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RESEARCH ARTICLE

Effects of high ammonium enrichment in water column on the clonal growth of submerged macrophyte *Vallisneria natans*

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Abstract

As we know, the survival of young ramets and stolons is essential for the clonal growth of many aquatic plants. However, few NH_4^+ enrichment experiments on clonal growth of submerged macrophytes have been conducted to provide possible evidences for their declines in eutrophic lakes. Here, the growth and physiological responses of *V. natans* to the enrichment of NH_4^+-N were examined under six inorganic nitrogen (IN, i.e., the sum of nitrate nitrogen (NO_3^--N) and ammonium nitrogen (NH_4^+-N)) concentrations (control, 2.5, 4.5, 6.5, 8.5, and 10.5 mg L^{-1}). When NH_4^+-N concentration increased over 0.5 mg L^{-1}, free amino acid (FAA) contents in leaves and stolons increased while soluble carbohydrate (SC) and starch contents decreased, and major growth indices (total biomass of plants, number of ramets, and stolon dry weight (DW)) also showed a degressive tendency. Remarkably, the stolon DW significantly declined with increasing FAA, but significantly positively related to SC and starch. These results indicated that clonal growth of *V. natans* was inhibited by high NH_4^+-N concentration, and imbalance of C-N metabolism of stolons partly explained the decline of submerged clonal macrophytes. In this study, the leaves of new and small (NS) ramets contained significantly more FAA and less SC than that of mature and mother (MM) plants, indicating that the C-N metabolism of young ramets was easier to be disrupted, consequently inhibiting the clonal growth of aquatic plants. Furthermore, under the condition of high NH_4^+-N concentration, FAA may be a useful indicator of both macrophyte growth and physiological stress of plants.

Keywords Clonal growth · Ammonium · Young ramets · Free amino acid · Carbohydrate · Submerged macrophyte

Abbreviations

V. natans	*Vallisneria natans*
IN	inorganic nitrogen
FAA	free amino acid
SC	soluble carbohydrates
NS ramets	new and small ramets
MM plants	mature and mother plants
DW	dry weight
TN	total nitrogen
TP	total phosphate
NO_3^--N	nitrate nitrogen
NH_4^+-N	ammonium nitrogen
PO_4^{3-}-P	orthophosphate
PAR	photosynthetically active radiation
SOD	superoxide dismutase
POD	guaiacol peroxidase
CAT	catalase
ROS	reactive oxygen species

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Introduction

The survival and growth of seedlings are considered as one of the most crucial stages of individual survival in early stages of plant life history (Schupp 1995), and passing through to the

next stages is critical to the establishment of population (Harper 1977). After initial colonization of a site by seedlings or asexual propagules, clonal growth appears to be a very important way to maintain and expand natural populations for aquatic clonal plants (Grace 1993; Xiao et al. 2007). For many aquatic clonal plants, they have rhizomes or stolons or both of them which elongate horizontally and form many small ramets at the nodes, then more ramets produce more stolons or rhizomes that interconnect with each other and spread to a larger area (Duarte and Sandjensen 1990; Xiao et al. 2006). Alternatively, the survival of new and small (NS) ramets and the growth of stolons or rhizomes may determine the clonal growth of aquatic plants.

However, the above reproduction processes are observed rarely in the eutrophic lakes, and submerged macrophytes are being reduced due to eutrophication, which have received more and more attention worldwide in shallow eutrophic lakes (Moss 1980; Korner 2002; Sand-Jensen et al. 2008). In the last several years, many studies have showed that the nitrogen loading in water column is one of the important factors for the reduction of species diversity and richness of macrophytes (Jeppesen et al. 2007; Moss et al. 2013; Barker et al. 2008; James et al. 2005). Nitrogen loading in the eutrophic lakes is not only the enrichment of nitrate in the water column, but also ammonium (Khan and Ansari 2005; Qin 2009). For instance, Lake Taihu, the third largest freshwater lake in China, has been seriously polluted and the total nitrogen (TN) and nitrate nitrogen (NO_3^--N) values of water samples collected from the whole lake ranged from 1.30 to 10.53 mg L^{-1} and 0.27 to 6.44 mg L^{-1} in late spring and early summer during 2009, 2010, and 2011. The ammonium nitrogen (NH_4^+-N) concentrations in some bays of Lake Taihu exceeded 3 mg L^{-1} (Ye et al. 2014). Meanwhile, the urban lakes have faced more serious eutrophication due to intensive human activities (Jin 2003). All these elucidate that high concentrations of NH_4^+ and NO_3^- may exist in the water column at the same time in the eutrophic lakes. In China, *V. natans* is a common submerged clonal macrophyte, and its rapid decline in many lakes of Yangtze floodplain has been put down to severe eutrophication and NH_4^+ enrichment in this region (Cao et al. 2007). As far as we know, few studies have investigated the influence of high NH_4^+-N concentrations on the clonal growth of *V. natans*, as many previous studies mainly focused on root-foraging behavior and biomass allocation of aquatic clonal macrophytes in heterogeneous sediments (Xie et al. 2005; Xiao et al. 2006; Chen et al. 2017).

The shoots and roots of submerged macrophytes are able to assimilate NH_4^+ and NO_3^- (Nichols and Keeney 1976), and the main source of nitrogen for submerged macrophytes depends not only on the relative contents of NH_4^+ and NO_3^- in the sediment and the water column, but also on species specific (Schuurkes et al. 1986). But the shoots/leaves of *V. natans* that live in eutrophic lakes or NH_4^+ enrichment water prefer to take up NH_4^+, and NH_4^+ is its dominant N source (Cao et al. 2011). Unfortunately, the leaves of submerged macrophytes tend to absorb excessive NH_4^+ even far beyond the requirement of plant growth (Yuan et al. 2013). To prevent the accumulation of NH_4^+ in plant organs, *V. natans* and many macrophytes usually incorporate it into some nitrogenous compounds (mainly free amino acids (FAA)), and/or transport it out of the organs, which processes consume carbohydrates and energy (Britto and Kronzucker 2002; Cao et al. 2009). Cao et al. (2007) also found that the FAA accumulation and soluble carbohydrate (SC) depletion of *V. natans* were not caused by the NO_3^- enrichment, but by the NH_4^+ enrichment in a 48-h acute experiment, and high NH_4^+-N concentrations (> 0.56 mg L^{-1}) declined the biomass of *V. natans* in lakes of Yangtze floodplain (Cao et al. 2007). However, in a 25-day experiment, there were four NO_3^--N concentrations in overlying water (0.5, 2.5, 5, and 10 mg L^{-1}), the total biomass of *V. natans* in no epiphytic algae group slightly increased with the increasing NO_3^--N concentrations (Min et al. 2017). Therefore, high NO_3^--N enrichment does not affect the growth of *V. natans* in a short time. Excessive NH_4^+ accumulated in plant organs combined with reduction of SC and starch contents can be detrimental to the plants (Cao et al. 2004, 2007). As we know, SC and starch in plant tissues are important nonstructural carbohydrates that support the survival and growth of plants under variable environmental conditions (Huber et al. 2012). Although the NH_4^+ toxicity has been widely recognized, we wonder if and to what degree these physiological processes (i.e., FAA accumulation, SC, and starch depletion) affect the growth of submerged macrophytes. Because few studies have found significant relationships between physiological indices (i.e., FAA, SC, and starch) and growth indices and examined the differences of physiological responses to NH_4^+ stress between young ramets and mature plants.

V. natans can elongate its stolons horizontally and produce new ramets at the nodes. The survival of NS ramets and stolons is vital for clonal propagation, and their growth can reflect the clonal growth of *V. natans*. Therefore, we established six target inorganic nitrogen (IN) concentrations (i.e., control, 2.5, 4.5, 6.5, 8.5, and 10.5 mg L^{-1}) by adding NH_4NO_3 to water column to clarify the different physiological responses between NS ramets and MM (i.e., mother and mature) plants and the growth and physiological responses of stolons to the enrichment of NH_4^+. The objectives of our study were threefold: (1) to explore the clonal growth of aquatic macrophytes at high NH_4^+-N concentration, (2) to compare the physiological responses to high NH_4^+-N concentrations between young ramets and mature plants, (3) and to explore whether and to what degree the clonal growth of aquatic plants is affected by these physiological processes (i.e., FAA accumulation, SC, and starch depletion).

Materials and methods

Experimental site and plant materials

The experimental platform of Lake Donghu Ecosystem Experimental Station (30°32′53.41″N, 114°21′15.63″E) was located on the lakeside of Lake Donghu in Wuhan city, China. The seedlings of *V. natans* were collected from Lake Tuanhu, a section of Lake Donghu that was divided into several areas in the 1960s. The seedlings were cultured in two aquaria (50 × 50 × 80 cm) that contained same sediment (clay) and were filled with 50 cm tap water for 2 weeks before the experiment.

Our experiment lasted for 8 weeks from 3 July to 27 August, 2017. One hundred and eighty young plants (no ramets, stolons, and rhizomes, height: 19.22 ± 0.25 cm, fresh weight: 1.75 ± 0.05 g) were uniformly transplanted into 36 plastic boxes (30 × 21 × 10 cm, five per box) and the position of each mother plant planted was marked. Every plastic box contained 10 cm sandy sediment which was washed by tap water until the outflow was clear. Every two plastic boxes were placed in an aquarium (50 × 50 × 80 cm).

Experimental design

A gradient of six target IN concentrations (i.e., control, 2.5, 4.5, 6.5, 8.5, and 10.5 mg L^{-1}) was established with three aquaria for each treatment. The IN concentrations of the control treatment were the IN concentrations of tap water. During the experimental period, NO_3^--N, NH_4^+-N, IN, and orthophosphate (PO_4^{3-}-P) concentrations of tap water were 1.43 ± 0.06, 0.10 ± 0.01, 1.53 ± 0.07, 0.01 ± 0.002 (mean ± SE) mg L^{-1}, respectively. NH_4NO_3 fertilizer (NH_4NO_3, ≥ 98.5%, Sinopharm Chemical Reagent Co., Ltd., Shanghai) was dissolved with tap water before being poured into aquarium. We poured out, in every week, all the water of each aquarium that filled with tap water to a height of 50 cm, then NH_4NO_3 fertilizer was added into them. The amounts of NH_4NO_3 fertilizer added to the aquaria could calculate by the volume and the IN concentrations of tap water. CK, T1, T2, T3, T4, and T5 indicated the treatment of target IN concentrations (i.e., control, 2.5, 4.5, 6.5, 8.5, and 10.5 mg L^{-1}), respectively, as shown in Fig. 1. A shelter covered with black shade net was established to prevent plants from intense sunlight and provided appropriate environmental conditions for the growth of *V. natans*. Therefore, 17% of ambient light could reach aquaria's water surface, adding tap water to aquaria to maintain the target water level.

Environmental parameter analysis

During the study, water samples were collected from overlying water for the measurements of NO_3^--N, NH_4^+-N,

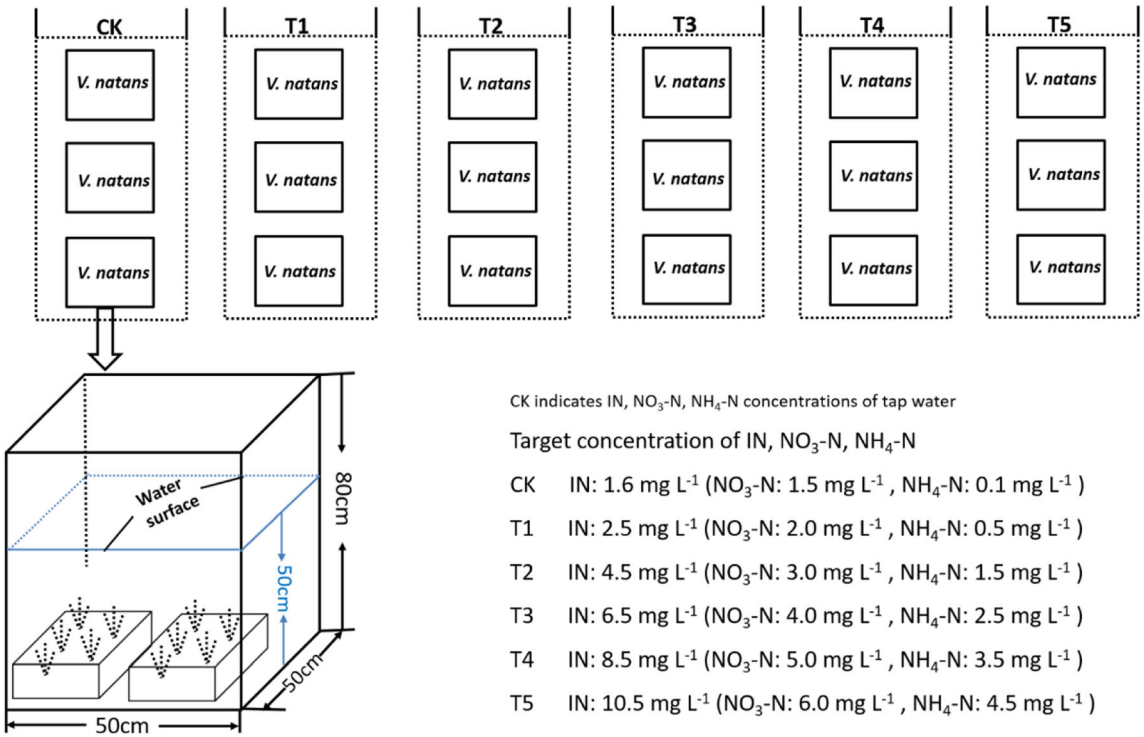

Fig. 1 Diagram showing the six treatments of target IN concentrations and *V. natans* cultured in the aquaria filled with 50 cm tap water and 10 cm sandy sediment for 56 days. CK, T1, T2, T3, T4, and T5 indicate the six treatments of target IN concentrations (i.e., control, 2.5, 4.5, 6.5, 8.5, and 10.5 mg L^{-1}), respectively

and PO_4^{3-}-P concentrations weekly. TN, total phosphate (TP), and total inorganic carbon (TIC) concentrations in the water column were analyzed every 14 days. At the 14th and 28th day after *V. natans* planted, NO_3^--N, NH_4^+-N, and PO_4^{3-}-P concentrations of pore water in the sandy sediment in each plastic box were measured by standard methods (Golterman 1969; Eaton et al. 1995). Ph, DO, T, and chlorophyll in the overlying water were measured with a YSI EXO2 (Yellow Spring Inc., USA) every 2 weeks. The Li-COR QUANTUM (Li-COR, USA) sensor was set up at the roof for the determination of photosynthetically active radiation (PAR) of ambient light that was collected by VAISALA APPK-SET110 (VAISALA, Finland) data logger and measured at twelve o'clock every day. The measurements of TN and TP of water samples were based on Chinese standard methods (Huang et al. 1999). Water samples were analyzed for the concentrations of NO_3^--N, NH_4^+-N, and PO_4^{3-}-P after filtration through GF/C filter (Whatman, Middlesex, UK). The NO_3^--N, NH_4^+-N, and PO_4^{3-}-P concentrations were determined by UV spectrophotometric method, the Nessler method, and the molybdenum blue method (Eaton et al. 1995; Golterman 1969), respectively. TIC was measured by the vario TOC analyzer (Elementar, German).

Plant harvest and measurements of growth and physiological indices of macrophytes

One marked mother plant was randomly chosen from each plastic box after they were cultured for 14, 28, and 42 days, and the rest of two mother plants were chosen at 56th day after *V. natans* were planted. The mother plants and all their ramets were carefully and gently harvested from the sandy sediment. We counted the ramets number of each mother plant, then ramets and mother plants were washed by tap water and dried with tissue paper. The mother plants and all its ramets were carefully divided into five parts which were: (1) leaves of MM (i.e., mother and mature) plants, (2) stolons of the whole plants, (3) leaves of NS (i.e., new and small) ramets, (4) roots of MM (i.e., mother and mature) plants, and (5) roots of NS (i.e., new and small) ramets. Mature plants represented that they had similar size with mother plants. For new and small ramets at the last few nodes, they were distinctly different in size when compared with mother plants. Above five parts were weighed to get fresh weight, dried to constant weight at 75 °C, followed by weighing to get the dry weight (DW). The dried plant organs were ground into fine power to determine FAA, SC, and starch contents. About 50 mg of the power was extracted twice with 5 mL 80% ethanol at 80 °C for 20 min. The extracts were pooled together and centrifuged at 10^4 g for 15 min. The measurements of FAA and SC contents in the supernatant and starch contents in the residue could refer to the methods of these articles (Yemm and Willis 1954; Yemm and Cocking 1955; Dirk et al. 1999; Cao et al. 2011).

Statistical analysis

Statistical analysis was performed using SPSS 18. Origin 8.0 was used to plot the graphs. All data were tested for normality and homogeneity of variance and non-normal data were Log_{10} (x) transformed before they were compared by one-way analysis of variance (ANOVA). The differences of pore water and overlying water chemical indices, plant growth indices, and plant physiological parameters among treatments were analyzed by ANOVA. Pearson correlation analysis was used to analyze the relationships between stolon DW and physiological parameters (i.e., FAA, SC, and starch contents) of stolons. Multiple stepwise regressions (forward procedure) were then conducted to analyze the relative importance of influencing factors according to their sequence of entering to explain variations of the stolon DW. All the indices were Log_{10} (x) transformed in the multiple stepwise regression analysis and Pearson correlation analysis.

The roots of *V. natans* contained lower SC, FAA, and starch contents than leaves and stolons; thus, data of roots were not included in this study.

Results

Environmental parameters and growth responses of *V. natans* to the various NH_4^+-N concentrations

IN concentrations in overlying water showed a significant gradient among six treatments, with mean values ranging from 0.38 to 8.1 mg L^{-1}. NH_4^+-N in the water column showed a similar variable pattern as IN, with mean values ranging between 0.04 and 2.81 mg L^{-1} (Table 1). The mean IN concentrations in pore water were much lower, ranging from 0.62 to 2.72 mg L^{-1} among six treatments (Table 1). There were no significant differences of TP in the overlying water and PO_4^{3-}-P of pore water among six treatments (Table 1). During the whole experiment, mean PAR of ambient light was 1436 ± 68 μmol m^{-2} s^{-1} (mean ± SE) at twelve o'clock every day, and the concentrations of TIC in the water column had no significant differences among six treatments. On days 14, 28, 42, and 56 of the experiment, the mean TIC concentrations (mean ± SE) of the six treatments were 9.46 ± 0.35, 13.67 ± 0.50, 17.25 ± 0.43, and 11.36 ± 0.41 mg L^{-1}, respectively.

On days 14 and 28 of the experiment, there were no significant differences of total biomass (DW), ramet number, and stolon mass (DW) of *V. natans* among six treatments. The

Table 1 The concentrations of TP, IN, NH_4^+-N, NO_3^--N, and PO_4^{3-}-P (mean ± SE; $n = 3$) in overlying water in six treatments on days 14, 28, 42, and 56 of the experiment. And the concentrations of IN, NH_4^+-N, NO_3^--N, and PO_4^{3-}-P (mean ± SE; $n = 6$) in pore water in six treatments on days 14 and 28 of the experiment. CK, T1, T2, T3, T4, T5 indicate the six treatments of target IN concentrations (i.e., control, 2.5, 4.5, 6.5, 8.5, 10.5 mg L^{-1}), respectively. Different letters indicate significant difference according to Duncan test ($p < 0.05$)

Sampling time	Water chemical indices	CK	T1	T2	T3	T4	T5
Overlying water							
14 days	TP (mg L^{-1})	0.039 ± 0.003a	0.046 ± 0a	0.049 ± 0.005a	0.044 ± 0.003a	0.041 ± 0.003a	0.041 ± 0.003a
	IN (mg L^{-1})	0.59 ± 0.067f	1.4 ± 0.11e	2.8 ± 0.16d	4.5 ± 0.11c	6.0 ± 0.18b	7.9 ± 0.10a
	NH_4^+-N (mg L^{-1})	0.04 ± 0.003d	0.05 ± 0.003d	0.19 ± 0.05d	0.86 ± 0.04c	1.22 ± 0.08b	2.19 ± 0.1a
	NO_3^--N (mg L^{-1})	0.54 ± 0.065f	1.34 ± 0.11e	2.59 ± 0.12d	3.63 ± 0.07c	4.73 ± 0.14b	5.66 ± 0.07a
	PO_4^{3-}-P (mg L^{-1})	0.0009 ± 0.0006a	0.0009 ± 0.0007a	0.0009 ± 0.0005a	0.002 ± 0a	0.002 ± 0.0007a	0.002 ± 0.0006a
28 days	TP (mg L^{-1})	0.046 ± 0.003a	0.045 ± 0a	0.054 ± 0.004a	0.051 ± 0.003a	0.049 ± 0.002a	0.052 ± 0.002a
	IN (mg L^{-1})	0.38 ± 0.064f	1.36 ± 0.072e	2.8 ± 0.049d	4.2 ± 0.093c	5.7 ± 0.13b	7.3 ± 0.11a
	NH_4^+-N (mg L^{-1})	0.13 ± 0.008d	0.09 ± 0.027d	0.23 ± 0.053d	0.49 ± 0.044c	1.08 ± 0.070b	1.58 ± 0.080a
	NO_3^--N (mg L^{-1})	0.25 ± 0.067f	1.27 ± 0.096e	2.57 ± 0.049d	3.70 ± 0.067c	4.58 ± 0.087b	5.71 ± 0.062a
	PO_4^{3-}-P (mg L^{-1})	0.004 ± 0.0006a	0.003 ± 0a	0.003 ± 0.0006a	0.003 ± 0a	0.003 ± 0.001a	0.003 ± 0a
42 days	TP (mg L^{-1})	0.02 ± 0.004a	0.02 ± 0.005a	0.02 ± 0.001a	0.02 ± 0.002a	0.03 ± 0.004a	0.03 ± 0a
	IN (mg L^{-1})	0.74 ± 0.027f	1.69 ± 0.056e	3.1 ± 0.089d	4.8 ± 0.061c	6.2 ± 0.15b	8.1 ± 0.14a
	NH_4^+-N (mg L^{-1})	0.086 ± 0.0095d	0.081 ± 0.020d	0.38 ± 0.03d	1.03 ± 0.0084c	1.71 ± 0.054b	2.81 ± 0.31a
	NO_3^--N (mg L^{-1})	0.65 ± 0.035f	1.61 ± 0.066e	2.71 ± 0.086d	3.75 ± 0.055c	4.45 ± 0.095b	5.37 ± 0.036a
	PO_4^{3-}-P (mg L^{-1})	0.004 ± 0.0001a	0.003 ± 0.0007a	0.002 ± 0.0005a	0.002 ± 0a	0.001 ± 0.001a	0.002 ± 0.0004a
56 days	TP (mg L^{-1})	0.047 ± 0.002a	0.048 ± 0.003a	0.045 ± 0.002a	0.043 ± 0.003a	0.052 ± 0.005a	0.052 ± 0.005a
	IN (mg L^{-1})	0.57 ± 0.099f	1.4 ± 0.096e	2.7 ± 0.21d	4.2 ± 0.14c	5.4 ± 0.26b	6.7 ± 0.25a
	NH_4^+-N (mg L^{-1})	0.11 ± 0.009c	0.11 ± 0.008c	0.13 ± 0.014c	0.34 ± 0.025c	0.71 ± 0.21b	1.3 ± 0.069a
	NO_3^--N (mg L^{-1})	0.46 ± 0.11f	1.31 ± 0.098e	2.56 ± 0.20d	3.91 ± 0.15c	4.65 ± 0.095b	5.34 ± 0.19a
	PO_4^{3-}-P (mg L^{-1})	0.004 ± 0.002a	0.002 ± 0.001a	0.002 ± 0a	0.002 ± 0.009a	0.001 ± 0.0006a	0.002 ± 0.0008a
Pore water							
14 days	IN (mg L^{-1})	0.62 ± 0.06c	1.12 ± 0.08c	1.91 ± 0.18b	2.03 ± 0.14b	2.65 ± 0.27a	2.72 ± 0.36a
	NH_4^+-N (mg L^{-1})	0.44 ± 0.07b	0.80 ± 0.11ab	0.90 ± 0.17a	0.98 ± 0.11a	1.00 ± 0.17a	1.07 ± 0.12a
	NO_3^--N (mg L^{-1})	0.18 ± 0.03b	0.32 ± 0.05b	1.00 ± 0.29a	1.05 ± 0.23a	1.35 ± 0.46a	1.65 ± 0.44a
	PO_4-P (mg L^{-1})	0.005 ± 0.001a	0.007 ± 0a	0.008 ± 0.001a	0.006 ± 0.001a	0.008 ± 0.001a	0.008 ± 0a
28 days	IN (mg L^{-1})	0.72 ± 0.06d	1.33 ± 0.14c	1.70 ± 0.06abc	1.51 ± 0.11bc	2.06 ± 0.30a	2.00 ± 0.27ab
	NH_4^+-N (mg L^{-1})	0.60 ± 0.07a	0.81 ± 0.20a	0.58 ± 0.20a	0.96 ± 0.16a	1.15 ± 0.41a	1.26 ± 0.11a
	NO_3^--N (mg L^{-1})	0.12 ± 0.02a	0.37 ± 0.12a	0.36 ± 0.06a	0.54 ± 0.09a	0.58 ± 0.42a	0.74 ± 0.27a
	PO_4-P (mg L^{-1})	0.005 ± 0.001a	0.008 ± 0.002a	0.02 ± 0.004a	0.01 ± 0.001a	0.01 ± 0a	0.01 ± 0.002a

average values (mean ± SE) of the six treatments for total biomass, ramet number, and stolon mass were 0.27 ± 0.02 g, 6.36 ± 0.53, and 0.04 ± 0.004 g, respectively, on day 14, and 0.33 ± 0.03 g, 10.33 ± 0.81, and 0.05 ± 0.02 g, respectively, on day 28. On days 42 and 56 of the experiment, the total biomass (Fig. 2a, b), ramet number (Fig. 2c, d) and stolon mass (Fig. 2e, f) of *V. natans* in CK and T1 treatments were similar, but showed generally declining trends with the increase of NH_4^+-N loading among T1 to T5 IN gradients. On day 56 (Fig. 2b, d, f) of the experiment, the average total biomass, ramet number, and stolon mass in the T2 (62.29%, 88.63%, 51.51%), T3 (63.74%, 79.61%, 63.86%), T4 (57.85%, 58.04%, 47.05%), and T5 (31.55%, 38.04%, 25.73%) treatments were consistently lower than the CK treatment.

The physiological responses of the leaves of NS ramets and MM plants and stolons of *V. natans* to the enrichment of NH_4^+

As for NS ramets and MM plants, FAA and SC contents in both leaves showed a similar pattern of variations among six treatments during the whole experiment. The pattern illustrated that FAA and SC contents shared similar values in the CK and T1 treatments, but showed a significant difference between CK treatment and T2, T3, T4, T5 treatments. Moreover, with the augment of NH_4^+-N concentrations, the FAA contents generally increased while the SC contents generally decreased among T1, T2, T3, T4, and T5 treatments (Fig. 3).

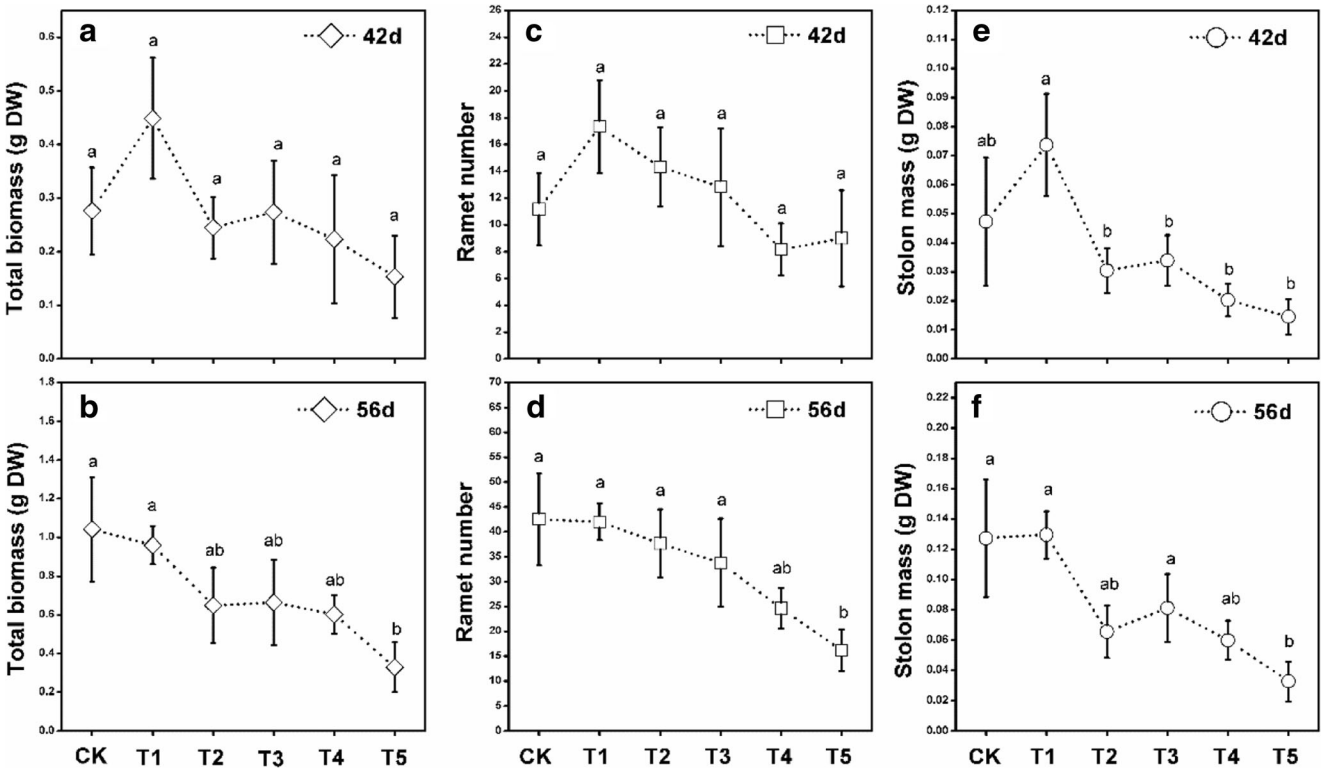

Fig. 2 Total biomass (mean ± SE) (**a**, **b**), ramet number (mean ± SE) (**c**, **d**), and stolon mass (mean ± SE) (**e**, **f**) of *V. natans* in six treatments on days 42 and 56 of the experiment. CK, T1, T2, T3, T4, T5 indicate the six treatments of target IN concentrations (i.e., control, 2.5, 4.5, 6.5, 8.5, 10.5 mg L^{-1}), respectively. Different letters indicate significant difference according to Duncan test ($p < 0.05$) ($3 \le n \le 6$, the mother plant died in some treatments and/or no ramet produced)

On days 14, 28, 42, and 56 of the experiment, FAA contents in the leaves of NS ramets were significantly higher while the SC contents were significantly lower than these indices in the leaves of MM plants for each treatment (Fig. 3). In summary, the leaves of NS ramets tended to accumulate more FAA and contain less SC than the leaves of MM plants under the conditions of NH_4^+ enrichment in the water column.

During the whole experiment, FAA, SC, and starch contents in stolons had no significant difference between CK and T1 treatments, but varied significantly between CK treatment and T2, T3, T4, T5 treatments. Similarly, with the increasing of NH_4^+ loading, these indices of the stolons showed same variational tendency compared with those changes of the leaves (Fig. 4).

The SC, FAA, and starch contents in stolons and leaves of NS ramets changed over time

SC contents in stolons and leaves of NS ramets slightly decreased with the extension of culture time, but FAA contents in both organs remained relatively stable (Fig. S2a, b, d, e). It is worth noting that the starch contents in both organs decreased distinctly along with the prolongation of the culture time (Fig. S2c, f). On day 42 of the experiment, the average starch contents in leaves of NS ramets had decreased to 2.7, 5.4, 2.9, 3.7, 0.9, 1.2 mg g^{-1} DW in the CK to T5 IN gradients, respectively. And the average starch contents in stolons had reduced to 30.3, 32.8, 7.9, 9.2, 4.8, 3.6 mg g^{-1} DW in the CK to T5 IN gradients, respectively.

Growth of stolon related to physiological indices of stolon

The results of Person correlations (Table 2) illustrated that stolon DW significantly negatively correlated with stolon FAA for the four sampling times. On days 14, 28, and 56 of the experiment, there was a significant positive correlation between stolon DW and stolon starch. In the multiple stepwise regression (Table 3), stolon DW was negatively related to stolon FAA on days 14, 28, 42, and 56 of the experiment, even when pooling data from all sampling times.

As can be seen from Fig. 5, stolon DW of *V. natans* also showed significantly declining trends with the increase of stolon FAA (Fig. 5b) for all the four sampling times. When pooling data from four sampling times, stolon DW was significantly related to all the physiological indices.

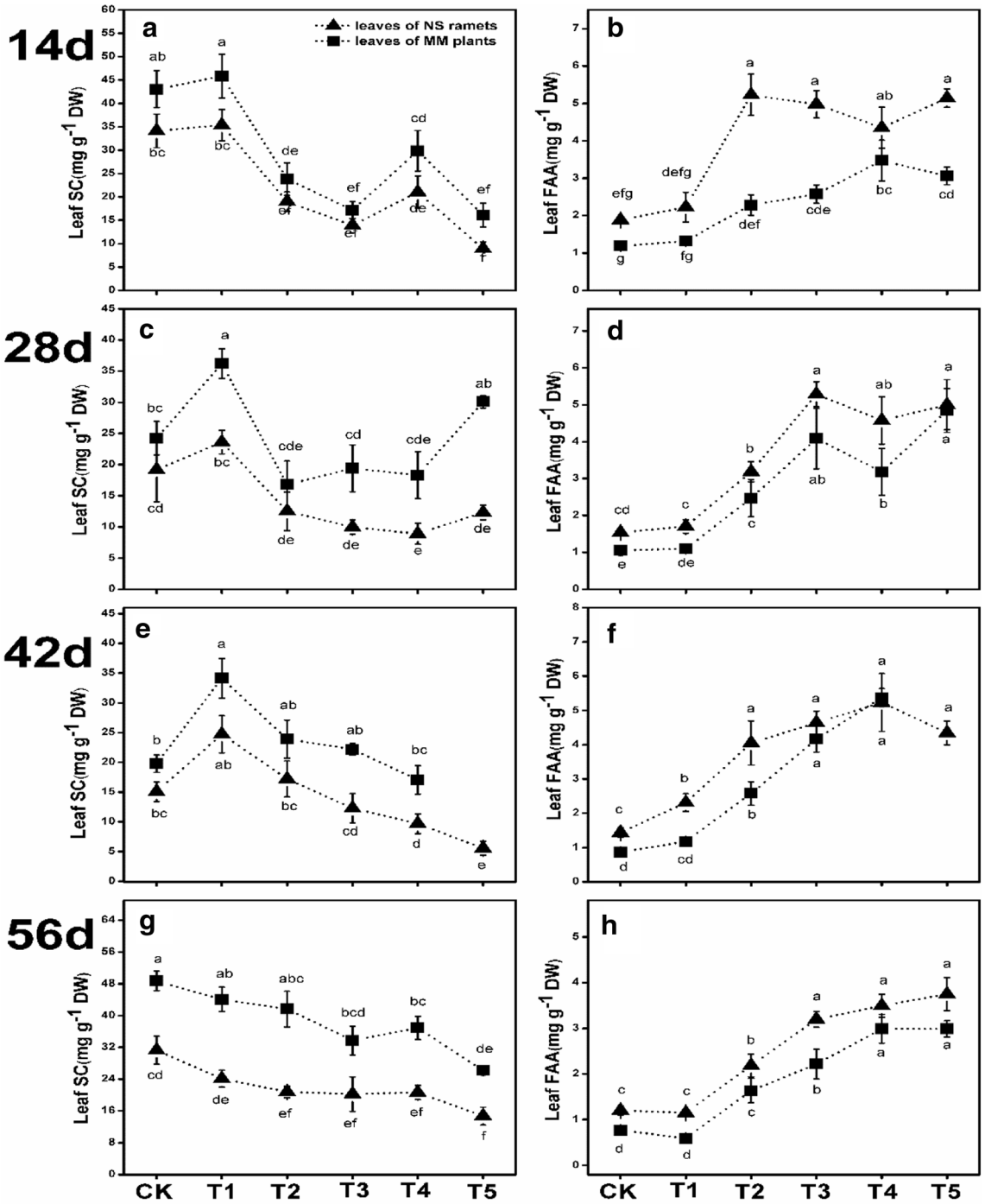

Fig. 3 On day 14 (**a**, **b**), 28 (**c**, **d**), 42 (**e**, **f**), and 56 (**g**, **h**) of the experiment, SC, and FAA contents of leaves (mean ± SE) of NS ramets and MM plants in six treatments. CK, T1, T2, T3, T4, T5 indicate the six treatments of target IN concentrations (i.e., control, 2.5, 4.5, 6.5, 8.5, 10.5 mg L^{-1}), respectively. Different letters indicate significant difference according to Duncan test ($p < 0.05$) ($3 \le n \le 6$, the mother plant died in some treatments and/or no ramet produced; just one mother plant left in T5 treatment on day 42 of the experiment)

Discussion

Our present study revealed that NH_4^+ enrichment in the water column negatively affected the clonal growth of *V. natans* such as the reduction of total biomass, ramet number, and stolon DW and resulted in physiological stress of NS ramets and stolons as indicated by the FAA accumulation, SC, and starch depletion. Remarkably, FAA accumulation and carbohydrate consumption in stolons could partially explain the decrease of stolon DW. FAA contents in leaves and stolons increased while the SC contents decreased with increasing NH_4^+-N concentrations, except for the T1 treatment. As the

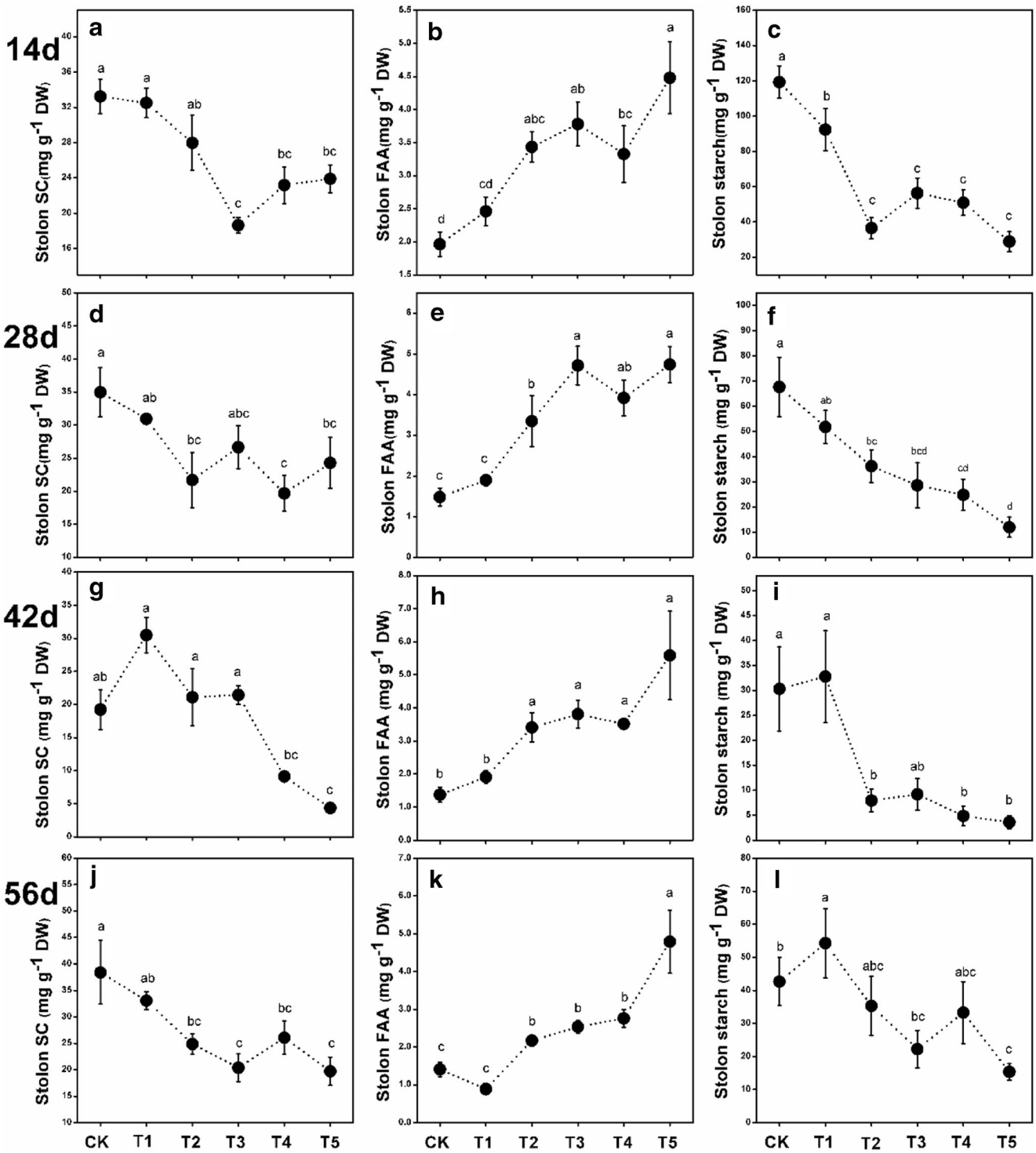

Fig. 4 On day 14 (**a**, **b**, **c**), 28 (**d**, **e**, **f**), 42 (**g**, **h**, **i**), and 56 (**j**, **k**, **l**) of the experiment, the contents of FAA, SC, and starch (mean ± SE) of stolons in six treatments. CK, T1, T2, T3, T4, T5 indicate the six treatments of target IN concentrations (i.e., control, 2.5, 4.5, 6.5, 8.5, 10.5 mg L^{-1}), respectively. Different letters indicate significant difference according to Duncan test ($p < 0.05$) ($3 \le n \le 6$, the mother plant died in some treatments and/or no stolon produced)

NH_4^+-N concentrations of the T1 treatment changed from target concentrations (0.5 mg L^{-1}) to the values (≥ 0.05 mg L^{-1}) that were similar to that of the CK treatment in a week. As depicted in Fig. 2, the growth indices of *V. natans* in the T1 treatment were similar to that of the CK treatment, which were consistent with the study that moderate NH_4^+ enrichment (0.16–0.25 mg L^{-1}) for 2 months did not affect the growth of *V. natans* (Cao et al. 2011).

Table 2 Person correlation coefficient of stolon DW and physiological indices of stolon; *significance < 0.05, **significance < 0.01. All data were Log_{10} (x) transformed ($27 \le n \le 36$, the mother plant died in some treatments and/or no stolon produced)

	Sampling time	Stolon SC	Stolon FAA	Stolon starch
Stolon DW	14 days	0.288	*− 0.658***	*0.489***
Stolon DW	28 days	− 0.126	*− 0.699***	*0.479**
Stolon DW	42 days	*0.423**	*− 0.488***	0.345
Stolon DW	56 days	*0.630***	*− 0.668***	*0.723***
Stolon DW	All data (four sampling times)	*0.357***	*− 0.642***	*0.390***

As for the physiological responses of *V. natans* to high concentration of NO_3^--N, we have already known that 10 mg L^{-1} NO_3^--N did not decrease the SC, total chlorophyll, and soluble protein contents of *V. natans* and did not increase the FAA contents in a 48-h acute and a 25-day experiment (Cao et al. 2007; Min et al. 2017). Although high concentration of NO_3^--N (> 2.5 mg L^{-1}) increased the activity of antioxidant enzyme (SOD, POD, CAT) in leaves of *V. natans*, the measurements of reactive oxygen species (ROS) were missing in the research of Min et al. (2017) (Min et al. 2017). Hence, it is needed in our future research to clarify whether high NO_3^--N concentration could induce the formation of ROS and damage the antioxidant system of *V. natans*. Moreover, if high NO_3^--N concentration could exacerbate the oxidative stress induced by ammonium, more extensive researches are required to evaluate combined toxicity of ammonium and nitrate to *V. natans*. In view of the growth of *V. natans* responding to the enrichment of NO_3^--N, the biomass of *V. natans* increased with the rise of nitrate nitrogen, even in the treatment of 10 mg L^{-1} NO_3^--N (Min et al. 2017). In the current study, the increase in antioxidant enzyme activity in leaves of *V. natans* induced by high levels of NO_3^--N did not cause decline of *V. natans* biomass. In summary, the physiological indices (FAA, SC) and growth indices of *V. natans* measured in our experiment were not affected by high NO_3^--N concentration in a short time.

Furthermore, compared with the leaves of MM plants, the leaves of NS ramets had significantly higher contents of FAA but lower contents of SC (Fig. 3), suggesting that the balance of C-N metabolism of NS ramets was easier to be disrupted. In our study, we poured out, in every week, all the water of each aquarium filled with 50 cm tap water. The overlying water contained low TP concentrations (≤ 0.054 mg L^{-1}), which ensured that *V. natans* could not be shaded by phytoplankton or periphyton. Meanwhile, in a 5-month experiment, Yu et al. (2015) found that the

Table 3 Multiple stepwise regressions of the stolon DW of *V. natans* relative to their physiological indices. F to enter is set as 0.05. All data represent the data from four sampling times. x = log_{10} (stolon SC), log_{10} (stolon FAA), and log_{10} (stolon starch) are independent variables

Sampling time			
14 days			
A:y = log_{10} (stolon mass)			
Step	R^2	P	Equation
1	0.433	< 0.001	y = − 0.964 (± 0.097) − 0.931 (± 0.191) log_{10} (stolon FAA)
28 days			
B:y = log_{10} (stolon mass)			
Step	R^2	P	Equation
1	0.489	< 0.001	y = − 1.162 (± 0.078) − 0.675 (± 0.138) log_{10} (stolon FAA)
42 days			
C:y = log_{10} (stolon mass)			
Step	R^2	P	Equation
1	0.238	0.01	y = − 1.242 (± 0.095) − 0.504 (± 0.180) log_{10} (stolon FAA)
56 days			
D: y = log_{10} (stolon mass)			
Step	R^2	P	Equation
1	0.523	< 0.001	y = − 2.506 (± 0.220) + 0.908 (± 0.151) log_{10} (stolon starch)
2	0.631	< 0.001	y = − 1.934 (± 0.272) + 0.643 (± 0.160) log_{10} (stolon starch) − 0.623 (± 0.204) log_{10} (stolon FAA)
All data			
E: y = log_{10} (stolon mass)			
Step	R^2	P	Equation
1	0.412	< 0.001	y = − 1.035 (± 0.44) − 0.819 (± 0.089) log_{10} (stolon FAA)
2	0.437	< 0.001	y = − 1.410 (± 0.169) − 0.750 (± 0.093) log_{10} (stolon FAA) + 0.252 (± 0.110) log_{10} (stolon SC)

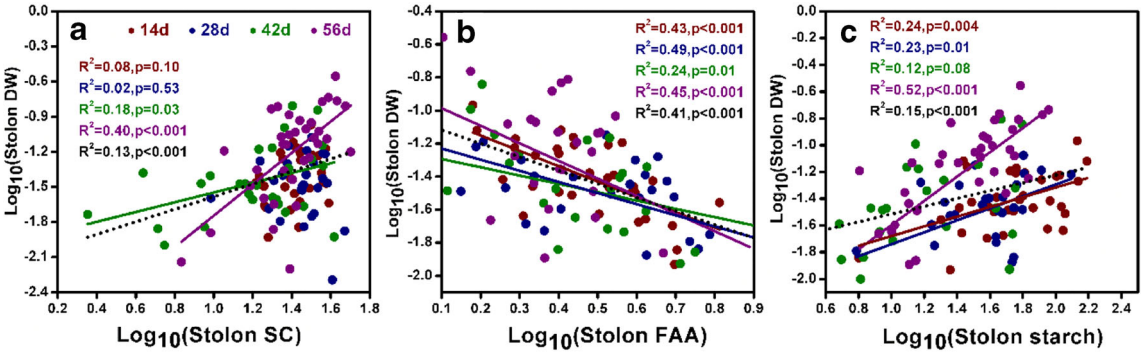

Fig. 5 Relationships between stolon DW of *V. natans* and the contents of stolon SC (**a**), FAA (**b**), starch (**c**). 14d, 28d, 42d, and 56d represent the sampling times on days 14, 28, 42, and 56 of the experiment. The dotted line indicates significant relationships of pooled data from four sampling times ($27 \le n \le 36$, the mother plant died in some treatments and/or no stolon produced)

ammonium toxic stress and phytoplankton shading were negatively affected by the growth of *V. natans*. In some bays of eutrophic lakes, it was common that the concentrations of NH_4^+-N reached mg L^{-1} magnitudes, especially in eutrophic urban lakes (Jin 2003; Ye et al. 2014). The growth and hence shading of phytoplankton promoted by nitrogen may cause the decline of macrophytes (Barker et al. 2008; Sayer et al. 2010a, b). Therefore, we concluded that the survival of NS ramets in eutrophic lakes or high NH_4^+ conditions may face more serious threat due to extra shading of phytoplankton, thus may lead to the decrease of ramet number of *V. natans*.

In the present experiment, the FAA accumulation in stolons could partially explain the decrease of stolon DW for the four sampling times, even when pooling data from all sampling times (Table 3; Fig. 5). Remarkably, many researchers had proposed that FAA accumulation in plant organs could negatively affect growth of macrophytes but without finding a significant relationship between the FAA contents and the growth indices of macrophytes (Smolders et al. 1996, 2000; Cao et al. 2004, 2007; Yu et al. 2017). The reasons why these researchers could not find such relationships were manifold, and it was no doubt that some other biochemical processes, such as induction of oxidative stress, inhibition of uptake and transport of cations, and inhibition of photosynthesis, may also be harmful to the growth of submerged macrophytes when they respond to NH_4^+ toxicity (Britto and Kronzucker 2002; Wang et al. 2008; Gong et al. 2018). This might be the cause for reduced R^2 of multiple stepwise regressions. Nevertheless, it was well known that submerged macrophytes tended to excessively synthesize FAA that was commonly used as physiological indicator at high levels of NH_4^+. Moreover, FAA accumulation is also found in many other submerged macrophytes, such as *Potamogeton crispus*, *Myriophyllum spicatum*, *Ceratophyllum demersum*, *Potamogeton pectinatus* (Cao et al. 2004; Yuan et al. 2013). Consequently, FAA may be a useful indicator of plant growth and physiological stress of macrophytes under NH_4^+ stress.

However, some studies found that NH_4^+ had no direct toxic effect on the growth of submerged macrophytes *Potamogeton lucens* and *Vallisneria spinulosa* even at high NH_4^+-N concentrations (Li et al. 2008; Olsen et al. 2015; Zhao et al. 2016). And Cao et al. (2018) proposed that species-specific characteristics of macrophytes might be a reason for this (Cao et al. 2018). Yet, it was very necessary to emphasize that the NH_4^+-N concentrations changed rapidly in the experiments of Olsen et al. (2015) and Zhao et al. (2016), because nitrogen source (NH_4NO_3) was added every 10 days. For example, in the high nitrogen (TN = 5 mg L^{-1}) treatments of these two experiments, the mean NH_4^+-N concentrations were less than 1 mg L^{-1} at the end of the tenth day and showed no significant difference with the control treatment. These results elucidated that the rapid change of NH_4^+-N concentrations might be one of the reasons for the contrasting results about NH_4^+ toxicity. In our experiment, the NH_4^+-N concentrations decreased rapidly in a week, especially dramatically in a month in the experiment of Yu et al. (2017), and we all found weak relationships between FAA contents in leaves and growth indices of *V. natans* (Fig. S1, detailed in supplementary material) (Yu et al. 2017). The leaf, as the photosynthetic organ of the plant, could produce carbohydrates by itself to modulate its growth under relatively low NH_4^+-N concentrations in the last period before the next round of nitrogen source addition, which may weaken the relationships between FAA contents in leaves and growth indices of plants. In conclusion, when we used FAA as an indicator of macrophyte growth and physiological stress of plants under NH_4^+ stress, we have to consider the following three points: (1) species-specific characteristics of macrophytes responding to NH_4^+ toxicity, (2) the rapid change of NH_4^+-N concentrations during the experiment, and (3) the ability of different organs regulating their own growth under conditions of changeable NH_4^+-N concentrations.

In the process of nitrification, NH_4^+ is first considered to be oxidized to nitrite, and subsequently to nitrate by nitrifying bacteria under aerobic condition. And the nitrifying bacteria is much more sensitive to low temperature (Guo et al. 2010).

Comparing to the temperature on the 14th (32.27 ± 0.09 °C), 28th (29.71 ± 0.07 °C), and 56th (31.15 ± 0.17 °C) days of the experiment, lower temperature (26.94 ± 0.02 °C) might be a reason why NH_4^+-N concentrations showed a maximum value in T2, T3, T4, T5 treatments on day 42 of the experiment. It was certain that higher NH_4^+-N concentration inhibited the accumulation of nonstructural carbohydrate more severely (Gao et al. 2015; Apudo et al. 2016). However, lower starch content in leaf and stolon of *V. natans* on day 42 of the experiment was caused not only by higher NH_4^+-N concentration, but also by accumulative effect of continuous ammonium stress. As the starch content in leaf and stolon decreased with the extension of culture time (Fig. S2), even the NH_4^+-N concentration was much lower on day 28 of the experiment. Therefore, high NH_4^+-N concentration on day 42 of the experiment may not be a trigger for the subsequent decline of growth indices of *V. natans* owing to no obvious physiological changes for the FAA and SC between these three sampling times (Fig. S2).

As can be seen from Fig. S2, the starch contents in leaves and stolons were relatively high at the beginning of the experiment. As the favorable environment for the growth of macrophytes led to an increase of nonstructural carbohydrates in the plants at early developmental stage, and the magnitude of the plants responding to different environment triggers may depend on carbohydrates accumulated previously (Groeneveld and Voesenek 2003; Huber et al. 2012). These results represented that after pre-culture for 2 weeks, starch accumulated in this period served as a source of energy to support carbohydrate and energy-demanding processes during detoxification of NH_4^+ under conditions of high NH_4^+-N concentrations on the 14th and 28th day of our experiment, which was further confirmed by the results of Person correlations that showed starch in stolons positively related to stolon DW (Table 2). At the end of the experiment, the SC and starch contents in stolons were so low, and Cao et al. (2007) found that low contents of carbohydrate in rhizomes of *V. natans* may result in their propagation failure at high levels of NH_4^+ (Cao et al. 2007). These results indicated that low contents of carbohydrate in plant tissues, especially in stolons and rhizomes, may affect the clonal growth of aquatic plants at high NH_4^+-N concentrations.

Conclusion

Our results clearly elucidated that the clonal growth of aquatic macrophytes was inhibited by high NH_4^+-N concentration. The growth of NS ramets and stolons was key to clonal growth of aquatic plants, and their imbalance of C-N metabolism partially explained the decline of submerged clonal macrophytes like *V. natans* at high NH_4^+-N concentrations. And the young ramets were more vulnerable to NH_4^+ stress owing to extreme imbalance of C-N metabolism, so we need to pay more attention to seedling stages of macrophytes in eutrophic lakes. This gives us insight about the common declines of submerged macrophytes in eutrophic lakes. Moreover, FAA may be a useful indicator of physiological stress of macrophytes and plant growth under high NH_4^+ stress, as the relationship between FAA in stolon and stolon DW of plants was significant.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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