DISTINGUISHED RESPONSES OF CURLED PONDWEED (*POTAMOGETON CRISPUS* L.) AND HORNWORT (*CERATOPHYLLUM DEMERSUM* L.) TO NH₄-N STRESS UNDER ELEVTED HCO₃⁻ CONDITIONS

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Abstract. In meso-eutrophic water, submerged plants responses to increased NH₄-N content by altering their free amino acid (FAA) and soluble sugar (SC). Both *Potamogeton crispus* L. and *Ceratophyllum demersum* L. are dominant species in meso-eutrophic waters. Both species can use HCO₃⁻ as an inorganic carbon source, but the differences in their affinities to HCO₃⁻ is still unclear. In this paper, under different levels of HCO₃⁻ and NH₄-N conditons, the changes in solution pH ($\triangle$pH), FAA and SC content of both species were measured to analyze the role of HCO₃⁻ utilization preference in the response of submerged plants to NH₄-N stress. Results revealed additional NH₄-N significantly increase solution $\triangle$pH, and increase FAA content and reduce SC content of both species. Additional HCO₃⁻ significantly reduced solution $\triangle$pH of both species, while only increased SC content of *P. crispus*. Due to the different HCO₃⁻ affinities of the two species, solution $\triangle$pH of *P. crispus* was lower than that of *C. demersum*. Since low $\triangle$pH decreased the ratio of NH₃ and NH₄⁺, additional HCO₃⁻ consequently alleviated the toxicity of NH₄-N to *P. crispus*. This may be one of the reasons why *P. crispu* is more tolerant to ammonia stress than *C. demersum* in nutrient-rich waters.
Keywords: *meso-eutrophic, submerged plants, NH₄-N toxicity, carbon and nitrogen metabolism, inorganic carbon source*

Introduction

Eutrophication has led to the degradation of many submerged vegetation in the world. NH₄-N toxicity is one of the important causes of submerged vegetation degradation because changes in environmental nutrient concentrations have a negative impact on the carbon and nitrogen (C-N) metabolism of submerged plants (Smolders et al., 2000, 2013). It is well known that high concentration levels above 1.0 mg L⁻¹ NH₄-N can be toxic to submerged plants (e.g. Cao et al., 2004, 2007, 2009a,b, 2011; Zhang et al., 2010, 2011). In eutrophic water, elevated NH₄-N concentration stresses the metabolism C-N of submerged plants. To alleviate the toxicity of NH₄-N, most plants reduce their accumulation in plants or transfer them out of plant cells by converting NH₄⁺ to free amino acids (FAA) and organic amines (Zhang et al., 2010). Among these response indicators, the importance of solution pH needs to be particularly emphasized, because solution pH adjusts the ratio of NH₃ and NH₄⁺, thereby affecting the NH₃ content in the plant (Heide et al., 2008). Soluble sugar (SC) is an important intermediate metabolite that links photosynthesis, respiration and carbohydrate storage, and is also a substrate for plant

ammonia assimilation. Therefore, changes in FAA/SC can reflect the effects of ammonia-nitrogen stress on carbon and nitrogen metabolism in plants (Zhang et al., 2011). Different submerged plants have different tolerance to NH$_4$-N stress, and some submerged species had higher NH$_4$-N tolerance than others (e.g. Cao et al., 2004, 2011).

Based on the specificity of the water environment, light and dissolved inorganic carbon (DIC) and O$_2$ are the most important factors limiting the photosynthesis rate and yield of submerged plants. A large number of field and laboratory studies have shown that HCO$_3^-$ utilization is an adaptive mechanism for many submerged plant species (Maberly and Madsen, 1998), but not all types (Iversen et al., 2019). Based on the different preferences of carbon source forms, the researchers divided the submerged large plants into three categories: HCO$_3^-$ utilization species using CO$_2$ and HCO$_3^-$ as carbon sources (Pronin et al., 2019); CO$_2$ utilization types can utilize CO$_2$ in water column and CO$_2$ in interstitial water (Wium-Andersen, 2010; Hussner et al., 2019); water column CO$_2$ utilization type can only use CO$_2$ in water column (Maberly and Madsen, 1998; Cao and Ruan, 2015). On this basis, the changes in water DIC content significantly affected the growth of different submerged plants and (e.g. Pagano and Titus, 2004) and inter-specific competition for HCO$_3^-$ (Van der berg et al., 2002). Because it is an important geological and geological history process that determines the distribution of lakes and rivers and the concentration of bicarbonate in freshwater ecosystems (Lauerwald et al., 2013; Marcé et al., 2015; Iversen et al., 2019), they are the chief determinants of plant distributions in fresh waters. The increase in bicarbonate (HCO$_3^-$) concentration have a significant impact on lakes lacking bicarbonate, because increased bicarbonate concentration significantly changed the species composition (Vestergaard and Sand-Jensen, 2000; Iversen et al., 2019).

Both *P. crispus* L. and *C. demersum* L. can uses HCO$_3^-$ as an inorganic carbon source (Iversen et al., 2019). Since the pH controls the DIC composition of the water (Wetzel, 2001), the affinities of submerged species to HCO$_3^-$ might be indicated by solution pH where plant grows. Furthermore, species with high affinities to HCO$_3^-$ usually outcompete in high solution pH and low HCO$_3^-$ concentration (Van der berg et al., 2002). Additional HCO$_3^-$ could alleviate the toxicity of NH$_4$-N on *Chara vulgaris* L. by decreased solution $\triangle$pH (Wang et al., 2013). In this study, NH$_4$-N and HCO$_3^-$ were added to the culture solution, and the pH of the solution, the FAA and SC content of the plant were tested to examine the interactive effect of the addition of HCO$_3^-$ and NH$_4$-N on *P. crispus* and *C. demersum*. We made the following assumptions: (1) the addition of NH$_4$-N increase the FAA and reduce the SC of plants; (2) with the addition of HCO$_3^-$, two species respond to NH$_4$-N stress differently.

Method and materials

Plant culture

P. crispus L. and *C. demersum* L. are submerged plants widely distributed throughout China and are often used for ecological restoration of eutrophic lakes (Xie and Yu, 2011; Dai et al., 2012). In the present study, *P. crispus* and *C. demersum* were collected from a depth of 1–1.5 m of Liangzi Lake (30° 05′ N–30° 18′ N, 114° 21′ E–114° 39′ E), a typical shallow lake located in the middle reach of the Yangtze River. These specimens were transplanted into a laboratory aquarium. These aquariums are filled with natural water and sediment from the Liangzi Lake. A month later, these branches developed to the surface of the water, growing well. More than 120 apical tips (weight 1.3 g-1.4 g) with

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three or four internodes (about 4–5 cm) were cut using scissors and pre-incubated in 4 1.0 L glass beakers filled with plant containing water (PCW). PCW was gotten by filtering water from those square aquaria through GF/C membranes, with dissolved inorganic carbon (DIC) 25 ppm and while NH$_4$-N undetectable). And all these beakers were put in the plant growth incubator, with a temperature of 25 ± 2 °C, an illumination at the water surface of 60 μmol m^{-2} s^{-1} and a light photoperiod of 12:12 (Light: Dark). After two weeks of pre-culture, all apical tips were moved to 1 L beakers with ultrapure water for 6 h under the same culture condition to remove the inorganic carbon accumulated inside the plants (Kahara and Vermaat, 2003).

Experimental design

It was a factorial design of 2×2 of dissolved inorganic carbon (DIC) and NH$_4$-N (NH$_4$-N). NH$_4$-N included 2 levels: low level (without additional NH$_4$-N) and high level (additional 1.0 mg L^{-1} NH$_4$-N as NH$_4$Cl); and HCO$_3^-$ level also included two levels: low level (without addition HCO$_3^-$) and high level (additional 50 ppm HCO$_3^-$ as NaHCO$_3$). Therefore, four treatments were recorded as CK (no addition), +N (additional 1.0 mg L^{-1} NH$_4$-N), +C (additional 50 ppm HCO$_3^-$) and +C+N (additional 1.0 mg L^{-1} NH$_4$-N and 50 ppm HCO$_3^-$). Under every treatment conditions, four flasks with plants were used to assess the difference of treatments and two flasks without plants were used to evaluate the changes of solution with time. In consequence, forty-eight 1000 mL beakers were placed in the plant incubator with an illumination at the water surface of 60 μmol (m^2 s)$^{-1}$ and with a 12:12 photoperiod under temperature condition of 25 ± 2 °C. The plants were transferred to 48 beakers with 800 mL of PCW at the beginning of the light. The pH of these solutions was adjusted to 8.2 by 0.1 M HCl or 0.1 M NaOH before moving to these beakers. And the culture medium is replenished daily at the beginning of the light period. The plants were washed with ultrapure water before transplanting to the new solution. After eight days, all the plants were harvested.

Experimental sampling and measurement

The pH of these solutions was measured daily using a pH meter (METTLER TOLEDO SevenCompact S210) at 12th and 24th hour of photoperiod. The drift of pH value was obtained by subtracting the average pH of the control groups from the pH of the experimental groups. The soluble sugar of the plants was determined by the Anthrone-sulfuric acid colorimetric method (Yemm and Willis, 1954), while the free amino acid of the sample was determined by the ninhydrin method (Yemm and Cocking, 1955); alanine and glucose were used as the standards.

Analysis

The drift of pH value of *P. crispus* and *C. demersum* was analyzed by three-way analysis of ANOVA with days, NH$_4$-N and HCO$_3^-$ as fixed factors While solution △pH was analyzed by three-way analysis of ANOVA with species, NH$_4$-N and HCO$_3^-$ as fixed factors. The difference between each treatment is obtained by t-test. The two-way analysis of ANOVA was used to analyze the impact of additional NH$_4$-N and HCO$_3^-$ on the FAA, SC and FAA/SC of each species. All data were analyzed with SPSS20.

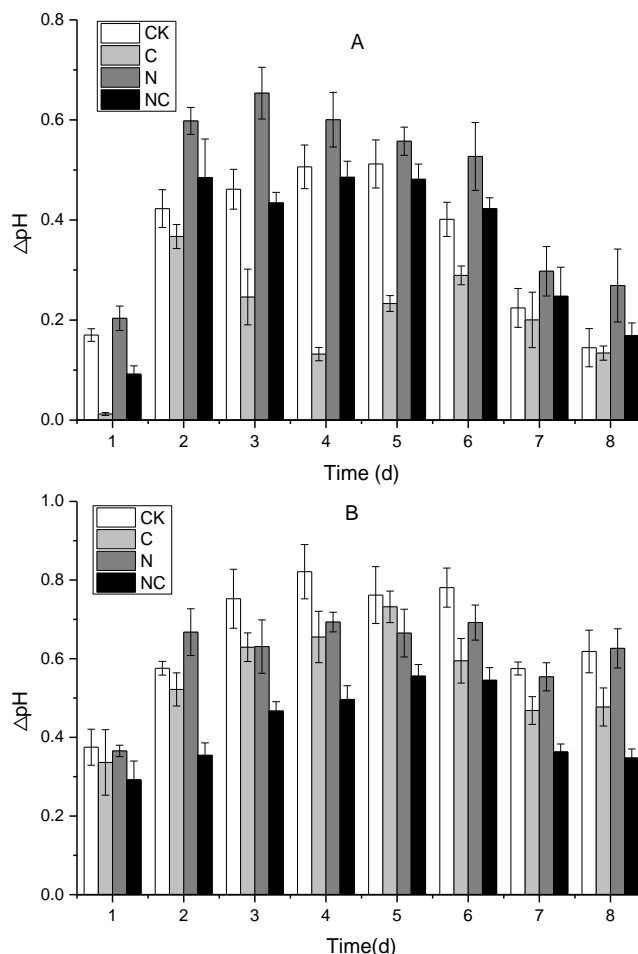Jin et al.: Distinguished responses of curled pondweed (*Potamogeton crispus* L.) and hornwort (*Ceratophyllum demersum* L.) to
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Results

The variation of solution pH

The three-way analysis of ANOVA with time, NH$_4$-N (+N), and HCO$_3^-$ (+C) as the influencing factors showed that time, the addition of NH$_4$-N and HCO$_3^-$ significantly affected the pH drift at the end of plant photosynthesis throughout the experiment. Plant photosynthesis can significantly increase the pH value of the solution. pH with *P. crispus* at 12th hour varied from 8.5 to 9.5, while solution pH of with *C. demersum* was from 8.7 to 9.6 (*Fig. 1*). At 24th hour of phothoperiod, solution pH with *P. crispus* ranged from 8.3 to 9.0, and solution pH with *C. demersum* ranged of from 8.6 to 9.2 (*Fig. 2*). pH with both *P. crispus* and *C. demersum* at 12th hour, the addition of NH$_4$-N significantly promotes the increase of pH ($\triangle$pH), and the addition of HCO$_3^-$ significantly reduced $\triangle$pH. Significant negative interaction on solution $\triangle$pH existed between +N and +C (*Figs. 1A* and *2A*, *Tables 1* and *2*). Three-way ANOVA with species, NH$_4$-N (+N), and HCO$_3^-$(+C) as the influencing factors revealed that species have a significant effect on $\triangle$pH. Among them, the addition of HCO$_3^-$ and the species are the two most important factors affecting $\triangle$pH. On the whole, the $\triangle$pH of *C. demersum* is higher than that of *P. crispus* (*Figs. 1* and *2*, *Tables 1, 2* and *3*).
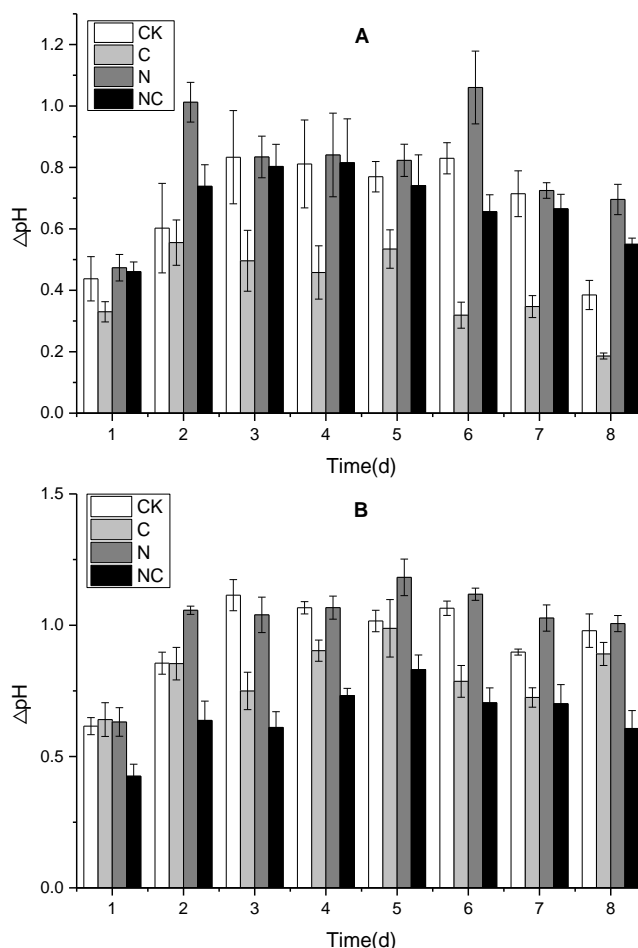
Figure 1. Temporal changes of pH drift of solution with (A) P. crispus and (B) C. demersum at 12th hours of photoperiod (The error bars in the figure is expressed by standard deviation, which is the mean of the distance of each data from the mean)

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Figure 2. *Temporal changes of pH drift of solution with (A) P. crispus and (B) C. demersum at 24th hours of photoperiod. (The error bars in the figure is expressed by standard deviation, which is the mean of the distance of each data from the mean)*

Table 1. *Results of the three-way ANOVA models for the effects of day (d.), additional NH$_4$-N (N.), additional HCO$_3^-$ (C.) and their interaction on the solution △pH at 12th hour of photoperiod. Significant effects (p < 0.05) are indicated in bold*

	P. crispus △pH			*C. demersum* △pH		
	d.f.	F	P	d.f.	F	P
d	7	31.166	**<0.001**	7	65.282	**<0.001**
N	1	154.198	**<0.001**	1	18.977	**<0.001**
d*N	7	3.717	**0.001**	7	4.359	**<0.001**
C	1	145.591	**<0.001**	1	500.149	**<0.001**
d*C	7	5.912	**<0.001**	7	8.97	**<0.001**
N*C	1	17.994	**<0.001**	1	104.548	**<0.001**
d*N*C	7	3.862	**0.001**	7	3.521	**0.002**
Error.(model)	96			96		

Table 2. *Results of the three-way ANOVA models for the effects of day (d.), additional NH$_4$-N (N.), additional HCO$_3^-$(C.) and their interaction on the solution △pH at 24th of photoperiod. Significant effects (p < 0.05) are indicated in bold*

	P. crispus △pH			*C. demersum* △pH		
	d.f.	F	P	d.f.	F	P
d	7	134.6	**<0.001**	7	69.942	**<0.001**
N	1	232.13	**<0.001**	1	76.231	**<0.001**
d*N	7	5.022	**<0.001**	7	3.05	**0.006**
C	1	212.7	**<0.001**	1	221.808	**<0.001**
d*C	7	9.471	**<0.001**	7	3.975	**0.001**
N*C	1	3.606	0.061	1	16.266	**<0.001**
d*N*C	7	6.497	**<0.001**	7	2.628	**0.016**
Error.(model)	96			96		

Table 3. *Results of the three-way ANOVA models for the effects of species (S.), additional NH$_4$-N (N.), additional HCO$_3^-$(C.) and their interaction on the solution △pH at 12th hour of light photoperiod. Significant effects (p < 0.05) are indicated in bold*
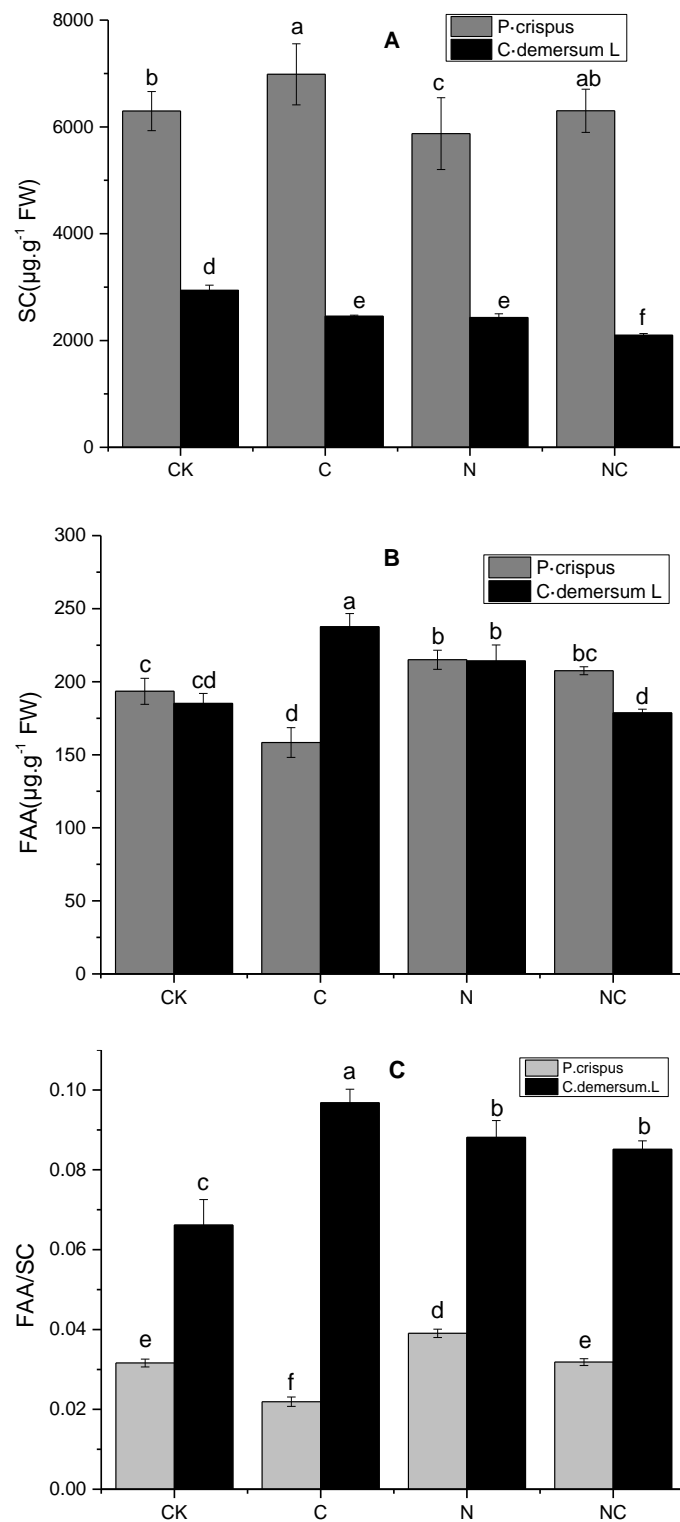
	d.f.	**F**	**P**
S	1	34.789	**<0.001**
N	1	4.4	**0.040**
S*N	1	11.461	**0.001**
C	1	35.886	**<0.001**
S*C	1	0.412	0.524
N*C	1	0.33	0.568
S*N*C	1	6.002	**0.017**
Error.(model)	56		

Impact of treatments on content of SC and FAA

The two-way analysis of ANOVA with +N and +C as the influencing factors showed that the addition of NH$_4$-N and HCO$_3^-$ had significant effects on the SC content of the two plants. The addition of NH$_4$-N significantly reduced the SC content of both species. However, the addition of HCO$_3^-$ significantly increased the SC content of *P. crispus*, while reduced the SC content of *C. demersum* (*Fig. 3A, Table 4*).

The addition of NH$_4$-N significantly increased the FAA content of both species, while the addition of HCO$_3^-$ significantly reduced the FAA content of *P. crispus*.

For these two plants, the addition of NH$_4^+$ significantly increased the FAA/SC of the two plants, while the addition of HCO$_3^-$ had different effects on the two plants. The addition of HCO$_3^-$ significantly reduced the FAA/SC of *P. crispus* while significantly increased the FAA/SC of *C. demersum* (*Fig. 3C, Table 4*).

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Figure 3. *SC (A) and FAA (B) contents and FAA/SC(C) of two plants under four different treatment. Different letters indicate significant differences between treatments. (The error bars in the figure is expressed by standard deviation, which is the mean of the distance of each data from the mean. The difference between each treatment is obtained by t-test)*

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Table 4. *Results of the two-way ANOVA models for the effects of four different treatments: N (additional NH_4-N), C (additional HCO_3^-), CN (additional NH_4-N and HCO_3^-). Their interaction on the SC, FAA and FAA/SC. Significant effects ($p < 0.05$) are indicated in bold*

			C	N	CN	Error
		df	1	1	1	12
	SC	F	5.82	5.75	1.86	
		P	**0.03**	**0.03**	0.2	
		df	1	1	1	12
P. crispus	FAA	F	23.3	64.3	9.78	
		P	**<0.001**	**<0.001**	**0.01**	
		df	1	1	1	12
	FAA/SC	F	21	26.6	2.27	
		P	**<0.001**	**<0.001**	0.16	
		df	1	1	1	12
	SC	F	129.58	145.44	5.74	
		P	**<0.001**	**<0.001**	**0.034**	
		df	1	1	1	12
C. demersum	FAA	F	3.35	10.51	91.135	
		P	0.92	**0.01**	**<0.001**	
		df	1	1	1	12
	FAA/SC	F	62.44	11.93	89.212	
		P	**<0.001**	**0.01**	**<0.001**	

Discussion

The general mechanism of submerged plants in response to NH_4-N stress

NHx is both valuable nutrient source and a toxic substance for freshwater plant species (Britto and Kronzucker, 2002). High NH_4-N concentration can inhibit photosynthesis, trigger oxidative stress, and cause carbon and nitrogen imbalance in submerged plants (Cao et al., 2004; Rudolph and Voigt, 2010; Zhang et al., 2010). Generally, at higher concentrations of NH_4-H, plants passively absorb NH_4-N (Day et al., 2017). To prevent NH_4-N from accumulating in plant tissues, plants actively transport NH_4-N from plant cells, and energy is consumed in the process (Britto and Kronzucker, 2002). Since FAA synthesis can also reduce the accumulation of NH_4-N at the expense of SC (Rare, 2015), so a high FAA/SC ratio can indicate that NH_4-N has a serious interference effect on plant carbon and nitrogen metabolism. In many experimental studies and field surveys, high concentrations of NH_4-N had increased the FAA content of many large submerged plants (e.g. *Vallisneria natans*, *Potamogeton maackianus Myriophyllum spicatum*, *Hydrilla verticillata*) and reduce their SC content (Cao et al., 2004, 2009a,b, 2011; Li et al., 2007; Zhang et al., 2010). In this experiment, the FAA content in both species increased significantly due to the addition of 1.0 mg L^{-1} of NH_4-N, and the SC content decreased significantly. This shows that 1.0 mg L^{-1} of NH_4-N has a stress effect on *P. crispus* and *C. demersum*. This conformed to our assumption 1. Similar results had been reported by Cao et al. (2009a,b). Furthermore, under the stress of 1 mg L^{-1} NH_4-N, the values of FAA and SC of different plants are different. The FAA and SC of *P maackianus* was

0.95 mg g⁻¹ DW and 61 mg g⁻¹ DW, respectively (Li et al., 2007). While the FAA and SC of *V. natans* was 6 mg g⁻¹ DW and 15 mg g⁻¹ DW, respectively (Cao et al., 2009a). The FAA of *P. crispus* was 0.5 mg g⁻¹ FW, SC is 10mg g⁻¹ FW (Cao et al., 2004). In the study, the FAA and SC of *P. crispus* was 0.2 mg g⁻¹ FW and 6 mg g⁻¹ FW, respectively. The lower value of FAA and SC might contribute to their distinguished grow conditions. Furthermore, two species exhibited different SC content under four different treatments (*Fig. 3*). This might mainly be ascribed to these species itself (Cao et al., 2004, 2009a,b, 2011).

Mechanism of HCO₃⁻ addition to buffer NH₄-N stress

The water body pH controls the DIC composition of the water body. When pH is less than 6.4, CO_2 is dominant; when pH is greater than 6.4 and 8.3, HCO_3^- is dominant; when pH is greater than 8.3, CO_3^{2-} is dominant. Based on this, the pH drift experiment is used to measure photosynthesis, and its drift value is used to measure the HCO_3^- utilization capacity and carbon enrichment capacity of the species (Kahara and Vermaat, 2003; Pierini and Thomaz, 2004). When the pH of the solution rises, NH₄-N ions will be converted into toxic gas NH_3 (Zhou et al., 2017). The suggested solution pH can control toxic gas NH_3 concentration. High solution pH indicated high toxic gas NH_3 concentration and low HCO_3^- concentration. In this study, the increase of solution pH of both species was significantly reduced in the +C treatment (*Figs. 1, 2, Tables 1, 2*), which confirmed the buffering effect of the addition of HCO_3^- on the increase of the solution pH. This is consistent with the results of Wang et al. (2013). For both *P. crispus* and *C. demersum* in the group added with NH₄-N and HCO_3^-, the accumulation of FAA in plant tissues was also significantly reduced (*Fig. 3, Table 4*). A possible reason is that the addition of HCO_3^- decreased toxic gas NH_3 concentration by buffering solution pH. When pH>8.2, high pH indicated low HCO_3^- concentration. In the present study, solution pH with *C. demersum* is above that with *P. crispus*. This suggesting lower HCO_3^- concentration in the solution with *C. demersum*. Therefore, the distinguished responses of two species to the addition of HCO_3^- (*Fig. 3, Table 4*) suggested different utilization to low HCO_3^- concentration when solution pH increase. This may partly explain the difference of SC content to the addition of HCO_3^-. As the consequence, additional HCO_3^- only alleviated the NH₄-N toxicity on *P. crispus*. This conformed to our assumption 2.

Inorganic carbon utilization preference of submerged plants and its influence on plant competitive advantage

Inorganic carbon may limit photosynthesis in aquatic systems because carbon dioxide diffuses 10^4 times faster in the air than in water. Therefore, the concentration of carbon dioxide required by plants in water to saturate photosynthesis is as high as 12 times the equilibrium concentration of air (Cao and Ruan, 2015). In addition, rapid photosynthesis will make the carbon dioxide content in water much lower than air saturation (Madsen et al., 2010). To cope with the carbon limitation, some aquatic angiosperms have evolved the same carbon dioxide enrichment mechanism as their terrestrial ancestors, but the most common mechanism is the extraction of bicarbonate (HCO_3^-) (Madsen et al., 2010; Cao and Ruan, 2015).

The assimilation and absorption capacity of some freshwater species far exceeds the content of dissolved inorganic carbon (DIC) in their living environment (Maberly and Madsen, 1998). This leads to that DIC may become a limiting factor for photosynthesis and even growth of other aquatic species (Van den Berg et al., 2002; Maberly and

Gontero, 2017). The DIC content and composition in the water affected the growth of submerged plants, the competition and distribution of plants, and then affect the community composition (Pagano and Titus, 2004, 2010). Since the pH controls the DIC composition of the water (Wetzel, 2001). Therefore, the pH drift value can be used to measure the ability of a species to utilize HCO_3^- in a close system (Kahara and Vermaat, 2003; Pierini and Thomaz, 2004). In the present study, additional HCO_3^- decreased solution $\triangle$pH of two submerged plant species. Comparing solution $\triangle$pH with plants, the overall $\triangle$pH with *P. crispus* was lower than that with *C. demersum*, which was controlled below pH 9.5. It is more conducive to *P. crispus* to deal with the toxicity caused by NH_4-N. This may be one of the reason why *P. crispu* is more tolerant to ammonia stress than *C. demersum* in nutrient-rich waters.

Conclusion

In the case where the inorganic carbon in the plant is exhausted, a factorial design of 2×2 is carried out by low or high level dissolve inorganic carbon (DIC) and NH_4-N (NH_4-N). During the 7-day test period, the addition of 1.0mg L^{-1} of NH_4-N significantly increased the FAA of *P. crispus* and *C. demersum*, and decreased the SC content, which made the FAA/SC increase. Different affinities for HCO_3^- of two plants resulted in different solution $\triangle$pH, which caused different toxic gas NH_3 concentration. This lead to distinguished responses to 1.0 mg L^{-1} NH_4-N of *P. crispus* and *C. demersum* under additional HCO_3^- conditions. Since plant calcification can alleviatd the increase of solution pH under additional HCO_3^- conditions, future studies will focus on the comparative responses of calcified plants and non-calcified plants to NH_4-N stress of more gradients under more different HCO_3^- levels.

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