



Higher Tolerance of Canopy-Forming *Potamogeton crispus* Than Rosette-Forming *Vallisneria natans* to High Nitrogen Concentration as Evidenced From Experiments in 10 Ponds With Contrasting Nitrogen Levels

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Specialty section:

This article was submitted to
Functional Plant Ecology,
a section of the journal
Frontiers in Plant Science

Received: 29 September 2017

Accepted: 28 November 2018

Published: 13 December 2018

Citation:

Yu Q, Wang H-Z, Xu C, Li Y, Ma S-N, Liang X-M, Jeppesen E and Wang H-J (2018) Higher Tolerance of Canopy-Forming *Potamogeton crispus* Than Rosette-Forming *Vallisneria natans* to High Nitrogen Concentration as Evidenced From Experiments in 10 Ponds With Contrasting Nitrogen Levels. *Front. Plant Sci.* 9:1845. doi: 10.3389/fpls.2018.01845

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Due to excess nutrient loading, loss of submersed macrophytes is a worldwide phenomenon in shallow lakes. Phosphorus is known to contribute significantly to macrophyte recession, but the role of nitrogen has received increasing attention. Our understanding of how high nitrogen concentrations affect the growth of submersed macrophytes, particularly under natural conditions, is still limited. In this study, we conducted experiments with canopy-forming *Potamogeton crispus* 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 of our earlier published experiments with rosette-forming *Vallisneria natans* performed 1 year before. Canopy-forming *P. crispus* was more tolerant than rosette-forming *V. natans* to exposure to high NH₄ concentrations. This is probably because canopy-forming species reach the water surface where there is sufficient light for production of carbohydrates, thereby allowing the plants to partly overcome high NH₄ stress. Both the canopy-forming *P. crispus* and the rosette-forming *V. natans* showed clear declining trends with increasing chlorophyll *a* in the water. Accordingly, shading by phytoplankton might be of key importance for the decline in submersed macrophytes in this experiment. Both experiments revealed free amino acids (FAA) to be a useful indicator of physiological stress by high ammonium but is not a reliable indicator of macrophyte growth.

Keywords: whole-ecosystem experiment, growth form, submersed macrophytes, ammonium, phosphorus, phytoplankton, periphyton

INTRODUCTION

The mechanisms behind the loss of submersed macrophytes in shallow lakes have received ample attention due to the important role that macrophytes play for maintaining healthy conditions in such ecosystems (Blindow, 1992; Jeppesen et al., 1998; Scheffer, 1998; Körner, 2002; Wang et al., 2014). In addition to excessive loading of phosphorus (P) (Scheffer, 1998; Carpenter, 2003), high nitrogen (N) loading has also been suggested to contribute importantly to the recession of macrophytes (Moss, 2001; Jeppesen et al., 2007; Moss et al., 2013). High N may cause physiological damage to the submersed macrophytes by generating oxidative stress (Wang et al., 2010; Zhang et al., 2011), disturbing the metabolism of carbon and nitrogen (Cao et al., 2009b; Gao et al., 2015; Yuan et al., 2015) and inhibiting photosynthesis (Wang et al., 2008; Su et al., 2012). High N may also affect the plants indirectly by promoting the growth of phytoplankton (Sayer et al., 2010a,b) or periphyton (Olsen et al., 2015; Zhao et al., 2016) and hence their shading effects. The effect of high N may, however, vary with the macrophyte species present (Cao et al., 2009a,b). Different key mechanisms have been identified in various studies and it has been suggested that such difference may partly reflect variation in temporal scale of the conducted experiments and timing (season). For example, toxic effects tend to play a key role in acute tests, while shading effects of algae are important in chronic tests (Cao et al., 2004; Nimptsch and Pflugmacher, 2007; Olsen et al., 2015; Zhao et al., 2016). In a long-term pond experiment carried out by Yu et al. (2015, 2017), shading by phytoplankton was identified as the main cause of declining growth for plants of *Vallisneria natans*, and high N-induced toxic stress seemed to have negative effects in both the growing season (summer and autumn) and the low-growth season (winter); however, active growth in the growing season enabled *V. natans* to partly overcome the stress.

Growth form may also influence the response of submersed macrophytes to the stress of high N loading. As to growth form, submersed macrophytes can be divided into rosette-forming species (e.g., *V. natans*), canopy-forming species (e.g., *P. crispus*), bottom-dwelling species (e.g., *Najas marina*), and erect species (e.g., *Hydrilla verticillata*) (Chambers, 1987; Chambers and Kalff, 1987). Canopy-forming and rosette-forming species are the two most common submersed macrophytes. Canopy-forming species form canopies on the water surface, whereas rosette-forming species spread under water through formation of ramets. Thus, canopy-forming species have better access to light than rosette-forming species. Low light may aggravate ammonium (NH_4)-related physiological stress, as suggested in a 2-month tank ($2\text{ m} \times 1\text{ m} \times 1\text{ m}$) experiment (Cao et al., 2011). This is because extra energy and carbohydrates are needed for the detoxication process (Rare, 1990; Krupa, 2003) and photosynthesis is limited under low light conditions (Cao et al., 2009a, 2011; Yuan et al., 2013, 2016). Therefore, we hypothesize that canopy-forming submersed macrophytes may be more tolerant to NH_4 stress than rosette-forming species.

The aim of this study was to explore the effects of high N concentrations on the growth of canopy-forming submersed macrophytes and compare their tolerance to high N with

that of the rosette-forming species *V. natans* reported in Yu et al. (2017), which showed that leaf length and dry mass of *V. natans* in summer declined with increasing ammonium (NH_4) concentrations. *P. crispus*, a typical canopy-forming and cosmopolitan species (Jackson and Kalff, 1993; Hudon et al., 2000), was selected and the experiment was performed 1 year before that of Yu et al. (2017). The two experiments were undertaken in the same pond system. The purposes of our study were twofold: (1) to test whether high N concentrations affect canopy-forming *Potamogeton crispus* through physiological stress or by promoting growth of phytoplankton or periphyton to form shading effects; (2) to compare the responses of canopy-forming and rosette-forming macrophytes to high N loading.

MATERIALS AND METHODS

Study Area and Experimental System

The experiments were conducted in 10 equally sized (ca. 0.08 ha) experimental ponds (N $30^\circ 17' 17''$, E $114^\circ 43' 45''$) being constructed from a lotus pond (culturing *Nelumbo nucifera*) by dredging surface sediments rich in nutrients and organic matter and introducing sediments and water from a nearby lake, Lake Bao'an (surface area 48 km², mean water depth, Z_M , and 1.9 m) (see Yu et al., 2015 for the initial environmental conditions of the 10 ponds). The experimental area is located in the middle Yangtze River Basin with a warm, humid subtropical climate (annual mean air temperature ca. 19°C and precipitation ca. 1030 mm). The monthly rainfall during the experiments was 125.7 mm in Yu et al. (2017) and 214.7 mm in this study (Supplementary Table 1). The mean air and water temperatures were 28.0 and 29.1°C in Yu et al. (2017) and 22.2 and 23.7°C in this study. Light intensity was 40998 and 38776 lux in this experiment and Yu et al. (2017), respectively. Mean Secchi depth (SD) in Yu et al. (2017) and this study were similar, being 52.1 and 51.9 cm, respectively.

Experimental Treatments

A gradient of five target concentrations of total nitrogen (TN) (control, 2, 10, 20, and 100 mg L⁻¹) and two duplicates were made. The target TN concentrations of the control ponds (ca. 0.5 mg L⁻¹) functioned as background concentrations. In China, a nitrogen concentration of 2 mg L⁻¹ TN refers to V type of water based on environmental quality standards for surface water (General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China [AQSIQ], 2002b), 10 mg L⁻¹ NO₃-N refers to standards for drinking water quality (Standardization Administration of the People's Republic of China [SAC], 2006), and 20 mg L⁻¹ TN refers to primary B based on the discharge standard of pollutants for municipal wastewater treatment plants (General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China [AQSIQ], 2002a). In part of the United States, 100 mg L⁻¹ NO₃-N refers to water quality standards for agriculture and livestock (Xia et al., 2004). Our gradient therefore covers the range observed in the real world. To maintain target concentrations, NH_4Cl fertilizer (NH_4Cl , $\geq 99.5\%$, Sinopharm Chemical Reagent Co., Ltd., Shanghai) was added every month

relative to the difference between the measured and target concentrations. No phosphate fertilizer was added to the ponds in the experiment.

On 15 March 2013, *P. crispus* were collected from Lake Biandantang (N 30°32', E 114°33'), a sub-area of Lake Bao'an. Similar-sized plants were selected and cut into a unified leaf length of 15 cm. The plants were then cultured in batches for 15 days in boxes (65 cm × 41 cm × 31 cm) filled with lake water. On 1 April 2013, similar-sized plants were selected and planted in plastic pots (23 cm in top diameter, 13 cm in bottom diameter, and 13 cm in height) filled with 10 cm sediments mixed with 1/3 washed sand (three plants in each pot). Sediment was

taken from the experimental pond of the control treatment, with an original total nitrogen concentration of 1.93 mg g⁻¹, a total phosphorus concentration of 0.67 mg g⁻¹, and an organic matter concentration of 39.8 mg g⁻¹. Three pots with plants were hung on bamboo racks (6 m in length, 2 m in width, and 3 m in height) at three water layers: 0.4, 0.8, and 1.2 m, and a total of nine pots were placed in each pond. Macrophytes with lower C: N ratios (N-enriched waters) may be more readily consumed by herbivorous or omnivorous fish (Dorenbosch and Bakker, 2011). Before the experiment published in Yu et al. (2015), we performed an experiment with the same treatments but without excluding fish. All the macrophytes had been removed by the fish at the beginning of the experiment. Therefore, to ensure sufficient exposure time of macrophytes to high ammonium stress, nets with a mesh size of 2 cm × 2 cm were fixed around the bamboo racks to prevent fish from entering. The experiment lasted around two and a half month, from 1 April to 12 June 2013.

Sampling and Measurement

All plants were harvested at the end of the experiment and washed with tap water. The number of shoots (N_{Shoot}) was counted and shoot height (H_{Shoot}) was measured. To prevent the accumulation of NH₄ in plant organs, macrophytes usually incorporate it into some nitrogenous compounds [mainly free amino acids (FAA)] and/or transport it out of the organs, which processes consume carbohydrate and energy. About 100 mg of fresh shoots were ground in 5 mL 10% acetic acid solution to measure the contents of FAA. The above solution was centrifuged at 10,000 g for 15 min. The supernatant was used to examine FAA using the ninhydrin colorimetric method with leucine as standard (Li et al., 2004).

The dry mass of shoots (DM_{Shoot}) was measured 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. Relative growth rates (RGRs, day⁻¹) of the macrophytes were calculated using the formula:

$$RGR = (\ln W_t - \ln W_0) / T$$

where T is the experimental period, W_t is the shoot number/shoot height/shoot biomass at the end of the experiment, and W₀

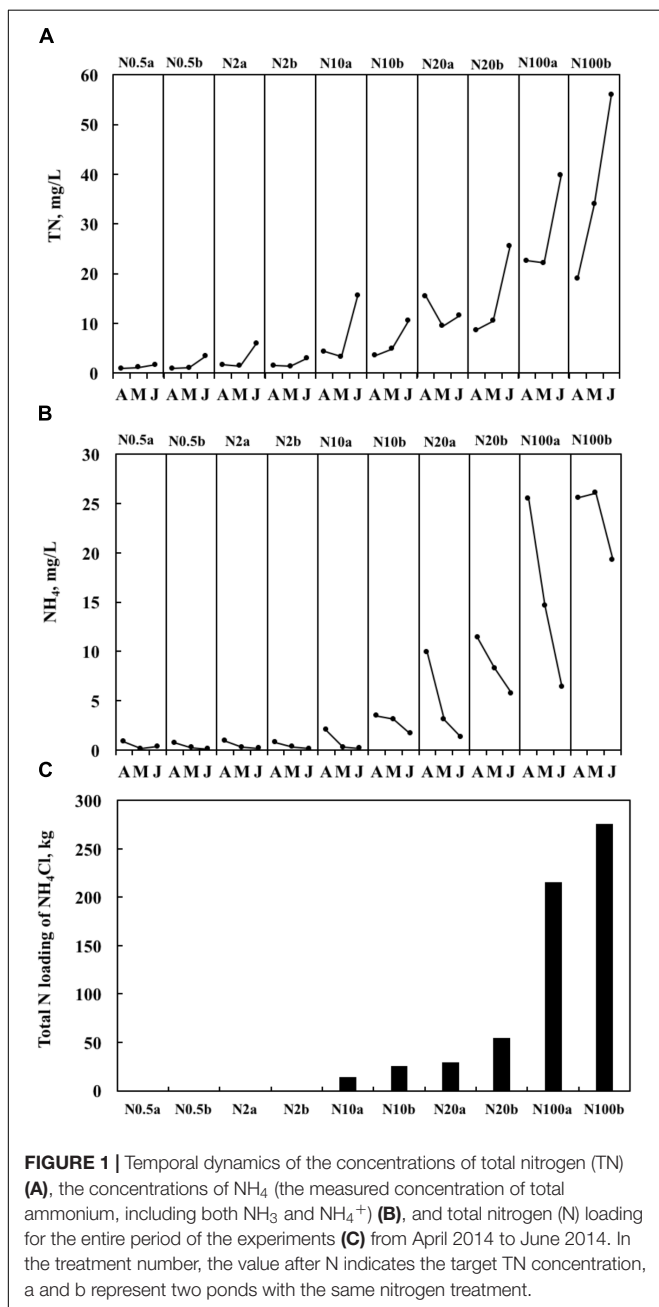


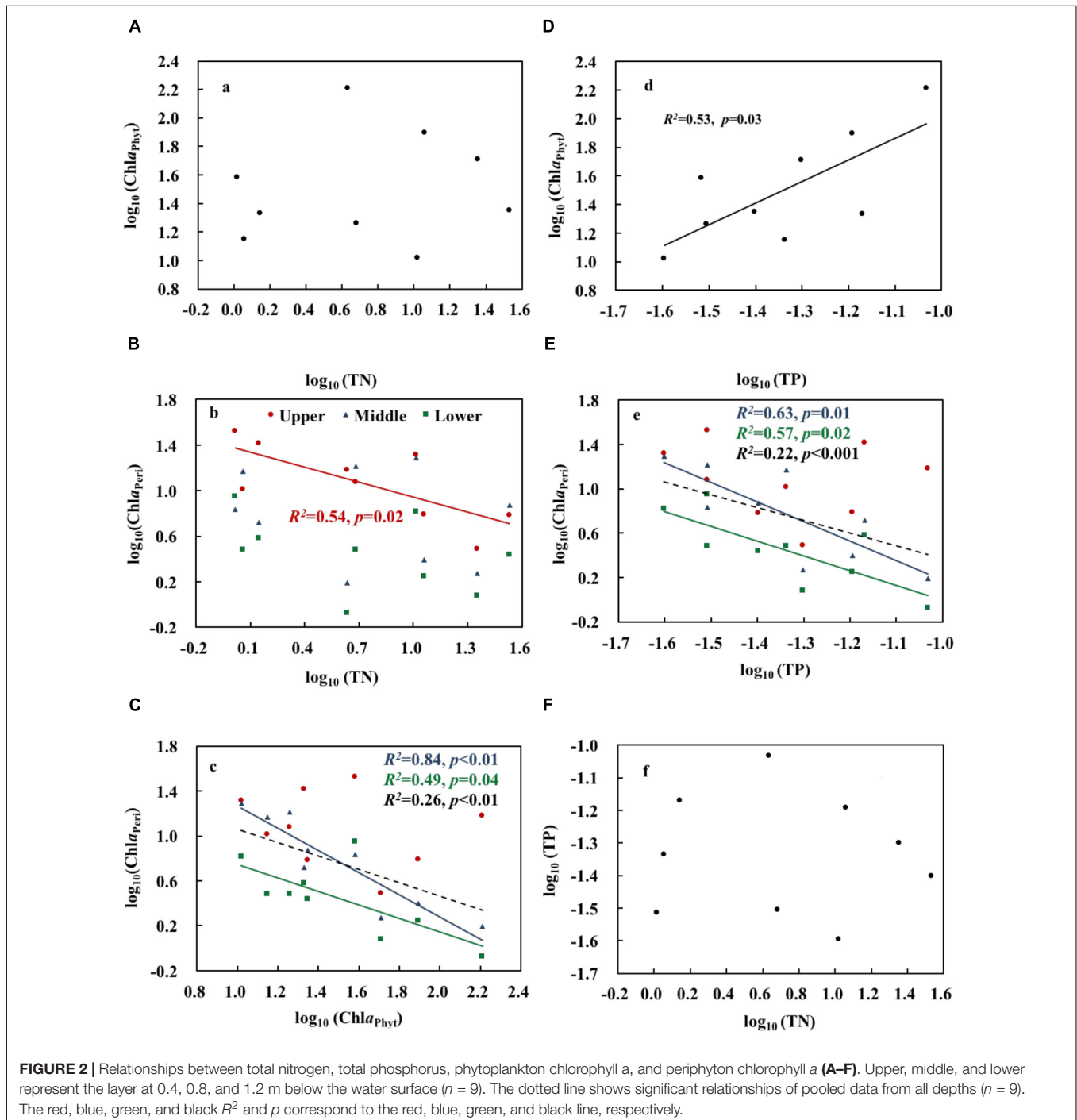
TABLE 1 | Pearson's correlations coefficients (*r*-values) among environmental variables; significant correlations are shown (**p* < 0.05 and ***p* < 0.01) in bold (*n* = 9).

	Layer	log ₁₀ (NH ₄)	log ₁₀ (Chla _{Phyt})	log ₁₀ (Chla _{Peri})
log ₁₀ (TN)	Upper	0.94**	0.12	-0.64*
	Middle			-0.37
	Lower			-0.26
log ₁₀ (NH ₄)	Upper		-0.19	-0.68*
	Middle			-0.11
	Lower			-0.01
log ₁₀ (Chla _{Phyt})	Upper			-0.21
	Middle			-0.86**
	Lower			-0.64*

is the initial shoot number/shoot height/shoot biomass of the macrophytes.

There was no water flow through the ponds during the experiments. Environmental parameters were measured every 20 days. Dissolved oxygen and water temperature at 0.4, 0.8, and 1.2 m from the water surface were measured *in situ* with a YSI ProPlus (Yellow Spring Inc., United States). The water temperature difference between 0.4 m (upper layer) and 1.2 m (lower layer) was less than 1°C. Light intensity was measured by

luminometer (KONICA MINOLTA, T-10, China) at the air-water interface (just above the water surface) and 0.4, 0.8, and 1.2 m below the water surface. Water samples for chemical analysis were collected at 5 randomly chosen locations within each pond by integrating the water column with a tube sampler (height 1.5 m and diameter 10 cm). Chlorophyll *a* of phytoplankton ($Chla_{phyt}$) was extracted with 90% acetone (at 4°C for 24 h) after filtration through GF/C filters (Whatman, GE Healthcare UK Limited, Buckinghamshire, United Kingdom), and absorbance was read



at 665 and 750 nm, both before and after acidification with 10% HCl using a spectrophotometer. The $Chla_{Peri}$ concentration was calculated following the equation in Zhang and Huang (1991):

$$C = \frac{(Eb - Ea) * R * K * V_e}{1000 * (R - 1) * S}$$

where C is the concentration of $Chla_{Peri}$ in $mg\ m^{-2}$; Eb is D-value of absorbance at 665 and 750 nm before acidification with HCl; Ea is D-value of absorbance at 665 and 750 nm after acidification with HCl; R = 1.7; K = 11.24; V_e is total volume of extract in mL; and S is surface area of the slides in m^2 .

Total nitrogen and total phosphorus (TP) were determined spectrophotometrically after digestion with $K_2S_2O_8$ solution (Huang et al., 1999). Total ammonium (NH_4) was determined with Nessler's reagent colorimetric method (ISO 5664, 1984; ISO 7150-1, 1984). NH_3 was calculated following the equation in

Emerson et al. (1975) and Wood (1993):

$$NH_3 = NH_4 - \frac{NH_4}{[1 + 10^{(pH - pKa)}]}$$

where $pKa = 0.09018 + 2729.92 / (273.2 + Temp)$, NH_4 is the measured concentration of total ammonium (including both NH_3 and NH_4^+), pH is the measured pH of the solution, and Temp is water temperature in °C. In Yu et al. (2017) and this study, NH_3 and NH_4^+ were expressed as total ammonium. Highly significant positive correlations were found between NH_3 and NH_4^+ in Yu et al. (2018) ($r = 0.90, n = 9, p < 0.001$) and in this study ($r = 0.98, n = 9, p < 0.001$), reflecting that the high concentrations of NH_3 were accompanied by high NH_4^+ concentrations. Consequently, the concentration of total ammonium (NH_4) in the two experiments serves as a combined indicator of toxicity, whether due to NH_3 , NH_4^+ , or both. Therefore, measured NH_4 was used to express toxicity in both Yu et al. (2017) and this study.

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 within the canopy of growing plants. Every 15 days, the glass slides were gently removed from the box for laboratory measurements after which a new set of three glass slides was embedded in the macrophytes. The periphyton growing on the glass slides was gently removed by a soft brush and flushed with 25 mL distilled water followed by filtered water, after which the concentration of periphyton chlorophyll a ($Chla_{Peri}$) was determined via acetone extraction.

Statistical Analyses

Median values were used for analyses to avoid deviation caused by extreme values of TN, TP, $Chla_{Phyt}$, etc. Pearson's correlations were used to test for relationships between macrophyte variables and environmental variables. Variables that did not follow normal distributions (Shapiro-Wilk test, $p < 0.05$) were $\log_{10}(x)$ -transformed and these included NH_4 , $Chla_{Phyt}$, and $Chla_{Peri}$. STATISTICA 8.0 and Microsoft Excel 2013 were used for all data

TABLE 2 | Characteristics of the growth and physiological variables of *Potamogeton crispus* (mean) in various experimental ponds (plants in pond N2b were grazed by invading fish because the net fell down).

Treatment number	Water layer	N_{Shoot}	H_{Shoot}	DM_{Shoot}	FAA	SC
N0.5a	Upper	11	77.0	2.27	0.076	2.13
	Middle	6	91.7	0.58	0.008	1.44
	Lower	4	74.7	2.55	0.062	2.03
N0.5b	Upper	11	82.0	1.44	0.041	12.35
	Middle	8	83.0	3.24	0.032	10.74
	Lower	3	104.7	1.69	0.051	14.04
N2a	Upper	7	71.7	4.13	0.037	8.37
	Middle	6	79.3	2.73	0.002	20.99
	Lower	2	54.3	0	0	0
N10a	Upper	6	49.7	3.33	0.160	2.14
	Middle	3	58.7	0	0	0
	Lower	0	0.0	0	–	–
N10b	Upper	17	109.3	11.13	0.042	4.29
	Middle	17	115.3	9.09	0.187	5.00
	Lower	9	124.3	6.54	0.156	7.15
N20a	Upper	11	80.3	5.62	0.100	5.94
	Middle	10	84.7	1.26	0.169	6.86
	Lower	2	52.7	0.56	0.090	8.54
N20b	Upper	19	120.0	7.02	0.204	1.83
	Middle	19	118.3	7.17	0.324	3.04
	Lower	8	126	3.76	0.160	12.83
N100a	Upper	4	62.7	0	0	0
	Middle	1	48.0	0	0	0
	Lower	0	0.0	0	–	–
N100b	Upper	12	86.3	7.83	0.204	4.76
	Middle	9	104.7	3.59	0.248	6.04
	Lower	5	117.3	1.27	0	0

N_{Shoot} , number of shoots; H_{Shoot} , height of shoots, cm; DM_{Shoot} , dry mass of shoots, g; FAA, free amino acids of plants, mg/g; SC, soluble carbohydrate, mg/g; “–” no sample of plants was obtained. In “treatment number” the value after N indicates the target concentration of total nitrogen, a and b represent two ponds subjected to the same nitrogen treatment. Due to its natural decay in early summer, *P. crispus* was fragile during the last measurement conducted on 12 June, preventing accurate measurements of number and height. Instead, data from 20th May (the period before the last measurement) were used in the analysis.

TABLE 3 | Pearson's correlation coefficients (r-values) between growth and physiological variables of *Potamogeton crispus* relative to environmental variables (see Tables 1, 2 for explanation of the abbreviations and units).

	Layer	$\log_{10}(NH_4)$	$\log_{10}(Chla_{Phyt})$	$\log_{10}(Chla_{Peri})$	FAA
N_{Shoot}	Upper	0.28	–0.65	0.34	0.26
	Middle	0.31	–0.58	0.91**	0.80*
	Lower	0.29	–0.77*	0.73*	0.88*
H_{Shoot}	Upper	0.40	–0.75*	0.26	0.13
	Middle	0.26	–0.75*	0.89**	0.81*
	Lower	0.22	–0.79*	0.82*	0.64
DM_{Shoot}	Upper	0.39	–0.38	0.29	0.22
	Middle	0.28	–0.62	0.81*	0.60
	Lower	0.16	–0.61	0.69	0.74

Significant differences are shown (* $p < 0.05$ and ** $p < 0.01$) in bold ($n = 9$).

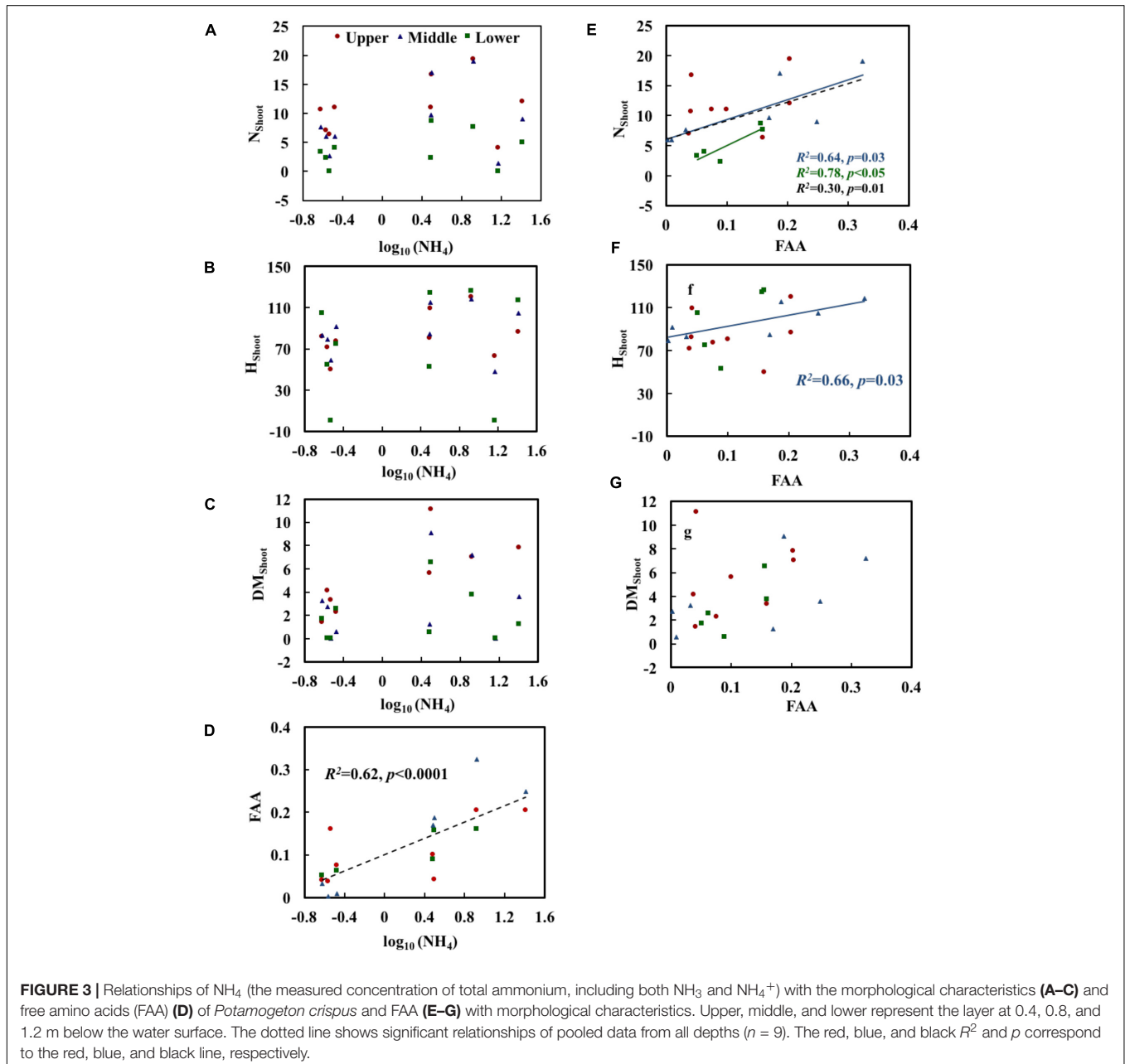
analyses. Plants in pond N2b were grazed by invading fish as the net fell down. We therefore only analyzed the data from the other nine treatments.

RESULTS

Growing Conditions of *P. crispus*

Both realized TN and ammonium (NH_4) formed a significant treatment gradient, with medians ranging from 1.0 to 34.0 mg L^{-1} (Figure 1A) and from 0.2 to 25.6 mg L^{-1} (Figure 1B), respectively. The total amounts of added fertilizer are shown in Figure 1C. $\text{Chla}_{\text{Phyt}}$ also increased significantly,

with medians ranging between 10.5 and $162.4 \mu\text{g L}^{-1}$. $\text{Chla}_{\text{Peri}}$ declined from the upper ($3.3\text{--}29.3 \text{ mg m}^{-2}$) over the middle ($1.8\text{--}17.0 \text{ mg m}^{-2}$) to the lower ($1.0\text{--}9.6 \text{ mg m}^{-2}$) layers in most treatments, except N0.5a and N100b ($\text{Chla}_{\text{Peri}}$ was a little bit higher in the middle layer than the upper layer in the two treatments). Pearson's correlations among the representative variables are given in Table 1. A highly significant positive relationship was found between TN and NH_4 ($p < 0.01$). There was no relationship between TN and $\text{Chla}_{\text{Phyt}}$ (Figure 2A). A significant negative correlation was found between TN and $\text{Chla}_{\text{Peri}}$ in the upper layer ($p < 0.05$) (Table 1 and Figure 2B) and between $\text{Chla}_{\text{Peri}}$ and $\text{Chla}_{\text{Phyt}}$ in the middle ($p < 0.01$) and lower layers ($p < 0.05$) (Table 1 and Figure 2C). A significant



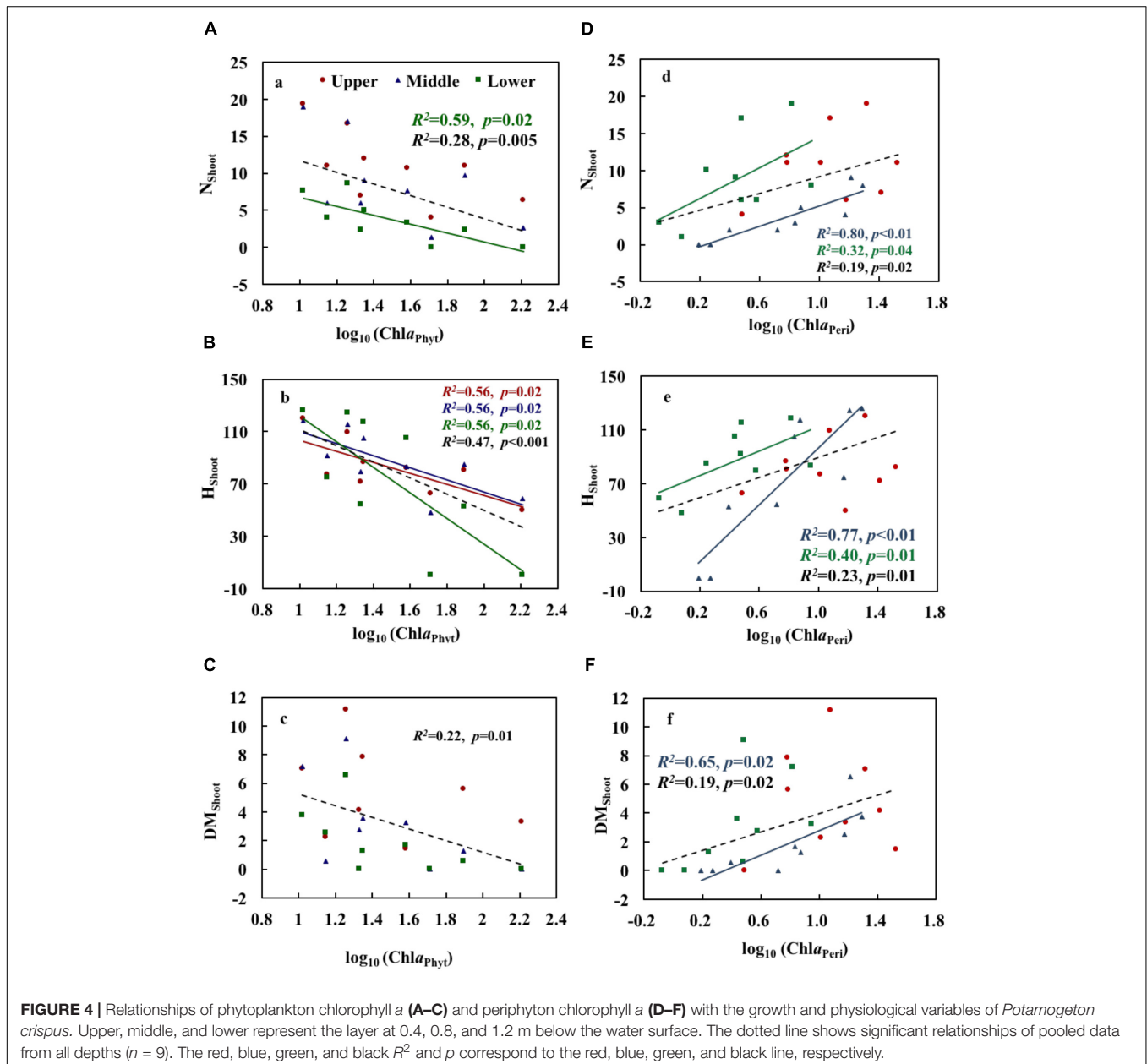
positive correlation was found between TP and $Chla_{phyt}$ ($p < 0.05$) (Figure 2D), and a negative correlation between $Chla_{peri}$ and TP, in the middle and lower layer ($p < 0.05$) (Figure 2E). No significant correlation was found between TN and TP (Figure 2F).

Growth of *P. crispus* at Contrasting Nitrogen Concentrations

Growth and physiological variables of plants are shown in Table 2. No significant correlations were found between NH_4 and the growth variables (Table 3). The scatterplots did not show any clear changes in growth variables with increasing NH_4 concentrations (Figures 3A–C). FAA showed a significant

increasing trend with NH_4 when pooling data from all depths ($p < 0.001$) (Figure 3D). When regressing FAA with growth variables of plants, N_{Shoot} demonstrated a significant positive relationship with increasing FAA in the middle and lower layers ($p < 0.05$). When pooling the data from all three layers, similar results were obtained (Figure 3E). H_{Shoot} in the middle layer exhibited a significant relationship with FAA ($p < 0.05$) (Figure 3F), while DM_{Shoot} was not related to increasing FAA (Figure 3G).

$Chla_{phyt}$ showed significant negative correlations with N_{Shoot} and H_{Shoot} in the lower layer and with H_{Shoot} in the upper and middle layers ($p < 0.05$) (Table 3). The scatterplots also showed generally declining trends of growth variables with increasing $Chla_{phyt}$, significantly so for N_{Shoot} in the lower layer, for H_{Shoot}



in all three layers, and for all growth indices when pooling the data from all three layers (Figures 4A–C).

$Chl_{a\text{peri}}$ showed significant positive correlations with N_{Shoot} and H_{Shoot} in the middle and lower layers, and with DM_{Shoot} in the middle layer ($p < 0.05$) (Table 3). The scatterplots also demonstrated clear increasing trends of growth variables with increasing $Chl_{a\text{peri}}$, significantly so for N_{Shoot} and H_{Shoot} in the

middle and lower layers, for DM_{Shoot} in the middle layer, and for all growth indices when pooling the data from all three layers (Figures 4D–F).

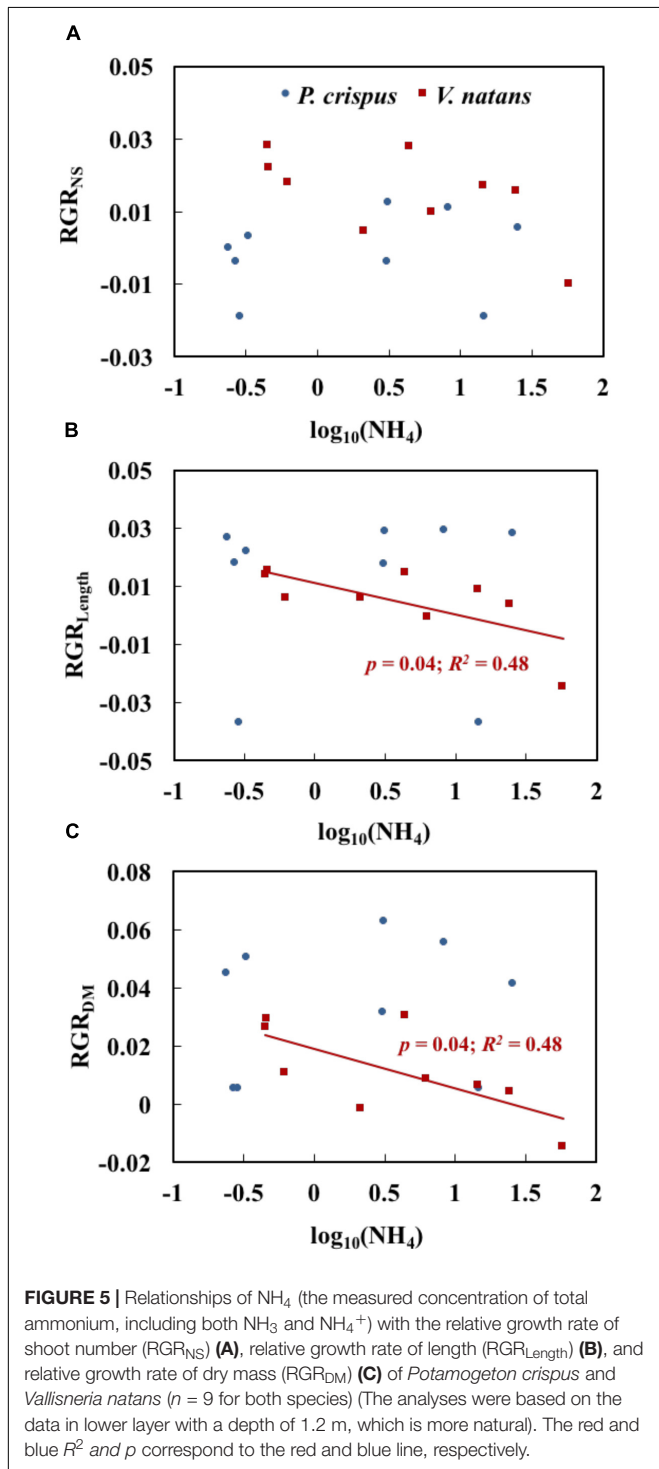
Comparison Between *P. crispus* and *V. natans*

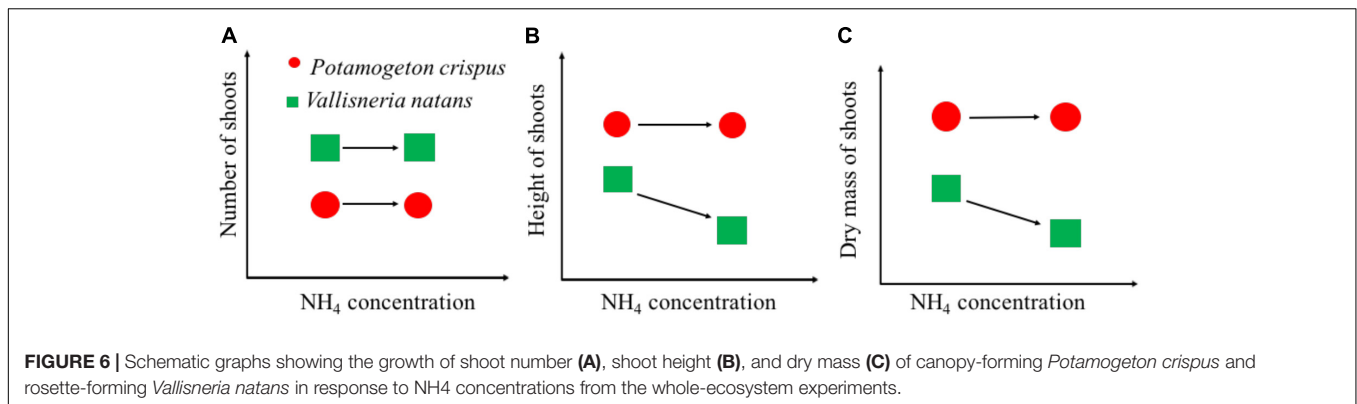
Although the precipitation varied in the experiments with *P. crispus* and *V. natans*, we maintained a fixed water level by pumping water out of the ponds after rainfall events. The average Secchi depth (SD) was similar in the two experiments, indicating that the two experiments apart from the temperature (see Discussion) were comparable despite the fact that they were conducted in two different years.

The relationships between NH_4 and growth variables for *P. crispus* and *V. natans* in the lower layer (1.2 m), a natural growth depth in the field, are compared in Figure 5. We found that 67% of the relative growth rates of shoot number (RGR_{NS}) was higher for *V. natans* than for *P. crispus*, while 78% of the relative growth rates of leaf length (RGR_{Length}) and 56% of dry mass (RGR_{DM}) of *P. crispus* were systematically higher than those of *V. natans* (Figures 5A–C). The RGR_{NS} of *P. crispus* and of *V. natans* demonstrated no significant relationships with increasing NH_4 (Figure 5A). RGR_{Length} and RGR_{DM} of *V. natans* both displayed a significant declining trend with NH_4 , while no such trend was found for *P. crispus* (Figures 5B,C).

DISCUSSION

In an earlier aquarium study by Cao et al. (2009b), an NH_4 concentration of $>1 \text{ mg L}^{-1}$ in the water column induced physiological stress on *P. crispus* at low light availability, while neither growth inhibition nor NH_4 accumulation was observed in the plant tissue of *P. crispus* at normal light availability (Cao et al., 2009b). In addition, high NH_4 concentrations ($>5 \text{ mg L}^{-1}$) produced significant acute biochemical changes in *P. crispus* (Cao et al., 2004). Moderate NH_4 concentrations ($0.16\text{--}0.25 \text{ mg L}^{-1}$) were not directly toxic to *V. natans* (Cao et al., 2011), but an NH_4 concentration of 1 mg L^{-1} may inhibit the growth and the carbon and nitrogen metabolism of *V. natans* (Cao et al., 2009a). However, in the present study, none of the growth variables of the canopy-forming *P. crispus* (including N_{Shoot} , H_{Shoot} , and DM_{Shoot}) decreased with increasing nitrogen loading, whereas all growth variables decreased with increasing phytoplankton chlorophyll *a*. Five ponds exhibited NH_4 concentrations ($3.1\text{--}25.6 \text{ mg L}^{-1}$) higher than the above thresholds of 1 and 5 mg L^{-1} . This suggests that *P. crispus* was able to overcome the stress induced by high nitrogen concentrations under field conditions. In our previous experiment with the rosette-forming *V. natans*, both leaf length and leaf mass decreased significantly with increasing NH_4 concentrations (Yu et al., 2017). These results suggest that the canopy-forming *P. crispus* is more tolerant than the rosette-forming *V. natans* when exposed to high NH_4 concentrations. A potential caveat is that the two experiments were undertaken in two years with different associated pond temperatures and precipitation. In the present experiment with *P. crispus*, the air and water temperature were lower than in





the previously published experiment with *V. natans*. In Yu et al. (2017), higher growth rates of *V. natans* were found in the summer-autumn experiment than in the winter-spring experiment, which suggests that *V. natans* growth benefits from higher temperatures, perhaps increasing its tolerance to damage induced by ammonium enrichment. Accordingly, the difference in tolerance of *V. natans* and *P. crispus* would expectedly have been even greater if they had been growing at similar temperatures, further substantiating our hypothesis that canopy-forming submersed macrophytes are more tolerant to NH₄ stress than rosette-forming species. The amount of precipitation may disturb pond conditions, cause resuspension of the sediment, and create shading effects. When phytoplankton abundance increases, so do the levels of detritus and inorganic suspended matter (see Yu et al., 2018). Although the precipitation in this study was much higher than that in Yu et al. (2017), the average Chl_a_{phyt} concentration (41.7 μg L⁻¹) did not differ significantly from those in Yu et al. (2017) (33.8 μg L⁻¹), indicating that the resuspension of the sediment caused by precipitation might not have had a significant effect on the macrophytes.

Macrophytes may excessively synthesize FAA to avoid NH₄ toxicity when experiencing high NH₄ stress. However, this process costs extra energy and consumption of carbohydrates (Rare, 1990; Krupa, 2003), which may reduce the growth of macrophytes, particularly at low light availability (Cao et al., 2009a, 2011; Yuan et al., 2013, 2016). FAA content is therefore commonly used as an indicator of nitrogen stress (Cao et al., 2009b; Zhang et al., 2010; Yuan et al., 2015). In our experiment, FAA tended to increase with increasing NH₄ concentrations. However, significant positive relationships were recorded between FAA and shoot number (N_{shoot}) (in the middle and lower layers) and shoot height (H_{shoot}) (in middle layer). These positive relationships of FAA with NH₄ and growth variables, together with weak relationships between NH₄ and growth variables, suggest that FAA might be a useful indicator of physiological stress by high ammonium but not of macrophyte growth. A similar conclusion was made in our previous pond experiment on *V. natans*, both for the low-growth season (in winter) (Yu et al., 2015) and the high growth season (in summer and autumn) (Yu et al., 2017).

All the growth variables of *P. crispus* declined with increasing concentrations of phytoplankton chlorophyll *a*, suggesting that the decline may mainly be due to shading of phytoplankton, which in our experiment was not significantly related to N loading. Similar results were found for the rosette-forming *V. natans* (Yu et al., 2017). In contrast, all the growth variables of *P. crispus* increased with increasing periphyton chlorophyll *a*. This finding probably reflects the fact that the development of periphyton and macrophytes both rely on underwater light conditions. If light is sufficient, both plant growth metrics and periphyton density will increase. In this experiment, Chl_a_{peri}, particularly in the middle and lower layers, tended to decrease with enhanced TP. Shading effects by phytoplankton, as suggested by the negative relation between Chl_a_{peri} and Chl_a_{phyt}, were likely promoted by TP. Periphyton on glass may not fully represent those on plants, but measurement of periphyton on glass has its advantages: (1) It does not cause damage to plant tissue and has little impact on plant growth; (2) it is possible to standardize measurement to ensure consistent results. Our results suggest that phytoplankton shading affected both the growth of submersed macrophytes and periphyton negatively.

Our findings can be summarized with a conceptual model of growth-NH₄ relationships between canopy-forming *P. crispus* and rosette-forming *V. natans* exposed to high N stress (Figure 6). Rosette-forming species tend to have a higher number of shoots but lower shoot height and mass than canopy formers, both in the presence and absence of high NH₄ stress. For canopy-forming species, high NH₄ stress may not significantly affect any growth variables (Figure 6). By contrast, for the rosette-forming species, high NH₄ stress may not necessarily affect the shoot number but may lead to a decline in shoot height and mass. The difference in the ability of canopy-forming and rosette-forming species to access underwater light might explain their contrasting tolerance to high NH₄ stress. In shallow lakes, canopy-forming species are often able to reach the water surface where there is sufficient light to produce carbohydrates and thereby avoid NH₄ toxicity. Rosette-forming species are often relatively short and therefore relatively prone to low-light conditions. At high N concentrations, rosette-forming species may therefore not produce enough carbohydrates to detoxify NH₄. Moreover, rosette-forming species become even more affected by high N loading which may cause higher phytoplankton growth or higher

periphyton growth as seen in some studies (Özkan et al., 2010; Olsen et al., 2015). Our findings are of practical importance and imply that canopy-forming species may potentially be preferred in the restoration of shallow lakes undergoing high N stress. Moreover, canopy-forming species, if covering the water surface, may block light penetration, limit gas exchange, adversely affect water oxygen concentrations, and probably alter biochemical processes (Turner et al., 2010). However, more tests, especially of different canopy-forming species are needed to draw firm conclusions regarding this study's conceptual model, so as to cover more species to help better understand the mechanisms underlying macrophyte decline in shallow lakes.

AUTHOR CONTRIBUTIONS

QY, H-JW, H-ZW, and EJ designed the research. QY, YL, S-NM, and X-ML carried out the research. QY and H-JW performed the data analyses. QY prepared the original draft of the paper. EJ, CX, and H-JW commented on the various drafts.

FUNDING

The project was supported by the State Key Laboratory of Freshwater Ecology and Biotechnology (2016FBZ09, 2014FB14,

and 2011FBZ14) and the Science and Technology Support Program of Hubei Province (2015BBA225). H-JW was supported by the Youth Innovation Association of Chinese Academy of Sciences (2014312). CX was supported by the National Natural Science Foundation of China (41271197). EJ received funding from the MARS project (Managing Aquatic ecosystems and water Resources under multiple Stress) funded under the 7th EU Framework Programme, Theme 6 (Environment including Climate Change), Contract No. 603378 (HYPERLINK "http://www.mars-project.eu"), Centre for Water Technology (WATEC), Aarhus University, and AnaEE, Denmark.

ACKNOWLEDGMENTS

We thank Shu-Ran Wang for assistance with water-quality measurements and Anne Mette Poulsen for valuable editing of the paper.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2018.01845/full#supplementary-material>

REFERENCES

- Blindow, I. (1992). Long- and short-term dynamics of submerged macrophytes in two shallow eutrophic lakes. *Freshw. Biol.* 28, 15–27. doi: 10.1111/j.1365-2427.1992.tb00558.x
- Cao, T., Ni, L. Y., and Xie, P. (2004). Acute biochemical responses of a submersed macrophyte, *Potamogeton crispus* L., to high ammonium in an aquarium experiment. *J. Freshw. Ecol.* 19, 279–284. doi: 10.1080/02705060.2004.9664542
- Cao, T., Ni, L. Y., Xie, P., Xu, J., and Zhang, M. (2011). Effects of moderate ammonium enrichment on three submersed macrophytes under contrasting light availability. *Freshwater Biol.* 56, 1620–1629. doi: 10.1111/j.1365-2427.2011.02601.x
- Cao, T., Xie, P., Li, Z. Q., Ni, L. Y., Zhang, M., and Xu, J. (2009a). Physiological stress of high NH₄⁺ concentration in water column on the submersed macrophyte *Vallisneria spiralis* L. *Bull. Environ. Contam. Toxicol.* 82, 296–299. doi: 10.1007/s00128-008-9531-5
- Cao, T., Xie, P., Ni, L. Y., Zhang, M., and Xu, J. (2009b). Carbon and nitrogen metabolism of a eutrophication tolerant macrophyte, *Potamogeton crispus*, under NH₄⁺ stress and low light availability. *Environ. Exp. Bot.* 66, 74–78. doi: 10.1016/j.envexpbot.2008.10.004
- Carpenter, S. R. (2003). Regime shifts in lake ecosystems: pattern and variation. *Excell. Ecol.* 15, i–195.
- Chambers, P. A. (1987). Light and nutrients in the control of aquatic plant community structure. II. In situ observations. *J. Ecol.* 75, 621–628. doi: 10.2307/2260194
- Chambers, P. A., and Kalf, J. (1987). Light and nutrients in the control of aquatic plant community structure. I. In situ experiments. *J. Ecol.* 75, 611–619.
- Dorenbosch, M., and Bakker, E. S. (2011). Herbivory in omnivorous fishes: effect of plant secondary metabolites and prey stoichiometry. *Freshw. Biol.* 56, 1783–1797. doi: 10.1111/j.1365-2427.2011.02618.x
- Emerson, K., Russo, R. C., Lund, R. E., and Thurston, R. V. (1975). Aqueous ammonia equilibrium calculations: effect of pH and temperature. *J. Fish. Res. Board Can.* 32, 2379–2383. doi: 10.1021/jo900391n
- Gao, J. Q., Li, L. S., Hu, Z. Y., Zhu, S. F., Zhang, R. Q., and Xiong, Z. T. (2015). Ammonia stress on the carbon metabolism of *Ceratophyllum demersum*. *Environ. Toxicol. Chem.* 34, 843–849. doi: 10.1002/etc.2866
- General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China [AQSIQ] (2002a). *Discharge Standard of Pollutants for Municipal Wastewater Treatment Plant*. Shanghai: AQSIQ.
- General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China [AQSIQ] (2002b). *Environmental Quality Standards for Surface Water*. Shanghai: AQSIQ.
- Huang, X. F., Chen, W. M., and Cai, Q. M. (1999). *Standard Methods for Observation and Analysis in Chinese Ecosystem Research Network-Survey, Observation and Analysis of Lake Ecology*. Beijing: Standards Press of China.
- Hudon, C., Lalonde, S., and Gagnon, P. (2000). Ranking the effects of site exposure, plant growth form, water depth, and transparency on aquatic plant biomass. *Can. J. Fish. Aquat. Sci.* 57, 31–42. doi: 10.1139/cjfas-57-S1-31
- ISO 5664 (1984). *Water Quality; Determination of Ammonium; Distillation and Titration Method*. Geneva: ISO.
- ISO 7150-1 (1984). *Water Quality; Determination of Ammonium. Part 1. Manual Spectrometric Method*. Geneva: ISO.
- Jackson, L. J., and Kalf, J. (1993). Patterns in metal content of submerged aquatic macrophytes: the role of plant growth form. *Freshw. Biol.* 29, 351–359. doi: 10.1111/j.1365-2427.1993.tb00769.x
- Jeppesen, E., Meerhoff, M., Jacobsen, B. A., Hansen, R. S., Søndergaard, M., Jensen, J. P., et al. (2007). Restoration of shallow lakes by nutrient control and biomanipulation—the successful strategy varies with lake size and climate. *Hydrobiologia* 581, 269–285. doi: 10.1007/s10750-006-0507-3
- Jeppesen, E., Søndergaard, M., Søndergaard, M., and Christoffersen, K. (1998). *The Structuring Role of Submerged Macrophytes in Lakes*. New York, NY: Springer. doi: 10.1007/978-1-4612-0695-8
- Körner, S. (2002). Loss of submerged macrophytes in shallow lakes in north-eastern Germany. *Int. Rev. Hydrobiol.* 87, 375–384. doi: 10.1002/1522-2632(200207)87:4<375::AID-IROH375>3.0.CO;2-7
- Krupa, S. V. (2003). Effects of atmospheric ammonia (NH₃) on terrestrial vegetation: a review. *Environ. Pollut.* 124, 179–221. doi: 10.1016/S0269-7491(02)004347
- Li, H. S., Sun, Q., Zhao, S. J., and Zhang, W. H. (2004). *Plant Physiology Biochemistry Principle and Experimental Technique*. Beijing: Higher Education Press.

- Moss, B. (2001). *The Broads. The People's Wetland. The New Naturalist*. London: Harper Collins Publishers.
- Moss, B., Jeppesen, E., Søndergaard, M., Lauridsen, T. L., and Liu, Z. W. (2013). Nitrogen, macrophytes, shallow lakes and nutrient limitation: resolution of a current controversy? *Hydrobiologia* 710, 3–21. doi: 10.1007/s10750-012-1033-0
- Nimptsch, J., and Pflugmacher, S. (2007). Ammonia triggers the promotion of oxidative stress in the aquatic macrophyte *Myriophyllum mattogrossense*. *Chemosphere* 66, 708–714. doi: 10.1016/j.chemosphere.2006.07.064
- Olsen, S., Chang, F. Y., Li, W., Zhao, S. T., Søndergaard, M., and Jeppesen, E. (2015). Strong impact of nitrogen loading on submerged macrophytes and algae: a long-term mesocosm experiment in a shallow Chinese lake. *Freshw. Biol.* 8, 1525–1536. doi: 10.1111/fwb.12585
- Özkan, K., Jeppesen, E., Johansson, L. S., and Beklioglu, M. (2010). The response of periphyton and submerged macrophytes to nitrogen and phosphorus loading in shallow warm lakes: a mesocosm experiment. *Freshw. Biol.* 55, 463–475. doi: 10.1111/j.1365-2427.2009.02297.x
- Rare, E. (1990). Stress physiology: the functional significance of the accumulation of nitrogen-containing compounds. *J. Hort. Sci.* 65, 231–243. doi: 10.1080/00221589.1990.11516052
- Sayer, C. D., Burgess, A. M. Y., Kari, K., Davidson, T. A., Peglar, S., Yang, H., et al. (2010a). Long-term dynamics of submerged macrophytes and algae in a small and shallow, eutrophic lake: implications for the stability of macrophyte-dominance. *Freshw. Biol.* 55, 565–583. doi: 10.1111/j.1365-2427.2009.02353.x
- Sayer, C. D., Davidson, T. A., and Jones, J. I. (2010b). Seasonal dynamics of macrophytes and phytoplankton in shallow lakes: an eutrophication-driven pathway from plants to plankton? *Freshw. Biol.* 55, 500–513. doi: 10.1111/j.1365-2427.2009.02365.x
- Scheffer, M. (1998). *Ecology of Shallow Lakes*. Dordrecht: Kluwer Academic Publishers.
- Standardization Administration of the People's Republic of China [SAC] (2006). *Standards for Drinking Water Quality*. Beijing: SAC.
- Su, S. Q., Zhou, Y. M., Qin, J. G., Wang, W., Yao, W. Z., and Song, L. (2012). Physiological responses of *Egeria densa* to high ammonium concentration and nitrogen deficiency. *Chemosphere* 86, 538–545. doi: 10.1016/j.chemosphere.2011.10.036
- Turner, A. M., Cholak, E. J., and Groner, M. (2010). Expanding American lotus and dissolved oxygen concentrations of a shallow lake. *Am. Midl. Nat.* 164, 1–8. doi: 10.1674/0003-0031-164.1.1
- Wang, C., Zhang, S. H., Wang, P. F., Hou, J., Li, W., and Zhang, W. J. (2008). Metabolic adaptations to ammonia-induced oxidative stress in leaves of the submerged macrophyte *Vallisneria natans* (Lour.) Hara. *Aquat. Toxicol.* 87, 88–98. doi: 10.1016/j.aquatox.2008.01.009
- Wang, C., Zhang, S. H., Wang, P. F., Li, W., and Lu, J. (2010). Effects of ammonium on the antioxidative response in *Hydrilla verticillata* (L.f.) Royle plants. *Ecotoxicol. Environ. Saf.* 73, 189–195. doi: 10.1016/j.ecoenv.2009.08.012
- Wang, H. J., Wang, H. Z., Liang, X. M., and Wu, S. K. (2014). Total phosphorus thresholds for regime shifts are nearly equal in subtropical and temperate shallow lakes with moderate depths and areas. *Freshw. Biol.* 59, 1659–1671. doi: 10.1111/fwb.12372
- Wood, C. M. (1993). "Ammonia and urea metabolism and excretion," in *The Physiology of Fishes*, ed. D. H. Evans (Ann Arbor, MI: CRC Press), 379–425.
- Xia, Q., Chen, Y. Q., and Liu, X. B. (2004). *Water Quality Criteria and Water Quality Standards*. Beijing: Standards Press of China.
- Yu, Q., Wang, H. J., Wang, H. Z., Li, Y., Liang, X. M., Xu, C., et al. (2017). Does the responses of *Vallisneria natans* (Lour.) Hara to high nitrogen loading differ between the summer high-growth season and the low-growth season? *Sci. Total Environ.* 60, 1513–1521. doi: 10.1016/j.scitotenv.2017.05.268
- Yu, Q., Wang, H. Z., Jeppesen, E., Xu, C., and Wang, H. J. (2018). Reply to Cao et al.'s comment on "Does the responses of *Vallisneria natans* (Lour.) Hara to high nitrogen loading differ between the summer high-growth season and the low-growth season? Science of the Total Environment 601-602 (2017) 1513-1521". *Sci. Total Environ.* 615, 1093–1094. doi: 10.1016/j.scitotenv.2017.09.306
- Yu, Q., Wang, H. Z., Li, Y., Shao, J. C., Liang, X. M., Jeppesen, E., et al. (2015). Effects of high nitrogen concentrations on the growth of submersed macrophytes at moderate phosphorus concentrations. *Water Res.* 83, 385–395. doi: 10.1016/j.watres.2015.06.053
- Yuan, G. X., Cao, T., Fu, H., Ni, L. Y., Zhang, X., Li, W., et al. (2013). Linking carbon and nitrogen metabolism to depth distribution of submersed macrophytes using high ammonium dosing tests and a lake survey. *Freshw. Biol.* 58, 2532–2540. doi: 10.1111/fwb.12230
- Yuan, G. X., Fu, H., Zhong, J. Y., Cao, T., Ni, L. Y., Zhu, T. S., et al. (2015). Nitrogen/carbon metabolism in response to NH₄⁺ pulse for two submersed macrophytes. *Aquat. Bot.* 21, 76–82. doi: 10.1016/j.aquabot.2014.12.001
- Yuan, G. X., Fu, H., Zhong, J. Y., Lou, Q., Ni, L. Y., and Cao, T. (2016). Growth and C/N metabolism of three submersed macrophytes in response to water depths. *Environ. Exp. Bot.* 122, 94–99. doi: 10.1016/j.envexpbot.2015.09.009
- Zhang, M., Cao, T., Ni, L., Xie, P., and Li, Z. (2010). Carbon, nitrogen and antioxidant enzyme responses of *Potamogeton crispus* to both low light and high nutrient stresses. *Environ. Exp. Bot.* 68, 44–50. doi: 10.1016/j.envexpbot.2009.09.003
- Zhang, M., Wang, Z., Xu, J., Liu, Y., Ni, L. Y., Cao, T., et al. (2011). Ammonium, microcystins, and hypoxia of blooms in eutrophic water cause oxidative stress and C-N imbalance in submersed macrophytes and floating-leaved aquatic plants in Lake Taihu, China. *Chemosphere* 82, 329–339. doi: 10.1016/j.chemosphere.2010.10.038
- Zhang, Z. S., and Huang, X. F. (1991). *Research Methods for Freshwater Plankton*. Beijing: Science Press of China.
- Zhao, S. T., Yin, L. Y., Chang, F. Y., Olsen, S., Søndergaard, M., Jeppesen, E., et al. (2016). Response of *Vallisneria spirulosa* (Hydrocharitaceae) to contrasting nitrogen loadings in controlled lake mesocosms. *Hydrobiologia* 766, 215–223. doi: 10.1007/s10750-015-2456-1

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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