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Ammonium, microcystins, and hypoxia of blooms in eutrophic water cause oxidative stress and C–N imbalance in submersed and floating-leaved aquatic plants in Lake Taihu, China

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ABSTRACT

The heavy bloom of cyanobacteria is a disastrous consequence of freshwater eutrophication, and the bloom is highly toxic due to its secondary metabolites called microcystins (MCs). The release of organic substances from dense blooms causes an increase in NH_4^+ and decrease in oxygen in lake water. In the present study, the dynamics of physio-biochemical responses of five aquatic macrophytes to MCs and NH_4^+ stresses in Meiliang Bay were evaluated. The bay is one of the most seriously eutrophized areas dominated by the toxic cyanobacteria of Lake Taihu, China. The results demonstrate that aquatic macrophytes in Meiliang Bay are subjected to successive external stresses. From January to May, they are subjected to high NH_4^+ stress (>0.56 mg L^{-1}), whereas from June to September or during dense blooms, the macrophytes experience both MC proliferation and moderate NH_4^+ toxicity (>0.3 mg L^{-1}). In August, high NH_4^+ stress occurs along with hypoxia stress, whereas from September to December, the macrophytes experience moderate NH_4^+ stress, causing a serious imbalance in C–N metabolism and oxidative stress. Between the two aquatic plant life forms, floating-leaved plants are more resistant to the stresses of eutrophication than are submersed plants. Elevated MCs in the water column can aggravate oxidative stress and suppress the soluble protein contents of aquatic plants. High NH_4^+ in the water causes severe C and N imbalance in submersed macrophytes because of considerable carbon consumption for free amino acid synthesis. The superoxide dismutase activities of submersed macrophytes are suppressed by low light penetrating the eutrophic water, which might impair the antioxidative function of the plants. The findings of this study provide mainly field evidence that reveals the physical, chemical, and biological stresses on aquatic plants in bloom-prevailed eutrophic lakes.

1. Introduction

The occurrence of heavy cyanobacterial blooms in eutrophic freshwater is a worldwide ecological problem (Carmichael, 1992). Lake Taihu in China is a well-known case of wide-ranging bloom occurrence, which causes serious problems in the drinking water quality of supply (Guo, 2007; Yang et al., 2008). The cyanobacterial blooms in Lake Taihu occurred since the early 1980s and then deteriorated in 2000 onward. The occurrence of cyanobacterial bloom has adverse effects on many aquatic animals, including zooplankton, zoobenthos, fish, amphibians, and water birds (e.g. Ghadouani et al., 2003; Gérard and Poullain, 2005; Buryskova et al., 2006; Qiu et al., 2007; Atencio et al., 2008; Leão et al., 2008; Chen et al., 2009), as well as on aquatic plants (Yamasaki, 1993); this also causes the degradation of shallow lake ecosystems (Harper et al., 1994).

The mechanisms of submersed plants inhibited by cyanobacterial blooms are characterized by a competition for resources, such as light (Casanova et al., 1999; Pokorný et al., 2002), total inorganic carbon (TIC) (Pokorný et al., 2002), and allelopathy of cyanobacterial toxins (Pflugmacher, 2002; LeBlanc et al., 2005). Apart from released toxins, cyanobacterial blooms can degrade water quality in nearby water sources when toxic cyanobacterial blooms decay; these harmful effects include the exhaustion of dissolved oxygen (DO) (Brownlee et al., 2005) and a sharp increase in nutrients (Buryskova et al., 2006). Low oxygen and NH_4^+ stresses during the

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period of bloom accumulation and decay can aggravate the adverse effect of blooms on aquatic macrophytes (Cizková-Koncalová et al., 1992; Buryskova et al., 2006). Previous experimental studies suggested that the decline of *Potamogeton maackianus* in eutrophic waters can be attributed to multiple environmental stresses, such as increased NH_4^+ loading and low DO (Li et al., 2007). However, no field study on the responses of macrophytes to the abovementioned stresses has been reported thus far.

Major cyanotoxins produced by the dominant cyanobacteria *Microcystis* in Lake Taihu are microcystins (MCs), which are among the most dangerous groups of potent hepatotoxins (Codd, 1995; Dawson, 1998). The inhibition effects of MCs on growth, photosynthesis, and/or biochemical–physiological processes have been detected in many aquatic macrophytes, including emergent plants (Pflugmacher et al., 2001; Pflugmacher, 2002), floating-leaved and free-floating plants (Weiss et al., 2000; LeBlanc et al., 2005; Mitrovic et al., 2005; Saqrane et al., 2007), and submersed plants (Pflugmacher et al., 1998a, 1999; Pflugmacher, 2002, 2004; Casanova et al., 1999; Pietsch et al., 2001; Romanowska-Duda et al., 2002; Romanowska-Duda and Tarczynska, 2002; Wiegand et al., 2002; Mitrovic et al., 2005; Yin et al., 2005a). The possible mechanism of MCs toxicity to aquatic macrophytes is attributed to MCs inhibition of protein phosphatases 1 and 2A (PP1 and 2A, Mackintosh et al., 1990; Ding and Ong, 2003). Oxidative stress in macrophytes was observed under MCs exposure, which was attributed to another important mechanism of MCs toxicity through the formation of reactive oxygen species (ROS, Pflugmacher, 2004; Peuthert et al., 2008). However, all of these studies were performed in the laboratory, and little evidence from field studies has been reported.

In addition, NH_4^+, one of the major components of domestic wastewaters and agricultural runoff, substantially increases during the eutrophication process. Many studies have shown that elevated NH_4^+ concentration is a primary stress factor in aquatic macrophytes because macrophytes are highly sensitive to its toxicity (Mulligan et al., 1976; Best, 1980; Roelofs et al., 1984; Rudolph and Voigt, 1986; Van Katwijk et al., 1997; Cao et al., 2004, 2007; Nimptsch and Pflugmacher, 2007). NH_4^+ enrichment can directly cause the decline in aquatic plant populations in natural waters (Ni, 2001; Brun et al., 2002; Cao et al., 2007). The mechanisms include the formation of ROS induced by NH_4^+ during its metabolism by the plants (Nimptsch and Pflugmacher, 2007) and the imbalance of C–N reserves in plants stemming from the incorporation of NH_4^+ into free amino acids (FAA) at the expense of soluble carbohydrates (SC) consumption (Smolders et al., 1996; Kohl et al., 1998; Saarinen and Haansuu, 2000; Cao et al., 2004, 2007). However, whether the effects of the stresses of MCs and NH_4^+ on macrophytes found in laboratory studies apply to natural waters remains uncertain as environmental factors are highly complicated and may interact with each other in the field. This diminishes the function of any single factor.

Few studies that investigate the dynamic patterns of aquatic plants exposed to long-term and/or frequent toxic cyanobacteria and NH_4^+ have been conducted *in situ*. This study aims to determine the seasonal physiological and biochemical responses of the aquatic macrophytes of different life forms to toxic blooms and high-NH_4^+ water in a large eutrophic Chinese lake, Lake Taihu, meanwhile, compare the sensitivity among plant species and life forms to toxic blooms and high-NH_4^+ water and discuss the possible mechanisms. The potential impacts of low oxygen stress are also discussed.

2. Materials and methods

2.1. Study area and sample collection

Lake Taihu (30°56′–31°34′N, 119°54′–120°36′E) is the third largest freshwater lake in China (with a catchment area of about 36 500 km^2, lake area of 2338 km^2, average depth of 1.9 m, and maximum depth of 2.6 m). During the past decades, rapid economic development in China has resulted in increased industrial and agricultural pollution, causing the lake to undergo steadily increasing eutrophication (Chang, 1996). In the recent decade, heavy cyanobacterial blooms have occurred every year during warm seasons over wide areas, sometimes covering approximately 1000 km^2 (Pu et al., 1998); such occurrence heavily influences the aquatic ecosystem of the lake. Meiliang Bay is one of the hyper-eutrophic areas in Lake Taihu. It is located in the northern region of the shallow lake and has an average depth of 1.8 m (Cai et al., 1997; Dokulil et al., 2000). Dense cyanobacterial blooms always cover this area from June to September.

Samples were taken monthly from large enclosures constructed in Meiliang Bay (Fig. 1) over the period December 2004–November. The enclosures covered a total area of about 2 km^2. Sampling was carried out in four sampling sites within the enclosures (Fig. 1). Their positions were located using a GPS (A: 31°30′21.5″N, 120°13′37.3″E, B: 31°30′29.6″N, 120°13′22.9″E, C: 31°30′16.1″N, 120°13′24.0″E and D: 31°29′55.8″N, 120°13′33.5″E). Water samples were a mixture of the surface (0–1 m), middle, and bottom layer samples of every site, and were collected with Tygon tubing fitted with a one-way valve. Water temperature (*T*), Secchi depth (SD), pH, conductivity, dissolved oxygen (DO), and soluble solids (SS) were measured *in situ*. Subsamples of phytoplankton were preserved with 1% acidified Lugol's iodine solution and concentrated to 30 mL after sedimentation for 48 h. After mixing, 0.1 mL of the concentrated samples was directly counted using a microscope at 600× magnification. Colonial *Microcystis* spp. cells were separated using a high-speed blender (Ultra-Turrax) and then counted. Taxonomic identification was processed according to Hu et al. (1979), and biomass was estimated from approximate geometric volumes of each taxon, assuming that 1 mm^3 equals 10^{-6} μg fresh weight (Shei et al., 1993). Water and algae samples in all the four sampling sites were determined individually.

The five species of aquatic macrophytes found in three sampling sites (A, B and D) belong to two life forms: submersed plants, including *Myriophyllum spicatum* (M. s), *Potamogeton malaianus* (P. m), and *Vallisneria natans* (V. n); and floating-leaved plants, including *Trapa bispinosa* (T. b) and *Nymphoides peltata* (N. p). All these plants are native to Lake Taihu. Ten individual plants per species were randomly harvested from these three sampling sites using a sickle. Five plants for each species were pooled together and stored at −20 °C for the analysis of soluble protein content and SOD (EC 1.15.1.1) activity (Cao et al., 2004). The rest of the plants were dried at 80 °C for 48 h for SC and FAA analyses (Yemm and Willis, 1954; Yemm et al., 1955).

2.2. Analysis of environmental factors

Water samples were kept in the dark in a refrigerator before laboratory analysis. Samples were filtered through a membrane filter (GF/C, Whatman, UK) for analysis of ammonium (NH_4^+–N), total nitrogen (TN), total phosphorus (TP), and chlorophyll *a* (Chl_*a*) content in the lake water. Biochemical oxygen demand (BOD_5) and chemical oxygen demand (COD_{Mn}) were assayed using the collected water in appropriate dilution ratios.

The NH_4^+–N, TN, TP, BOD_5 and COD_{Mn} were analyzed using standard methods (SEPA, 2002). The determination of BOD_5 followed the procedures of the standard methods for sample pretreatment, dilution, incubation, and determination of initial and final DO. Chl_*a* was determined using a spectrophotometer (Lorenzen, 1967) after 24 h extraction of the residue on the glass-fiber filter in 90% acetone. The residue on the glass-fiber filter and the filtrate of the 500 mL water sample were used to detect intracellular and extracellular MCs, respectively. MCs were

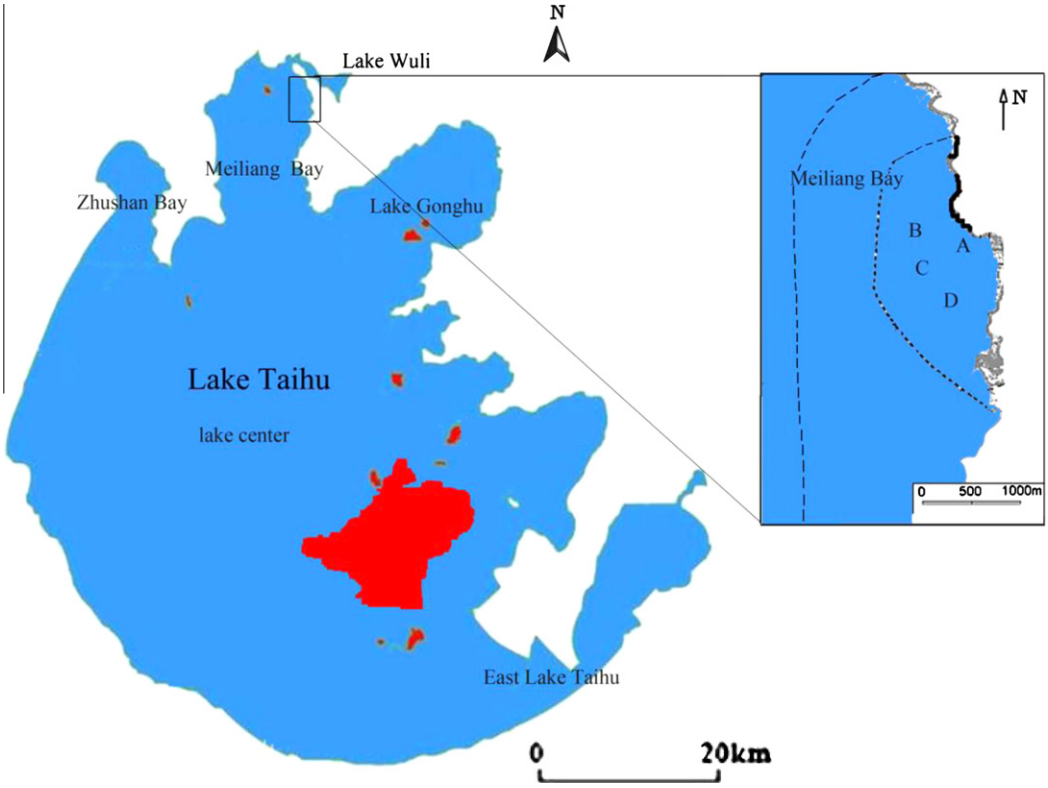

Fig. 1. The sketch of Lake Taihu (left) and the location of the plant enclosures in Meiliang Bay (right), and A–D were the sampling sites.

measured according to the methods of Park et al. (1998) using a reverse-phase high performance liquid chromatography (HPLC) equipped with an ODS column (Cosmosil 5C18-AR, 4.6 × 150 mm, Nacalai, Japan) and a SPD-20A UV–Vis spectrophotometer set at 238 nm. MCs concentrations were determined by comparing the peak areas of the test samples with those of the standards available (Wako Pure Chemical Industries – Japan, purity ⩾95.0%). The limit of detection for the MCs was 0.02 μg mL^{-1}.

2.3. Biochemical analyses

For enzyme measurements, the frozen shoots were ground into fine powder in liquid nitrogen with a mortar and pestle. The fine powder (1 g) was homogenized for 20 min in a cold (4 °C) buffer solution containing 50 mM potassium phosphate with 1 mM EDTA, 1 mM PMSF, 1% (w/v) PVP, 0.5% (v/v) Triton X-100, and 5 mM 2-mercaptoethanol, with pH adjusted to 7.8. Homogenates were centrifuged at 15 000*g* (4 °C) for 10 min, and the obtained supernatants from the final centrifugation were employed for analyses.

Superoxide dismutase activity (SOD) assay was based on the method described by Beyer and Fridovich (1987). One unit of the enzyme activity is defined as the amount of enzyme required to generate a 50% inhibition of the rate of nitro blue tetrazolium reduction measured at 560 nm. The activity of antioxidants was calculated in terms of the protein content of the sample (Bedford, 1969).

For determination of resource indices, dry samples were ground into fine powder with an automatic miller equipped with a 150 μm screen. About 50 mg powder was extracted twice with 5 mL 80% ethanol at 80 °C for 20 min, then centrifuged at 15 000*g* for 10 min. The supernatants were collected, decolorized using 10 mg activated charcoal, and then filtered using filter papers (microvoid filter film, *Φ*20 mm). The SC and FAA in the supernatants were determined by the anthrone (Yemm and Willis, 1954) and ninhydrin methods (Yemm et al., 1955) using a spectrophotometer (SHIMADZU UV-1601), with glucose and alanine as standards, respectively. All determinations were performed in triplicate.

2.4. Statistical analyses

Values are expressed as means ± standard deviation (SD). All data were tested for normality and homogeneity using Levene's test. Analysis of variance (ANOVA) was used to compare the statistical differences of averages among three different periods of the bloom stages. The three periods were divided as the periods "before the blooms" (January to May), "during the blooms" (June to September), and "after the blooms" (October through the end of the year). The data sets were SOD, FAA, SC, soluble protein concentrations, and FAA/SC ratio in the five aquatic plants. Correlation was used to present the relationship between the environmental factors and biochemical components in the aquatic plants ($n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Multiple comparisons of the pos hoc test were performed using Tukey HSD's test or independent samples *t*-test at the 0.05 significance level. Principal component analysis (PCA) was used as an ordination method to classify the plants according to SOD activities, SC, FAA, and soluble protein concentrations of the plants during cyanobacterial blooming. PCA was also applied to explore the relationships between the environmental variables of the lake and the biochemical index of aquatic macrophytes during the study period, and identify a small number of factors that explain most of the variance observed in a much larger number of manifest variables. To ensure that all variables were given equal weight, the data were standardized before PCA analyses. The abovementioned statistical analysis was carried out using SPSS 17.0.

3. Results

The average surface temperature of the lake water is 17.4 °C, and ranges from 4.6 to 29.6 °C, with the lowest temperature

recorded in January and the highest in July. All physical and chemical parameters in the water column are significantly different among or between different bloom stages ($p < 0.05$), except for BOD_5. Among these parameters, NH_4–N, TN, conductivity, pH, DO, and biomass of cyanobacteria are significantly different in the periods "during the blooms" and "after the blooms" ($p < 0.05$) compared with the period "before the blooms". The *T*, SD, TP, Chl_*a*, and biomass of cyanobacteria and *Microcystis*, as well as the MCs, are all significantly different during blooming ($p < 0.05$) compared with the two other stages (Table 1).

The NH_4^+ concentration of the lake water averages 0.92 mg L^{-1} during the entire study period, and reaches a peak value of 4.06 mg L^{-1} in March (Fig. 2a). It is 1.77 ± 1.18 mg L^{-1} on average (±SD) in the period "before the blooms" 0.30 ± 0.15 mg L^{-1} "during the blooms" and 0.31 ± 0.33 mg L^{-1} "after the blooms" (Table 1). *Microcystis* dominates the cyanobacteria and phytoplankton "during the blooms". The maximum biomass of cyanobacteria and *Microcystis* in August reaches 14.5 mg L^{-1} and 12.5 mg L^{-1}, respectively, when total MCs content is up to 1.78 μg L^{-1} (Fig. 2b and Table 1). The biomass of *Microcystis* and total MCs content are respectively 1.60 ± 2.62 mg L^{-1} and 0.09 ± 0.09 μg L^{-1} on average (±SD) in the period "before the blooms", 5.11 ± 4.01 mg L^{-1} and 0.91 ± 0.70 μg L^{-1} "during the blooms", and 0.19 ± 0.08 mg L^{-1} and 0.17 ± 0.14 μg L^{-1} "after the blooms", respectively (Table 1). DO is relatively stable in the lake water throughout the study year, which is likely due to frequent mixing of lake water by strong monsoons (Qiu et al., 2007). The annual average concentration of DO in the lake water is 8.87 mg L^{-1} and the lowest 2.72 mg L^{-1} in August (the hottest month in a year) with the decay of the blooms (Fig. 2c).

According to OECD (1982) standards, the water in Meiliang Bay of Lake Taihu was hyper-eutrophic during the study period (Ke et al., 2007). Chl_*a* content averages 0.060 mg L^{-1} during the entire period and reaches a peak value of 0.140 mg L^{-1} in August, and the lowest value of 0.0077 mg L^{-1} in December. The mean ± SD of Chl_*a* content "before", "during", and "after the blooms" are 0.038 ± 0.034, 0.109 ± 0.057, and 0.033 ± 0.024 mg L^{-1}, respectively. Similarly, the mean ± SD of biomass of cyanobacteria "before", "during", and "after the blooms" are 4.60 ± 4.21, 7.26 ± 3.20, and 1.23 ± 1.13 mg L^{-1}, respectively (Table 1).

The biochemical values of the five aquatic macrophytes (Fig. 3 and Table 2), generated using ANOVA analyses or *t*-tests, show that the activities of antioxidative enzyme SOD and three C–N reserve indices are statistically different among different periods of bloom stages (Table 2).

The soluble protein contents in all macrophytes significantly decrease "during the blooms" ($p < 0.05$) compared with the period "before the blooms". The soluble protein contents of two floating-leaved plants, *T. bispinosa* and *N. peltata*, and a submersed plant, *V. natans*, are significantly lower "after the blooms" than "before the blooms" ($p < 0.05$).

For two submersed macrophytes, *M. spicatum* and *P. malaianus*, the SC contents increase significantly by 215% ($p < 0.001$) and 44% ($p < 0.001$) "during the blooms", and by 150% ($p < 0.001$) and 45% ($p < 0.001$) "after the blooms", respectively, when compared with the period "before the blooms". The SC contents of *V. natans*, however, increase significantly only in the period "after the blooms" ($p < 0.001$) compared with the other bloom stages. The FAA contents in the three submersed plants decrease both "during the blooms" and "after the blooms". The FAA contents of *M. spicatum*, *P. malaianus*, and *V. natans* significantly decrease by 28% ($p < 0.01$), 68% ($p < 0.001$), and 24% ($p < 0.01$), respectively "during the blooms" than in the previous period. However, the FAA content is still significantly lower by 54% ($p < 0.001$) "after the blooms" than "before the blooms" only in *P. malaianus*.

In floating-leaved macrophytes *T. bispinosa* and *N. peltata*, the contents of SC are significantly reduced by 64% ($p < 0.001$) and 51% ($p < 0.01$) "during the blooms" and by 64% ($p < 0.001$) and 42% ($p < 0.01$) "after the blooms", respectively, compared with those observed "before the blooms". The FAA of two floating-leaved *T. bispinosa* and *N. peltata* also decrease significantly "during the blooms" ($p < 0.001$) and "after the blooms" ($p < 0.001$) when compared with the previous period. No significant difference between the latter periods ("during the blooms" and "after the blooms" $p > 0.05$) are observed.

A significant change in the FAA/SC ratio of all the plants is observed "during the blooms", except for *V. natans*. The FAA/SC ratio of the four submersed macrophytes sharply decreases both "during the blooms" and "after the blooms", whereas that in *V. natans* also declines in both of the stages by 26% and 47%, respectively, when compared with the previous stage. No remarkable change is observed between the period "after blooms" and "during the blooms" in all the aquatic plants. The lowest FAA/SC ratio of the five macrophytes is observed in August, whereas the peaks are observed in April for most of the plants and in May for *M. spicatum*.

The SOD activities of the five plants all peak in August. During the study period, SOD activity is at its highest level from July to September for all the macrophytes. The SOD activities of all the five plants are significantly different between the period "during the blooms" and "before the blooms", and also between "during the

Table 1
The mean values (±SD) of physical and chemical parameters in the enclosures during the periods of different stages regarding the blooms and their differences between stages (ANOVA).

	Before cyanobacterial blooms	During the blooms	After the blooms
T (□)	12.17 ± 7.73	28.50 ± 1.14***	11.48 ± 5.37$^{\#\#\#}$
pH	7.21 ± 0.37	8.20 ± 0.48***	7.74 ± 0.81*
DO (mg L^{-1})	10.24 ± 1.25	7.03 ± 2.29***	9.03 ± 1.13*
SD (cm)	33 ± 16	29 ± 10*	51 ± 20$^{\#\#}$
Conductivity (ms m^{-1})	69.2 ± 2.7	53.1 ± 3.3***	57.1 ± 7.0***
COD_{Mn} (mg L^{-1})	5.85 ± 1.09	6.61 ± 1.18	4.81 ± 0.70$^{*\#\#\#}$
SS (mg L^{-1})	31.9 ± 20.3	29.3 ± 11.6	18.4 ± 11.2*
NH_4–N (mg L^{-1})	1.77 ± 1.18	0.30 ± 0.15***	0.31 ± 0.33***
BOD_5 (mg L^{-1})	4.51 ± 1.50	4.62 ± 1.98	3.30 ± 1.00
Chl_*a* (mg L^{-1})	0.038 ± 0.034	0.109 ± 0.057***	0.033 ± 0.024$^{\#\#\#}$
TP (mg L^{-1})	0.089 ± 0.021	0.133 ± 0.053**	0.064 ± 0.017$^{\#\#\#}$
TN (mg L^{-1})	4.61 ± 1.46	2.25 ± 0.40***	1.82 ± 1.00***
Biomass of cyanobacteria (mg L^{-1})	4.60 ± 4.21	7.26 ± 3.20*	1.23 ± 1.13$^{*\#\#\#}$
Biomass of *Microcystis* (mg L^{-1})	1.60 ± 2.62	5.11 ± 4.01***	0.19 ± 0.08$^{\#\#}$
MCs (μg L^{-1})	0.09 ± 0.09	0.91 ± 0.70***	0.17 ± 0.14$^{\#\#\#}$
Time (month)	January–May	June–September	October–December

The significance levels are $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, compared with the values before the blooms, while $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$, compared with the value during the blooms.

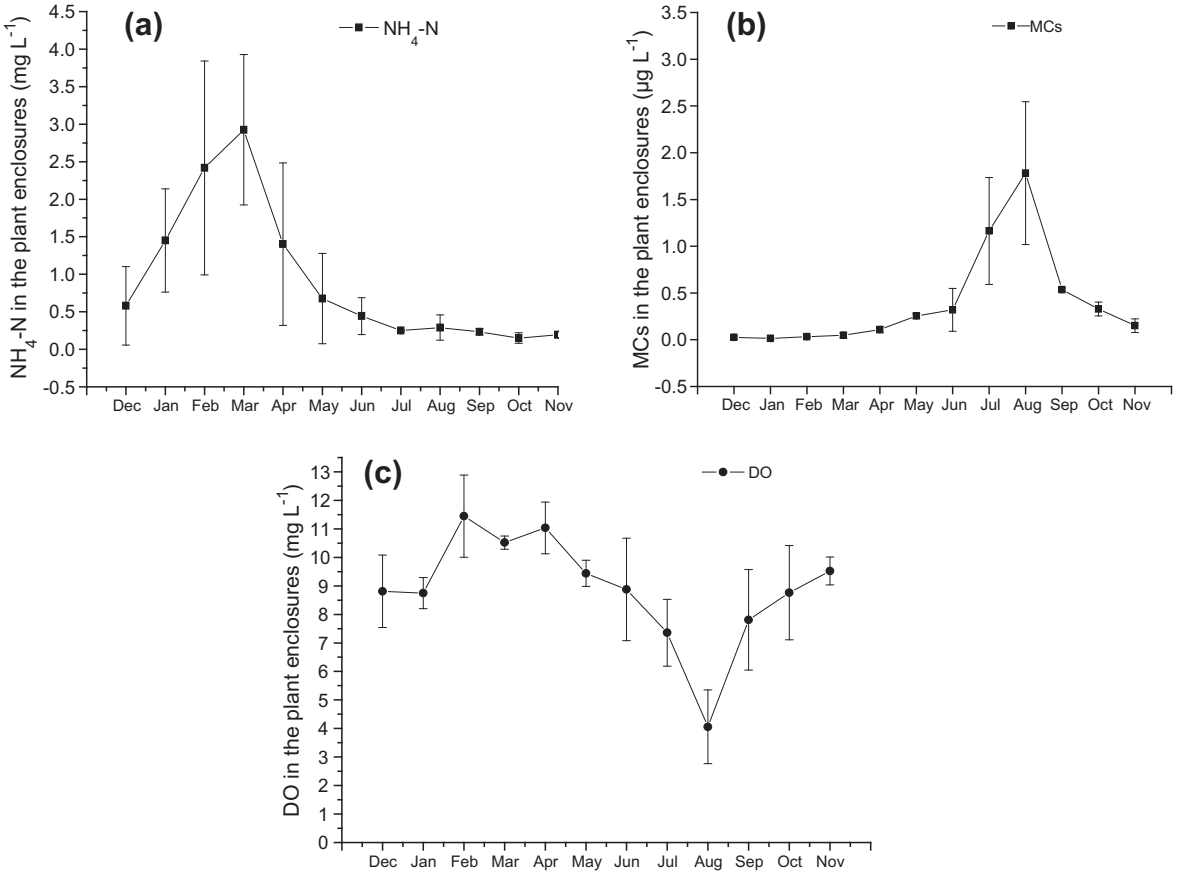

Fig. 2. Temporal changes of NH_4–N concentrations (a) and MCs contents (b) and DO concentrations (c) in the water column of these plant enclosures.

blooms" and "after the blooms", except for submersed plant *M. spicatum* ($p > 0.05$). The SOD activities of submersed plants *M. spicatum* and *P. malaianus* are enhanced less sharply "during blooms" than that of floating-leaved plants *T. bispinosa* and *N. peltata*, and submersed plant *V. natans*. In the present study, the SOD activities are low in submersed plants, and particularly very low in *P. malaianus* and *V. natans* when compared with their normal levels.

The correlations between the environmental factors and biochemical parameters in the five species of aquatic macrophytes are shown in Table 3. Close relationships, such as FAA (positive relationship, +), SC (negative relationship, −), and soluble protein (+), are observed between NH_4^+ and C–N reserves. Close relationships are observed as well between MCs and stress indices, such as SOD activity (+) and soluble protein (−), suggesting that both NH_4^+ and MCs affect the normal growth and metabolism of major aquatic plants in Lake Taihu. Among these relationships, both NH_4^+-to-C–N reserves and MCs-to-oxidative stress are most remarkable. In addition to these relationships, the SOD activities in these plants are closely related to water temperature (+), DO (−), *Microcystis* biomass (+), and cyanobacteria biomass (+), rather than with NH_4^+ in Meiliang Bay. The soluble protein contents of the plants are closely related to pH (−), except for *M. spicatum* and *P. malaianus*, DO (+), conductivity (+), and TN (+) in the water column. The SC contents in the five plants are strongly correlated with conductivity (−), but not for *V. natans*, TN (−) and *Microcystis* biomass (+), except for *V. natans* and *N. peltata* in the water. The FAA contents in these plants are highly correlated with TN (+), except for *M. spicatum* and *V. natans*, and with conductivity (+) in the lake water. These relationships indicate that the N reserves of the plants are positively associated with TN in the water column ($p < 0.05$), and the carbohydrate reserve of the plants is negatively associated with TN and *Microcystis* biomass in the water column ($p < 0.05$).

The correlation between the major biochemical parameters in the plants shows different response patterns in the five plants (Table 3). For *M. spicatum*, both the C–N reserve and SOD cooperate well with each other, but not SOD–FAA. For *P. malaianus*, all indices cooperate well with each other, but not SOD–FAA and SOD–SC. For *V. natans*, the correlations are weak and only the FAA contents are closely related with SC (+) and SOD activity (−). For the two floating-leaved plants, the correlations are strong, except for SC–SOD (*T. bispinosa*) and protein–SOD (*N. peltata*).

PCA analysis on the data scores shows that submersed macrophytes with perennial growth periods (*M. spicatum* and *P. malaianus*) are similar in PCA scores; floating-leaved plants *T. bispinosa* and *N. peltata* are also similar. *V. natans*, which grows from spring to autumn, differs from the abovementioned plants, suggesting that the response pattern in the C–N metabolites and antioxidants of the tissue to environmental stresses of aquatic plants differ according to their life forms and growth periods (Fig. 4a).

To identify the principal factor that affects the biochemical parameters of the aquatic plants in Meiliang Bay that exhibit different response patterns to stresses, PCA analyses on the data scores of biochemical indices and their environmental factors were performed on two submersed plants (*M. spicatum* and *P. malaianus*) and two floating-leaved plants (*T. bispinosa* and *N. peltata*). PC1 and PC2 account for 57.2% and 67.5% of the total variance of the data in the submersed macrophytes and floating-leaved plants, respectively (Fig. 4b and c).

The plots show that the SC and SOD of the two submersed macrophytes are closer to factors related to blooms, such as MCs, pH, cyanobacteria density *Microcystis* biomass, Chl_*a*, and T. The soluble protein, FAA, and FAA/SC ratio are affected mainly by nitrogen in the water, TN, NH_4^+, EC, and DO (Fig. 4b), indicating that the bloom mass, its metabolite, and its effects on DO are the principal

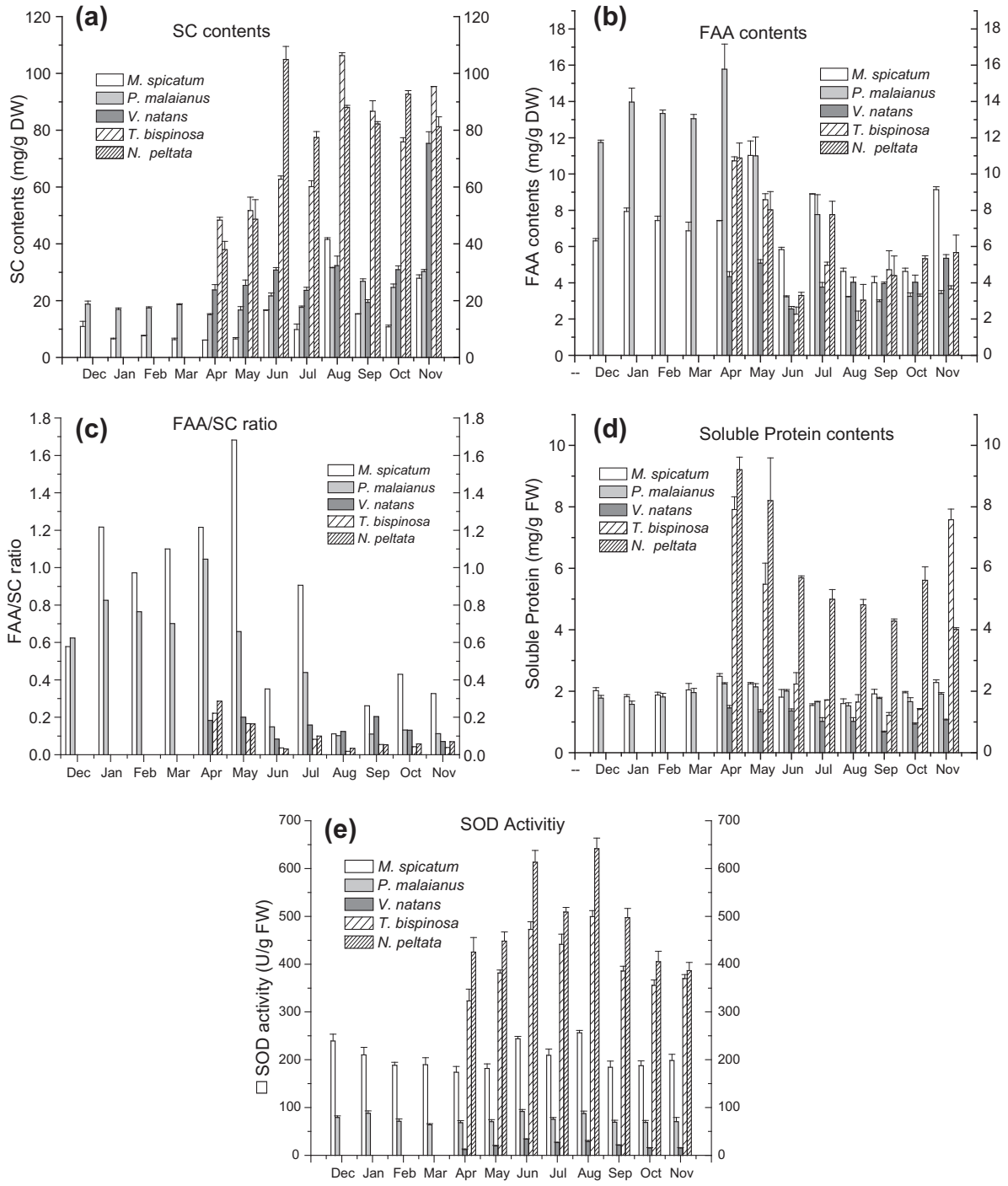

Fig. 3. Biochemical changes of five aquatic macrophytes during the year. (a) Temporal changes of SC contents of five vascular macrophytes *Myriophyllum spicatum*, *Potamogeton malaianus*, *Vallisneria natans*, *Trapa bispinosa* and *Nymphoides peltata*; SC contents are expressed in mg g^{-1} dry weight (DW). (b) Temporal changes of FAA contents in these five vascular macrophytes. (c) Temporal changes of FAA/SC ratio in these five vascular macrophytes. (d) Temporal changes of soluble protein contents in these five vascular macrophytes. (e) Temporal changes of SOD activities in these five vascular macrophytes. SOD activity is expressed in unit min^{-1} mg^{-1} fresh weight (FW). Data are expressed as mean values ± standard deviation. Vertical bars show standard deviation of each data.

causes of stress in macrophytes. In two floating-leaved plants, SC and SOD are also close to the same factors related to blooms, as in submersed macrophytes. However, the SOD activities of floating-leaved plants are much closer to those of the bloom mass and MCs than those in submersed macrophytes, indicating that the response of the former is more sensitive. The soluble protein, FAA, and FAA/SC ratio of the floating-leaved plants are also close to the factors similar to that in the macrophytes. However, they are closer to cyanobacteria biomass, but less close to DO than that in submersed macrophytes. These results indicate that blooms might supply nitrogen to the floating-leaved plants, whereas hypoxia causes less stress (Fig. 4c).

4. Discussion

A number of investigations have observed a decline in the abundance and diversity of macrophytes in eutrophic lakes where cyanobacterial blooms present (e.g. Morgan, 1970; Jupp et al.,

Table 2
Contents of resource matters and activities of antioxidant enzymes (Mean ± SD) in the five aquatic plants and their differences between different stages regarding the blooms.

	Before cyanobacterial blooms	During the blooms	After the blooms
Protein			
M. spicatum	2.09 ± 0.28	1.71 ± 0.17***	2.08 ± 0.18$^{\#\#}$
P. malaianus	1.95 ± 0.27	1.74 ± 0.20*	1.78 ± 0.12
V. natans	1.40 ± 0.09	1.02 ± 0.28**	1.00 ± 0.10**
N. peltata	8.71 ± 0.71	4.95 ± 0.58***	4.81 ± 1.13***
T. bispinosa	6.70 ± 1.72	1.70 ± 0.42***	4.49 ± 4.36$^{*\#}$
SC			
M. spicatum	6.61 ± 0.60	20.81 ± 14.12***	16.53 ± 9.85***
P. malaianus	16.96 ± 1.27	24.43 ± 6.06***	24.57 ± 5.73***
V. natans	24.56 ± 1.16	26.52 ± 6.07	53.12 ± 31.39$^{***\#\#\#}$
N. peltata	43.32 ± 7.54	88.15 ± 11.96***	86.95 ± 8.16***
T. bispinosa	50.03 ± 2.40	78.95 ± 21.77**	85.62 ± 13.75**
FAA			
M. spicatum	8.13 ± 1.66	5.84 ± 2.17**	6.69 ± 2.28
P. malaianus	13.43 ± 1.72	4.30 ± 2.31***	6.14 ± 4.85***
V. natans	4.72 ± 0.53	3.58 ± 0.68**	4.69 ± 0.93$^{\#\#}$
N. peltata	9.46 ± 2.02	4.63 ± 2.17***	5.50 ± 0.24$^{**\#}$
T. bispinosa	9.65 ± 1.51	3.47 ± 1.59***	3.46 ± 0.28***
FAA/SC ratio			
M. spicatum	1.24 ± 0.27	0.41 ± 0.35**	0.45 ± 0.13**
P. malaianus	0.80 ± 0.15	0.20 ± 0.16**	0.29 ± 0.29**
V. natans	0.19 ± 0.01	0.14 ± 0.05	0.10 ± 0.04
N. peltata	0.23 ± 0.09	0.05 ± 0.03**	0.06 ± 0.009*
T. bispinosa	0.19 ± 0.04	0.05 ± 0.03**	0.04 ± 0.003**
SOD			
M. spicatum	188.77 ± 13.46	223.48 ± 32.77**	208.46 ± 27.12*
P. malaianus	72.40 ± 9.19	81.04 ± 10.44*	72.76 ± 5.63$^{\#}$
V. natans	15.98 ± 5.22	27.60 ± 5.07***	15.63 ± 0.70$^{\#\#\#}$
N. peltata	436.79 ± 16.27	565.32 ± 72.70***	395.86 ± 13.46$^{\#\#\#}$
T. bispinosa	352.23 ± 41.44	449.86 ± 48.96***	362.65 ± 9.87$^{\#\#\#}$

The significance levels are $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{**^{*}}p < 0.001$, compared with the values before the blooms, while $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$, compared with the value during the blooms.

1974; Jupp and Spence, 1977; Harper et al., 1994; Li et al., 2009). The physiological stresses were found significant to aquatic plants in relation to the toxicity of MCs (e.g. Pflugmacher et al., 1998a,b; Yin et al., 2005a) and NH_4^+ (Smolders et al., 2000; Cao et al., 2004, 2007; Nimptsch and Pflugmacher, 2007). Studies suggest that MCs have an adverse effect on the growth of the *Vallisneria* genus (Yin et al., 2005a) and *Myriophyllum* (Casanova et al., 1999; Pflugmacher, 2002), whereas NH_4^+ is found harmful to the growth of plants in the *Potamogeton* (Cao et al., 2004, 2007) and *Stratiotes* genera (Smolders et al., 2000). High levels of NH_4^+—N (>0.30 mg L^{-1}) played an important role in the decline of a *Vallisneria* species in lakes in the Yangtze River Basin (Cao et al., 2007). Buryskova et al. (2006) suggested that other factors including non-specific parameters, such as toxic NH_4^+ released during bacterial decay of blooms, are also important. The present study provides field evidence of the imbalance of C–N reserves and oxidative stress in aquatic plants caused by the occurrence of toxic cyanobacterial blooms.

4.1. Responses of C–N reserves to the NH_4^+ stress in the five aquatic plants

High NH_4^+ concentration in the water column is toxic to aquatic macrophytes, as indicated by the imbalance in C–N metabolism in plant tissues (Smolders et al., 1996, 2000; Cao et al., 2004, 2007). In this field study, the decrease in SC content and increase in FAA content are related significantly to the NH_4^+ increase in the lake water. Low SC content and high FAA content simultaneously occur during the period "before the blooms" which is the period of high NH_4^+ concentration (average, 0.56 mg L^{-1}) in the lake water. NH_4^+ is previously the inorganic nitrogen for aquatic macrophytes (Best, 1980) and is copiously absorbed by macrophytes when external nutrient levels are high (Gertoff and Krombholz, 1966). In the laboratory experiments, FAA is found to accumulate and SC is reduced simultaneously in aquatic macrophytes *Potamogeton crispus*, *V. natans* and *Stratiotes aloides* under high NH_4^+ treatment (Smolders et al., 1996; Cao et al., 2004, 2007, 2009a,b), which can be attributed to the consumption of carbohydrates for incorporating large amount of NH_4^+ into FAA (Rabe, 1990; von Wiren et al., 2000; Coruzzi and Zhou, 2001). FAA assimilation is considered effective for detoxification of NH_4^+ in macrophyte tissues (von Wiren et al., 2000; Coruzzi and Zhou, 2001; Cao et al., 2004).

Our field results suggest that SC is substantially consumed under high ambient NH_4^+ stress (NH_4^+—N > 0.3 mg L^{-1}), which is in agreement with previous experimental results (Cao et al., 2007). The overall low NH_4^+ concentration in the water during the entire period of the blooms shows that cyanobacteria absorbs more NH_4^+ than the amount released in the water during the decay of blooms. Thus, the blooms can play a role in relieving the effect of NH_4^+ on aquatic macrophytes in summer, which can be confirmed by the significant increase in SC content of the plants "during the blooms" compared with "before the blooms" ($p < 0.05$).

In a previous study, increased FAA content was observed when NH_4^+—N > 0.3 mg L^−1 (Cao et al., 2007). In this study, NH_4^+—N concentration in the water column is higher than 0.3 mg L^{-1} most of the year, and the FAA contents of these five aquatic plants (7.01 ± 2.08, 8.56 ± 5.08, 4.14 ± 0.85, 5.01 ± 3.10, and 6.06 ± 2.68 mg g^{-1} DW on annual average for *M. spicatum*, *P. malaianus*, *V. natans*, *T. bispinosa* and *N. peltata*, respectively) are much higher than those reported by Cao et al. (2008) (FAA: 4.11 ± 3.33, 3.69 ± 2.21, 2.33 ± 1.42, 1.74 ± 0.68, and 3.05 ± 2.05 mg g^{-1} DW, respectively) for the plants growing in the lakes of this area. The FAA of the plants in this study are even higher especially during the period "before the blooms" when MCs in the water column are low and NH_4^+ is high, indicating that the increase in FAA is due mainly to NH_4^+ stress in the lake, but not to the occurrence or decay of the blooms. Although the FAA contents of the submersed macrophytes are considerably reduced in periods during and after the blooms, the FAA contents in the macrophytes are still higher than the levels reported in another study (Cao et al., 2008), indicating that in hyper-eutrophic Lake Taihu, the NH_4^+ concentration of the water column is also significantly reduced by the effective utilization of phytoplankton during the occurrence of heavy blooms. However, the concentration of NH_4^+ in the water at the time is still high enough to affect the metabolism of macrophytes.

The FAA/SC ratio was used for the evaluation of growth and resource balance (Smolders et al., 1996; Kohl et al., 1998; Saarinen and Haansuu, 2000; Cao et al., 2007; Zhang et al., 2010). In this study, the FAA/SC ratios in the plants belonging to both life forms are much higher than those recorded in another study (Cao et al., 2008), especially during the period of high NH_4^+ concentration. An imbalance in the C–N reserves of aquatic plants were found, which is attributed to high NH_4^+ in the water (Smolders et al., 1996; Cao et al., 2004, 2007; Zhang et al., 2010). Our study shows that in field conditions, the FAA/SC ratio is a sensitive indicator for monitoring the status of the C–N metabolism of aquatic macrophytes.

The soluble protein contents are significantly suppressed in all the aquatic plants during the bloom period. In previous studies, a decrease in soluble protein contents was also observed in high NH_4^+ conditions (Rabe, 1990; Praba et al., 2004; Cao et al., 2004), ascribed to the inhibition of protein synthesis by the shortage of energy and carbohydrates (Rabe, 1990), or to protein decomposition (Barker et al., 1966). The latter might occur under severe car-

Table 3
Correlation between biochemical indicators in aquatic macrophytes and environmental factors during the study period, and correlation results among physio-biochemical indicators in same aquatic macrophytes.

	Soluble protein					SC					FAA					SOD				
	M. s	P. m	V. n	T. b	N. p	M. s	P. m	V. n	T. b	N. p	M. s	P. m	V. n	T. b	N. p	M. s	P. m	V. n	T. b	N. p
T	−0.23	−0.04	−0.11	**−0.53****	−0.19	**0.38***	0.33	**−0.65****	−0.07	0.29	−0.16	**−0.61****	**−0.68*****	−0.26	−0.35	0.19	0.2	**0.86****	**0.77*****	**0.84*****
pH	−0.04	−0.16	**−0.71*****	**−0.57****	**−0.60****	**0.36***	**0.49****	−0.03	0.30	0.33	−0.16	**−0.68*****	−0.06	−0.34	−0.11	−0.11	−0.17	−0.01	0.05	−0.11
DO	**0.49****	**0.52****	**0.47***	**0.56****	**0.53****	**−0.65*****	**−0.56*****	0.12	**−0.59****	**−0.43***	**0.35***	**0.54*****	0.22	**0.57****	**0.57****	**−0.59****	**−0.47****	**−0.54****	**−0.70*****	**−0.63*****
SD	0.17	−0.18	−0.05	0.05	−0.23	−0.01	0.12	**0.72*****	0.11	0.21	0.22	−0.13	0.26	−0.19	0.06	−0.12	0.05	−0.39	−0.37	**−0.59****
Conductivity	**0.41***	**0.48****	**0.81*****	**0.88*****	**0.92*****	**−0.59****	**−0.74*****	−0.13	**−0.69****	**−0.81*****	**0.43****	**0.91*****	0.27	**0.84*****	**0.73*****	−0.28	−0.14	−0.37	**−0.44***	−0.27
COD_{Mn}	−0.03	0.14	0.25	0.04	0.22	0.31	0.16	**−0.44***	−0.04	−0.23	−0.09	−0.18	−0.15	0.15	0.08	0.01	−0.06	0.26	0.36	**0.48***
SS	0.06	0.17	0.11	0.04	0.27	−0.13	−0.14	**−0.47***	−0.32	−0.34	0.16	0.07	0.12	0.30	0.29	−0.25	**−0.37***	0.04	0.06	0.04
NH_4–N	0.40	**0.64****	**0.55****	**0.59****	**0.65****	**−0.42***	**−0.47****	−0.22	**−0.48***	**−0.58****	0.14	**0.64****	0.08	**0.62****	**0.55****	−0.29	−0.26	−0.27	−0.32	−0.13
BOD_5	0.09	**0.36***	**0.55****	0.15	0.34	−0.28	**−0.42***	−0.33	**−0.56****	−0.13	0.18	0.23	−0.40	0.23	0.22	0.13	0.11	0.37	0.22	0.29
Chl_*a*	−0.22	−0.16	−0.29	−0.30	−0.24	0.42*	**0.38***	−0.35	0.24	0.04	−0.20	**−0.46****	−0.12	−0.11	−0.18	0.11	0.05	0.32	0.38	**0.42***
TP	−0.27	−0.23	−0.23	−0.35	−0.22	0.27	0.18	−0.42*	0.13	0.04	−0.13	−0.25	−0.20	−0.11	−0.09	0.09	−0.01	0.38	**0.46***	**0.45***
TN	0.21	**0.35***	**0.64****	**0.64****	**0.80*****	**−0.49****	**−0.64****	**−0.45***	**−0.61****	**−0.72****	0.16	**0.80****	−0.02	**0.76*****	**0.68****	−0.28	−0.25	−0.21	−0.26	0.00
Microcystis	**−0.37***	−0.27	−0.06	−0.23	−0.15	**0.71*****	**0.45****	−0.21	**0.41***	0.07	−0.21	**−0.37***	−0.12	−0.25	−0.29	**0.50****	**0.37***	**0.48***	**0.68****	**0.70****
MCs	−0.26	−0.21	−0.18	−0.44	−0.33	0.13	0.02	−0.26	−0.12	0.34	0.00	−0.34	**−0.55***	−0.29	−0.12	0.34	0.31	**0.64****	**0.57***	**0.46***
Cyanobacteria	**−0.48****	**−0.38***	−0.39	**−0.49***	−0.37	**0.56****	**0.40***	−0.22	0.36	0.25	−0.19	**−0.46****	−0.23	−0.37	−0.30	**0.39***	0.26	**0.49***	**0.65****	**0.58****

	FAA–SC	FAA–SP	FAA–SOD	SC–SOD	SC–SP	SP–SOD
M. s	**−0.34***	**0.48****	−0.32	**0.60****	**−0.35***	**−0.63*****
P. m	**−0.85*****	**0.33***	−0.22	0.14	**−0.39***	**−0.38***
V. n	**0.49***	−0.01	**−0.72****	−0.24	0.00	−0.03
T. b	**−0.72*****	**0.79*****	**−0.67****	0.37	**−0.49***	**−0.59****
N. p	**−0.89*****	**0.76****	**−0.60****	**0.48***	**−0.82*****	−0.27

SP stands for soluble protein. M. s stands for *M. spicatum*, P. m for *P. malaianus*, V. n for *V. natans*, T. b for *T. bispinosa* and N. p for *N. peltata*. Black numbers are significant at $p < 0.05$ and the significance levels are $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

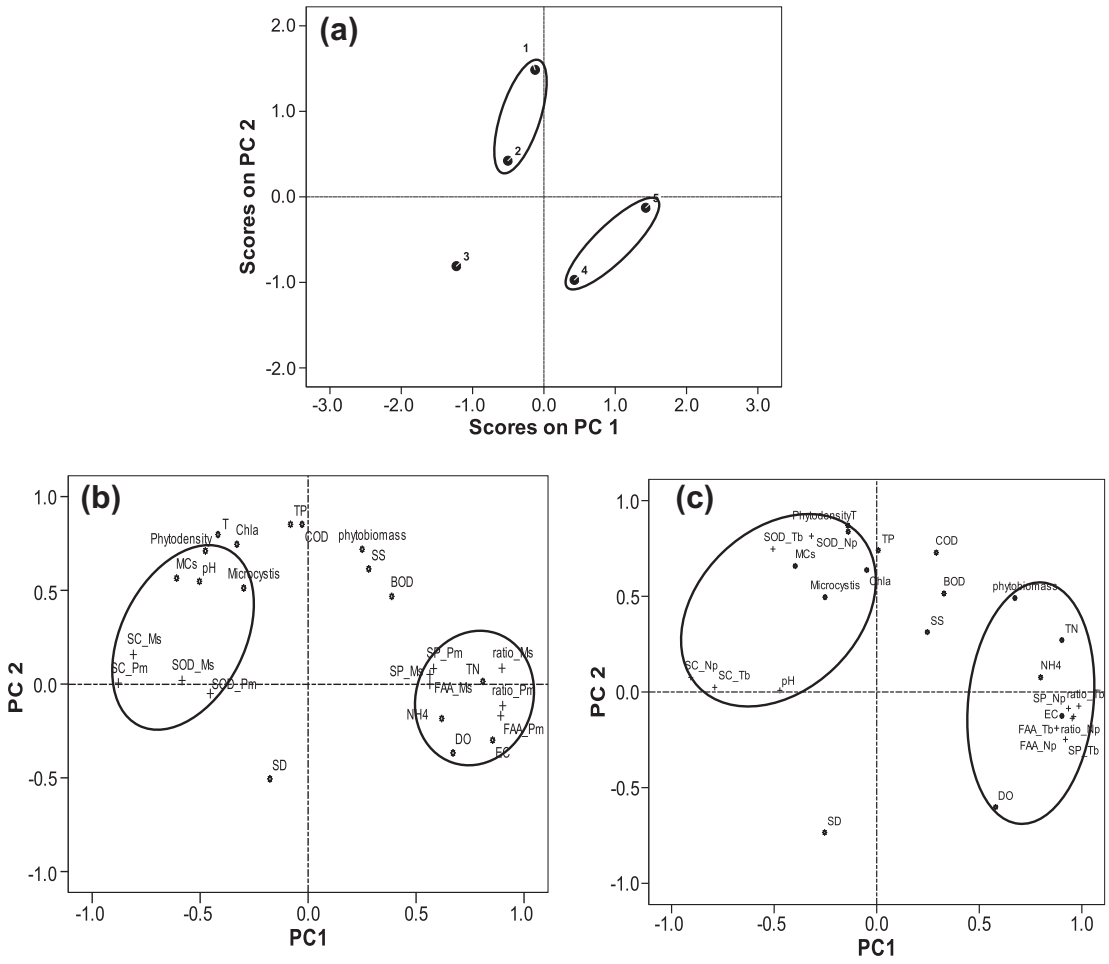

Fig. 4. (a) PCA of the antioxidant activity and C, N metabolites in five aquatic macrophytes during the cyanobacterial blooms showing the data scores labeled as species. The two PCA factors explain 85.4% of variation (PC1: 53.4%; PC2: 32.0%). Numbers 1–5 stand for submersed plants [*Myriophyllum spicatum*, *Potamogeton malaianus* and *Vallisneria natans*] and leaf-floating plants [*Trapa bispinosa* and *Nymphoides peltata*], respectively. PCA on the biochemical indicators of the plants and environmental factors of water. (b) Submersed macrophytes *M. spicatum* and *P. malaianus* (PC1 35.1% and PC2 22.1%). (c) floating-leaved plants *T. bispinosa* and *N. peltata* (PC1 42.1% and PC2 25.4%). In the figure, phytodensity and phytobiomass mean cyanobacteria density and cyanobacteria biomass, respectively. "EC" means conductivity. "Ratio" stands for "FAA/SC ratio", and "SP" for "soluble protein". M. s stands for *M. spicatum*, P. m for *P. malaianus*, Tb for *T. bispinosa* and Np for *N. peltata*. *Represents the environmental variables, and + the biochemical ones in different macrophytes.

bon starvation (Elamrani et al., 1994; Brouquisse et al., 1998). In the present study, however, NH_4^+ in the water column is positively correlated with the protein contents in all the submersed and floating-leaved macrophytes, suggesting that NH_4^+ might not suppress protein in field conditions. Instead, MCs might be the major factors that inhibit the soluble protein of the plants because the MCs and soluble protein are negatively correlated. Although the actual MCs concentration remains low throughout this study (max in enclosure: 2.6 μg L^{-1}), MCs could still have adverse effects on growth and metabolism of the plants according to the results of laboratory studies. In previous experiments, MCs is found to inhibit the protein phosphatase in a terrestrial plant *Brassica napus* at concentration of 0.9 μg L^{-1} (MacKintosh et al., 1990). The mechanism referred to might be attributed to the toxicity of MCs to protein synthesis enzymes that consequently cause the reduction in protein synthesis of the plants (Deng et al., 2010). And around the concentration of 5.0 μg L^{-1}, MCs is found to inhibit germination and root growth of Alfalfa (*Medicago sativa*), markedly trigger its oxidative stress and cause damage in 7 d (Pflugmacher et al., 2006), to increase the lipid antioxidant strongly in 3 d (Peutherta and Pflugmacher, 2010), and to induce the physiological stress significantly in a submersed macrophyte *Ceratophyllum demersum* in 2 h (Pflugmacher, 2004). In a field investigation of aquatic animals, fishes even present serious histological injury at MCs concentration of less than 1.6 μg L^{-1} in the enclosures of Meiliang Bay, Taihu Lake (Qiu et al., 2007), which locate in the middle of the Bay and not far from ours.

4.2. Responses of SOD to MCs, low light, and hypoxia in the five aquatic plants

The SOD activities of aquatic macrophytes and MCs concentration in this study are closely correlated, indicating that MCs induce considerable oxidative stress in the aquatic plants of the eutrophic lake during the occurrence of blooms. This result is consistent with those of previous laboratory studies using aquatic plants (Pflugmacher et al., 1998a,b, 1999; Pietsch et al., 2001; Pflugmacher, 2002, 2004; Wiegand et al., 2002; Hu et al., 2004, Babica et al., 2006), non-aquatic plant Alfalfa (Pflugmacher et al., 2006; Peuthert et al., 2008), Tobacco BY-2 cells (Yin et al., 2005b), seedlings of rape and rice (Chen et al., 2004), and *Arabidopsis thaliana* suspension cells (Mitrovic et al., 2004; Yin et al., 2005c) (see Supplementary material).

In the present study, the negative relationship between the SOD activities and soluble protein in the five plants is consistent with previous results (e.g. Wu et al., 2009), indicating that changes in SOD activity are not caused mainly by the changes in enzyme amounts in aquatic plants under field conditions.

In comparison with other studies, the SOD activities in this work are much lower only for submersed macrophytes, but are comparable to those of floating-leaved plants in other studies (Wu et al., 2009). The low SOD activities found in submersed macrophytes in Meiliang Bay of Lake Taihu are probably due to very low SD levels in this area (36 cm on annual average) during the study period. As suggested in the study of Wu et al. (2009), light deficiency is the primary factor suppressing the SOD activities of macrophytes in eutrophic waters. Low SOD activities in submersed macrophytes should impair the scavenging systems of ROS (Bowler et al., 1992; Alscher et al., 2002; Blokhina et al., 2003), causing more injury to the plants that suffer from multiple oxidative stresses, such as MCs and NH_4^+. By contrast, stresses of eutrophic waters in floating-leaved plants were reported to be nonsubstantial (Cao et al., 2004), indicating that floating-leaved macrophytes are more resistant to eutrophication than are submersed macrophytes. The present results show that the SOD activities of the floating-leaved macrophytes markedly increase in the period "during the blooms" compared with those in the two other stages ($p < 0.001$). This finding indicates that the antioxidative enzyme might help develop stress tolerance in floating-leaved macrophytes during the toxic blooms.

The negative correlation of SOD activities with DO in the five aquatic plants are significant during the period of the study, indicating that elevated SOD activities are induced by low oxygen stress. A similar observation was found in another field study (Cizková-Koncalová et al., 1992). Usually, DO levels higher than 7.0 mg L^{-1} should be satisfied to ensure the survival of aquatic organisms. In this work, DO concentration in August is much lower than 7.0 mg L^{-1} when the surface blooms are dense and begin to decay due to the high summer temperature. The peak of the SOD activities in all the plants simultaneously occur with the lowest DO in the water, implying the elevation of SOD activity by hypoxia stress in aquatic macrophytes.

5. Conclusions

In Meiliang Bay of Lake Taihu, serious NH_4^+ stress in submersed macrophytes occurs during the period "before the blooms". Moderate NH_4^+ stress in both submersed and floating-leaved macrophytes occur in the period "during the blooms". MCs stress is significant in aquatic plants "during the blooms". Low DO induces stress in aquatic plants only in August. Underwater low light as indicated by the low transparency of the lake water suppresses the SOD level of submersed macrophytes, which might impair their antioxidative function. The combination of the abovementioned stresses could result in serious oxidative stress and C–N metabolic imbalance in aquatic plants, particularly in submersed macrophytes. The present study may help distinguish stresses and mechanisms that cause physiological injury to aquatic macrophytes in bloom-prevailing lakes.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2010.10.038.

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