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Trends of Superoxide Dismutase and Soluble Protein of Aquatic Plants in Lakes of Different Trophic Levels in the Middle and Lower Reaches of the Yangtze River, China

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Abstract

A limnological study was carried out to determine the responses of superoxide dismutase (SOD) activities and soluble protein (SP) contents of 11 common aquatic plants to eutrophication stress. Field investigation in 12 lakes in the middle and lower reaches of the Yangtze River was carried out from March to September 2004. Our results indicated that non-submersed (emergent and floating-leafed) plants and submersed plants showed different responses to eutrophication stress. Both SOD activities of the non-submersed and submersed plants were negatively correlated with their SP contents ($P < 0.000\ 1$). SP contents of non-submersed plants were significantly correlated with all nitrogen variables in the water ($P < 0.05$), whereas SP contents of submersed plants were only significantly correlated with carbon variables as well as ammonium and Secchi depth (SD) in water ($P < 0.05$). Only SOD activities of submersed plants were decreased with decline of SD in water ($P < 0.001$). Our results indicate that the decline of SOD activities of submersed plants were mainly caused by light limitation, this showed a coincidence with the decline of macrophytes in eutrophic lakes, which might imply that the antioxidant system of the submersed plants were impaired under eutrophication stress.

Key words: eutrophication; macrophytes; soluble protein; superoxide dismutase.

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Reactive oxygen species (ROS) are produced in both unstressed and stressed plant cells (Alscher et al. 2002). The production of ROS has been shown in chloroplasts, mitochondria, peroxisome, the plasma membrane and the apoplastic space (Fridovich 1986) and production of ROS is a normal part of aerobic metabolism (Cavas and Yurdakoc 2005). Although all compartments of a cell are possible sites for O_2^- formation, chloroplasts, mitochondria and peroxisome are considered to be the most important generators of ROS (Fridovich 1986; Bowler et al. 1994). Plants have well developed defense systems against ROS, involving both limiting the formation of ROS as well as instituting its removal. Under unstressed conditions, the formation and removal of ROS are in balance. However, when exposed to increased ROS formation induced by stress conditions, the defense systems can be overwhelmed (Alscher et al. 2002) because they are unable to remove increased ROS with increased enzymatic or non-enzymatic antioxidant processes (Alscher and Hess 1993). Uncontrolled activation of ROS might damage some important parts of the cell such as DNA (Imlay and Linn 1988), proteins (Gardner and Fridovich 1991) and carbohydrates (Bradley and Min 1992). Direct attacks of ROS to cell membrane cause a complex process called lipid peroxidation (LPO). Damages in the polyunsaturated fatty acids of cell membrane may result in a failure in the permeability of cell membrane and cause cell death (Gutteridge and Halliwell 2000). Plants are well equipped with both enzymatic (superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), dehydro-ascorbate reductase (DHAR), glutathione reductase (GR)) and non-enzymatic

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(carotenoid, ascorbate, glutathione, α-tocopherol) antioxidants to overcome the oxidative stress (Upadhyay and Panda 2005). SOD forms the first step in the removal of ROS; it rapidly removes O_2^-, and hence decreases the risk of the formation of hydroxy (.OH) from O_2^-. This reaction is 10 000-fold faster than spontaneous dismutation (Bowler et al. 1992). So its role in providing protection to plants against oxidative damage is very important (Wang et al. 1989; Rout and Shaw 2001). SOD is a kind of protein that accounts for 1.6% to 2.4% of the water-soluble protein in seedlings of corn, peas, and oats (Giannopolitis and Ries 1977). Four types of this enzyme, classified by their metal cofactor, can be found in living organisms, and they are structurally similar Fe-SOD (prokaryotic organisms, chloroplast stroma) and Mn-SOD (prokaryotic organisms and mitochondria); and structurally unrelated Cu/Zn-SOD (crytosolic and chloroplast enzyme, Gram-negative bacteria) and a new type of SOD (Kim et al. 1996) with Ni in the active center. Plants are prone to suffer multifarious biotic and abiotic stresses, and the effect of SOD on the ability of plants to cope with stresses of anoxia, salt, metal pollution, polycyclic aromatic hydrocarbons pollution, nutrient deficiency, and cyanobacterial toxin microcystin-LR has been well studied (Roy et al. 1996; Yu and Rengel 1999; Rout and Shaw 2001; Blokhina et al. 2003; Ratkevicius et al. 2003; Ederli et al. 2004; Pflugmacher 2004; Upadhyay and Panda 2005; Singh et al. 2006). However, the SOD reaction of macrophyte to eutrophication stress has thus far been overlooked.

Freshwater lakes, rivers, wetlands and the coastal marine environment have been documented as having excess nutrient inputs (Smith et al. 1999), and eutrophication is recognized as a global problem. Many shallow aquatic ecosystems suffer from eutrophication and species diversity is low. On the contrary, deep plankton-dominated aquatic ecosystems are supported by various autotrophs such as vascular plants, epiphytes (algae attached to sediment, rocks, plants and other substrata), macroalgae and phytoplankton (Havens et al. 2001). When a water body receives increased additional nutrients (mainly nitrogen (N) and phosphorus (P)), autotrophs increase their biomass, and even taxonomic structure and functional groups can be dramatically changed (Valiela et al. 1997; Smith et al. 1999; Phlippart et al. 2000). These changes can further affect primary and secondary consumers through the food web (Valiela et al. 1992; Moeller et al. 1998). Excessive nutrient loading can lead to algae bloom (Paerl 1988; Burkholder and Glasgow 1997). It has been suggested that macrophytes are physiologically inhibited or even suffer from toxic effects from high nitrogen and phosphorus concentrations (Forsberg 1965; Smolders et al. 1996). Aquatic plants can generate ROS and subsequently activate the defense mechanism under eutrophication stress. There are some studies on the defense mechanism of macrophytes under eutrophication stress (Ni 2001; Song et al. 2006). However, studies on SOD changes in macrophytes under eutrophication stress are few (Cao et al. 2004; Wang et al. 2005), and are usually acute experiments that last for a short time and only focus on single species. These studies are therefore not suitable for determining a general rule for SOD in aquatic plants under eutrophication stress. Furthermore, lake eutrophication that lasts for extended periods of time in most places of the world is considered the main reason for retreat and retrogression of aquatic plants (Balls et al. 1989; Ni 1997), and SOD protects plants against oxidative damage under moderate stress (Rout and Shaw 2001). However, the way in which SOD in aquatic plants responds to long-term eutrophication is almost unknown. Therefore, the change of SOD of aquatic plants with eutrophication in natural lakes is academically necessary and important.

In the present study, a total of 11 species of aquatic plants of 12 lakes common in the middle and lower reaches of the Yangtze River were investigated from March to September 2004. As SOD is a kind of enzyme and related to total soluble protein (SP), both SOD activities and soluble protein contents of the macrophytes were measured. In this experiment, we wanted to test: (i) whether SOD activities of aquatic plants increased with increasing trophic levels of the studied lakes; (ii) whether SP contents of aquatic plants increased with increasing trophic levels of the studied lakes; and (iii) whether there were different responses of SOD activities and SP concentrations to eutrophication stress between submersed and non-submersed (floating-leafed and emergent) plants.

Results

Trophic levels of the investigated lakes

According to the standard by OECD (1982), our results of water physical chemical indices indicated the trophic level of the studied lakes to be as follows (Figure 1, Table 1): no ultra-oligotrophic and oligotrophic lakes were presented; two lakes were mesotrophic, seven were eutrophic and the other three were hyper-eutrophic. According to the trophic levels of the investigated lakes, trophic ranks from low to high were arranged using the numbers one to 12 (Figure 1, Table 1).

Correlations of SOD and SP of the plants with physical and chemical indices

A total of 11 aquatic plants, common in the middle and low reaches of the Yangtze River, were sampled at the sample stations, including three emergent plants: *Phragmites communis, Typha angustifolia, Zizania caduciflora*; one floating-leafed plant: *Trapa bispinosa*; and seven submerged plants: *Ceratophyllum demersum*, *Hydrilla verticilata*, *Myriophyllum spicatum*, *Vallisnera natans*, *Potamogeton malaianus*, *P. maakianus*, and *P. crispus*. The correlations between SP contents and

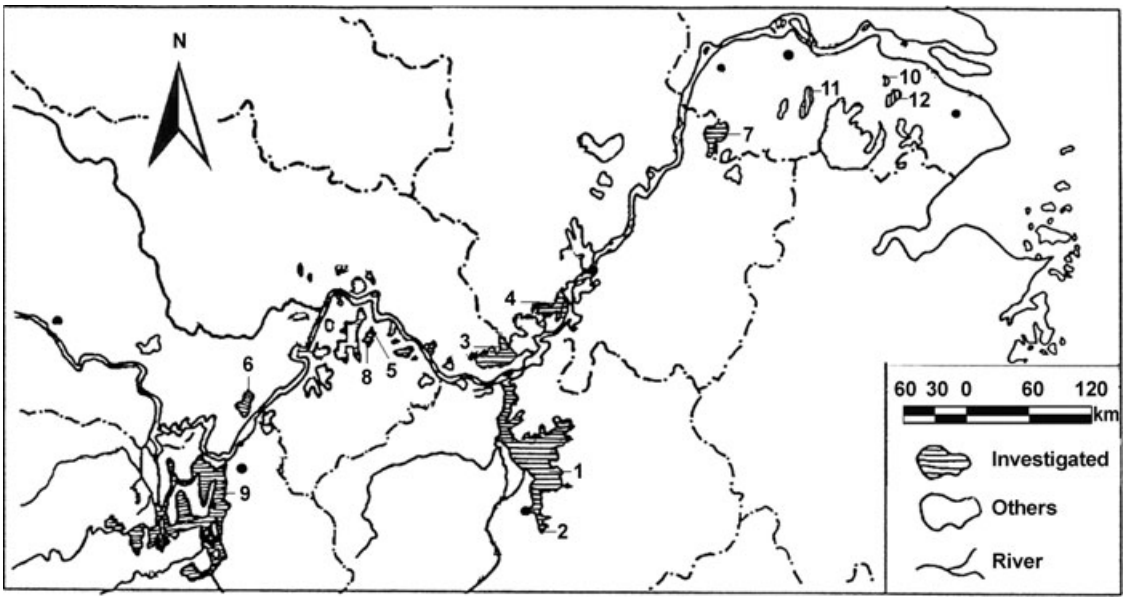

Figure 1. The investigated lakes in the middle and lower reaches of the Yangtze River.

1, Lake Poyang; 2, Lake Junshan; 3, Lake Longgan; 4, Lake Wuchang; 5, Lake Qiaodun; 6, Lake Hong; 7, Lake Shijiu; 8, Lake Baoan; 9, Lake Dongting; 10, Lake Yangcheng; 11, Lake Ge; 12, Lake Dianshan. The sequence of the number means the trophic level of the investigated lakes from low to high.

activities of SOD of the plants, both for submersed and non-submersed plants, were very strong and significant (Table 2, Figure 2A,C, $P < 0.01$). For submersed plants, activities of SOD were decreased with decline of Secchi depth (SD) in water (Table 2, Figure 2B, $P < 0.001$), SP contents of the plants were only significantly correlated with carbon variables, as well as ammonium and SD in the water (Table 2, $P < 0.05$). While for non-submersed plants, activities of SOD were not significantly correlated with any physical and chemical indices in water (Table 2, Figure 2D, $P > 0.05$), SP contents of the plants were significantly correlated with all nitrogen variables in the water (Table 2, $P < 0.05$).

Variation of SP contents and activities of SOD of the plants among life forms and seasons

As shown in Figure 3, SP contents of the non-submersed (emergent and floating-leafed) plants were higher than the submersed plants (ANOVA, $P < 0.01$), but the activities of SOD were not significantly different among life forms (ANOVA, $P > 0.05$). Both SP contents of the submersed (except *Vallisnera natans*) and non-submersed (except *Typha angustifolia*) plants were significantly different between spring and summer ($P < 0.05$). SOD activities of non-submersed plants (except *Z. caduciflora*) were significantly different ($P < 0.05$, *t*-test) between the seasons (higher in spring), while those of submersed species were not significantly different between two seasons ($P > 0.05$, *t*-test).

Discussion

Response of SOD activities of the plants to eutrophication

With the expansion of the economy, macrophytes in the middle and lower reaches of the Yangtze River suffer from durative eutrophication stress due to increasing loads of nitrogen and phosphorus (Tong et al. 2005). Eutrophication stress even results in the severe retreat and retrogression of macrophytes in this region (Ni 1997). It has been proved that some aquatic plants, such as *P. malaianus* and *C. demersum*, can be aroused to acute antioxidative responses under mesotrophical stress in this region (Wang and Li 2002; Wang et al. 2005).

The present results did not show any consistent changes between the SOD activities and the increase of soluble protein of both the submersed and the non-submersed plants, which implied that the decline of SOD activities of the plants was due to enhanced degradation or inactivation of the enzymes rather than the decrease of total protein level of the plant (Yu and Rengel 1999). With the trophic levels of lakes increasing, on one hand, accumulation of nitrogen was found in aquatic plants (Wu et al. 2005), which would result in producing more ROS by accelerating nitrogen metabolism in the plant (Foyer et al. 1994), and on the other hand, the activity of SOD enzymes declined as found in the present study, which caused the accumulation of produced ROS in cells and caused further cell damage (Imlay and Linn 1988; Gardner and Fridovich 1991; Bradley and Min 1992; Gutteridge and Halliwell 2000). Therefore, the contrasting

Table 1. Limological characters and sampled plants for the 12 studied lakes in the Yangtze River area

Lakes	Poyang	Junshan	Longgan	Wuchang	Qiaodun	Hong	Shijiu	Bao'an	Dongting	Yangcheng	Ge	Dianshan
Phragmites communis	+		+	+		+	+	+	+	+	+	+
Typha angustifolia			+	+						+		
Zizania caduciflora			+			+		+		+		
Trapa bispinosa	+	+	+	+	+	+	+	+		+		
Ceratophyllum demersum			+	+		+	+	+	+	+		
Hydrilla verticilata	+	+	+			+	+		+	+		
Myriophyllum spicatum	+		+	+	+	+	+	+	+	+		
Vallisnera natans	+	+	+	+		+		+	+	+		
Potamogeton malaianus	+	+	+			+	+		+	+		
P. maakianus					+	+	+			+		
P. crispus	+		+	+		+		+	+	+		
Latitude	29°16′N	28°37′N	29°57′N	30°16′N	30°15′N	29°49′N	31°30′N	30°12′N	29°15′N	31°25′N	29°16′N	31°10′N
Longitude	115°58′E	116°18′E	116°10′E	116°40′E	114°41′E	113°22′E	118°53′E	114°43′E	113°02′E	120°49′E	119°49′E	120°58′E
Area (km^2)	3583.7	192.5	316.2	100.5	8.0	348.4	210.4	3.6	2915.8	119.1	146.5	63.7
SD (cm)	133	221	67	109	138	58	113	48	83	78	35	33
TP (mg/L)	0.02	0.05	0.03	0.06	0.04	0.08	0.07	0.09	0.05	0.09	0.25	0.25
TN (mg/L)	1.21	0.62	0.52	0.48	0.59	1.69	0.69	0.92	0.79	0.63	1.22	1.52
pH	6.4	7.5	7.8	7.9	7.9	8.0	7.8	7.9	8.4	8.3	nd	8.5

"+", sampled; nd, no data; SD, Secchi depth; TN, total nitrogen; TP, total phosphorus.

Table 2. Relationships between soluble protein (SP) contents, activities of superoxide dismutase (SOD) of aquatic plants and physical and chemical indices in lake water

	Submersed ($n = 189$)				Non-submersed ($n = 46$)			
	SOD		SP		SOD		SP	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
SOD			−0.42	<0.001			−0.68	<0.001
SP	−0.42	<0.001			−0.68	<0.001		
TIC	−0.12	ns	0.17	<0.05	−0.01	ns	0.04	ns
TOC	−0.07	ns	0.18	<0.05	−0.05	ns	0.06	ns
DIC	−0.12	ns	0.21	<0.01	−0.06	ns	0.12	ns
DOC	−0.09	ns	0.19	<0.01	−0.05	ns	0.08	ns
TN	−0.01	ns	0.12	ns	−0.16	ns	0.43	<0.01
TDN	−0.08	ns	0.01	ns	−0.16	ns	0.35	<0.05
NO_3	−0.09	ns	0.03	ns	−0.23	ns	0.38	<0.01
NH_4	−0.02	ns	0.23	<0.05	−0.19	ns	0.41	<0.01
NO_2	−0.08	ns	0.04	ns	−0.33	ns	0.45	<0.01
TP	−0.00	ns	0.13	ns	0.19	ns	−0.34	<0.05
TDN	−0.05	ns	0.01	ns	0.14	ns	−0.29	ns
SD	0.51	<0.001	−0.44	<0.001	0.21	ns	−0.04	ns

"−", negative correlative; ns, not significant ($P > 0.05$); DIC, dissolved inorganic carbon; DOC, dissolved organic carbon; NH_4, ammonium; NO_2, nitrite; NO_3, nitrate; SD, Secchi depth; TDN, total dissolved nitrogen; TIC, total inorganic carbon; TN, total nitrogen; TOC, total organic carbon.

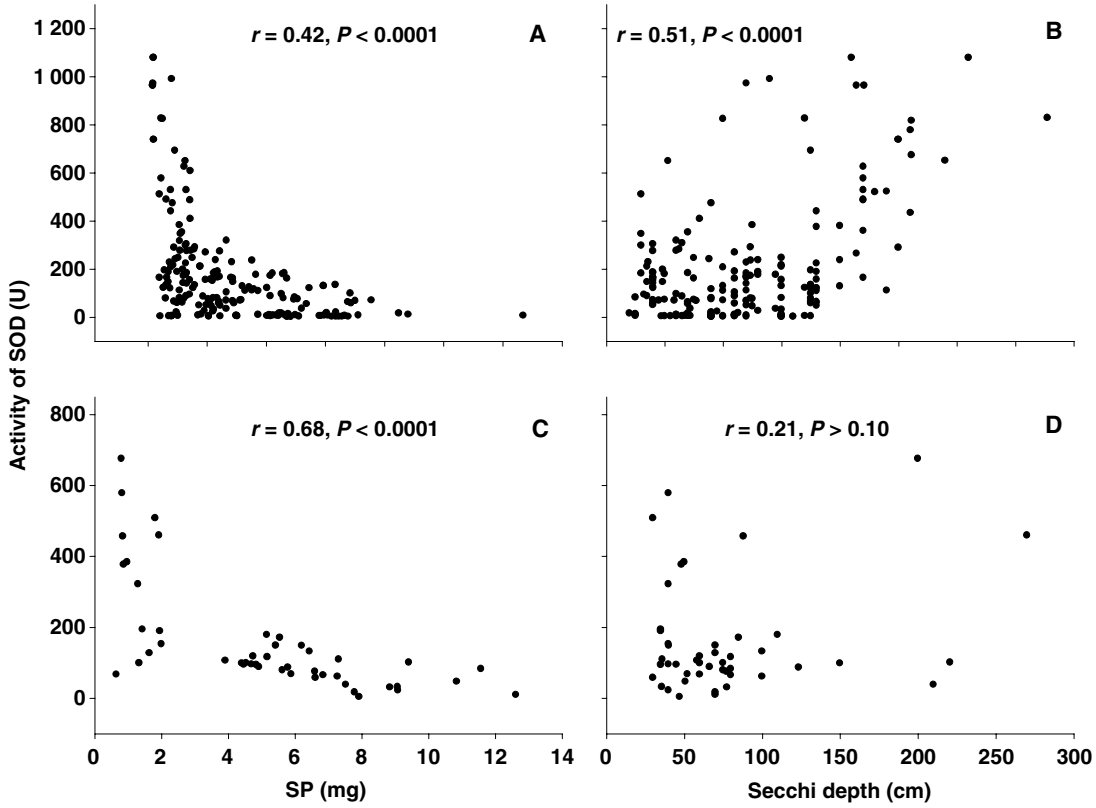

Figure 2. Correlations between superoxide dismutase (SOD) activities and soluble protein (SP) concentrations of submersed (A and B) and non-submersed (C and D) plants and Secchi depth (SD) in water.

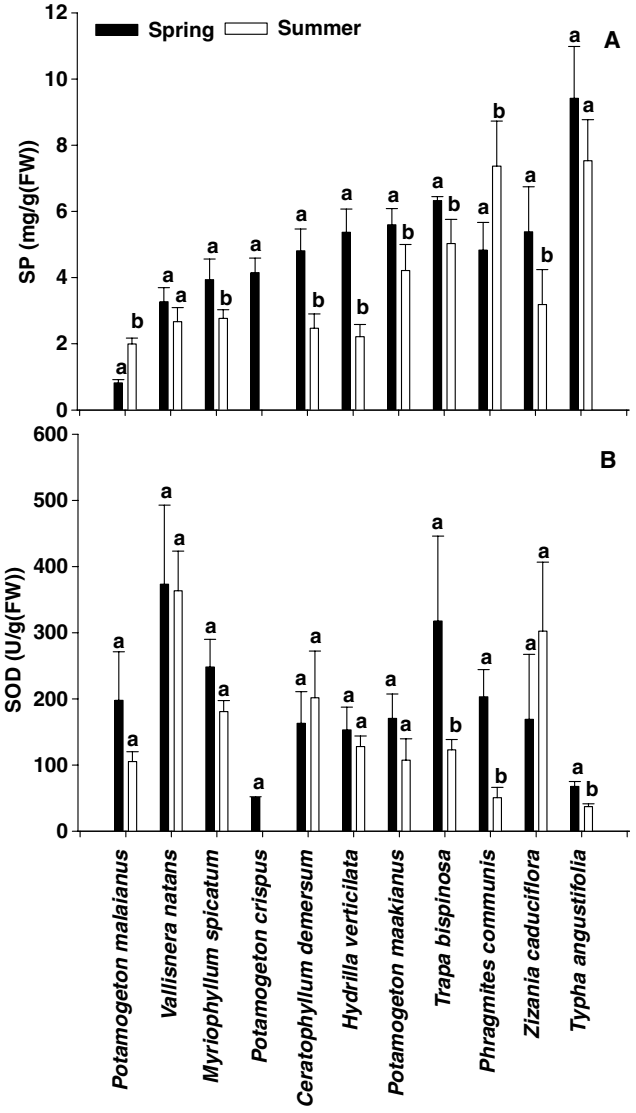

Figure 3. Soluble protein (SP) **(A)** contents (± standard deviation (*SD*)) and superoxide dismutase (SOD) **(B)** activities (± *SD*) of the investigated plants in spring and summer.

trends of soluble protein contents of the plants increased with increasing trophic levels of lakes, while SOD activities of the aquatic plants decreased, might lead to an imbalance between the producing and scavenging systems of reactive oxygen species.

Both SOD activities of the submersed and non-submersed plants in the present study did not show any significant correlation with carbon, phosphorus and nitrogen variables, indicating that no oxidative stress was caused by elevation of phosphorus and nitrogen concentration in the water, which was in agreement with Blindow (1988). Under stress conditions, the activities of antioxidative enzymes (including SOD) of the plant was reported to increase in the early stage, providing a certain degree of protection from oxidative damage, and then decline with the duration of the stress (Yu and Rengel 1999). Our results showed that SOD activities of non-submersed plants were not significantly correlated with Secchi depth (SD), while SOD activities of the submersed plants were decreased with decline of (SD), which proved that activities of SOD of the submersed macrophytes on the whole were in the stage of decline with the decline of SD during the process of eutrophication. Therefore our results denoted that light limitation in the water rather than carbon, phosphorus and other nitrogen variables was a critical factor causing oxidative stress to the submersed plants. This was consistent with other former experiments (Phillips et al. 1978; Balls et al. 1989; Sand-Jensen et al. 2000; Riis and Sand-Jensen 2001). In eutrophic lakes, both SOD activities of submersed plants and diversity and quantity of macrophytes are declining with increasing trophic levels of lakes, which might imply that the antioxidant system of the submersed plants were impaired under the stress of eutrophication. The different responses to SD between submersed and non-submersed plants were presumably caused by their different life forms and different responses to increased eutrophication (Riis et al. 2000; Sand-Jensen et al. 2000; Riis and Sand-Jensen 2001).

Changes of soluble protein of the plants with eutrophication

It was reasonable that SP contents of the non-submersed macrophytes were positively correlated with various nitrogen species in the water, because macrophytes could luxuriously absorb more nitrogen when external nutrient levels were higher (Gertoff and Krombholz 1966; Wetzel 1975). However, SP contents of the submersed macrophytes were only positively correlated with ammonium in the water, which was explained in that ammonium was the most direct form of nitrogen for submersed macrophytes (Best 1980). Furthermore, it was reported that an increasing supply of inorganic nitrogen (ammonium and nitrate) promoted synthesis of amino acid and thus increased the amount of nitrogen stored in plants as free amino acids and protein nitrogen (Singh and Srivastava 1986; Limpens and Berendse 2003). The present result showed that carbon concentrations in the water and SP content of the non-submersed plants were not significantly correlated while the correlations between carbon concentrations and SP content of the submersed plants were significant. It might be that non-submersed plants can acquire their carbon source from air while submersed plants can not. When dissolved inorganic carbon (DIC) supply is higher under eutrophication conditions, carboxylation activity of Rubisco of submersed plant is accordingly higher, which increased the critical tissue-N concentration of submersed plant (Madsen et al. 1998; Madsen and Cedergreen 2002). Abundant inorganic carbon supply was also found to promote the rate at which N assimilated into organic compounds (Limpens and

Berendse 2003). Our results confirmed that the correlation between DIC in water and SP of aquatic plants also existed on a regional scale for macrophytes in natural lakes.

Materials and Methods

Study area

Along the Yangtze River many lakes centralize in China. In this region, the climate consists of subtropical monsoons with rainfall primarily in the summer. The region suffers from durative eutrophication due to increasing loadings of nitrogen and phosphorus drained by agriculture, industry and household waste (Tong et al. 2005). The retreat of macrophytes with progressive eutrophication is very severe in this region (Ni 1997). In the present study, a total of 12 shallow lakes along the river were investigated during May to September 2004 (Figure 1). The lakes are either large or small, connected to or separated from the Yangtze River, and near to or far away from cities. These lakes represented a gradient in trophic status (Figure 1, Table 1).

Sample collection and measurements

According to area, shape and heterogeneities of the studied lakes, three or more stations were set evenly at each lake by GPS in order to obtain accurate results. There were in total 123 sample stations and every station was visited twice in spring (March to May) and summer (June to September), respectively. Physical indices (including pH and Secchi depth) were measured immediately at each station, water and aquatic plants were sampled at the same time. Immediately on return to the laboratory a known volume (1 L) of each water sample was filtered through prewashed glass fiber filters. The filtered water representing the dissolved fraction was used to measure total dissolved nitrogen (TDN), total dissolved phosphorus (TDP), ammonium (NH_4-N), nitrite (NO_2-N) and nitrate (NO_3-N). Total phosphorus (TP) and TDP in the lake water were measured by colorimetry after digestion with $K_2S_2O_8$ + NaOH to orthophosphate (Ebina et al. 1983). Total nitrogen (TN) and TDN were digested simultaneously with TP and TDP. After digestion, TN and TDN were determined by the alkaline potassium persulfate digestion-UV spectrophotometric method (Nydahl 1978). Ammonium was determined by the Nessler method, nitrite by the α-naphthy-method and the concentrations of nitrate were determined using the automated Korolev (Dionex-100 Ion Chromatography, Sunnyvale, CA, USA). Dissolved inorganic carbon (DIC), total inorganic carbon (TIC), dissolved organic carbon (DOC) and total organic carbon (TOC) were measured with O. I. Analytical WIN TOC1010 Total Organic Carbon analyzer (O. I. Corporation, College Station, TX, USA).

In each sample site, three apical shoots (10 cm in length and similar in morphology) of a submersed macrophyte were collected, three apical shoots (5 cm in length and similar in morphology) of a floating-leafed macrophyte (*Trapa bispinosa*) were collected, and three intact, young shoots with open leaves of emergent macrophytes adjacent to the sample site (< 20 m) were collected (pH and SD were recorded at this site). All of the samples were then preserved in liquid nitrogen immediately and brought back to the laboratory. The samples were then frozen at $-80\,^{\circ}$C until they were measured. In each sample site and sampling season, we used three parallel samples for each species at the same time. Activities of SOD and contents of soluble protein were also detected three times. The frozen samples were ground into fine powder in liquid nitrogen with a mortar and a pestle. To determine the enzyme and SP, 1.0 g fine powder was extracted for 20 min at $4\,^{\circ}$C in 5 mL ice cooled buffer (50 mM potassium phosphate (pH 7.8) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ascorbate, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1% (w/v) PvP, 0.5% (v/v) Trition X-100 and 5 mM 2-mercaptoethanol), and centrifuged (JOUAN BR 4i, Centrifuge, St Herblain, France) at 15 000 *g* and $4\,^{\circ}$C for 10 min. The supernatant was used for analysis. SP content was determined by the Coomassie brilliant blue-bye binding method (Bradford 1976) using bovine serum albumin (BSA) as standard. The assay of SOD (EC 1.15.1.1) was based on a method described by Bayer and Fridovich (1987), which measured spectrophotometrically the inhibition in the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm. Each 3 mL reaction mixture containing 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 μM 4-nitroblue tetrazolium chloride (NBT), 2 μM riboflavin, and 20 μL of enzyme extract, was transferred to a transparent test tube. Reaction was carried out at $25\,^{\circ}$C under illumination with two 15 W fluorescent tubes providing a photon flux density of around 40 μmol/m^2 per s for 20 min. The absorption was measured at 560 nm and denatured protein extracts were used as blank. One unit of SOD was defined as 50% inhibition of the NBT photo reduction to blue formazan. SOD activity of the extracts was expressed as units of SOD per g fresh weight (FW).

Data analyses

Seasonal dynamics in SP concentrations and activities of SOD of each species were tested by the Student's *t*-test. The differences of SP concentrations and SOD activities between submersed and non-submersed plants were tested by a simple two-way ANOVA. The relationships between SP concentrations and activities of SOD of the plants and physical and chemical indices in water were analyzed with least-squares regression of the raw data using the software package STATISTICA 6.0 (Statsoft Inc., Tulsa, OK, USA).

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