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# Time-Dependent Oxidative Stress Responses of Submerged Macrophyte *Vallisneria natans* Seedlings Exposed to Ammonia in Combination with Microcystin Under Laboratory Conditions

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**Abstract** We studied the antioxidant responses of macrophyte *Vallisnerria natans* seedlings to combined ammonia (0, 0.21, and 0.85 mg $L^{-1}$) and microcystin-LR (MC-LR) (0, 10, and 50 μg $L^{-1}$) for 7 days. Results showed that superoxide dismutase, catalase (CAT), peroxidase, and glutathione were significantly induced by the mixtures of ammonia and MC-LR, and there were significant interactions between ammonia and MC-LR. Specially, CAT increased about fivefold at ammonia 0.85 mg $L^{-1}$ and MC-LR 50 μg $L^{-1}$ on day 3. Malondialdehyde fluctuated with both ammonia and MC-LR, and significant interactions were detected between the two stressors. Changes in all the measured variables were time-dependent.

**Keywords** Ammonia · Microcystin · Oxidative stress · *Vallisneria natans*

Toxic cyanobacterial blooms occur worldwide in eutrophic lakes and reservoirs and have harmful influences on aquatic ecosystems (Codd 1995). In order to reduce cyanobacterial blooms, nutrient removal from eutrophic waters is an promising way; therefore, restoring submerged macrophytes has received much attention in recent years (Hilt et al. 2006), as aquatic macrophytes have an outstanding ability to assimilate nutrients and to purify water.

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However, before these macrophytes can be applied on a large scale, their ability to survive in the harsh aquatic environment has to be assessed. Senescence of the cyanobacteria along with cyanobacterial decomposition causes rapid decay and cell lysis leading to the release of toxins into the water. The most common toxins produced by cyanobacteria are the cyclic heptapeptide hepatotoxins called microcystins, especially the variant microcystin-LR (MC-LR) among more than 80 microcystin variants (Dittmann and Wiegand 2006). Microcystins induce oxidative stress and inhibit protein phosphatases, which is considered to be the main mechanism of its toxicity (MacKintosh et al. 1990; Ortiz-Rodríguez and Wiegand 2010). Because microcystins are extremely stable and resist common chemical breakdown such as hydrolysis or oxidation under conditions found in most natural water bodies (Duy et al. 2000), the released toxins could then come into contact with a wide range of aquatic organisms including invertebrates, fish, and aquatic plants (Pflugmacher 2004; Wiegand and Pflugmacher 2005). Furthermore, during the degradation of cyanobacterial blooms, elevated concentrations of ammonia usually occur in the eutrophic lakes. It is well known that high ammonia levels inhibit photosynthesis, trigger oxidative stress and cause internal carbon–nitrogen imbalance in submersed macrophytes (Rudolph and Voigt 1986; Cao et al. 2009), although it is also an important nitrogen source for plant growth. The mixtures of the two prevailing toxicants, ammonia and microcystin, released from the process of degradation of cyanobacterial blooms may impact the growth of macrophytes in blooming waters.

Although there are several studies on oxidative stress responses in submerged macrophyte to ammonia (Nimptsch and Pflugmacher 2007; Wang and Zhang 2008) and microcystin (Pflugmacher et al. 1997; Pflugmacher 2004; Jiang

et al. 2011) separately, little is known about the responses of the antioxidant activities in the submerged macrophytes to the mixtures of ammonia and MC-LR. Here, we focused on the combined effects of the two main toxins, dissolved ammonia and dissolved MC-LR that arise as a result of cyanobacterial bloom decay, a process that seriously impact fresh waters. In the experiments, therefore, we used submerged macrophyte *Vallisnerria natans*, an ecologically important species widely used in nutrient removal in eutrophic waters (Xiao et al. 2007), as a test aquatic plant to determine the antioxidant responses to the combined toxicity of microcystin and ammonia. We hypothesized that: (1) oxidative stress parameters are significantly influenced by ammonia and microcystin respectively as literature reported; (2) there is a significant interaction between ammonia and microcystin on oxidative stress parameters of *V. natans* seedlings; (3) oxidative stress responses of *V. natans* seedling to the mixtures of ammonia and microcystin are time dependant. The results should be helpful to evaluate the physiological stress state of restoring submerged macrophytes in eutrophic waters.

## Materials and Methods

*Vallisneria natans* seeds, obtained from Lake Taihu, were surface-sterilized in a 2 % NaClO solution for 10 min, followed by three 10-min washes in sterile water (Yin et al. 2005). Then, the seeds were cultured on the surface of semisolid medium (5 cm deep) containing pond sludge and tap water, including necessary nutritional elements for germination and growth. When the seedlings grew up to 10 cm in average length, those of uniform growth were randomly selected to be treated with test solutions of ammonia and microcystin.

For the stress exposure experiments, the *V. natans* seedlings which grew up to 10 cm in average length were treated with the mixtures of ammonia and microcystin for 7 days. The concentration gradients for un-ionized ammonia nitrogen ($NH_3$–N) and purified MC-LR (Express, Beijing, China) were: 0, 0.21, 0.85 mg $L^{-1}$ and 0, 10, 50 μg $L^{-1}$, respectively. To retain necessary nutritional elements for the seedlings growth during the treatments, *V. natans* seedlings were cultured with modified 10 % Hoagland nutrient solution (containing 94.5 mg $L^{-1}$ Ca $(NO3)_2$, 50.6 mg $L^{-1}$ $KNO_3$, 8.0 mg $L^{-1}$ $NH_4NO_3$ and 13.6 mg $L^{-1}$ $KH_2PO_4$, 49.3 mg $L^{-1}$ $MgSO_4$ and 0.25 ml $L^{-1}$ iron salt solution, pH 5.5–6.0), under a light: dark cycle of 16 h:8 h, a light intensity of 40 μmol photons $m^{-2}$ $s^{-1}$, and at a temperature of 24 ± 0.5°C.

Experiments were carried out using a full-factorial design; i.e. there were 9 combinations of treatments. Ammonia test solutions were prepared by dissolving ammonium chloride $NH_4Cl$ (Sinopharm, Shanghai, China) in de-chlorinated tap water. $NH_3$–N concentrations were calculated using the general equation of bases (Emerson et al. 1975): $NH_3 = \frac{[NH_3+NH_4^+]}{[1+10^{(pKa-pH)}]}$. The calculation of $pK_a$ is based on the equation developed by Emerson et al. (1975) $pK_a = 0.09018 + 2729.92/T$, (T in Kelvin = 273 + T°C). Plant seedlings were planted in 500-mL beakers with sand culture (5 cm thick) at the bottom; each treatment has three replicates. The experiment was conducted under the conditions as described above. Throughout the experimental period, the solutions were replaced every 2 days to maintain constant concentrations of $NH_3$–N and MC-LR (Jiang et al. 2011). The pH of the solution was adjusted to 7.5 ± 0.1 twice each day using 0.5 M $H_2SO_4$ or 1 M NaOH.

To determine the antioxidant responses of *V. natans* seedlings, the seedling leaves were harvested randomly after 3, 5, and 7 days. The samples were stored at −70°C until analyses. About 0.2 g frozen seedling leaf tissues were ground into a slurry with a mortar and pestle procedure with 2 mL phosphate buffer solution (pH = 7.8) on ice during the entire process. Then, the extracts were centrifuged at 1,530*g* for 10 min at 4°C. The supernatants were collected and stored at −70°C for biochemical assays. The protein content of the extract was determined using the Coomassie Brilliant Blue dye binding technique with bovine serum albumin (BSA) as the standard. Biochemical parameters, including catalase (CAT, U $mg^{-1}$ protein), superoxide dismutase (SOD, U $mg^{-1}$ protein), peroxidase (POD, U $mg^{-1}$ protein), glutathione (GSH, mg $g^{-1}$ protein), and malondialdehyde (MDA, nmol $mg^{-1}$ protein) were determined using the Diagnostic Reagent Kits purchased from Nanjing Jian Cheng Bioengineering Institute (China).

All biochemical data are presented as mean ± 1 SE. The data on biochemical parameters were evaluated by three-way ($NH_3$–N concentration, MC-LR concentration, and time) analysis of variance (ANOVA) followed by Duncan's multiple range test ($\alpha = 0.05$). All statistical analyses were carried out with SigmaPlot 11.0.

## Results and Discussion

In this study, we found in *V. natans* seedlings that substantial antioxidant responses from the selected antioxidant enzymes and MDA levels are induced by $NH_3$–N, MC-LR, separately and together; and such responses change over a period of 7 days. Significant interactions were found among $NH_3$–N, MC-LR, and time on the SOD activity of the plant in the study period (Table 1, Fig. 1). During the experiment, SOD activity of *V. natans* seedlings exposed to

**Table 1** Summary of three-way ANOVA on the interactions among $NH_3$–N (0, 0.21, 0.85 mg $L^{-1}$), MC-LR (0, 10, 50 μg $L^{-1}$), and time (day 3, 5, 7) on oxidative stress of the *V. natans* seedlings which grew up to 10 cm in average length

| Parameters | Source of variation | DF | F | P |
|---|---|---|---|---|
| SOD | Time | 2 | 8.944 | <0.001 |
| | $NH_3$–N | 2 | 185.386 | <0.001 |
| | MC-LR | 2 | 1.027 | 0.365 |
| | Time × $NH_3$–N | 4 | 146.616 | <0.001 |
| | Time × MC-LR | 4 | 12.700 | <0.001 |
| | $NH_3$–N × MC-LR | 4 | 75.018 | <0.001 |
| | Time × $NH_3$–N × MC-LR | 8 | 37.813 | <0.001 |
| CAT | Time | 2 | 71.153 | <0.001 |
| | $NH_3$–N | 2 | 58.940 | <0.001 |
| | MC-LR | 2 | 44.879 | <0.001 |
| | Time × $NH_3$–N | 4 | 32.283 | <0.001 |
| | Time × MC-LR | 4 | 31.047 | <0.001 |
| | $NH_3$–N × MC-LR | 4 | 63.417 | <0.001 |
| | Time × $NH_3$–N × MC-LR | 8 | 38.451 | <0.001 |
| POD | Time | 2 | 219.715 | <0.001 |
| | $NH_3$–N | 2 | 246.897 | <0.001 |
| | MC-LR | 2 | 56.666 | <0.001 |
| | Time × $NH_3$–N | 4 | 32.333 | <0.001 |
| | Time × MC-LR | 4 | 24.317 | <0.001 |
| | $NH_3$–N × MC-LR | 4 | 109.945 | <0.001 |
| | Time × $NH_3$–N × MC-LR | 8 | 33.591 | <0.001 |
| MDA | Time | 2 | 6.250 | 0.004 |
| | $NH_3$–N | 2 | 26.283 | <0.001 |
| | MC-LR | 2 | 4.386 | 0.017 |
| | Time × $NH_3$–N | 4 | 27.796 | <0.001 |
| | Time × MC-LR | 4 | 4.724 | 0.002 |
| | $NH_3$–N × MC-LR | 4 | 5.998 | <0.001 |
| | Time × $NH_3$–N × MC-LR | 8 | 6.341 | <0.001 |
| GSH | Time | 2 | 859.604 | <0.001 |
| | $NH_3$–N | 2 | 297.656 | <0.001 |
| | MC-LR | 2 | 26.388 | <0.001 |
| | Time × $NH_3$–N | 4 | 67.177 | <0.001 |
| | Time × MC-LR | 4 | 15.091 | <0.001 |
| | $NH_3$–N × MC-LR | 4 | 53.602 | <0.001 |
| | Time × $NH_3$–N × MC-LR | 8 | 4.070 | <0.001 |

0.85 mg $L^{-1}$ $NH_3$–N in combination with 10 μg $L^{-1}$ MC-LR increased apparently and was three times of that when exposed to the 10 μg $L^{-1}$ MC-LR treatment alone. Meanwhile, the SOD activity of *V. natans* when exposed to 0.85 mg $L^{-1}$ $NH_3$–N in combination with 50 μg $L^{-1}$ MC-LR was about 1.5 times of that when exposed to the 50 μg $L^{-1}$ MC-LR treatment alone.

Catalase activity was significantly induced by ammonia and microcystin separately and there was a significant interaction between them in inducing CAT activity

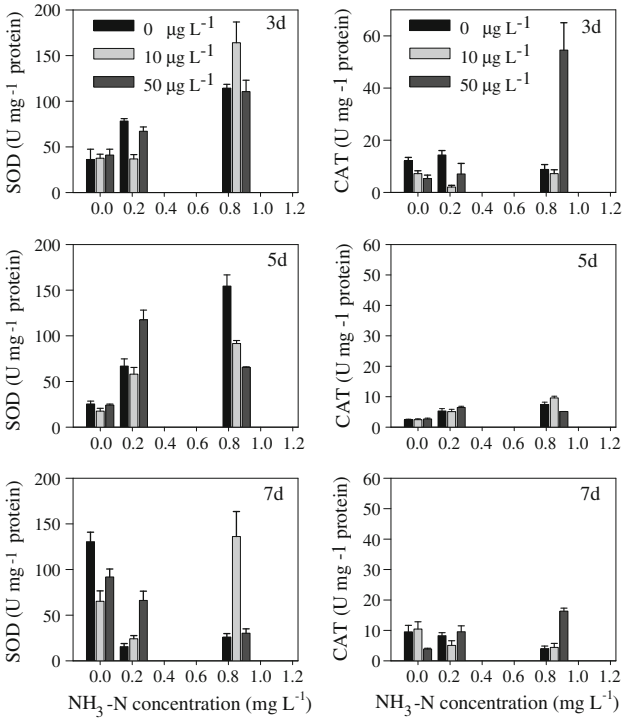


**Fig. 1** Changes in SOD and CAT of *Vallisneria natans* seedlings exposed to purified microcystin (0, 10, 50 μg $L^{-1}$) in combination with ammonia (0, 0.21, 0.85 mg $L^{-1}$)

(Table 1, Fig. 1). Catalase activities increased significantly to a high level at the highest $NH_3$–N and MC-LR combinations on day 3 and decreased evidently with longer treatment time, suggesting the seedlings exposed to multiple toxins suffered oxidative damage, which was time dependent.

Peroxidase showed a similar trend to that observed for SOD, except for the difference in response to each MC-LR concentration (Table 1, Fig. 2). On day 3, the POD showed the highest activity in the exposure to 10 μg $L^{-1}$ MC-LR in combination with 0.85 mg $L^{-1}$ $NH_3$–N, among other treatments (Fig. 2). Then, POD, along with SOD, showed the highest activities respectively in the mixtures of high $NH_3$–N and MC-LR (POD on day 7, SOD on day 3; Figs. 1, 2), suggesting oxidative-stress response occurred sensitively in multiple toxins.

Superoxide dismutase and POD play important roles in scavenging reactive oxygen species (ROS), therefore, the variations of their activities indicated oxidative stress in plant seedlings (Bowler et al. 1992; Blokhina et al. 2003). Previous studies indicated that in *Spinacia oleracea*, *Lepidium sativum*, and *Ceratophyllum demersum*, the antioxidant enzymes can be induced by MC exposure (Pflugmacher 2004; Pflugmacher et al. 2007; Stüven and Pflugmacher 2007). In our experiment, SOD and POD activities of *V. natans* seedlings exposed to mixes of MC-LR and $NH_3$–N

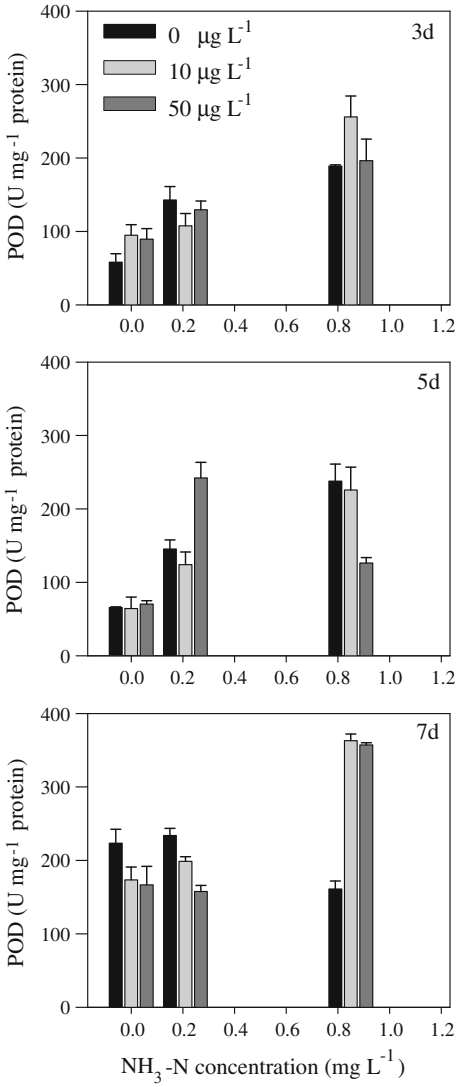


**Fig. 2** Changes in POD of *Vallisneria natans* seedlings exposed to purified microcystin (0, 10, 50 μg $L^{-1}$) in combination with ammonia (0, 0.21, 0.85 mg $L^{-1}$)

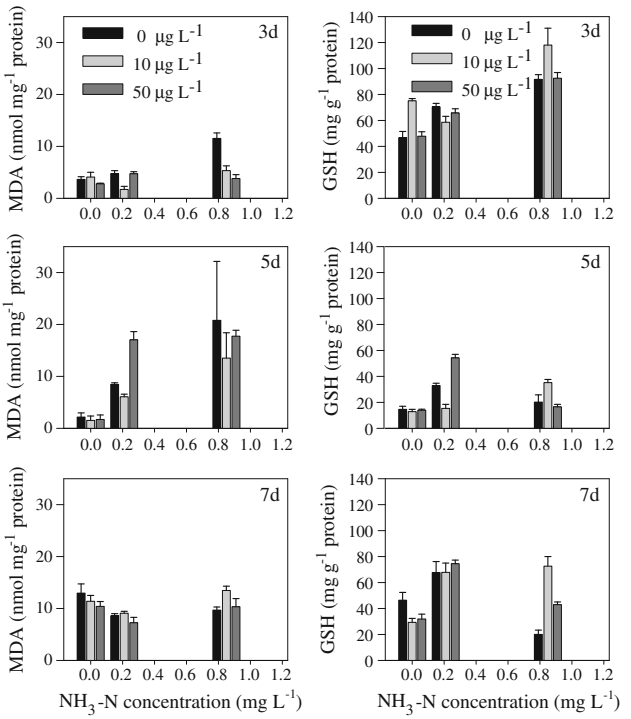


**Fig. 3** Changes in MDA and GSH of *Vallisneria natans* seedlings exposed to purified microcystin (0, 10, 50 μg $L^{-1}$) in combination with ammonia (0, 0.21, 0.85 mg $L^{-1}$)

increased significantly, which suggests severe oxidative stress was triggered by the combined stressors. With the extending of the treatment time, CAT activity increased and hence mitigated oxidative damage, suggesting CAT is an important enzyme in reducing relatively high ROS concentration during the degradation of cyanobacterial blooms (Ortiz-Rodríguez and Wiegand 2010). However, CAT activities kept a low and stable level in single $NH_3$–N and MC-LR treatments on days 3 and 5, suggesting CAT is insensitive to low toxin concentrations, as it was explained that CAT just has a poor efficiency in removing $H_2O_2$ at low level single toxin (Asada 1999). In general, the mixtures of ammonia and microcystin can induce oxidative stress response in *V. natans* seedlings. The antioxidative enzymes provide the first defense against oxygen toxicity (Pandey et al. 2003), i.e. several antioxidant enzymes cooperate together, providing an effective defense system (Pflugmacher 2004).

Significant interactions among ammonia, microcystin, and time in inducing GSH were also detected (Table 1, Fig. 3). GSH provides high resistance to MC toxicity in different aquatic organisms (Takenaka and Otsu 1999). At the beginning of the experiment, the content of GSH was high in the combination groups, because of synthesis of new GSH triggered by exposure to the mixtures of toxins, and then became low, which is considered to be related to forming glutathione conjugate via the GST system (Wiegand and Pflugmacher 2005). Decreased GSH levels were observed after exposure to 5 and 7 days, suggesting that the detoxification of MC-LR by the glutathione pathway would cause a shock in the GSH level. The GSH content in the treatment of 0.83 mg $L^{-1}$ $NH_3$–N decreased more than one time of that exposed to the control on day 7, which may be due to that the cell membrane damage contributed to the depletion of intracellular GSH.

Malondialdehyde content changed significantly with ammonia, microcystin, and time respectively (Table 1, Fig. 3), and three-way ANOVA indicated that there were significant interactions between any two factors and among all three factors (time, $NH_3$–N, and MC-LR). Malondialdehyde (MDA) is the final product of lipid peroxidation, and MDA content is positively correlated with the content of ROS (Wang and Zhang 2008); thus increased levels of

MDA indicate toxic activity caused by free radicals (Doyotte et al. 1997). The content of MDA was determined by the balance between the production of oxidants, the removal and scavenging of those oxidants by antioxidant defenses. It has been proposed that in ammonia-treated bryophyte, membrane damage is attributed to lipid peroxidation of membranes, which is triggered through intracellular accumulation of ROS (Pearce et al. 2003; Nimptsch and Pflugmacher 2007). In *V. natans* seedlings exposed to the mixtures of ammonia and microcystin, the content of MDA fluctuated significantly with time and the two toxins, suggesting that oxidative stress occurred and that changes in MDA content were direct evidence of toxic processes caused by free radicals (Doyotte et al. 1997).

In conclusion, the activities of SOD, POD, CAT, and the production of GSH in *V. natans* seedlings were strongly induced by the combined ammonia and microcystin, and there were significant interactions between ammonia and microcystin. Such results indicated that the antioxidases cooperated with each other and were in dynamic equilibrium to protect the organism from oxidative damage. MDA fluctuated significantly with both ammonia and microcystin, and significant interactions were detected between the two toxicants, which was the direct evidence of toxic activity caused by free radicals. Additionally, changes in all the parameters (SOD, CAT, POD, GSH, MDA) of *V. natans* seedlings exposed to ammonia in combination with microcystin were time-dependent. Our experiment demonstrated that the harmful pollutants (such as ammonia, microcystin) derived from cyanobacterial blooms can have potential negative impact on aquatic macrophytes, which is in according with the finding that the abundance and diversity of macrophytes in eutrophic water bodies suffering cyanobacterial blooms have decreased significantly (Casanova et al. 1999).

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**Conflict of interest** We declare that there is no potential.

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