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Does the responses of *Vallisneria natans* (Lour.) Hara to high nitrogen loading differ between the summer high-growth season and the low-growth season?



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

^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 School of Life Sciences, Nanjing University, Nanjing 210093, China

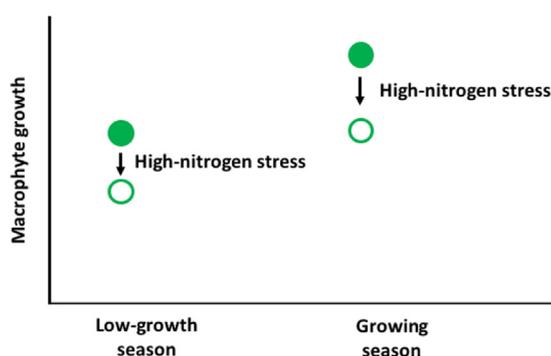
^d Department of Bioscience and Arctic Research Centre, Aarhus University, 8600 Silkeborg, Denmark

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

HIGHLIGHTS

- Growth of *Vallisneria natans* declines with increasing NH_4 concentrations.
- Phytoplankton biomass was of key importance for differences in *Vallisneria* growth.
- Free amino acid content might not reliably indicate high N effect on plant growth.
- Active growth in growing season may enable plants to overcome N stress to some extent.

GRAPHICAL ABSTRACT



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ABSTRACT

Loss of submersed macrophytes is a world-wide phenomenon occurring when shallow lakes become eutrophic due to excess nutrient loading. In addition to the well-known effect of phosphorus, nitrogen as a trigger of macrophyte decline has received increasing attention. The precise impact of high nitrogen concentrations is debated, and the role of different candidate factors may well change over the season. In this study, we conducted experiments with *Vallisneria natans* during the growing season (June–September) in 10 ponds subjected to substantial differences in nitrogen loading (five targeted total nitrogen concentrations: control, 2, 10, 20, and 100 mg L^{-1}) and compared the results with those obtained in our earlier published study from the low-growth season (December–April). Like in the low-growth season, growth of *V. natans* in summer declined with increasing ammonium (NH_4) concentrations and particularly with increasing phytoplankton chlorophyll *a* ($\text{Chl}_{a\text{Phyt}}$). Accordingly, we propose that shading by phytoplankton might be of key importance for macrophyte decline, affecting also periphyton growth as periphyton chlorophyll *a* ($\text{Chl}_{a\text{Peri}}$) decreased with increasing $\text{Chl}_{a\text{Phyt}}$. Free amino acid contents (FAA) of plants tended to increase with increasing NH_4 concentrations, while the relationships between FAA with growth indices were all weak, suggesting that FAA might be a useful indicator of the physiological stress of the plants but not of macrophyte growth. Taken together, the results from the two seasons

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

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

indicate that although a combination of high nitrogen concentrations (ammonium) and shading by phytoplankton may cause severe stress on macrophytes, active growth in the growing season enabled them to partly overcome the stress.

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1. Introduction

Worldwide, with the increasing eutrophication of shallow lakes, a decline in the abundance of submersed macrophytes has often been seen (Körner, 2002; Ibelings et al., 2007; Wang et al., 2014), producing substantially negative impacts on the ecosystem structure, functioning, and services of shallow lakes (Scheffer, 1998; Jeppesen et al., 1998). Most scientists agree that phytoplankton shading promoted by increasing inputs of phosphorus is an underlying cause of the decreasing abundance, but the role of nitrogen has received increasing attention in recent years (Moss, 2001; Jeppesen et al., 2007; Moss et al., 2013). In previous studies, two main mechanisms have been identified to explain the effects of nitrogen on submersed macrophytes: 1) it promotes the growth and hence shading of phytoplankton (Sayer et al., 2010a, 2010b) or periphyton (Olsen et al., 2015; Zhao et al., 2016) that uses nitrogen as its nutrient source; 2) it causes physiological stress on the submersed macrophytes and limits the metabolism of carbohydrates (Cao et al., 2009a; Yuan et al., 2015; Gao et al., 2015), inducing oxidative stress (Wang et al., 2010; Zhang et al., 2011) and inhibiting photosynthesis (Wang et al., 2008; Su et al., 2012). Experimental studies have revealed that ammonium (NH_4)-related physiological stress may be aggravated by low light conditions (Cao et al., 2011). However, there is little agreement on the relative importance of these two effects of high nitrogen concentrations on submersed macrophytes compared with the effects of phosphorus that stimulates shading by enhancing phytoplankton or periphyton growth (Moss et al., 2013; Yu et al., 2015). Little attention has been paid to the varied effects of high nitrogen concentrations and the potential factors inducing such variation. Previous studies have revealed that the role of different influencing mechanisms might depend on the experiment scale. Toxic effects, for example, have been found to play a key role in acute tests, whereas shading effects from algae have been emphasized in long-term chronic tests (Li et al., 2008; Olsen et al., 2015; Zhao et al., 2016). In a long-term whole-ecosystem experiment carried out in 10 ponds in the low-growth season, shading by phytoplankton was identified as the main cause of declined plant growth, and toxic stress induced by high N levels may to a certain extent have negative effects (Yu et al., 2015).

Seasonality is also expected to impact the sensitivity of submersed macrophytes to stress factors such as high N loading, although this has so far not been extensively investigated. Growth of phytoplankton is typically higher in summer, implying stronger shading. This may affect the NH_4 stress on submersed macrophytes because the physiological stress of NH_4 is light-dependent (Cao et al., 2009b, 2011). The negative effects of nitrogen will therefore be expected to be strongest during the warm season. However, higher photosynthesis in summer favors the formation of carbohydrates and thus NH_4 detoxification of macrophytes, as demonstrated for terrestrial plants subjected to high NH_4 exposure (Krupa, 2003), and this may benefit plant growth.

In the experiment conducted by Yu et al. (2015) during the months December to May (a relatively cold period and mainly the low-growth season), leaf length and leaf dry mass were found to decline with increasing concentrations of total nitrogen and ammonium. How macrophyte growth will respond to high nitrogen concentrations in the growing season is less clear (see Fig. 1) and the N-stressed growth could be 1) higher than or similar to the stress-free growth in the low-growth season; 2) similar to the growth in low-growth season subjected to N stress or 3) lower than the N-stressed growth in the low-growth season. Since both NH_4 toxicity and plant detoxification are

expected to increase in the growing season, it is uncertain which scenario will be most realistic.

In this study, we conducted in situ growth experiments with *Vallisneria spiralis* as test plant during the growing season (June–September) in 10 ponds subjected to substantial differences in nitrogen loading (five targeted total nitrogen concentrations: control (0.5), 2, 10, 20, and 100 mg L^{-1}) and compared them with earlier published results from the winter season (December–April). The objectives were twofold: 1) to test whether nitrogen impacts macrophytes through physiological stress or by promoting growth and thereby the shading effects of phytoplankton or periphyton in the growing season; 2) to compare the responses of macrophytes to high nitrogen loading between growing and low-growth seasons. Our work contributes to solving the puzzle of submersed macrophyte decline but also provides important useful information regarding restoration of submersed macrophyte and water quality management of shallow lakes.

2. Materials and methods

2.1. Treatments

The experiments were conducted in ten ponds with a depth of $1.78 \pm 0.2 \text{ m}$ (mean \pm standard error) and an area of ca. 0.08 ha. The ponds were located to the northeast of Lake Bao'an (N $30^\circ 17' 17''$, E $114^\circ 43' 45''$; area 48 km^2 and mean depth 1.9 m; more details in Yu et al., 2015) on the south bank of the middle Yangtze River, which is characterized by a warm humid subtropical climate. The experimental treatments included a gradient of five target concentrations of total nitrogen (TN) (control (0.5), 2, 10, 20, and 100 mg L^{-1}) with two ponds (replicates) for each treatment. The TN concentration of the control ponds (0.5 mg L^{-1}) served as background concentrations. 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 to maintain the target concentrations. The fertilizer was dissolved in pond water

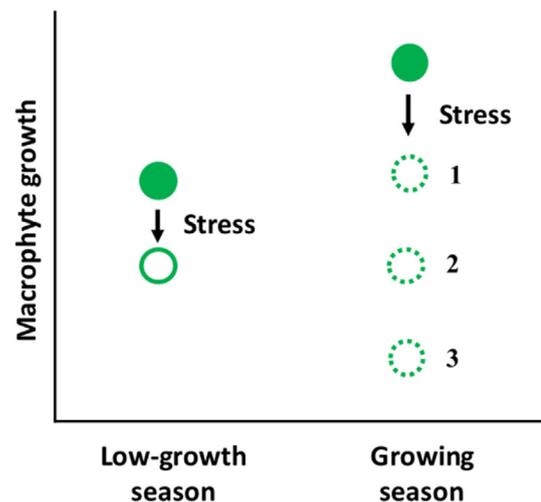


Fig. 1. Hypothesized scenarios of macrophyte growth change when stress of high nitrogen is present (open circles). Closed circles signify that nitrogen stress is absent. Dashed open circles indicate three potential scenarios: 1) higher than or similar to the stress-free growth rate in low-growth season; 2) similar to the stressed growth rate in low-growth season; 3) lower than the stressed growth rate in low-growth season.

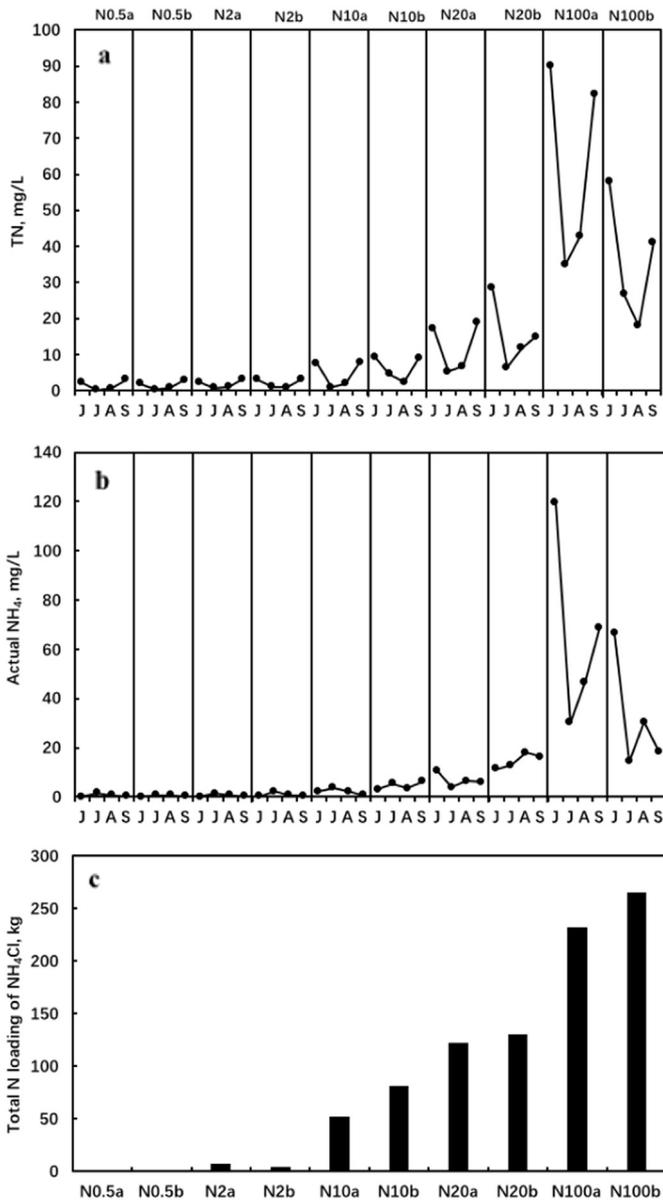


Fig. 2. Temporal dynamics of the concentrations of total nitrogen (a), actual concentrations of ammonium (NH₄) (b), total nitrogen (N) loading for the entire period of the experiments (c) from June 2014 to September 2014. In the treatment number, the value after N indicates the target TN concentration, a and b represent two ponds with the same nitrogen treatment.

Table 1

Pearson's correlation coefficients (*r* values) between the environmental variables; significant correlations are shown (**p* < 0.05, ***p* < 0.01) in bold (*n* = 9).

| | log ₁₀ (NH ₄) | log ₁₀ (Chl _a _{Phyt}) | log ₁₀ (Chl _a _{Peri}) |
|---|--------------------------------------|---|--|
| log ₁₀ (TN) | 0.99** | 0.52 | -0.51 (upper) -0.44 (middle) 0.19 (lower) |
| log ₁₀ (NH ₄) | | 0.44 | -0.50 (upper) -0.41 (middle) 0.27 (lower) |
| log ₁₀ (Chl _a _{Phyt}) | | | -0.34 (upper) -0.78* (middle) -0.64 (lower) |

Notes: TN, total nitrogen of pond water, mg L⁻¹; NH₄, ammonium of pond water, mg L⁻¹; Chl_a_{Phyt}, chlorophyll *a* of phytoplankton, μg L⁻¹; Chl_a_{Peri}, chlorophyll *a* of periphyton, mg m⁻².

in a polyethylene bucket before being poured into the ponds. No phosphate fertilizer was added throughout the experiment. The experiment lasted from 1st June to 18th September 2013, covering mainly the growing season. Our previous experiment conducted in the same ponds lasted from 15th December 2012 to 12th May 2013, including mainly a period of the low-growth season (Yu et al., 2015).

In the current experiment, *Vallisneria natans* (Lour.) Hara was selected as test plant as in the low-growth season experiment (Yu et al., 2015). The plants were collected from Lake Yandong (N 30°32', E 114°33'). Plants of similar size were chosen and cut into a unified leaf

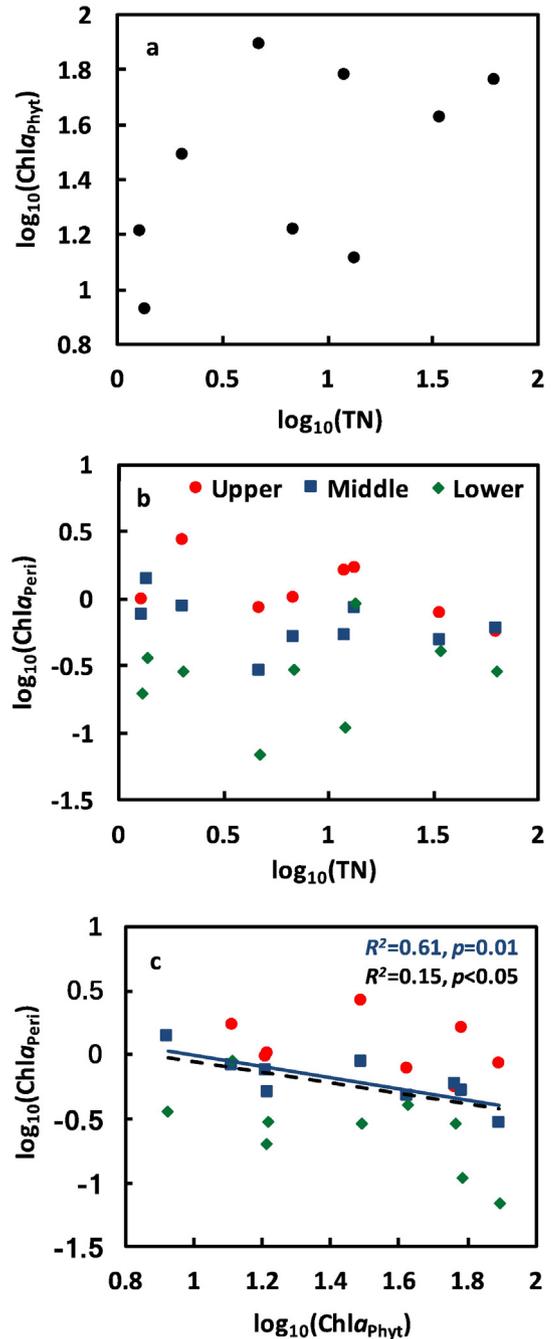


Fig. 3. Relationships between total nitrogen, phytoplankton chlorophyll *a*, and periphyton chlorophyll *a* (a–c). Upper, middle, and lower represent the layer at 0.4 m, 0.8 m, and 1.2 m below water surface. The dotted line shows significant relationships of pooled data from all depths (*n* = 9). The blue and black *R*² and *p* correspond to the blue and black line, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2

Mean value of the growth variables and free amino acid contents of *Vallisneria natans* (Lour.) Hara in the experimental ponds. One pond was left out (N2a) as the net fell down and left the pond open to invasion by grazing fish.

| Treatment number | Water layer | N _{Shoot} | L _{Leaf} | DM _{Leaf} | FAA |
|------------------|-------------|--------------------|-------------------|--------------------|------|
| N0.5a | Upper | 89 | 67.3 | 34.57 | 0.06 |
| | Middle | 77 | 74.7 | 23.34 | 0.03 |
| | Lower | 66 | 68.3 | 8.90 | 0.07 |
| N0.5b | Upper | 81 | 71.3 | 45.05 | 0.06 |
| | Middle | 53 | 87.0 | 25.65 | 0.07 |
| | Lower | 33 | 80.7 | 12.72 | 0.04 |
| N2b | Upper | 96 | 60.3 | 17.83 | 0.07 |
| | Middle | 51 | 63.0 | 9.96 | 0.10 |
| | Lower | 21 | 28.3 | 1.56 | 0.08 |
| N10a | Upper | 58 | 31.7 | 8.38 | 0.11 |
| | Middle | 26 | 33.0 | 4.90 | 0.08 |
| | Lower | 4 | 27.7 | 0.32 | 0.07 |
| N10b | Upper | 183 | 48.0 | 35.34 | 0.56 |
| | Middle | 124 | 66.0 | 27.33 | 0.39 |
| | Lower | 64 | 75.7 | 14.08 | 0.40 |
| N20a | Upper | 126 | 56.0 | 22.01 | 0.34 |
| | Middle | 62 | 42.3 | 8.53 | 0.19 |
| | Lower | 8 | 13.0 | 1.21 | 0.09 |
| N20b | Upper | 116 | 56.7 | 45.85 | 0.48 |
| | Middle | 64 | 63.3 | 15.33 | 0.50 |
| | Lower | 19 | 39.3 | 0.91 | 0.31 |
| N100a | Upper | 0 | 0 | 0 | – |
| | Middle | 0 | 0 | 0 | – |
| | Lower | 0 | 0 | 0 | – |
| N100b | Upper | 56 | 24.0 | 2.66 | 0.14 |
| | Middle | 37 | 18.3 | 1.02 | 0.10 |
| | Lower | 16 | 22.3 | 0.69 | 0.23 |

Notes: N_{Shoot}, number of shoots; L_{Leaf}, leaf length, cm; DM_{Leaf}, dry mass of leaves, g; FAA, free amino acid of fresh weight, mg g⁻¹ FW. In the treatment number, the value after N indicates the target TN concentration, a and b represent two ponds with the same nitrogen treatment.

length of 15 cm and then cultured in batches in boxes (65 cm × 41 cm × 31 cm) filled with water from Lake Bao'an. On 1st June 2013, after 45 days of cultivation, three similar-sized plants were selected and each plant was planted in a plastic pot (23 cm in top diameter, 13 cm in bottom diameter, 13 cm in height) filled with 10 cm sediment. 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), i.e. nine pots in each pond in total.

Fish occurred naturally in the ponds and were thus not stocked. In order to prevent fish grazing on plants, fish nets (2 cm × 2 cm in mesh size) were fixed around the bamboo racks.

2.2. Sampling and measurements

The experiment lasted 110 days after which some pots had become overgrown by plants. All pots were then removed from the ponds and

Table 3

Pearson's correlation coefficients (*r* values) between the morphological variables of *Vallisneria natans* and the environmental variables; significant differences are shown (**p* < 0.05, ***p* < 0.01) in bold (*n* = 9).

| | Layer | log ₁₀ (NH ₄) | log ₁₀ (Chl _a _{phyt}) | log ₁₀ (Chl _a _{peri}) | log ₁₀ (FAA) |
|---|--------|--------------------------------------|---|---|-------------------------|
| log ₁₀ (N _{Shoot}) | Upper | -0.29 | -0.46 | 0.54 | 0.66 |
| | Middle | -0.36 | -0.65 | 0.27 | 0.40 |
| | Lower | -0.52 | -0.83** | 0.30 | 0.17 |
| L _{Leaf} | Upper | -0.79* | -0.76* | 0.74* | -0.24 |
| | Middle | -0.80* | -0.78* | 0.56 | -0.09 |
| | Lower | -0.65 | -0.80* | 0.16 | -0.06 |
| log ₁₀ (DM _{Leaf}) | Upper | -0.66 | -0.69* | 0.66 | -0.16 |
| | Middle | -0.72* | -0.72* | 0.46 | 0.09 |
| | Lower | -0.53 | -0.74* | 0.18 | -0.08 |

Notes: N_{Shoot}, number of shoots; L_{Leaf}, length of leaf, cm; DM_{Leaf}, dry mass of leaf, g; FAA, free amino acid of fresh weight, mg g⁻¹ FW.

washed with tap water to record the number of plants (N_{Shoot}) and to measure the length of leaves (L_{Leaf}). Next, leaves and roots in each pot were separated and dry mass of leaves (DM_{Leaf}) was measured with an electronic balance after drying at 80 °C for 48 h to constant mass (Yu et al., 2015). Water dissolved oxygen (DO), pH, and conductivity (Cond) were measured in situ with a YSI ProPlus (Yellow Spring Inc., USA) every twenty days. Light intensity was measured with an illuminometer at the three depths where plants were growing. Water samples for chemical analysis were collected by a tube sampler (1.5 m in height, 10 cm in diameter) (Yu et al., 2015). TN and total phosphorus (TP) were determined using a spectrophotometric method after digestion with K₂S₂O₈ solution (Huang et al., 1999). Chlorophyll *a* of phytoplankton (Chl_a_{phyt}) and chlorophyll *a* of periphyton (Chl_a_{peri}) were 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. For analysis of ammonium (NH₄), water was filtered through a Whatman (Middlesex, U.K.) GF/C glass fibre filter (1.2 μm bore diameter).

About 0.5–1.0 g fresh leaves of *V. natans* were ground in 10% acetic acid solution to examine the free amino acid (FAA) in the plant tissues. The above solution was centrifuged at 10,000g for 15 min. The supernatant was used to examine the FAA by the ninhydrin colorimetric method using leucine as standards (Li et al., 2004).

In order to study the possible effects of periphyton on plants, artificial substrates were used to monitor the growth of periphyton. Three glass slides were embedded into a box with an open top and bottom, which was hung among the canopy of growing plants. Every 20 d, three 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.

2.3. Statistical analyses

Median values were used for analyses to avoid deviation caused by the extreme values in NH₄, Chl_a_{phyt}, Chl_a_{peri}, and other variables. Pearson's correlations were used to test for relationships between environmental variables and between macrophyte growth variables. The variables that did not follow normal distributions (Shapiro-Wilk test, *p* < 0.05) were log₁₀-transformed, including TN, NH₄, Chl_a_{phyt}, Chl_a_{peri}, FAA, N_{Shoot}, and DM_{Leaf}. One pond (with target TN of 2 mg L⁻¹) was left out in the analysis as the net fell down, leaving the plant treatments open to invasion by grazing fish. STATISTICA 8.0 and Microsoft Excel 2013 were used to analyze the data.

3. Results

3.1. Growing conditions of *V. natans* in the growing season

Both TN and NH₄ showed a significant increase along the treatment gradient, with medians ranging from 1.3 to 62.5 mg L⁻¹ and from 0.5 to 57.8 mg L⁻¹, respectively (Fig. 2a, b). The total nitrogen (TN) loadings for the entire experimental period are shown in Fig. 2c. Chl_a_{phyt} also increased significantly, with medians ranging between 8.4 and 60.6 μg L⁻¹. Chl_a_{peri} declined from the upper (0.56–2.69 mg m⁻²) layers over the middle (0.29–1.68 mg m⁻²) layers to the lower (0.07–0.92 mg m⁻²) layers in most treatments. A significant positive relationship was found between NH₄ and TN (Table 1). Neither NH₄ nor TN was significantly related to Chl_a_{peri} or Chl_a_{phyt} (Table 1, Fig. 3a, b), but Chl_a_{phyt} tended to increase with TN. Negative relationships were found between Chl_a_{phyt} and Chl_a_{peri}, being significant in the middle layer (Table 1, Fig. 3c).

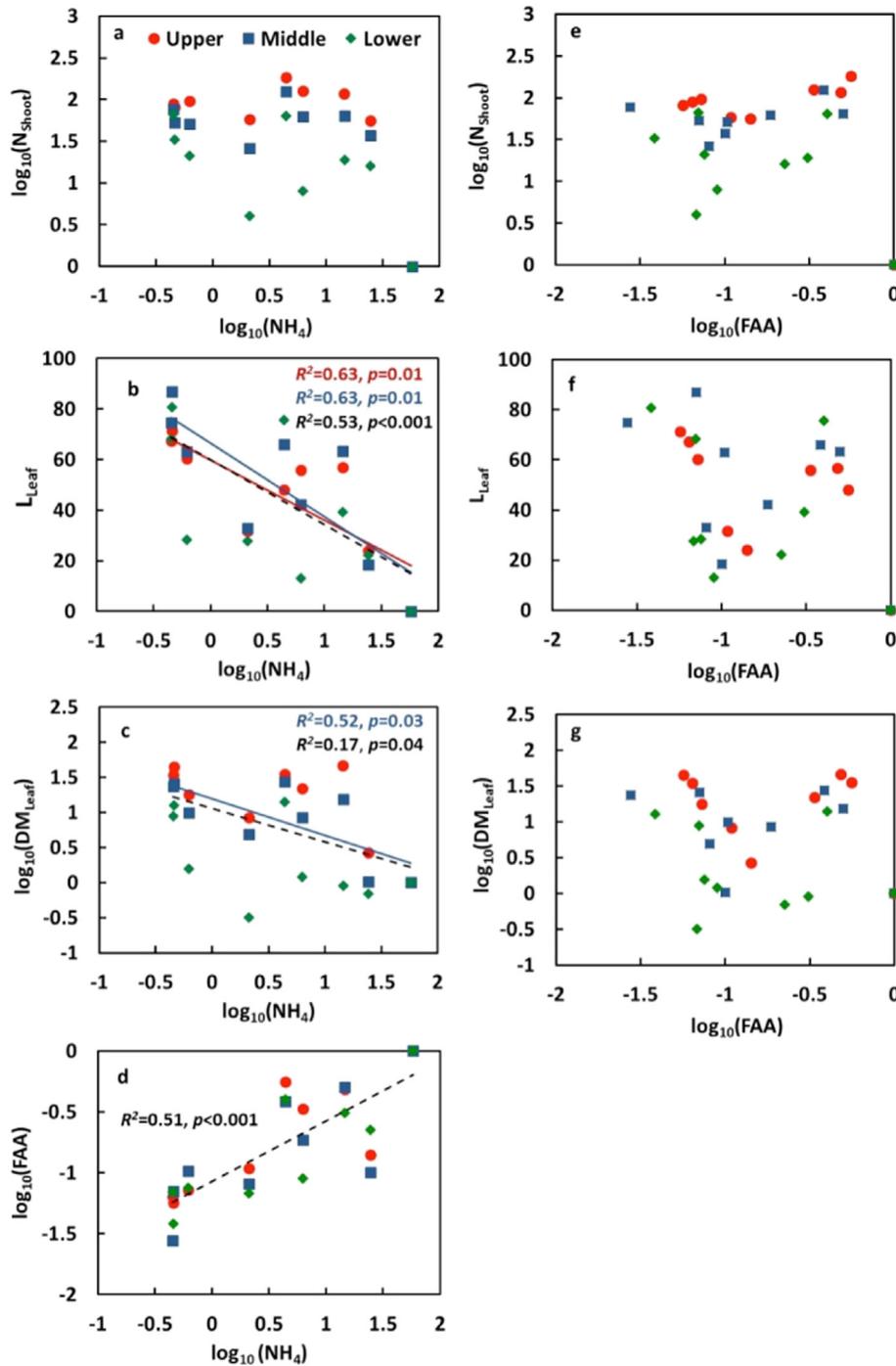


Fig. 4. Relationships between ammonium (a–c) with the morphological characteristics and free amino acid (FAA) (d) of *V. natans* and between FAA (e–g) and the morphological characteristics. Upper, middle, and lower represent the layer at 0.4 m, 0.8 m, and 1.2 m below water surface. The dotted line shows significant relationships of pooled data from all depths ($n = 9$). The red, blue, and black R^2 and p correspond to the red, blue, and black line, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Growth of *V. natans* in the growing seasons

Growth variables and the free amino acid (FAA) contents of *V. natans* at the end of the experiment in the different experimental ponds are shown in Table 2. Pearson's correlations (Table 3) demonstrated a significant negative relationship between NH_4 and leaf length (L_{Leaf}) in the upper and middle layers and with leaf dry mass (DM_{Leaf}) in the middle layer. The scatterplots showed, however, that N_{Shoot} and DM_{Leaf} had no relationship with increasing NH_4 concentrations, while L_{Leaf} declined

with increasing NH_4 in both the upper and middle layers (Fig. 4b). When pooling the data from all the three layers, similar results were obtained, with the exception of a significant relationship between DM_{Leaf} and NH_4 . When regressing NH_4 to FAA (Fig. 4d), a significant relationship emerged only when the data were pooled together, but not for any of the three layers individually. No significant relationship between FAA and the three growth variables was found (Table 3; Fig. 4e–g).

Chl_{Phyt} correlated significantly negatively with N_{Shoot} in the lower layer and with L_{Leaf} and DM_{Leaf} in all three layers (Table 3). In the

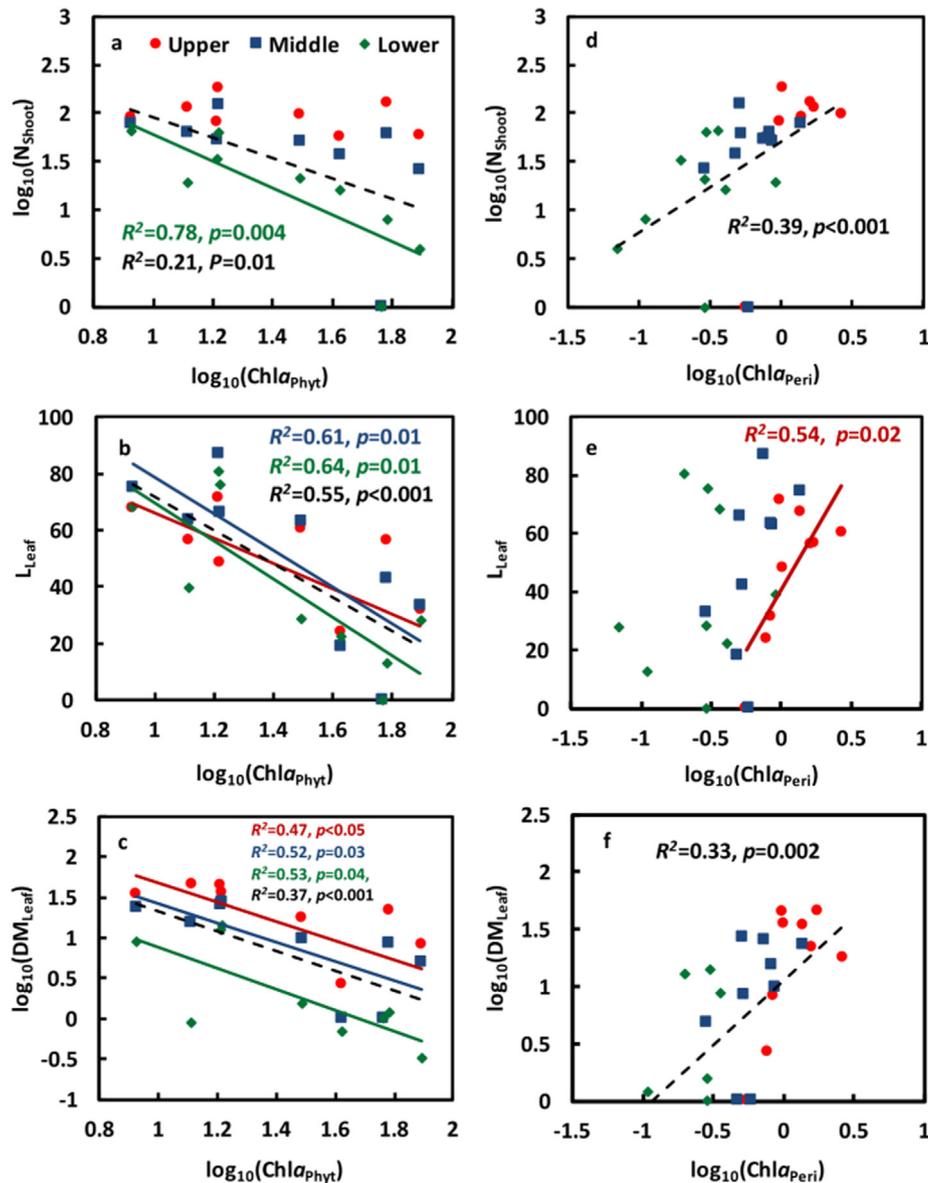


Fig. 5. Relationships between chlorophyll *a* concentration of phytoplankton (a–c) and periphyton (d–f) with the growth characteristics of *V. natans*. Upper, middle, and lower represent the layer at 0.4 m, 0.8 m, and 1.2 m below water surface. The dotted line shows relationships of pooled data from all depths ($n = 9$). The red, blue, and black R^2 and p correspond to the red, blue, and black line, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

scatterplots, the growth indices of macrophytes also showed declining trends with increased $\text{Chla}_{\text{Phyt}}$, significantly so for N_{Shoot} in the lower layer (Fig. 5a) and for L_{Leaf} and DM_{Leaf} in all three layers (Fig. 5c). When pooling the data from all three layers, all the growth indices were significantly related to $\text{Chla}_{\text{Phyt}}$. No significant relationship between $\text{Chla}_{\text{Peri}}$ and growth variables was detected, excepting L_{Leaf} in the upper layer from the Pearson's correlations analyses (Table 3). When pooling the data of all the three layers, significant positive relationships emerged between $\text{Chla}_{\text{Peri}}$ and N_{Shoot} and DM_{Leaf} (Fig. 5d–f). Partial correlations between nitrogen and growth variables with a control for $\text{Chla}_{\text{Phyt}}$ showed significant effects of TN only for L_{Leaf} and only in the upper and middle layers ($p < 0.05$).

3.3. Comparison between growing and low-growth seasons

NH_4 and $\text{Chla}_{\text{Phyt}}$ were selected for a comparison of the growth responses between growing and low-growth seasons (Fig. 6). The comparison was based on the data from the lower layer (1.2 m), being a

more natural growth depth in the field. In the low-growth season, leaf length (L_{Leaf}) exhibited a declining trend with increasing NH_4 , while none of the growth indices demonstrated significant relationships with $\text{Chla}_{\text{Phyt}}$ (Fig. 6). In the growing season, none of the growth indices displayed a significant declining trend with NH_4 , while all declined significantly with increasing $\text{Chla}_{\text{Phyt}}$. Although the responses of plant growth to the stress were different between the two seasons and the stress is potentially more severe in summer, the growth of macrophytes was overall higher in summer than in winter at comparable ranges of NH_4 and $\text{Chla}_{\text{Phyt}}$. N_{Shoot} , L_{Leaf} , and DM_{Leaf} averaged 26 (0–66) individuals, 39.6 (0–80.7) cm, and 4.6 (0–14.1) mg, respectively, in the growing season and were 3 (range, 2–5) individuals, 29.6 (9–46.3) cm, and 0.7 (0.08–2.0) mg in the low-growth season.

4. Discussion

In our experiment conducted in the growing season, the growth of *V. natans* was found to decline with increasing NH_4 concentrations

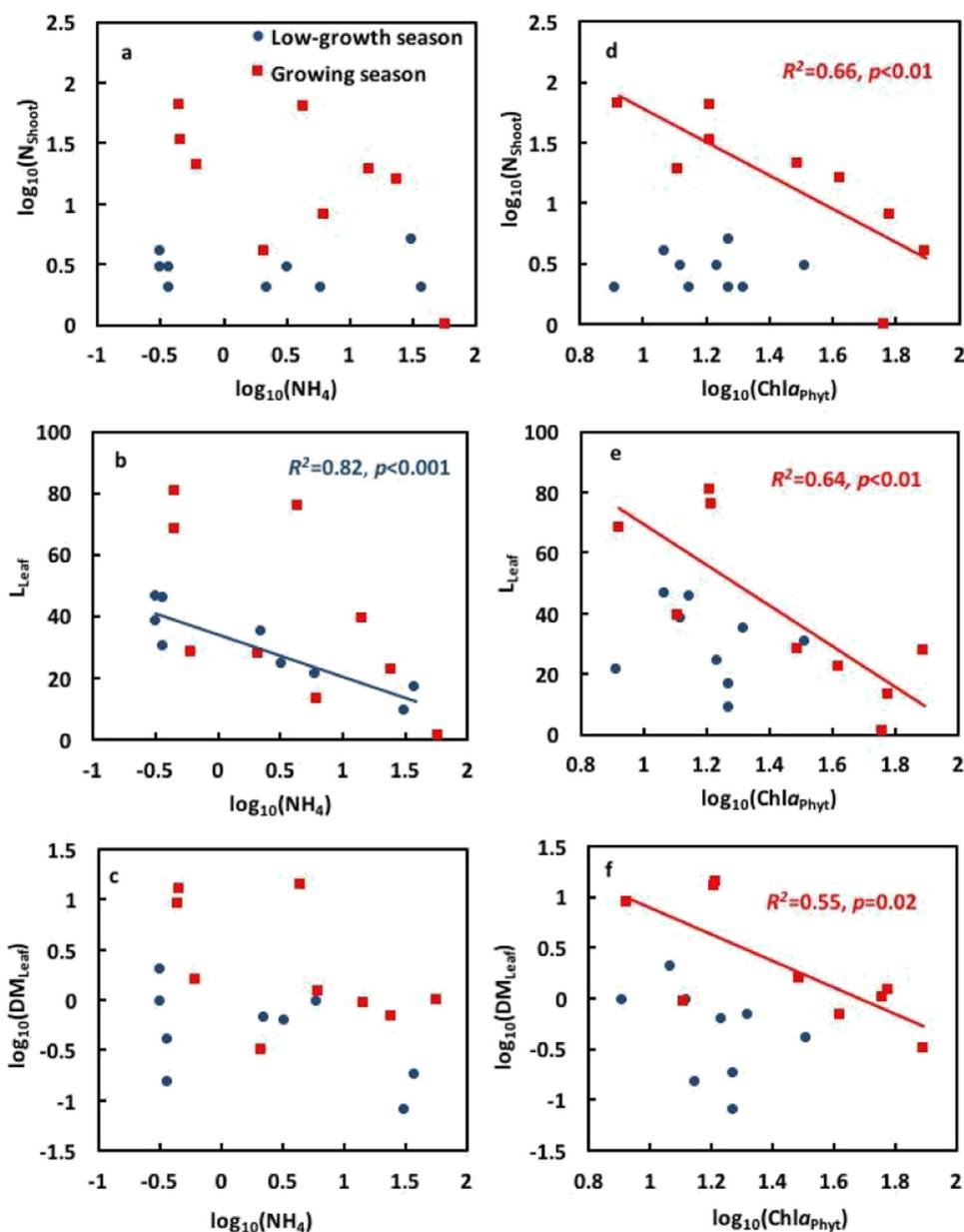


Fig. 6. Relationships between ammonium (NH_4) and phytoplankton chlorophyll *a* ($\text{Chla}_{\text{Phyt}}$) with the growth of *V. natans* in growing and low-growth seasons ($n = 9$ for both seasons) (the analyses were based on the data on the lower layer with a depth of 1.2 m, which is more natural). The red and blue R^2 and p correspond to the red and blue line, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and particularly with increasing phytoplankton chlorophyll *a* ($\text{Chla}_{\text{Phyt}}$), whereas it did not decline with increasing periphyton chlorophyll *a* (which was negatively related to $\text{Chla}_{\text{Phyt}}$). This suggests that the decline in macrophyte growth was induced mainly by shading of phytoplankton, but that it was only weakly related to N loading as indicated by the weak and insignificant TN- $\text{Chla}_{\text{Phyt}}$ relationship, as well as by the partial correlation between nitrogen and growth variables with a control for water column $\text{Chla}_{\text{Phyt}}$. This concurs with experimental results from the low-growth season in the same system (Yu et al., 2015). In both growing and low-growth season experiments, $\text{Chla}_{\text{Phyt}}$ was close to or higher than the level (0.01 mg L^{-1}) suggested to entail high risk of macrophyte loss in Yangtze shallow lakes (Wang et al., 2014) and the level (0.018 mg L^{-1}) suggested as a threshold for a dominance shift from macrophytes to phytoplankton in Canadian lakes (Bayley et al., 2007). The relatively minor effect of nitrogen may be due to a weak development of periphyton compared with other studies showing a strong positive effect of nitrogen on periphyton growth

(Özkan et al., 2010) and an associated decline in macrophyte growth (Olsen et al., 2015; Zhao et al., 2016).

Macrophytes may benefit from sufficient supply of HCO_3^- which is often the dominant form of dissolved inorganic carbon (DIC) in eutrophic lakes (Rantakari and Kortelainen, 2008). Macrophytes may utilize HCO_3^- to synthesize carbohydrates (which is important for production of FAA detoxification) and alleviate the stress of NH_4 (Dou et al., 2013). Although we did not measure HCO_3^- in this study, in a two-month aquarium experiment, Dou et al. (2013) found the FAA content in *Vallisneria natans* leaves to be lower in the treatment with high HCO_3^- concentrations.

Stress exerted by high nitrogen concentrations may also have contributed to a reduction of macrophyte growth. Four ponds in the growing season and three ponds in the low-growth season exhibited NH_4 concentrations higher than 5 mg L^{-1} , a level causing biochemical damage to *Potamogeton crispus* in acute tests (Cao et al., 2004). In the other ponds, NH_4 exceeded 5 mg L^{-1} for some months in both the present

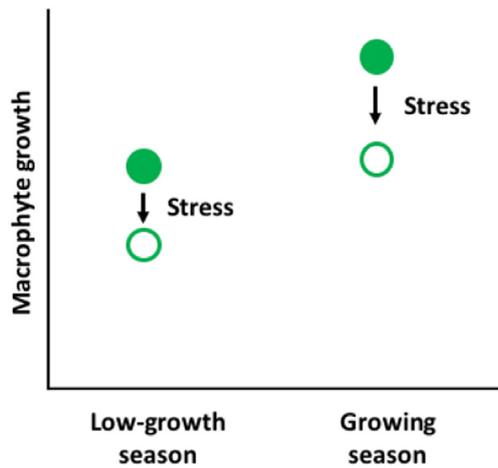


Fig. 7. A synthesis of growth of submersed macrophytes in low-growth and growing seasons in response to high nitrogen stress from the whole-ecosystem experiments. Closed circles: stress free; open circles: stress present.

and the previous experiment. When experiencing high NH_4 stress, macrophytes may excessively synthesize FAA to avoid NH_4 toxicity. Such a process costs energy and consumes carbohydrates (Rare, 1990; Krupa, 2003), which may reduce the growth of macrophytes, particularly at low light availability (Cao et al., 2009a, 2009b, 2011; Yuan et al., 2013, 2016). The content of FAA is therefore commonly used as an indicator of nitrogen stress (Cao et al., 2009a; Yuan et al., 2015; Zhang et al., 2010). In our experiment, FAA tended to increase with increasing NH_4 concentrations; however, a significant FAA- NH_4 relationship was only found for the pooled data from three layers, and the relationships of FAA with the various growth indices used were all weak. This implies that N stress on macrophytes may have occurred in our experiments but with stronger impacts on physiological activities than on growth indices. Growth of macrophytes was recorded in most treatments even when TN and NH_4 were as high as 33.8 and 24.4 mg L^{-1} , respectively. Active growth may explain why the toxic effect of N is less obvious in longer running experiments (Li et al., 2008; Olsen et al., 2015; Zhao et al., 2016) where there is sufficient time for macrophytes to be released from high N stress through growth than in short-term lab experiments.

Active macrophyte growth may also explain the differential responses of *V. natans* to high N loading between summer and winter at otherwise comparable NH_4 concentrations (Fig. 6a–c). Phytoplankton chlorophyll *a* was higher in the growing season than in the low-growth season (Fig. 6d–f), concurring with the expectedly higher phytoplankton shading in summer. The higher growth indices (N_{Shoot} , L_{Leaf} , and DM_{Leaf}) in the growing season than in the low-growth season in the low nitrogen concentration treatment (Fig. 6a–c) also support the hypothesized difference between seasons in macrophyte growth. We therefore suggest that scenario 1 (in Fig. 1) is the most likely scenario, i.e. the growth rate of macrophytes in the growing season at high N stress can be equal to or higher than growth in the low-growth season at low N concentrations (without N stress) (Fig. 7). This is of practical importance and implies that the growing season (summer) will be the best time to transplant submersed macrophytes when restoring shallow lakes (e.g. after biomanipulation by fish removal, Jeppesen et al., 2012), even though phytoplankton shading and nitrogen stress potentially may be higher during this season.

5. Conclusions

Based on the results of our 4-month whole-ecosystem experiment conducted in the growing season, our conclusions are:

- In both the high and the low-growth season, the decline in macrophyte growth appeared to be mainly due to shading of phytoplankton (which was only weakly and not significantly related to nitrogen concentrations), perhaps because periphyton growth was modest. Stress by high ammonium levels may also have contributed to the decline in macrophyte growth, especially regarding the length and mass of leaves.
- FAA (free amino acid contents of plants) might be a useful indicator of physiological stress by high ammonium but not of macrophyte growth, as suggested by the weak relationships between FAA and the various growth indices used.
- Although a combination of high nitrogen concentrations and shading by phytoplankton may create severe stress on macrophytes in summer, active growth may to some extent release the plants from such stress.

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