *Zostera marina* L., particularly when grown in sandy sediment (Burkholder, Glasgow & Cooke, 1994). Compared with *Z. marina* plants grown in ambient water, plants of NO_3^- enriched water had elevated concentrations of total N and amino acids and decreased C-levels indicating that the energy- and C-demanding process of NO_3^- uptake and assimilation to amino acids had led to an internal 'carbon drain' (Touchette & Burkholder, 2000).

Apart from high N loading, rooted submerged species may suffer from reduced light availability in eutrophic systems (Sand-Jensen & Søndergaard, 1981; Goldsborough & Kemp, 1988). Many aspects of the sediment type including nutrient availability, organic content and redox potential may additionally affect rooted submerged plants. For example, organic matter additions to sediments negatively influenced the growth of *Myriophyllum spicatum* L. (Barko & Smart, 1983).

High water column NO_3^- concentrations, low light availability and anoxic, muddy sediments are hypothesised to be key factors hampering growth of rooted submerged plants in shallow, eutrophied fresh waters, the combined effects of which are poorly understood. In this study, we investigated the relative roles and interacting effects of these potential stressors on survival, growth, allocation of biomass and foliar nutrient and free amino acid content of *Potamogeton alpinus* Balbis, a rooted, mostly submerged species from moderately nutrient-rich sediments (De Lyon & Roelofs, 1986). The species is declining in several parts of western Europe, presumably because of ongoing eutrophication (Preston & Croft, 1997; Sand-Jensen *et al.*, 2000; Riis & Sand-Jensen, 2001). We determined the effects by using contrasting values of each stressor: 500 versus 0 μmol NO_3^- L^{-1}, low irradiance, corresponding to the eutrophic environment, versus ambient irradiance, and a muddy versus a sandy sediment.

Methods

Species

Potamogeton alpinus is a circumboreal species of slowly running waters, although it may inhabit a wider range of freshwater habitats less than 1.5 m deep (Wiegleb

& Todeskino, 1983; Preston, 1995; Preston & Croft, 1997). It grows in slightly acidic, neutral or moderately alkaline waters that may be mesotrophic or eutrophic and is found over peaty sand, loam and clay (Wiegleb & Todeskino, 1983; Preston & Croft, 1997). Its life cycle starts in spring from turions producing vertical shoots and the subsequent growth of either stoloniferous or rhizomatous lower horizontal shoots. Submerged leaves are produced when plants start to grow and both floating leaves and inflorescences may arise in late spring or summer. Stands decline in autumn and only turions remain as hibernating organs. New stands usually arise from turions and not from seeds (Brux, Todeskino & Wiegleb, 1987).

Sediments and plant material used

Sediments were obtained from eutrophic backwaters along the Twentekanaal (52°10′N, 6°23′E), the Netherlands, where *P. alpinus* had occurred for only 1 year (Boedeltje *et al.*, 2001). They consist on the one hand of sandy sediment which makes up the original bottom, and on the other hand of relatively organic, muddy sediment (hereafter called 'muddy sediment'), deposited on the sandy sediment. Percentage loss-on-ignition and nutrient concentrations were higher in the muddy than in the sandy sediment (Table 1). In the second week of April 2002, the sediments were collected, submerged by canal water and stored at 5 °C until the experiment started.

Table 1 Mean values and SE of parameters measured in the sandy and muddy sediment at the start of the experiment: in mmol kg^{-1} DW unless indicated otherwise

	Sandy sediment		Muddy sediment		
Parameter	Mean	SE	Mean	SE	Significance
Substrate ($n = 4$)					
Dry matter (%)	78	(2.6)	45.3	(0.5)	***
Organic matter (% DW)	0.78	(0.02)	7.2	(0.1)	***
N	14.3	(7.1)	192.9	(21.4)	***
P	8.2	(2.3)	84.7	(6.4)	***
S	10.7	(3.7)	78.7	(2.5)	**
K	14	(3.9)	31.8	(1.6)	*
Ca	232	(74)	808	(28)	*
Mg	49.2	(6.9)	136.6	(6.2)	**
Fe	101	(20)	656	(48)	***

ANOVA, *$P \leq 0.01$; **$P \leq 0.001$; ***$P \leq 0.0001$.

In mid-April, young plants each consisting of *c.* 10 cm vertical shoot and *c.* 5 cm 'stolon', were collected from a population in the Hagmolenbeek (52°13′N, 6°44′E), a lowland stream in the Netherlands. They were obtained from a single site in order to minimise genetic discontinuities. Plant material was transferred to the laboratory in stream water and stored for 16 h at 5 °C until transplantation. Prior to allocating to black plastic pots (12 cm diameter × 20 cm deep), each sediment type was mixed. Before they were individually planted to a depth of *c.* 4 cm into either sand or mud, fresh masses of plants were determined. Thereafter, substrates were covered with a 1-cm layer of washed silica sand to minimise the introduction of suspended sediment and nutrients into the overlying water (Barko & Smart, 1981). An additional 19 plant individuals were dried (48 h at 70 °C) and weighed. The dry masses (dm) were regressed on the fresh masses (fm) for the estimation of initial dry mass of the transplants: $dm = 0.00703 \times fm$ ($F_{1,18} = 407.4$, $P < 0.0001$, $r^2 = 0.96$).

Experimental design

The plants were grown in 24 glass aquaria (30 cm long × 30 cm wide × 60 cm deep), placed in a water-filled basin and maintained at 18 °C in a climate control room with a photosynthetically active radiation (PAR) of 100 μmol m^{-2} s^{-1} at the water surface and a daily photoperiod of 16 h. The aquaria were filled with nutrient solutions without phosphorus (P) to minimise algal growth. It was assumed that P would be obtained from the substrates by roots (Barko & Smart, 1980). Two different NO_3^- treatments were applied in addition to the basic medium composition (Table 2): 0 μmol NO_3^- L^{-1} and 500 μmol NO_3^- L^{-1}, the latter corresponding to winter and spring concentrations in eutrophic waters (ILEC, 2004). Each NO_3^- treatment consisted of 12 aquaria, randomly distributed over the water bath. The amounts of other constituents added were selected to obtain concentrations similar to those in eutrophic waters (ILEC, 2004). Each aquarium, equipped with an overflow system, received its medium from its own opaque, polyethylene stock container through black silicon tubes, at a flow rate of 0.22 L h^{-1} which was maintained by peristaltic pumps.

Two pots with sandy sediment and two with muddy sediment, including the plants, were positioned into

Table 2 Solution chemistry: N = NO_3^- added; 0 = without NO_3^-

Solution and constituent	Concentration (μmol L^{-1})	Determination	
Solution N + 0			
$CaCl_2$	1000		
$CaSO_4$	500		
$MgSO_4$	400		
$NaHCO_3$	2000		
Solution N			
KNO_3	300	pH	7.55 (0.08)
$Ca(NO_3)_2$	100	alk	2205 (52)
Solution 0			
KCl	300	pH	7.57 (0.07)
$CaCl_2$	100	alk	2249 (69)

Determination of pH and alkalinity (in μeq L^{-1}) made 24 h after aquaria were filled: means and (SE) are given; $n = 7$.

each aquarium. After 1 week of acclimatisation in ambient light, light intensity was reduced for 12 aquaria (randomly allocated to six aquaria with NO_3^- and six without NO_3^-) using neutral-density shade fabric whereas ambient light conditions were maintained for the other 12 aquaria over 6 weeks. At a depth of 40 cm below water surface, PAR of the unshaded treatment was on average 62.3 ± 2.7 μmol m^{-2} s^{-1}, of the shaded treatment 17.2 ± 1.1 SE μmol m^{-2} s^{-1} the latter corresponding to light conditions in eutrophic waters during the growth season. For example, PAR in backwaters along the Twentekanaal was 19.6 ± 1.8 μmol m^{-2} s^{-1} ($n = 20$) on a cloudless day (30 May 2002, 12.00 hours CET at a depth of 40 cm). PAR was measured with an underwater quantum sensor (LI-COR Biosciences, model LI-192SB, Lincoln, NE, U.S.A.) connected to a quantum photometer (LI-COR, model LI-185B).

Sampling

A Rhizon Soil Moisture Sampler (Eijkelkamp, Agrisearch Equipment, Giesbeek, The Netherlands) was inserted to a depth of 10 cm into one of each sediment type of each aquarium. Pore and surface water were sampled after 1, 2, 3, 5 and 6 weeks from the start of the experiment. After the addition of citric acid to a final concentration of 0.6 mmol L^{-1} to prevent metal precipitation, water samples were stored at −20 °C until further analyses. Polyethylene bottles used for storage were *a priori* iodinated and acid-cleaned. Seven weeks after the start of the experiment, plants were harvested. Each plant was washed with water over a 0.4 mm sieve and the shoot numbers per plant were counted. If present, the upper seven green, undamaged, submerged leaves excluding the youngest three were selected to be analysed for free amino acids. After weighting, these leaves were frozen with liquid nitrogen and stored at −20 °C until analysis. The fresh and dry masses of additionally collected green leaves of 11 individuals were determined separately. Dry masses (dm) were then regressed on the fresh masses (fm) for the estimation of dry mass of the leaves used for amino acid analysis: $dm = 0.00703 \times fm$ ($F_{1,10} = 2814$, $P < 0.0001$, $r^2 = 0.99$). In addition, each plant was separated into below-ground (roots and stolons) and above-ground (leaves, stems, spikes) parts which were dried (48 h at 70 °C) and weighed. Absolute growth (increase of biomass on dry weight basis) instead of relative growth is reported because: (i) the initial weight of the plants did not vary among the treatments ($P > 0.2$); and (ii) final masses were up to 40 times greater than initial masses (cf. Cronin & Lodge, 2003).

Physico-chemical analyses

Turbidity was measured by means of a Denton FN5 turbidity meter, pH with a WTW SenTix 41-3 electrode. Total inorganic carbon (TIC) concentrations of water samples were determined by infrared gas analysis (model PIR-2000, Horiba Instruments, Irvine, CA, U.S.A.). NO_3^-–N and NH_4^+–N were determined colorimetrically with a Traacs 800+ auto-analyser, using hydrazine sulphate (Technicon, 1969) and salicylate (Grasshoff & Johannsen, 1977), respectively. Ortho-phosphate (PO_4^{3-}) concentrations were determined colorimetrically with a Technicon AA II system, using ammonium molybdate (Henriksen, 1965). Total P and Fe were determined by ICP (Spectro Analytical Instruments, type Spectroflame, Kleve, Germany).

N and C concentrations of leaves were measured in dried samples using a CNS analyser (type NA1500; Carlo Erba Instruments, Milan, Italy). Additional dried material was ground in liquid nitrogen and digested with nitric acid and hydrogen peroxide in Teflon vessels in a Milestone microwave oven (type mLs 1200 Mega, Sorisole, Italy). P and Fe concentrations were then determined according to the method described above. Free amino acids were extracted according to

Van Dijk & Roelofs (1988) and quantified by measuring fluorescence after precolumn derivation with 9-fluorenylmethyl-chloroformate and measured using High Performance Liquid Chromatography (with a Star 9050 variable wave length UV-VIS and Star 9070 fluorescence detector; Varian Liquid Chromatography, Palo Alto, CA, U.S.A.) with norleucine as the internal standard. Twenty-two amino acids were determined (alanine, arginine, asparagine, aspartic acid, gamma-aminobutyric acid, glutamine, glutamic acid, glycine, histidine, homoserine, hydroxylysine, isoleucine, leucine, lysine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine). Concentrations are expressed on a dry weight basis.

Data analyses

Data were tested for normality and equality of variance and, when necessary, $\log_{10}$-transformed prior to analysis. Effects of the treatments (sediment type, irradiance and NO_3^-) on survival, biomass increase, amino acid content and pore water nutrient concentrations were analysed by a three-way ANOVA, using GLM. The effects of the treatments on the final below-ground dry mass (R), the above-ground dry mass (S), the R : S ratio, the stem length and the number of shoots were tested using three-way ANCOVAs with final plant dry weight as covariate. As R and S biomass, shoot length, and the number of shoots are related to plant size (biomass) (McConnaughay & Coleman, 1999), we included final plant biomass as a covariate after confirming the relationships between these characteristics and final dry mass. All analyses were performed using the SPSS package 11.0.

Results

Pore water and water column

Total dissolved P concentrations were significantly lower in NO_3^- enriched water than in water without NO_3^- addition and higher in muddy than in sandy sediment (Table 3). NO_3^- enrichment of the water column led to enhanced pore water NO_3^- concentrations (Fig. 1), but this effect was strongest in sandy sediment as is indicated by the significant substrate × NO_3^- interaction (Table 3). Pore water NH_4^+ concentration was significantly higher in muddy than in sandy sediment (Table 3; Fig. 1). Pore water TIC concentrations were solely affected by sediment type (Table 3) which were on average higher in muddy (15.15 ± 0.25 mmol SE L^{-1}) than in sandy (12.89 ± 0.23 mmol L^{-1}) sediment. Treatments had no effect on pore water pH which was on average 7.44 ± 0.03 ($n = 156$).

Throughout the experiment, the concentration of water column PO_4^{3-} was significantly lower in NO_3^- enriched water (0.19 ± 0.01 μmol L^{-1}) than in water without NO_3^- (0.28 ± 0.02 μmol L^{-1}), whereas it was not affected by irradiance (Table 3). Water column NH_4^+ concentration did not differ between the treatments and was overall low (6.1 ± 0.6 μmol L^{-1}). Turbidity was not affected by light, but significantly decreased by NO_3^- enrichment (Table 3). Differences were, however, small: 2.9 ± 0.1 ppm at low NO_3^- and 2.5 ± 0.1 ppm at high NO_3^-. In contrast, water column TIC and pH were significant affected by light whereas NO_3^- had no effect (Table 3). Under high and low irradiance, mean TIC concentrations were 1.56 ± 0.11 and 2.04 ± 0.06 mmol L^{-1}, respectively, whereas pH was on average 8.50 ± 0.09 and 8.08 ± 0.03, respectively.

Survival, growth and allocation of biomass

The survival of *P. alpinus* (Fig. 1) was not significantly affected by the treatments (Table 3). The increase of biomass was lowest in NO_3^- enriched water, on sandy sediment and under low irradiance (Fig. 1; Table 3). Negative growth effects of water column NO_3^- enrichment were more pronounced in sandy than in muddy sediments (Fig. 1). The treatments had similar effects on final root and shoot biomass (Table 3). The root to shoot ratio, however, was mainly affected by the sediment type (Table 3). Plants grown in muddy sediment had lower root : shoot ratios (0.21 ± 0.02) than plants from sandy sediment (0.41 ± 0.04) (Fig. 1). The number of shoots per plant was lower in the sandy sediment, at low irradiance and in NO_3^- enriched water (Fig. 1). Shoot length was mainly affected by light availability (Table 3), shoots being shorter under unshaded (31.0 ± 1.3 cm) than under shaded (40.3 ± 2.0 cm) conditions. However, on sandy sediment, NO_3^- enriched water resulted in shorter shoots (27.1 ± 3.2 cm) than water without NO_3^- (33.0 ± 2.1 cm) as indicated by the significant substrate × NO_3^- interaction (Table 3).

Table 3 Effects of substrate type (S), irradiance (I) and nitrate concentration (N) on: (a) pore and surface water concentrations of some parameters (three-way ANOVA); (b) survival and biomass increase (three-way ANOVA); (c) final root (below-ground) and shoot (above-ground) biomass, the root (R): shoot (S) ratio, the number of shoots and length of shoots (three-way ANCOVA), and: (d) foliar nutrient and free amino acid concentration (three-way ANOVA)

	S	I	N	S × I	S × N	I × N	S × I × N	d.f. (error)	Biomass
d.f. (effect)	1	1	1	1	1	1	1		
(a)									
Pore water									
Ortho-PO_4^{3-}	95.0***	1.8^{NS}	2.1^{NS}	1.9^{NS}	0.2^{NS}	0.3^{NS}	0.1^{NS}	148	
P	573.0***	0.3^{NS}	5.3*	0.4^{NS}	0.3^{NS}	0.4^{NS}	0.1^{NS}	148	
NO_3^-	43.7***	7.4**	92.4***	0.5^{NS}	47.5***	5.1*	0.5^{NS}	148	
NH_4^+	816.1***	0.2^{NS}	3.0^{NS}	1.6^{NS}	0.9^{NS}	1.3^{NS}	0.0^{NS}	148	
Total inorganic carbon	1161.1***	5.0*	0.1^{NS}	3.9*	0.2^{NS}	0.3^{NS}	1.0^{NS}	148	
Surface water									
Turbidity		0.6^{NS}	4.5*			0.3^{NS}		92	
Total inorganic carbon		14.6***	0.0^{NS}			0.0^{NS}		92	
pH		19.7***	1.4^{NS}			0.4^{NS}		92	
Ortho-PO_4^{3-}		0.1^{NS}	11.1**			0.2^{NS}		92	
NH_4^+		0.0	0.5			0.4^{NS}		92	
(b)									
Survival %	0.9^{NS}	2.6^{NS}	0.8^{NS}	0.8^{NS}	0.3^{NS}	0.8^{NS}	0.3^{NS}	40	
Biomass increase†	27.0***	18.5***	7.1**	1.9^{NS}	0.2^{NS}	0.1^{NS}	0.3^{NS}	79	
(c)									
Root biomass	5.2*	6.8*	4.4*	1.3^{NS}	2.7^{NS}	3.7^{NS}	0.9^{NS}	78	124***
Shoot biomass	5.2*	6.8*	4.4*	1.3^{NS}	2.7^{NS}	3.7^{NS}	0.9^{NS}	78	5149***
R : S ratio	14.8***	1.3^{NS}	1.2^{NS}	5.1*	1.1^{NS}	1.4^{NS}	0.2^{NS}	78	4.1*
Number of shoots	7.7**	20.8***	14.0***	2.3^{NS}	1.3^{NS}	2.7^{NS}	1.0^{NS}	78	148***
Length of shoots	0.9^{NS}	35.9***	0.0^{NS}	0.1^{NS}	4.4*	0.0^{NS}	0.0^{NS}	287	628***
(d)									
N†	21.5***	2.9^{NS}	30.0***	3.4^{NS}	8.0**	0.5^{NS}	8.8**	33	
C	4.7*	0.1^{NS}	6.1*	1.6^{NS}	0.5^{NS}	2.4^{NS}	0.3^{NS}	33	
C : N ratio	14.5***	3.8^{NS}	19.1***	3.2^{NS}	8.5**	3.5^{NS}	8.2**	33	
P	7.3*	1.7^{NS}	0.4^{NS}	0.2^{NS}	1.2^{NS}	0.2^{NS}	2.1^{NS}	33	
N : P ratio	0.4^{NS}	0.4^{NS}	1.4^{NS}	1.6^{NS}	4.7*	0.2^{NS}	0.0^{NS}	33	
Total free amino acids†	5.0*	6.6*	17.8***	0.0^{NS}	6.3*	0.1^{NS}	1.1^{NS}	22	
% Amino acids of N	0.7^{NS}	5.4*	7.1*	0.2^{NS}	2.7^{NS}	0.9^{NS}	0.0^{NS}	22	
% ASN of amino acids	0.1^{NS}	6.1*	3.5^{NS}	0.7^{NS}	0.7^{NS}	0.0^{NS}	0.7^{NS}	22	

For all main effects and their interactions, the d.f., *F*-ratios and significance levels (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; NS = not significant) are shown. ASN, asparagine.
†$\log_{10}$-transformed.

Foliar concentrations of N, C, P and free amino acids

Growth in NO_3^- enriched water and on a sandy sediment resulted in significant lower foliar N and C concentrations than in the contrasting treatments (Table 3; Fig. 1). The adverse NO_3^- effect on foliar N was most pronounced on sand as indicated by the significant substrate × NO_3^- interaction (Table 3). The C : N ratio was significantly enhanced by NO_3^- enrichment and on sand (Table 3; Fig. 1). Foliar P concentration was higher on a muddy (175 ± 8 μmol g^{-1} DW) than on a sandy sediment (145 μmol ± 6.0 μmol g^{-1} DW) (Table 3). The N : P ratio ranged on average from 7.5 to 9.9 and was lowest in NO_3^- enriched water on a sandy sediment as indicated by the significant substrate × NO_3^- interaction (Table 3). Light availability did not affect the N, C and P concentrations (Table 3).

Foliar free amino acid concentration was lowest under high NO_3^- and high irradiance and on a sandy sediment$^-$ (Table 3; Fig. 1). The free amino acid concentration showed a positive relationship with the total N concentration [equation: amino acid concentration = 167.84 × Ln (N-concentration) −1236.71; $F_{1,28} = 27.51$, $P \leq 0.0001$, $R^2 = 0.50$]. The fraction of N present in free amino acids ranged from 2.9 to 4.3% and was highest at low irradiance and low NO_3^- conditions (Fig. 1; Table 3). Asparagine dominated the free amino

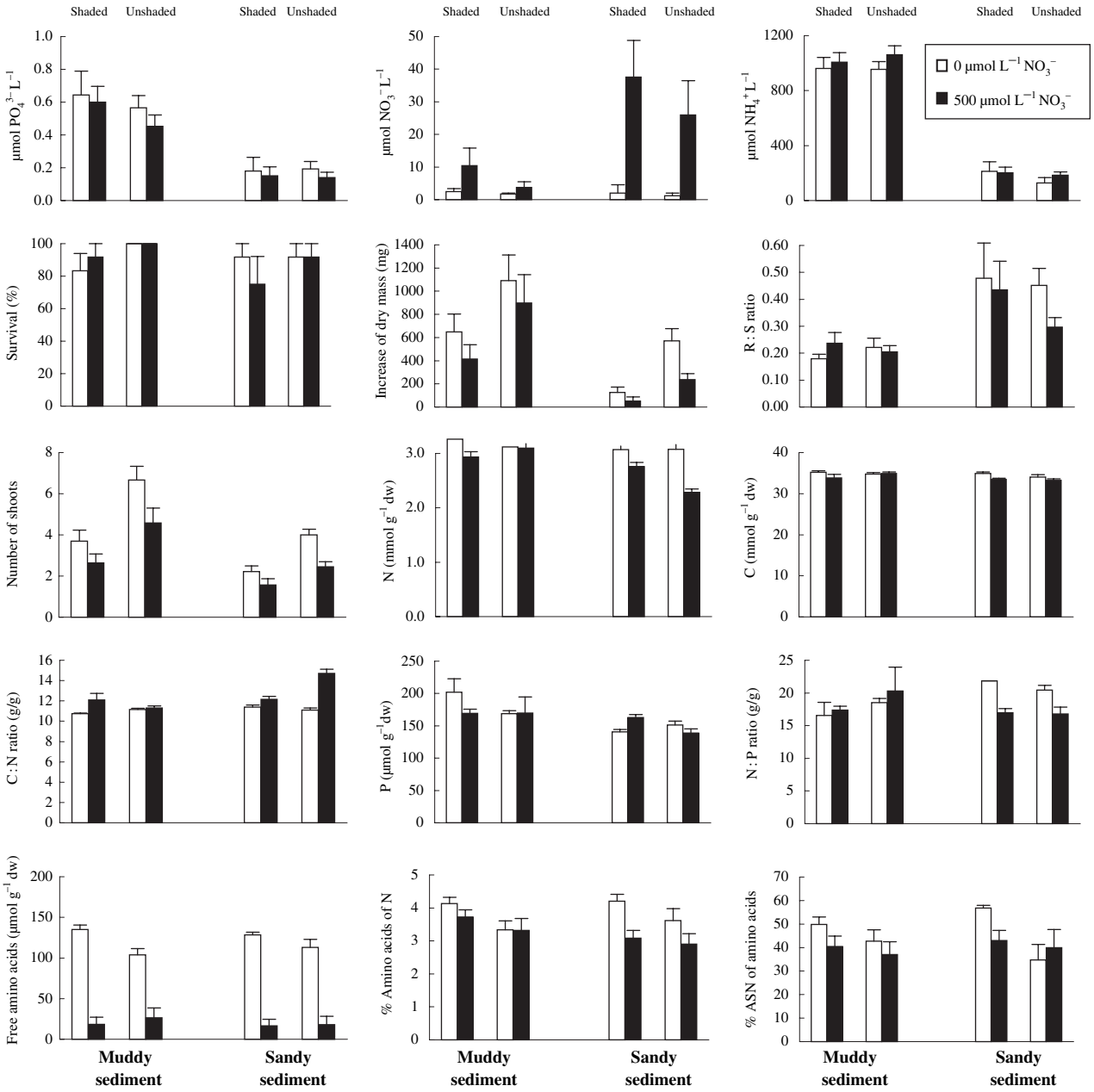

Fig. 1 The effect of sediment type, irradiance and water column NO_3^- concentration on pore water PO_4^{3-}, NO_3^- and NH_4^+ concentrations (per L sediment; $n = 20$ for each histogram), survival ($n = 6$ for each histogram), biomass, root : shoot ratio and shoot numbers ($n = 10$,11, 12,12, 11,9 and 11,11), foliar N, C, P concentrations and ratios ($n = 4$,6, 5,5, 3,4 and 5,12) and free amino acid concentrations ($n = 3$,4, 5,5, 3,4 and 3,3) of *P. alpinus* plants. Values are means + SE. Shaded: *c.* 17 µmol m^{-2} s^{-1}; unshaded: *c.* 62 µmol m^{-2} s^{-1}, at a depth of 40 cm. ASN, asparagine.

acid pool (42.6 ± 2.0% of the total concentration), aspartic acid (10.2 ± 0.4%), glutamine (9.8 ± 0.4%) and gamma-aminobutyric acid (9.7 ± 0.7%) coming after. The total foliar free amino acid concentration and biomass increase were not correlated (Pearson's correlation coefficient 0.20, $P = 0.290$).

Discussion

Growth reduction as a result of nitrate enrichment

When exposed to elevated NO_3^- concentrations, *P. alpinus* showed a marked reduction in growth, shoot density and foliar N and C concentrations, and this

effect was most pronounced on the sandy sediment. Three potential explanations for these observations will be discussed: (i) lower availability of PO_4^{3-}; (ii) enhanced turbidity at high NO_3^-; and (iii) metabolic disturbances.

(i) As it is an energetically more favourable electron acceptor than Fe, high NO_3^- concentrations in the upper part of sediments may prevent the reduction of Fe resulting in decreased PO_4^{3-} pore water concentrations (Ripl, 1978; Lucassen *et al.*, 2004). In our experiment, pore water PO_4^{3-} concentrations did not significantly differ between the NO_3^- treatments, although water column PO_4^{3-} concentrations were indeed lower in NO_3^- enriched water. However, shoot tissue P-concentrations were not significantly affected at high water column NO_3^- concentrations. In fact, tissue P concentrations remained within the range that is considered to be optimal for plant growth (Marschner, 1998). Thus we can reject this hypothesis.

(ii) Turbidity was lower in NO_3^- enriched water than in water without NO_3^- addition. Therefore, increased phytoplankton was not the likely cause for the reduced growth of *P. alpinus*. Macroscopically there we no indications for enhanced epiphytic algae growth on the leaves in the nitrate treatment. These observations are in line with the experiments of Burkholder, Mason & Glasgow (1992) who found no differences in epiphytic growth on *Z. marina* plants from NO_3^- enriched water and water without NO_3^-.

(iii) Although NH_4^+ is the preferred source of inorganic N for several rooted submerged plants including *P. alpinus* (Jorga & Weise, 1981), NO_3^- can be absorbed by roots and shoots as well (Melzer & Exler, 1982; Cedergreen & Madsen, 2003). In their natural environment, however, species such as *P. alpinus* grow on sediments with high NH_4^+ concentrations (De Lyon & Roelofs, 1986). Typically, sediment NH_4^+ concentrations are more than 10 times higher than NO_3^- concentrations in the water layer (De Lyon & Roelofs, 1986). NH_4^+, taken up by the roots, will therefore be the natural N source for this species. In well-buffered sediments, NH_4^+ uptake by the roots will have important advantages as NH_4^+ assimilation involves low metabolic costs while the required release of H^+ will not lead to unfavourable decreases of the pH in the rooting medium or rhizosphere (Runge, 1986).

A high NO_3^- availability in the water layer may induce nitrate reductase activity in the shoots of species that are normally adapted to NH_4^+ assimilation (Nichols & Keeney, 1976; Schuurkes, Kok & Den Hartog, 1986; Marschner, 1998). Therefore it is likely that *P. alpinus* plants subjected to high water column NO_3^- concentrations assimilated NO_3^- by their shoots. However, NH_4^+ or the products of NH_4^+ assimilation can inhibit the induction of nitrate reductase or even inactivate it (Schwoerbel & Tillmanns, 1974; Nichols & Keeney, 1976; Runge, 1986; Marschner, 1998). In our experiment, NH_4^+ and its assimilation products were probably transported to the shoots after uptake by the roots. Assimilation of NO_3^- by the shoots may thus be expected to have been highest on the sandy sediment where the dissolved NH_4^+ concentration in the sediment was lowest.

In the sandy sediment, high NO_3^- concentrations resulted in the strongest inhibition of plant growth. Uptake, reallocation and reduction of NO_3^- by plants have a much higher energy and carbon requirement than NH_4^+ uptake and assimilation (Runge, 1986; Marschner, 1998). NO_3^- versus NH_4^+ assimilation will greatly affect the levels of organic anions in the plants. It is suggested that individual plant species need a specific level of organic anions for optimal growth and that they will be impaired if they are unable to maintain their optimal level (Runge, 1986). We hypothesise that NO_3^- dominated N-assimilation may lead to strong metabolic disturbances in species adapted to NH_4^+ uptake by the roots (such as *P. alpinus*) resulting in the observed reduction in growth and foliar N-content under high water column NO_3^- concentrations. The higher pore water NO_3^- concentrations in sandy sediment relative to the muddy sediment, might have aggravated these negative effects.

The leaves of *P. alpinus* display an amino acid pattern similar to that of *P. pectinatus* L., *P. natans* L. and *Zannichellia palustris* L. in which asparagine is the dominant amino acid (Janauer, 1976). Neither the foliar N content, nor the foliar free amino acid concentration, nor the amount of N stored in free amino acids was higher in NO_3^- treated leaves. Overall, the fractions of N present in free amino acids (2.9–4.3%) are far below the percentages (>20%) that are found in plants subjected to a relative N-overload (Smolders *et al.*, 1997; Smolders, Van Riel & Roelofs, 2000). These results indicate that a relative N-overload

was unlikely the mechanism that led to reduced growth of *P. alpinus* at high NO_3^-.

Sediment and irradiance effects

Pore water concentrations of NH_4^+ and PO_4^{3-} in muddy sediment were significantly higher than those in sandy sediment. Since both plant growth and tissue nutrient concentration tend to be positively correlated with nutrient supply when other resources are sufficiently available (Güsewell & Koerselman, 2002), it is likely that the reduced growth and relatively low foliar N and P concentrations of sand-grown plants relative to mud-grown plants reflect a lower availability of interstitial inorganic N and P. Previous studies have reported similar substrate effects on the growth of rooted submerged species (Idestam-Almquist & Kautsky, 1995; Hangelbroek, Santamaria & De Boer, 2003). Although a negative relationship is known between aquatic plant growth and the proportion of organic matter in sediment, the organic matter content of muddy sediment in our study (7.2%) is well below the concentration (≈15%) at which growth inhibition of rooted submerged species occurred at lake sediments (Barko & Smart, 1983).

On the sandy sediment, plants allocated more resources to the growth of below-ground structures than on muddy sediment, resulting in a higher root : shoot ratio which is generally associated with plants growing in nutrient-poor habitats (Chapin, 1980; Tilman, 1988). This feature is consistent with allocation patterns observed in other aquatic (Idestam-Almquist & Kautsky, 1995; Cronin & Lodge, 2003) and terrestrial (Wilson, 1988) species. Our results imply that a low root : shoot ratio may be expected for rooted submerged species growing in shallow waters involving the accumulation of nutrient-rich, muddy sediment. As such waters are frequently exposed to wind or boat-induced water currents (Boedeltje *et al.*, 2001), this feature may be disadvantageous for plant survival because a high root : shoot ratio is a prerequisite to resist the uprooting effects of water currents (Barrat-Segretain, 2001).

Low light availability resulted in reduced photosynthetic activity as can be deduced from the higher TIC concentrations of the water column at low irradiance relative to high light conditions. Therefore, growth at reduced irradiance resulted in significantly decreased biomass yield. As the low light conditions in our experiment correspond to those observed in backwaters along navigation canals and other eutrophic shallow waters of temperate regions, these results confirm that low light availability is a key factor hampering the growth of rooted submerged species in these systems (Sand-Jensen *et al.*, 1989; Weisner, Strand & Sandsten, 1997). Shoot elongation under low irradiance, whereby photosynthetically active tissue is concentrated at or near the water surface, has been generally observed in other freshwater species (Pilon & Santamaria, 2002; Cronin & Lodge, 2003). The reduction in the number of shoots per plants at low light is consistent with the observation of Barko & Smart (1981) at other submerged macrophytes.

Low light availability hampers the growth of submerged plants in eutrophic fresh waters. High water column NO_3^- concentrations, however, can also significantly reduce the growth of such species as was demonstrated for *P. alpinus*, particularly on sediments with a relatively low nutrient availability. Until now, the potential direct negative effects of very high NO_3^- concentrations on the growth of rooted submerged aquatic macrophytes have been largely overlooked. Although water column concentrations as high as 500 μmol L^{-1} are uncommon under natural conditions, such values are no longer the exception, because of anthropogenic causes. Further experiments are needed to assess the impacts of water column NO_3^- enrichment on other rooted submerged species and to further elucidate the underlying physiological mechanisms of growth reduction.

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References

Barko J.W. & Smart R.M. (1980) Mobilization of sediment phosphorus by submersed freshwater macrophytes. *Freshwater Biology*, **10**, 229–238.

Barko J.W. & Smart R.M. (1981) Comparative influences of light and temperature on the growth and metabolism of selected submersed freshwater macrophytes. *Ecological Monographs*, **51**, 219–235.

Barko J.W. & Smart R.M. (1983) Effects of organic matter additions to sediment on the growth of aquatic plants. *Journal of Ecology*, **71**, 161–175.

Barrat-Segretain M.-H. (2001) Biomass allocation in three macrophyte species in relation to the disturbance level of their habitat. *Freshwater Biology*, **46**, 935–945.

Best M.D. & Mantai K.E. (1978) Growth of *Myriophyllum*: sediment or lake water as the source of nitrogen and phosphorus. *Ecology*, **59**, 1075–1080.

Boedeltje G., Smolders A.J.P., Roelofs J.G.M. & Van Groenendael J.M. (2001) Constructed shallow zones along navigation canals: vegetation establishment and change in relation to habitat characteristics. *Aquatic Conservation: Marine and Freshwater Ecosystems*, **11**, 453–471.

Brux H., Todeskino D. & Wiegleb G. (1987) Growth and reproduction of *Potamogeton alpinus* Balbis growing in disturbed habitats. *Archiv für Hydrobiologie Beihefte*, **27**, 115–127.

Burkholder J.M., Glasgow H.B. Jr & Cooke J.E. (1994) Comparative effects of water-column nitrate enrichment on eelgrass *Zostera marina*, shoalgrass *Halodule wrightii*, and widgeongrass *Ruppia maritima*. *Marine Ecology Progress Series*, **105**, 121–138.

Burkholder J.M., Mason K.M. & Glasgow H.B. Jr (1992) Water-column nitrate enrichment promotes decline of eelgrass *Zostera Marina*: evidence from seasonal mesocosm experiments. *Marine Ecology Progress Series*, **81**, 163–178.

Cedergreen N. & Madsen T.V. (2003) Nitrate reductase activity in roots and shoots of aquatic macrophytes. *Aquatic Botany*, **76**, 203–212.

Chapin F.S. (1980) The mineral nutrition of wild plants. *Annual Reviews of Ecology and Systematics*, **11**, 233–260.

Cronin G. & Lodge D.M. (2003) Effects of light and nutrient availability on the growth, allocation, carbon/nitrogen balance, phenolic chemistry, and resistance to herbivory of two freshwater macrophytes. *Oecologia*, **137**, 32–41.

De Lyon M.J.H. & Roelofs J.G.M. (1986) *Waterplanten in relatie tot waterkwaliteit en bodemgesteldheid. Deel 1 & 2*. Laboratorium voor Aquatische Oecologie, Katholieke Universiteit, Nijmegen.

Goldsborough W.J. & Kemp W.M. (1988) Light responses of a submersed macrophyte: implications for survival in turbid tidal waters. *Ecology*, **69**, 1775–1786.

Grasshoff K. & Johannsen H. (1977) A new sensitive method for the determination of ammonia in sea water. *Water Research*, **2**, 516.

Güsewell S. & Koerselman W. (2002) Variation in nitrogen and phosphorus concentrations of wetland plants. *Perspectives in Plant Ecology, Evolution and Systematics*, **5**, 37–61.

Hangelbroek H.H., Santamaria L. & De Boer T. (2003) Local adaptation of the pondweed *Potamogeton pectinatus* to contrasting substrate types mediated by changes in propagule provisioning. *Journal of Ecology*, **91**, 1081–1092.

Henriksen A. (1965) An automated method for determining low-level concentrations of phosphate in fresh and saline waters. *Analyst*, **90**, 29–34.

Idestam-Almquist J. & Kautsky L. (1995) Plastic responses in morphology of *Potamogeton pectinatus* to sediment and above-sediment conditions at two sites in the northern Baltic proper. *Aquatic Botany*, **52**, 205–216.

International Lake Environment Committee (ILEC) (2004) World Lakes Database, Survey of the State of World Lakes, available at: http://www.ilec.or.jp/database/database.html.

Iversen T.M., Grant K. & Nielsen K. (1998) Nitrogen enrichment of European inland and marine waters with special attention to Danish policy measures. *Environmental Pollution*, **102**, 771–780.

Janauer G.A. (1976) Amino acids in aquatic macrophytes. *Zeitschrift für Pflanzenphysiologie*, **82**, 45–50.

Jorga W. & Weise G. (1981) Wasserinhaltsstoffe als Haupteinflussgrössen für Massenentwicklungen von Wasserpflanzen. *Limnologica*, **13**, 363–372.

Labadz J.C., Butcher D.P. & Sinnott D. (2002) Wetlands and still waters. In: *Handbook of Ecological Restoration* (Eds M.P. Perrow & A.J. Davy), Volume 1, Principles of Restoration, pp. 106–132. Cambridge University Press, Cambridge.

Lucassen E.C.H.E.T., Smolders A.J.P., Van der Salm A.L. & Roelofs J.G.M. (2004) High groundwater nitrate concentrations inhibit eutrophication of sulphate-rich freshwater wetlands. *Biogeochemistry*, **67**, 249–267.

Marschner H. (1998) *Mineral Nutrition of Higher Plants*, pp. 232–242. Academic Press, London.

McConnaughay K.D.M. & Coleman J.S. (1999) Biomass allocation in plants: ontogeny or optimality? A test along three resource gradients. *Ecology*, **80**, 2581–2593.

Melzer A. & Exler D. (1982) Nitrate- and nitrite-reductase activities in aquatic macrophytes. In: *Studies on Aquatic Vascular Plants* (Eds J.J. Symoens, S.S. Hooper & P. Compère), pp. 128–135. Royal Botanical Society of Belgium, Brussels.

Nichols D.S. & Keeney D.R. (1976) Nitrogen nutrition of *Myriophyllum spicatum*: uptake and translocation of ^{15}N by shoots and roots. *Freshwater Biology*, **6**, 145–154.

Pilon J. & Santamaria L. (2002) Clonal variation in morphological and physiological responses to irradiance and photoperiod for the aquatic angiosperm *Potamogeton pectinatus*. *Journal of Ecology*, **90**, 859–870.

Preston C.D. (1995) *Pondweeds of Great Britain and Ireland*, pp. 164–167. B.S.B.I. Handbook No. 8, Botanical Society of the British Isles, London.

Preston C.D. & Croft J.M. (1997) *Aquatic Plants in Britain and Ireland*. Harley Books, Colchester, Essex, 209 pp.

Riis T. & Sand-Jensen K. (2001) Historical changes in species composition and richness accompanying perturbation and eutrophication of Danish lowland streams over 100 years. *Freshwater Biology*, **46**, 269–280.

Ripl W. (1978) *Oxidation of Lake Sediments with Nitrate: A Restoration Method for Former Recipients*. Institute of Limnology, University of Lund, Lund.

Runge M. (1986) Physiology and ecology of nitrogen nutrition. In: *Physiological Plant Ecology III: Responses to the Chemical and Biological Environment* (Eds O.L. Lange, P.S. Nobel, C.B. Osmond & H. Ziegler), pp. 163–200. Springer-Verlag, Heidelberg-NewYork.

Sand-Jensen K. & Søndergaard M. (1981) Phytoplankton and epiphyton development and their shading effect on submerged macrophytes in lakes of different nutrient status. *Internationale Revue der Gesamten Hydrobiologie*, **66**, 539–552.

Sand-Jensen K., Riis T., Vestergaard O. & Larsen S.E. (2000) Macrophyte decline in Danish lakes and streams over the past 100 years. *Journal of Ecology*, **88**, 1030–1040.

Sand-Jensen K., Jeppesen E., Nielsen K., Van der Bijl L., Hjermind L., Wiggers Nielsen L. & Moth Iversen T. (1989) Growth of macrophytes and ecosystem consequences in a lowland Danish stream. *Freshwater Biology*, **22**, 15–32.

Santamaria L., Dias C. & Hootsmans M.J.M. (1994) The influence of ammonia on the growth and photosynthesis of *Ruppia drepanensis* Tineo from Doñana National Park (SW Spain). *Hydrobiologia*, **276**, 219–231.

Schuurkes J.A.A.R., Kok C.J. & Den Hartog C. (1986) Ammonium and nitrate uptake by aquatic plants from poorly buffered and acidified water. *Aquatic Botany*, **24**, 131–146.

Schwoerbel J. & Tillmanns G.C. (1974) Assimilation of nitrogen from the medium and nitrate reductase activity in submerged macrophytes: *Fontinalis antipyretica* L. *Archiv für Hydrobiologie Supplement*, **47**, 282–294.

Smolders A.J.P., Van Riel M.C. & Roelofs J.G.M. (2000) Accumulation of free amino acids as an early indication for physiological stress (nitrogen overload) due to elevated ammonium levels in vital *Stratiotes aloides* L. stands. *Archiv für Hydrobiologie*, **150**, 169–175.

Smolders A.J.P., Den Hartog C., Van Gestel C.B.L. & Roelofs J.G.M. (1996) The effects of ammonium on growth, accumulation of free amino acids and nutritional status of young phosphorus deficient *Stratiotes aloides* plants. *Aquatic Botany*, **53**, 85–96.

Smolders A.J.P., Hendriks R.J.J., Campschreur H.M. & Roelofs J.G.M. (1997) Nitrate induced iron deficiency chlorosis in *Juncus acutiflorus*. *Plant and Soil*, **196**, 37–45.

Technicon (1969) *Industrial Method 33–69W, Nitrate + Nitrite in Water. Technicon Autoanalyser Methodology*, pp. 1–2. Technicon Corporation, Karrytown, NY.

Tilman D. (1988) *Plant Strategies and the Dynamics and Structure of Plant Communities*. Princeton University Press, Princeton, NJ.

Touchette B.W. & Burkholder J.M. (2000) Review of nitrogen and phosphorus metabolism in seagrasses. *Journal of Experimental Marine Biology and Ecology*, **250**, 133–167.

Van Dijk H.F.G. & Roelofs J.G.M. (1988) Effects of excessive ammonium deposition on the nutritional status and conditions of pine needles. *Physiologia Plantarum*, **73**, 494–501.

Weisner S.E.B., Strand J.A. & Sandsten H. (1997) Mechanisms regulating abundance of submerged vegetation in shallow eutrophic lakes. *Oecologia*, **109**, 592–599.

Wetzel R.G. (2001) *Limnology, Lake and River Ecosystems*. Academic Press, London.

Wiegleb G. & Todeskino D. (1983) Habitat conditions of *Potamogeton alpinus* Balbis stands and relations to the plants characters. *Proceedings International Symposium on Aquatic Macrophytes*, Nijmegen, pp. 311–316.

Wilson J.B. (1988) A review of evidence on the control of shoot:root ratio, in relation to models. *Annals of Botany*, **61**, 433–449.

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