



Effects of high nitrogen concentrations on the growth of submersed macrophytes at moderate phosphorus concentrations



Qing Yu ^{a, b}, Hong-Zhu Wang ^a, Yan Li ^{a, b}, Jian-Chun Shao ^{a, c}, Xiao-Min Liang ^a, Erik Jeppesen ^{d, e}, Hai-Jun Wang ^{a, *}

^a State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China

^b University of the Chinese Academy of Sciences, Beijing 100049, China

^c Huazhong Agricultural University, Wuhan 430070, China

^d Department of Bioscience and Arctic Research Centre, Aarhus University, 8600 Silkeborg, Denmark

^e Sino Danish Centre for Education and Research (SDC), Beijing, China

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ABSTRACT

Eutrophication of lakes leading to loss of submersed macrophytes and higher turbidity is a worldwide phenomenon, attributed to excessive loading of phosphorus (P). However, recently, the role of nitrogen (N) for macrophyte recession has received increasing attention. Due to the close relationship between N and P loading, disentanglement of the specific effects of these two nutrients is often difficult, and some controversy still exists as to the effects of N. We studied the effects of N on submersed macrophytes represented by *Vallisneria spiralis* (Lour.) Hara in pots positioned at three depths (0.4 m, 0.8 m, and 1.2 m to form a gradient of underwater light conditions) in 10 large ponds having moderate concentrations of P (TP $0.03 \pm 0.04 \text{ mg L}^{-1}$) and five targeted concentrations of total nitrogen (TN) (0.5, 2, 10, 20, and 100 mg L^{-1}), there were two ponds for each treatment. To study the potential shading effects of other primary producers, we also measured the biomass of phytoplankton ($\text{Chl}_{\text{aPhyt}}$) and periphyton ($\text{Chl}_{\text{aPeri}}$) expressed as chlorophyll *a*. We found that leaf length, leaf mass, and root length of macrophytes declined with increasing concentrations of TN and ammonium, while shoot number and root mass did not. All the measured growth indices of macrophytes declined significantly with $\text{Chl}_{\text{aPhyt}}$, while none were significantly related to $\text{Chl}_{\text{aPeri}}$. Neither $\text{Chl}_{\text{aPhyt}}$ nor $\text{Chl}_{\text{aPeri}}$ were, however, significantly negatively related to the various N concentrations. Our results indicate that shading by phytoplankton unrelated to the variation in N loading and perhaps toxic stress exerted by high nitrogen were responsible for the decline in macrophyte growth.

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1. Introduction

Submersed macrophytes play a key structuring role in ecosystems and help to maintain the clear-water state in shallow lakes (Moss, 1990; Jeppesen et al., 1998; Scheffer, 1998). Along with the intensification of human activities, loss of submersed macrophytes and higher turbidity in shallow lakes have occurred worldwide (Jeppesen et al., 1998; Scheffer, 1998; Körner, 2002; Wang et al., 2014). Excessive input of phosphorus is a well-known underlying

cause (Scheffer, 1998; Carpenter, 2003; Sand-Jensen et al., 2008), but the role of nitrogen (N) for macrophytes recession has recently received increasing attention (Moss, 2001; Jeppesen et al., 2007; Moss et al., 2013). For instance, the reduced species diversity of macrophytes observed in Polish and British lakes has been related to increased concentrations of nitrate (James et al., 2005). A declining species richness of submersed macrophytes with increasing N loading was also suggested by Barker et al. (2008). Nitrogen loading has been observed to prompt a shift in macrophyte assemblages from dominance of submersed macrophytes to dominance of floating-leaved macrophytes in mesocosm experiments (Feuchtmayr et al., 2009). Mesocosm experiments and field surveys conducted in shallow lakes in Denmark (González Sagrario

* Corresponding author. 7 South Donghu Road, Wuchang District, Wuhan 430072, Hubei Province, China.

E-mail address: wanghj@ihb.ac.cn (H.-J. Wang).

et al., 2005) suggest that the risk of submersed macrophyte loss increases significantly when the concentration of total nitrogen (TN) exceeds $1.2\text{--}2\text{ mg L}^{-1}$ and when total phosphorus (TP) exceeds $0.1\text{--}0.2\text{ mg L}^{-1}$. While the global consumption of phosphate fertilizers tends to be stable (FAOSTAT, 2014), the consumption of nitrogenous fertilizers is still rising, which may potentially have detrimental effects of nitrogen on submersed macrophytes.

The mechanisms underlying the effects of nitrogen on submersed macrophytes could be threefold. Nitrogen may 1) promote growth and hence shading of phytoplankton (González Sagrario et al., 2005; Barker et al., 2008; Sayer et al., 2010a, b); 2) promote shading of periphyton (Phillips et al., 1978; González Sagrario et al., 2005; Roberts et al., 2003; Barker et al., 2008; Olsen et al., 2015); and 3) exert toxic stress on plant metabolism, by generating oxidative stress (Nimptsch and Flugmacher, 2007), disturbing carbon and nitrogen metabolisms by accumulating free amino acids (FAA) and losing soluble carbohydrates (SC) (Cao et al., 2004, 2009a, b), and by inhibiting photosynthesis (Su et al., 2012), resulting in chlorosis of leaves and suppressed growth (Zaman and Asaeda, 2013). Controversy exists, however, on the effects of increasing N concentrations on submersed macrophytes and the mechanisms behind. First, it is difficult to distinguish the effects of N from those of P due to the close N–P relationship (Downing, 1992; Wang et al., 2008b; Kosten et al., 2009), and the role of N versus P in limiting phytoplankton *per se* has long been debated (Elser et al., 2007; Lewis and Wurtsbaugh, 2008; Schindler et al., 2008; Conley et al., 2009; Wang et al., 2008b, Wang and Wang, 2009). Secondly, some field experiments suggest that periphyton abundance is determined by the abundance of grazers rather than by the concentrations of nutrients (Jones et al., 2002; Jones and Sayer, 2003). Thirdly, as to toxic stress, a wide range of N concentrations over which the effect occurs has been proposed, the stress being much higher in chronic (e.g., $10\text{ mg L}^{-1}\text{ NH}_4^+ - \text{N}$ and $\text{NO}_3^- - \text{N}$, 5-month, Li et al., 2008; $>105\text{ mg L}^{-1}\text{ NH}_4^+ - \text{N}$, 21 days, Best, 1980) than in acute experiments ($5\text{ mg L}^{-1}\text{ NH}_4^+ - \text{N}$, 2 days, Cao et al., 2004).

Most studies have so far been conducted at small scale and may not necessarily mimic the real effects of N on macrophytes in natural systems. Aiming at exploring the effects of N on submersed macrophytes under conditions closer to those of natural systems, a whole-ecosystem experiment lasting about 5 months was carried out in 10 experimental ponds. A combination of a gradient of five target TN concentrations and three growing depths was established and P concentrations were kept at a control level without any artificial fertilization. The objectives of this study were twofold: 1) to explore the growth of submersed macrophytes at high nitrogen and moderate phosphorus concentrations; 2) to test whether nitrogen promotes growth and hence shading effects of phytoplankton or periphyton on macrophytes.

2. Materials and methods

2.1. Study area and experimental system

The experimental ponds (N $30^\circ 17' 17''$, E $114^\circ 43' 45''$) were located to the northeast of Lake Bao'an on the south bank of the middle Yangtze River. The region is dominated by a warm, humid subtropical climate, with an annual mean air temperature of ca. 19°C and a precipitation level about 1031 mm. Lake Bao'an has a surface area of 48 km^2 and a mean water depth (Z_M) of 1.9 m. According to our surveys from 2011 through 2012, annual mean Secchi depth (Z_{SD}) is 0.6 m, pH 8.6, dissolved oxygen (DO) 8.9 mg L^{-1} , conductivity (Cond) $474.8\text{ }\mu\text{S cm}^{-1}$, total nitrogen (TN) 1.41 mg L^{-1} , total phosphorus (TP) 0.09 mg L^{-1} , and chlorophyll *a* (Chl *a*) $50.36\text{ }\mu\text{g L}^{-1}$. The pond, previously supporting cultures of lotus, was dredged to remove the surface sediments rich in

nutrients and organic matter before separation into 10 equally-sized parts (ca. 0.8 ha) by constructed embankments. The experiment began on 15th December 2012, 13 months after introducing sediments and water from Lake Bao'an with the aim to create a natural lake system. The initial environmental parameters of the 10 experimental ponds were $1.78 \pm 0.20\text{ m}$ (mean \pm standard error) in Z_M , $0.57 \pm 0.19\text{ m}$ in Z_{SD} , $4.86 \pm 0.38\text{ mg L}^{-1}$ in DO, $447.7 \pm 92.3\text{ }\mu\text{S cm}^{-1}$ in Cond, 8.62 ± 0.16 in pH, $7.8 \pm 10.5\text{ mg L}^{-1}$ in TN, $1.73 \pm 2.6\text{ mg L}^{-1}$ in NH_4 , $0.01 \pm 0.008\text{ mg L}^{-1}$ in TP, and $11.9 \pm 6.3\text{ }\mu\text{g L}^{-1}$ in Chl *a*.

2.2. Treatments of experiments

A gradient of five target concentrations of TN (i.e. control, 2, 10, 20, and 100 mg L^{-1}) was established with two ponds for each treatment. The target TN concentrations of the control ponds (0.5 mg L^{-1}) were the background concentrations. In China, nitrogen concentration of 2 mg L^{-1} TN refers to V type of water based on environmental quality standards for surface water (AQSIQ, 2002a), $10\text{ mg L}^{-1}\text{ NO}_3^- - \text{N}$ refers to standards for drinking water quality (SAC, 2006), and 20 mg L^{-1} TN refers to primary B based on the discharge standard of pollutants for municipal wastewater treatment plants (AQSIQ, 2002b). In part of US, $100\text{ mg L}^{-1}\text{ NO}_3^- - \text{N}$ refers to water quality standards for agriculture and livestock (Xia et al., 2004). Our gradient therefore cover the range observed in the real world. NH_4Cl fertilizer (NH_4Cl , $\geq 99.5\%$, Sinopharm Chemical Reagent Co., Ltd, Shanghai) was added as a nitrogen source to the ponds every month relative to the difference between the measured and the target concentration of TN to maintain the target concentrations. The fertilizer was dissolved in pond water in a polyethylene bucket before being poured evenly into the ponds. No phosphate fertilizer was added to the ponds throughout the experiment.

Vallisneria natans (Lour.) Hara, a widely distributed submersed macrophyte in lakes along the mid-lower Yangtze River, was selected for the pot experiment in the ponds. In October 2012, plants were collected from Lake Yandong (N $30^\circ 32'$, E $114^\circ 33'$), a middle Yangtze lake with a surface area of 13 km^2 and a mean water depth of about 1.3 m. Similar-sized plants were selected and cut to into a unified leaf length of 15 cm before being transported to the experimental area. The plants were then cultured in batches in boxes ($65\text{ cm} \times 41\text{ cm} \times 31\text{ cm}$) filled with water from Lake Bao'an. On 15th

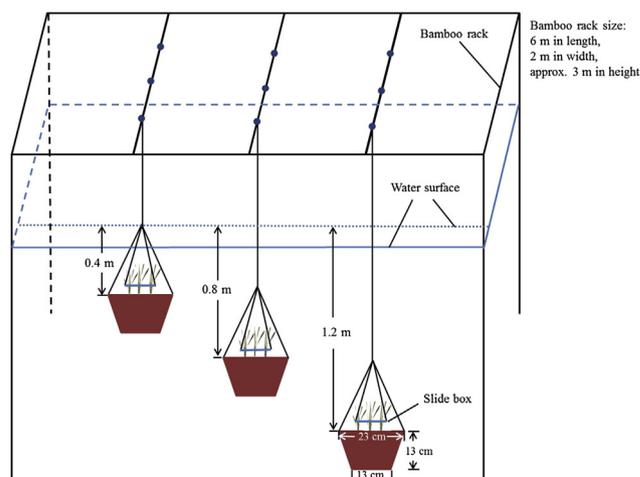


Fig. 1. Diagram showing the experimental design used in one of the experimental ponds. The fish net fixed around each bamboo rack is not shown.

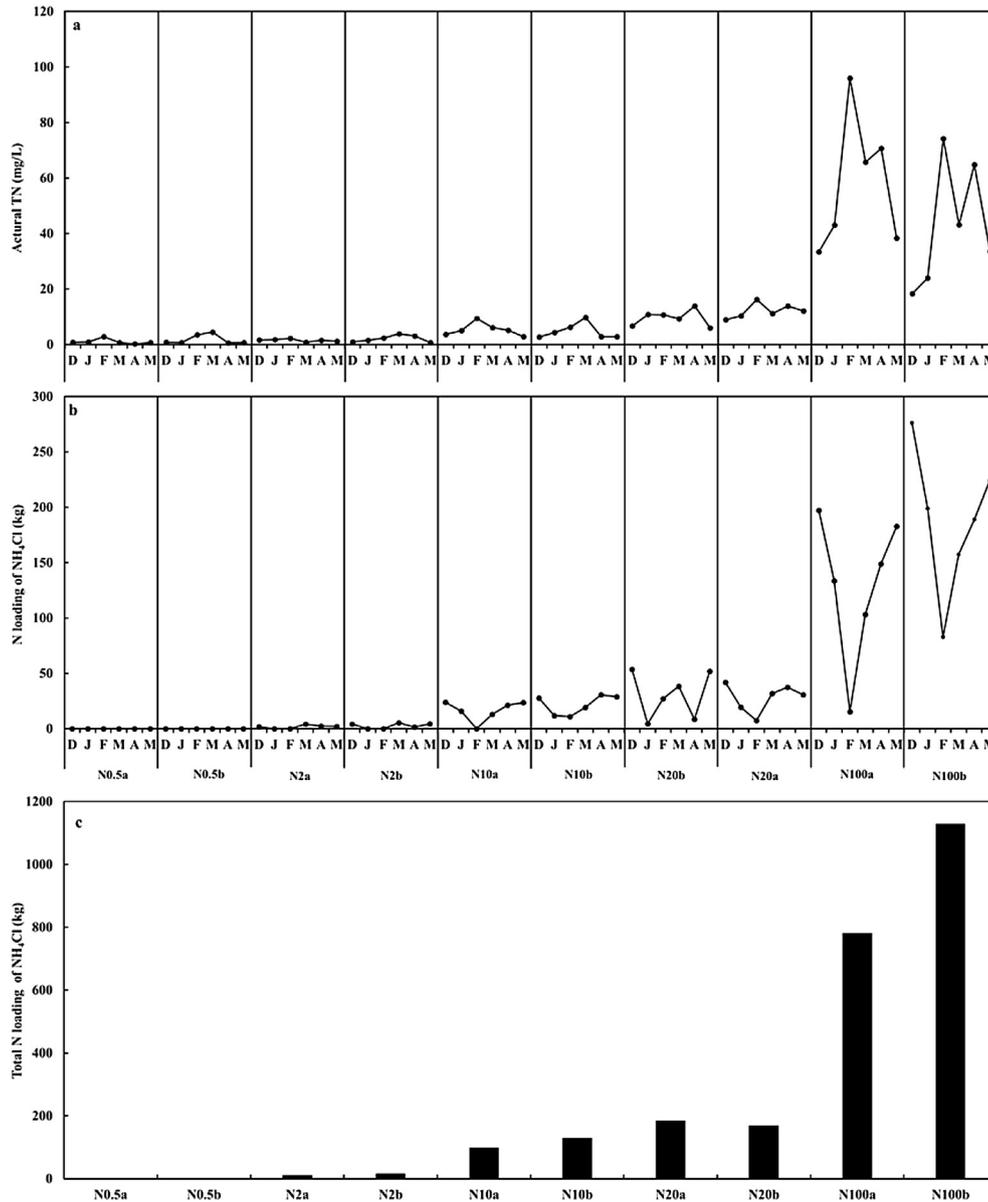


Fig. 2. Temporal dynamics of the actual concentrations of total nitrogen (TN) (a), nitrogen (N) loading for each month (b), and total N loading for the entire period of the experiments (c) from December 2012 to May 2013. In the treatment number, the value after N indicates the target TN concentration, a and b represent two ponds with the same nitrogen treatment.

December 2012, after 45 days of cultivation, similar-sized plants were selected and planted in plastic pots (23 cm in top diameter, 13 cm in bottom diameter, 13 cm in height) filled with 10 cm sediments (three plants for each pot). Sediment (originally, total nitrogen 1.93 mg g⁻¹, total phosphorus 0.67 mg g⁻¹, organic matter 39.8 mg g⁻¹) was taken from the control experimental pond and mixed with washed sand. The ratio of sand to sediment was 1/3. Three pots with plants were hung on bamboo racks (6 m in length, 2 m in width and approx. 3 m in height) at each water depth (0.4 m, 0.8 m, 1.2 m, the distance between the sediment surface and the water surface), nine pots in each pond in total. The experiment lasted 140 days during which some pots had become overgrown by plants.

Fish occurred naturally in the ponds and were thus not stocked. In a pilot experiment, the plants were removed by the fish. In our experiment, fish nets (2 cm × 2 cm in mesh size) were, therefore, fixed around the bamboo racks to prevent grazing by large fish (Fig. 1).

Table 1

Spearman rank correlations (*r* values) among environments; significant correlations are shown (**p* < 0.05, ***p* < 0.01, ****p* < 0.001) in bold (*n* = 9).

	NH ₄	Chl _a _{phyt}	Light	Chl _a _{peri}
TN	0.98***	0.22	-0.08 (Upper)	-0.87**
			-0.05 (Middle)	-0.07
			-0.08 (Lower)	0.03
NH ₄		0.30	0.03	-0.85**
			0.07	-0.15
			0.00	-0.08
Chl _a _{phyt}			0.28	-0.15
			0.20	-0.38
			0.17	-0.65
Light				0.27
				-0.23
				-0.13

Notes: TN, total nitrogen of pond water, mg L⁻¹; NH₄, ammonium of pond water, mg L⁻¹; Light, light intensity, lux; Chl_a_{phyt}, chlorophyll *a* of phytoplankton, μg L⁻¹; Chl_a_{peri}, chlorophyll *a* of periphyton, mg m⁻².

2.3. Sampling and measurement

At the end of the experiment, all pots were removed from the ponds and washed with tap water to record the number of plants and to measure the length of leaves and roots. Then, the plants in each pot were separated into leaves and roots to measure dry mass with an electronic balance (0.01 g, BL-2200H, Shimadzu Corporation, Japan) after drying at 80 °C (Jinghong, DHG-9071A, Shanghai) for 48 h to constant mass.

During the experiment, environmental parameters were measured every twenty days. Water DO, pH, and Cond were measured *in situ* with a YSI ProPlus (Yellow Spring Inc, USA). Light intensity was measured with an illuminometer (KONICA MINOLTA, T-10, China) at the three depths where plants were growing. Water samples for chemical analysis were collected at 5 randomly chosen locations within each pond by integrating the water column with a tube sampler (1.5 m in height, 10 cm in diameter). TN was determined following an alkaline potassium persulphate digestion-UV spectrophotometric method (PERSEE, TU-1810, Beijing), NH₄

using a Nessler's reagent colorimetric method and TP using an ammonium molybdate-ultraviolet spectrophotometric method after digestion with K₂S₂O₈ solution (Huang et al., 1999). Chl *a* was extracted using 90% acetone (at 4 °C for 24 h) after filtration through GF/C filters (Whatman, GE Healthcare UK Limited, Buckinghamshire, UK), and absorbance was then read at 665 nm and 750 nm, both before and after acidification with 10% HCl using a spectrophotometer.

In order to study the possible effects of periphyton on plants, artificial substrates were used to monitor the growth of periphyton. Three pieces of glass slides were embedded into a box with an open top and bottom, which was hung among the canopy of growing plants. Every 15 days, three pieces of glass slides were gently removed from the box for laboratory measurements after which a new set of glass slides was embedded. The periphyton growing on the glass slides was gently removed by a soft brush, flushed with 20 ml distilled water followed by filtered water, after which the concentration of chlorophyll *a* was determined via acetone extraction.

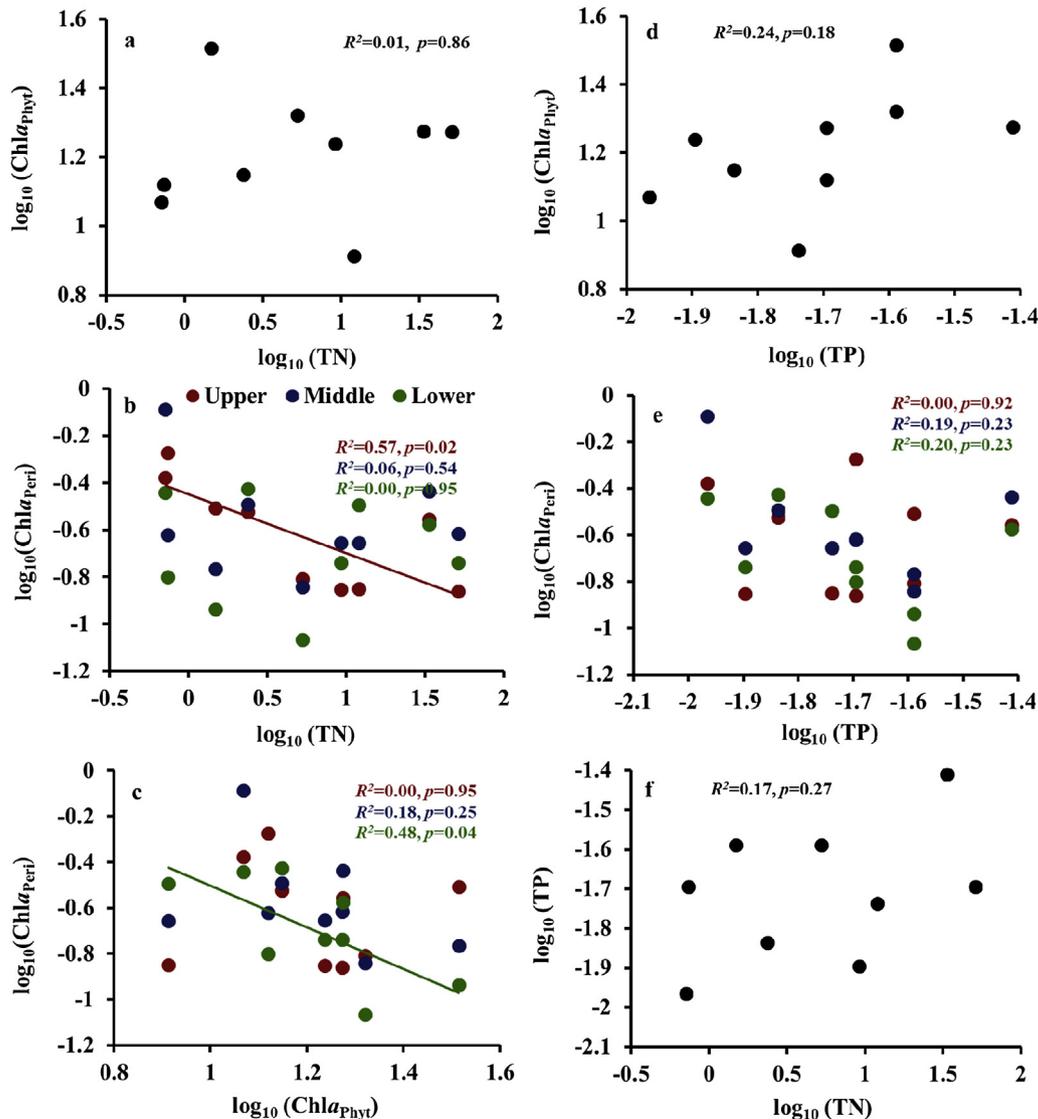


Fig. 3. Relationships between total nitrogen, total phosphorus, phytoplankton chlorophyll *a*, and periphyton chlorophyll *a* (a–f). Upper, middle, and lower represent the layer at 0.4 m, 0.8 m, and 1.2 m below the water surface ($n = 9$).

Table 2
Characteristics of the growth variables of *Vallisneria natans* (Lour.) Hara (mean) in various experimental ponds.

Treatment number	Water layer	N _{Shoot}	L _{Leaf} (cm)	DM _{Leaf} (g)	L _{Root} (cm)	DM _{Root} (g)
N0.5a	Upper	6	17.9	2.08	11.5	0.97
	Middle	4	17.3	0.98	9.1	0.46
	Lower	3	21.5	0.96	10.6	0.36
N0.5b	Upper	4	19.5	1.34	11.7	0.56
	Middle	5	24.7	1.70	14.5	0.66
	Lower	4	26.8	2.03	12.5	0.89
N2a	Upper	4	12.1	0.54	6.4	0.23
	Middle	3	18.7	0.64	10.4	0.34
	Lower	3	19.1	0.40	7.8	0.31
N2b	Upper	9	18.4	2.21	13.5	0.85
	Middle	5	30.0	1.96	10.8	0.73
	Lower	2	10.4	0.30	9.8	0.53
N10a	Upper	4	13.6	1.03	9.2	0.65
	Middle	2	17.4	0.82	11.0	0.46
	Lower	2	17.4	0.67	9.8	0.24
N10b	Upper	16	26.9	4.37	12.0	1.35
	Middle	11	26.7	2.88	11.5	1.56
	Lower	6	24.6	1.29	10.7	1.32
N20a	Upper	5	14.2	0.67	6.8	0.29
	Middle	4	15.3	0.68	8.3	0.30
	Lower	3	15.7	0.61	5.1	0.32
N20b	Upper	14	18.8	3.84	10.8	0.87
	Middle	5	22.5	1.91	10.3	0.61
	Lower	2	17.6	0.94	9.0	0.31
N100a	Upper	2	6.2	0.20	7.8	0.35
	Middle	2	6.7	0.20	4.1	0.45
	Lower	2	7.4	0.18	11.1	0.45
N100b	Upper	3	7.6	0.20	8.7	0.22
	Middle	3	8.0	0.41	4.2	0.51
	Lower	4	9.6	0.08	6.7	0.12

Notes: N_{Shoot}, number of shoots; L_{Leaf}, length of leaf, cm; DM_{Leaf}, dry mass of leaf, g; L_{Root}, length of root, cm; DM_{Root}, dry mass of root, g. In the treatment number, the value after N indicates the target concentration of total nitrogen, a and b represent two ponds of the same nitrogen treatment.

2.4. Statistical analyses

Microsoft Excel 2010 and STATISTICA 8.0 were used to process and analyze the data. Median values were used for analyses to avoid deviation caused by the extreme values in TN, TP, Chl_a_{Phyt}, etc. Spearman rank correlations were used to test for relationships between environmental variables and between macrophyte growth variables and these environmental variables. Multiple stepwise regressions (forward procedure), were then performed to analyze relative importance of the influencing factors according to their sequence of entering to explain variations of the growth variables of macrophytes. In the regression analyses, all the parameters of skewed distribution were log₁₀(x)-transformed. One pond was left out in the analysis (N10b) as it had a unusually low TP level, potentially violating the test of N treatment effects.

3. Results

3.1. Growing conditions of *V. natans*

TN showed a significant treatment gradient, with medians ranging from 0.7 to 51.5 mg L⁻¹ (Fig. 2a). The amounts of added fertilizer for each month and the whole period of the experiment are shown in Fig. 2b and c, respectively. Ammonium exhibited a similar pattern of variation as TN and constituted a high proportion of the TN (NH₄-N %), on average 47.2%, ranging from 15.4% to 94.0%. Wide ranges were also found in Chl_a_{Phyt}, light, and Chl_a_{Peri}. The underwater light intensity declined from the upper through the middle to the lower layers in all the treatments with effects on the periphyton in the different layers.

Spearman rank correlations among the environmental variables revealed a significant positive relationship between NH₄ and TN and a negative relationship between Chl_a_{Peri} and NH₄ and TN

(upper layer) (Table 1, Fig. 3b). A close relationship was observed between NH₄ and TN ($R^2 = 0.63$, $p < 0.001$). As no relationship was found with light, this variable was excluded in the subsequent analyses.

3.2. Growth of *V. natans* related to nitrogen

Growth variables of *V. natans* at the end of the experiment in various experimental ponds are shown in Table 2. Spearman rank correlations (Table 3, more details in Appendix Table A1) showed significant negative relationships between TN and both leaf length

Table 3

Spearman rank correlations (r values) among morphological variables of *Vallisneria natans* relative to the environment; significant differences are shown (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) in bold ($n = 9$).

	Layer	TN	NH ₄	Chl _a _{Phyt}	Chl _a _{Peri}
N _{Shoot}	Upper	-0.32	-0.40	-0.69*	0.18
	Middle	-0.28	-0.35	-0.87**	0.38
	Lower	-0.26	-0.19	0.03	0.16
L _{Leaf}	Upper	-0.60	-0.67*	-0.82**	0.42
	Middle	-0.63	-0.68*	-0.42	0.03
	Lower	-0.85**	-0.78*	-0.37	-0.13
DM _{Leaf}	Upper	-0.43	-0.52	-0.75*	0.28
	Middle	-0.52	-0.62	-0.70*	0.07
	Lower	-0.68*	-0.67*	-0.60	-0.05
L _{Root}	Upper	-0.43	-0.57	-0.68*	0.47
	Middle	-0.73*	-0.75*	-0.15	-0.10
	Lower	-0.36	-0.40	-0.37	0.22
DM _{Root}	Upper	-0.35	-0.42	-0.57	0.38
	Middle	-0.20	-0.32	-0.52	0.50
	Lower	-0.40	-0.47	-0.28	0.25

Notes: N_{Shoot}, number of shoots; L_{Leaf}, length of leaf, cm; DM_{Leaf}, dry mass of leaf, g; L_{Root}, length of root, cm; DM_{Root}, dry mass of root, g.

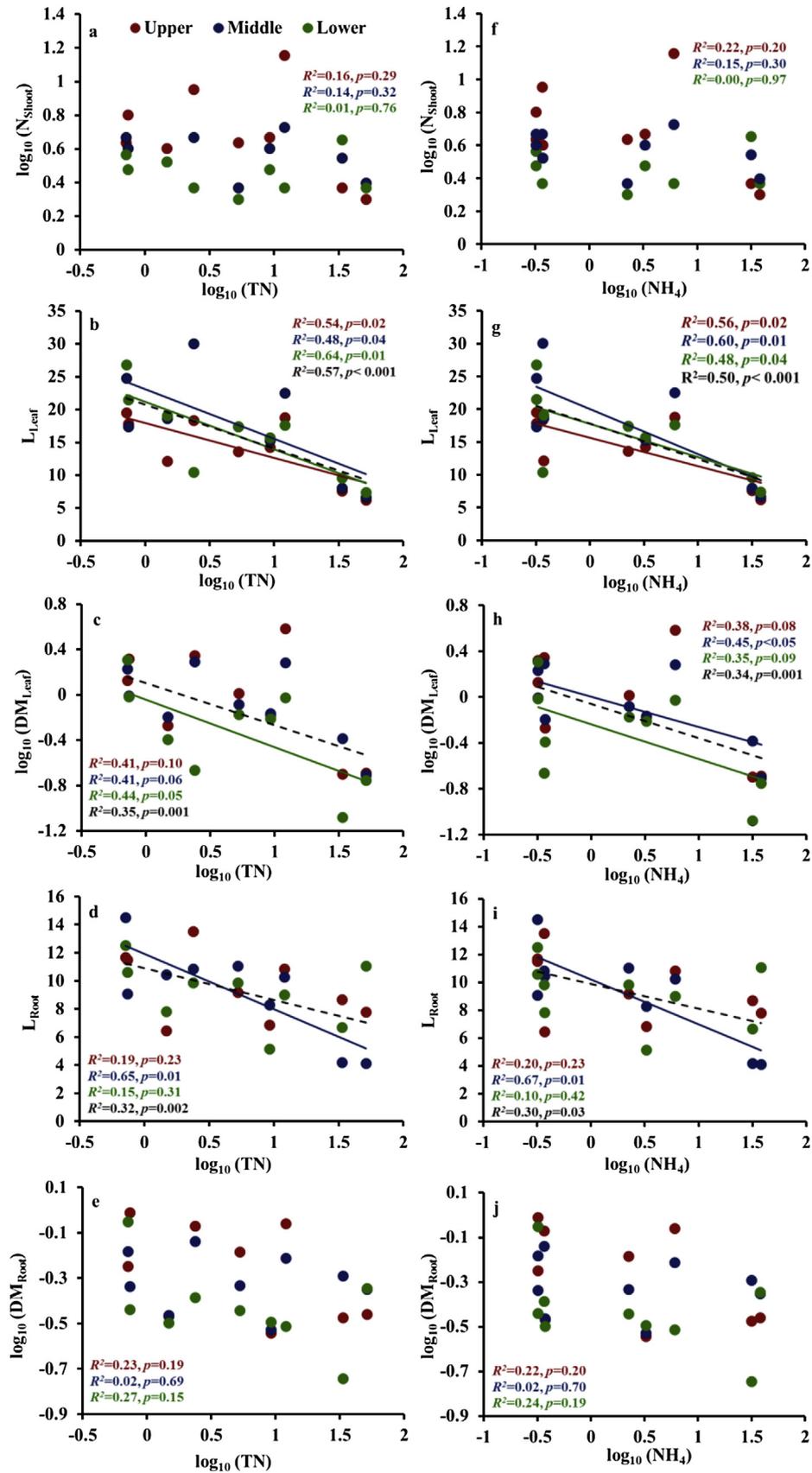


Fig. 4. Relationships between total nitrogen (a–e) and ammonium (f–j) with the morphological characteristics of *V. natans*. Upper, middle, and lower represent the layer at 0.4 m, 0.8 m, and 1.2 m below the water surface. The dotted line shows significant relationships of pooled data from all depths ($n = 9$).

(L_{Leaf}) and leaf dry mass (DM_{Leaf}) in the lower layers and with root length (L_{Root}) in the middle layer. In contrast, no significant relationship was found between TN and shoot number (N_{Shoot}) or root dry mass (DM_{Root}) for any of the three layers (Fig 4). The relationships of NH_4 with growth indices were similar with the relationships between TN and growth indices, with an exception of additional significant correlations with L_{Leaf} in all the three layers (Table 3). In the multiple stepwise regressions (Table 4), NH_4 was negatively related to L_{Leaf} in all the three layers, to DM_{Leaf} in the upper and middle layers, and to L_{Root} in the middle layer.

The scatterplots in Fig. 4 show that the growth indices of plants generally decline with increasing TN and NH_4 concentrations, significantly so for L_{Leaf} versus both TN and NH_4 in all the three layers (Fig. 4b, g), for DM_{Leaf} with TN in the lower layer and with NH_4 in the middle and lower layers (Fig. 4c, h), and for L_{Root} with both TN and NH_4 in the middle layer (Fig. 4d, i). When pooling the data from all the three layers, similar results were obtained. Neither N_{Shoot} nor DM_{Root} was significantly related to TN or NH_4 ; L_{Leaf} , DM_{Leaf} , and L_{Root} were all significantly related to both TN and NH_4 .

3.3. Growth of *V. natans* related to phytoplankton

The results of Spearman rank correlations (Table 3, more details in Appendix Table A1) showed that $\text{Chla}_{\text{Phyt}}$ correlated significantly negatively with N_{Shoot} , L_{Leaf} , DM_{Leaf} , and L_{Root} in the upper layers and with N_{Shoot} and DM_{Leaf} in the middle layers. The relationship between $\text{Chla}_{\text{Phyt}}$ and DM_{Root} for all the three layers was not significant. In the multiple stepwise regressions (Table 4), $\text{Chla}_{\text{Phyt}}$ was negatively related to N_{Shoot} in the middle layer, to L_{Leaf} in the upper layer, to DM_{Leaf} in the upper and middle layers, and to L_{Root} in the upper layer.

In the scatterplots in Fig. 5, the growth indices of plants also showed generally declining trends with the increases of $\text{Chla}_{\text{Phyt}}$, significantly so for N_{Shoot} in the middle layer (Fig. 5a) and for DM_{Leaf} and L_{Root} in the upper layer (Fig. 5c, d). When pooling the data from

all the three layers, all the growth indices were significantly related to $\text{Chla}_{\text{Phyt}}$.

3.4. Growth of *V. natans* related to periphyton

No significant relationship was detected between the growth indices of plants with $\text{Chla}_{\text{Peri}}$ from the Spearman rank correlation analyses (Table 3, more details in Table A1) or in the multiple stepwise regressions (Table 4). When pooling the data of all the three layers, significant positive relationships emerged between $\text{Chla}_{\text{Peri}}$ and L_{Root} and DM_{Root} (Fig. 5i, j).

4. Discussion

Our 5-month experiment revealed that various growth indices of *V. natans*, such as leaf length, leaf mass, and root length, declined with increasing nitrogen concentrations, most obvious for leaf length. This may in part be attributed to toxic effects as NH_4 in three of the treatments (i.e. N20b, N100a, and N100b) exceeded 5 mg L^{-1} . In an acute experiment conducted by Cao et al. (2004), an ammonium level of $>5 \text{ mg L}^{-1}$ was found to cause biochemical damage of macrophytes. Likewise, oxidative stress and inhibition of photosynthesis were also on plant leaves in studies with high ammonia ($>10 \text{ mg L}^{-1}$) (Wang et al., 2008a; Su et al., 2012). Moreover, in three of the other high N treatments, i.e. N10a, N10b, and N20a, NH_4 exceeded 5 mg L^{-1} for some months during the experiment (data not shown). In addition, ammonium constituted a high proportion of the TN, especially at the highest TN treatments (N100a and N100b), contributing 94% of TN. It must be emphasized, however, that overall weaker toxic effects of N have been recorded in chronic experiment (Best, 1980; Li et al., 2008) than in acute experiments (Saunkaew et al., 2011; Cao et al., 2004; Wang et al., 2008a, 2010) as the plants may adapt to the enhanced N concentrations in chronic experiments (Wan et al., 2006; Xu et al., 2010; Xu, 2012).

An alternative explanation of the declined growth of

Table 4

Multiple stepwise regressions of the morphological variables of *V. natans* relative to their environment. See Tables 1 and 2 for explanation of the abbreviations and units; F to enter is set as 4. Independent variables in the equation are $x = \log_{10}(\text{NH}_4)$, $\log_{10}(\text{Chla}_{\text{Phyt}})$, and $\log_{10}(\text{Chla}_{\text{Peri}})$.

Upper layer			
A: $y = L_{\text{Leaf}}$			
Step	R^2	p	Equation
1	0.56	0.02	$y = 15.63 (\pm 1.26) - 4.33 (\pm 1.47) \log_{10}(\text{NH}_4)$
2	0.95	<0.001	$y = 37.24 (\pm 3.34) - 4.23 (\pm 0.56) \log_{10}(\text{NH}_4) - 17.91 (\pm 0.274) \log_{10}(\text{Chla}_{\text{Phyt}})$
B: $y = \log_{10}(DM_{\text{Leaf}})$			
Step	R^2	p	Equation
1	0.46	<0.05	$y = 2.10 (\pm 0.90) - 1.78 (\pm 0.74) \log_{10}(\text{Chla}_{\text{Phyt}})$
2	0.81	0.006	$y = 2.15 (\pm 0.57) - 1.74 (\pm 0.46) \log_{10}(\text{Chla}_{\text{Phyt}}) - 0.32 (\pm 0.09) \log_{10}(\text{NH}_4)$
C: $y = L_{\text{Root}}$			
Step	R^2	p	Equation
1	0.50	0.03	$y = 21.62 (\pm 4.61) - 9.95 (\pm 3.78) \log_{10}(\text{Chla}_{\text{Phyt}})$
Middle layer			
D: $y = \log_{10}(N_{\text{Shoot}})$			
Step	R^2	p	Equation
1	0.50	0.002	$y = 1.18 (\pm 0.23) - 0.50 (\pm 0.19) \log_{10}(\text{Chla}_{\text{Phyt}})$
E: $y = L_{\text{Leaf}}$			
Step	R^2	p	Equation
1	0.60	0.01	$y = 20.03 (\pm 1.82) - 6.87 (\pm 2.12) \log_{10}(\text{NH}_4)$
F: $y = \log_{10}(DM_{\text{Leaf}})$			
Step	R^2	p	Equation
1	0.45	<0.05	$y = 0.002 (\pm 0.09) - 0.26 (\pm 0.11) \log_{10}(\text{NH}_4)$
2	0.81	0.01	$y = 1.39 (\pm 0.42) - 0.26 (\pm 0.074) \log_{10}(\text{NH}_4) - 1.15 (\pm 0.35) \log_{10}(\text{Chla}_{\text{Phyt}})$
G: $y = L_{\text{Root}}$			
Step	R^2	p	Equation
1	0.67	0.01	$y = 10.22 (\pm 0.74) - 3.22 (\pm 0.86) \log_{10}(\text{NH}_4)$
Lower layer			
H: $y = L_{\text{Leaf}}$			
Step	R^2	p	Equation
1	0.48	0.04	$y = 17.78 (\pm 1.70) - 5.10 (\pm 2.00) \log_{10}(\text{NH}_4)$

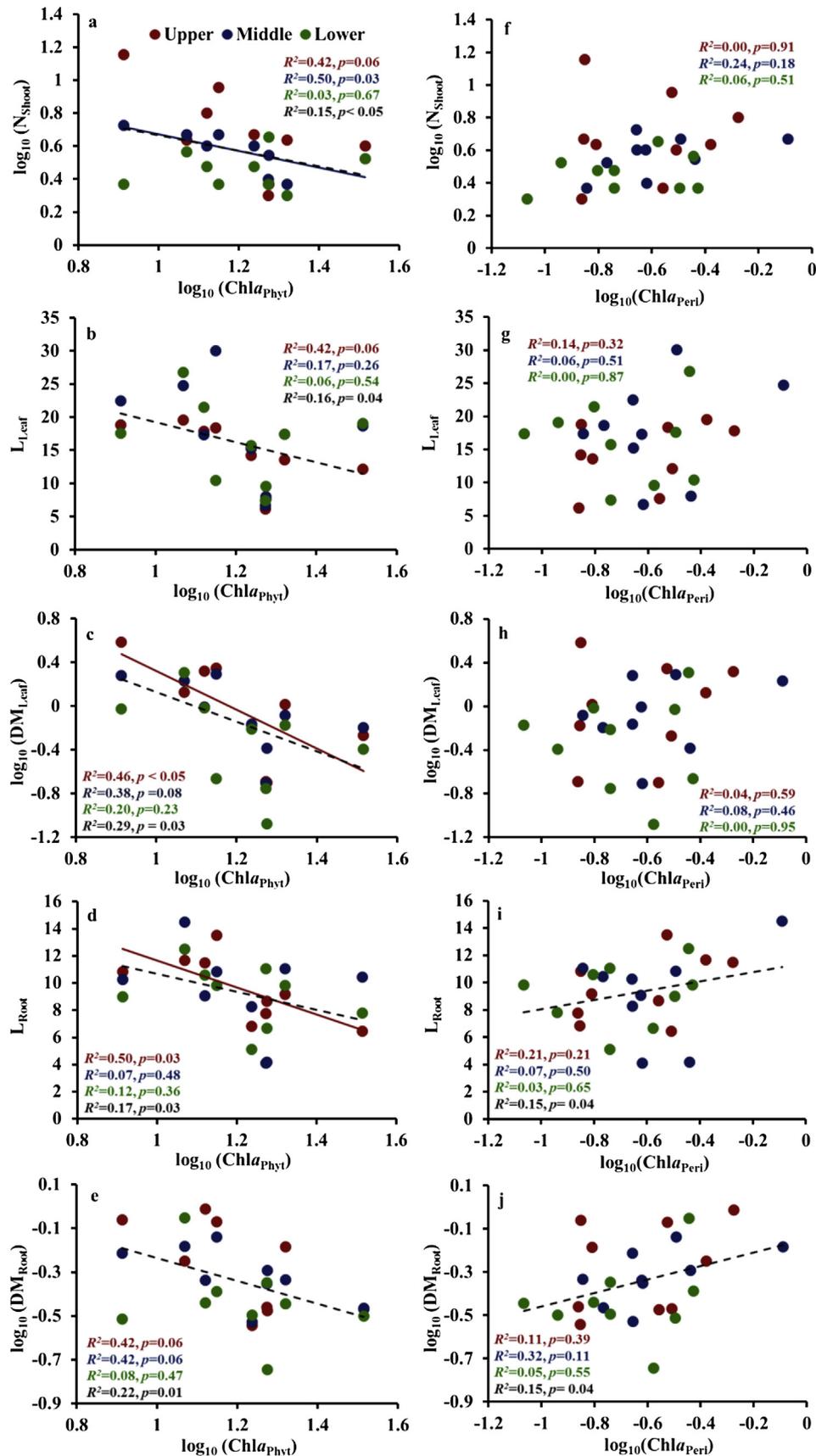


Fig. 5. Relationships between phytoplankton chlorophyll a (a–e) and periphyton chlorophyll a (f–j) with the morphological characteristics of *V. natans*. Upper, middle, and lower represent the layer at 0.4 m, 0.8 m, and 1.2 m below the water surface. The dotted line shows significant relationships of pooled data from all depths ($n = 9$).

macrophytes could be shading by phytoplankton or periphyton, being stimulated at increasing N loading. However, no relationship was found between Chl_{Phyt} and TN or NH_4 or TP (Fig. 2a, d). Moreover, TP concentrations in the treatments were all lower than 0.05 mg L^{-1} , which is suggested as an approximate threshold for the shift from a phytoplankton to a macrophyte-dominated state in Yangtze lakes (Wang et al., 2014). Significant negative effects of Chl_{Phyt} were found on the growth of macrophytes, i.e. on N_{Shoot} in the middle layer, DM_{Leaf} and L_{Shoot} in the upper layer, and on all the indices when data from the three depths were pooled. Previously, the shading effect of phytoplankton has been suggested to impede plant growth (Jackson, 2003; González Sagrario et al., 2005; Barker et al., 2008; Sayer et al., 2010b; Le Bagousse-Pinguet et al., 2012). According to 76 lake-year investigations conducted in Yangtze shallow lakes (Wang et al., 2014), Chl_{Phyt} in 85% lakes dominated by macrophytes was less than 0.01 mg L^{-1} , while it was above 0.01 mg L^{-1} in 83% lakes that had few or no macrophytes. In Canadian lakes, the corresponding Chl_{Phyt} threshold for a shift was $18 \mu\text{g L}^{-1}$ (Bayley et al., 2007). In our experiment, Chl_{Phyt} was generally higher than these levels.

As for some of the macrophyte growth indices, Chl_{Peri} was negatively related to TN and NH_4 . Accordingly, no significant negative relationship was revealed between Chl_{Peri} and the various growth indices of macrophytes. Actually, the relationships with L_{Root} and DM_{Root} were positive when pooling the data from the three growing depths. These results contrast the findings in some previous studies (Roberts et al., 2003; Barker et al., 2008; Bécares et al., 2008; Özkan et al., 2010; Olsen et al., 2015). The realized Chl_{Peri} in our experiment ranged from 0.08 to 0.81 mg m^{-2} , which is lower than the levels reported to cause plant loss (ranging from approximately 1 to 25 mg m^{-2}) (Barker et al., 2008; Roberts et al., 2003). Whether these low values are due to high grazing by fish or invertebrates cannot be determined with certainty. Grazing by larger fish can be excluded as the plants were protected, but small larvae fish may have entered the fences with the plants. Grazing effects from gastropods on macrophytes or on periphyton can also be excluded since no gastropods were observed during the experiment; however, other grazing invertebrates may have been present. The larger scale of our experiment may potentially also have

been important as the periphyton was less protected from wave-induced disturbance than in smaller-scale enclosure experiment conducted so far. Whatever the reason, the low periphyton development in our experiment may explain the low impact of periphyton on macrophyte growth compared with other similar studies having higher biomasses of periphyton.

5. Conclusions

Based on the results of our 5-month whole-ecosystem experiment, the following conclusions can be drawn:

- High nitrogen concentrations may negatively affect the growth of macrophytes, especially leaf length, leaf mass, and root length. This is in our study most likely attributable to toxic stress and phytoplankton shading, the latter being unrelated to TN in our experiment, though.
- Low development of periphyton may explain the lower effect of N in our experiment compared with other experiments, although the larger scale of our experiments than used in other similar N effect studies may have been a contributory factor.
- Further large-scale studies of the N effect in lakes are encouraged to better elucidate the role of nitrogen in shallow lakes.

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Appendix

Table A1

Spearman rank correlations between morphological variables of *V. natans* and the environment. See Table 1 for explanation of the abbreviations and units; significant correlations are shown in bold ($n = 9$).

		N_{Shoot}	L_{Leaf}	DM_{Leaf}	L_{Root}	DM_{Root}
A: Upper layer						
TN	<i>r</i>	−0.32	−0.60	−0.43	−0.43	−0.35
	<i>p</i>	0.40	0.09	0.24	0.24	0.36
NH_4	<i>r</i>	−0.40	−0.67	−0.52	−0.57	−0.42
	<i>p</i>	0.28	<0.05	0.15	0.11	0.26
TP	<i>r</i>	−0.51	−0.74	−0.55	−0.44	−0.13
	<i>p</i>	0.16	0.02	0.13	0.24	0.73
Chl_{Phyt}	<i>r</i>	−0.69	−0.82	−0.75	−0.68	−0.57
	<i>p</i>	0.04	0.01	0.02	0.04	0.11
Light	<i>r</i>	−0.28	−0.23	−0.48	−0.52	−0.53
	<i>p</i>	0.46	0.55	0.19	0.15	0.14
Chl_{Peri}	<i>r</i>	0.18	0.42	0.28	0.47	0.38
	<i>p</i>	0.64	0.26	0.46	0.21	0.31
B: Middle layer						
TN	<i>r</i>	−0.28	−0.63	−0.52	−0.73	−0.20
	<i>p</i>	0.47	0.07	0.15	0.02	0.61
NH_4	<i>r</i>	−0.35	−0.68	−0.62	−0.75	−0.32
	<i>p</i>	0.35	0.04	0.08	0.02	0.41
TP	<i>r</i>	−0.69	−0.47	−0.58	−0.31	−0.25
	<i>p</i>	0.04	0.20	0.10	0.42	0.51
Chl_{Phyt}	<i>r</i>	−0.87	−0.42	−0.70	−0.15	−0.52
	<i>p</i>	0.002	0.26	0.04	0.70	0.15

(continued on next page)

Table A1 (continued)

		N _{Shoot}	L _{Leaf}	DM _{Leaf}	L _{Root}	DM _{Root}
Light	r	-0.02	-0.32	-0.35	-0.30	-0.62
	p	0.97	0.41	0.36	0.43	0.08
Chl _a _{Peri}	r	0.38	0.03	0.07	-0.10	0.50
	p	0.32	0.93	0.86	0.80	0.17
C: Lower layer						
TN	r	-0.26	-0.85	-0.68	-0.36	-0.40
	p	0.51	0.004	0.04	0.34	0.29
NH ₄	r	-0.19	-0.78	-0.67	-0.40	-0.47
	p	0.63	0.01	0.05	0.28	0.21
TP	r	0.05	-0.29	-0.50	-0.29	-0.55
	p	0.90	0.44	0.17	0.45	0.12
Chl _a _{Phyt}	r	0.03	-0.37	-0.60	-0.37	-0.28
	p	0.93	0.33	0.09	0.33	0.46
Light	r	0.76	0.18	-0.12	-0.71	-0.72
	p	0.02	0.64	0.77	0.03	0.03
Chl _a _{Peri}	r	0.16	-0.13	-0.05	0.22	0.25
	p	0.68	0.73	0.90	0.57	0.52

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