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Physiological responses of *Vallisneria spiraslis* L. induced by different hydraulic conditions when exposed to copper and nitrogen

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A two-flume experiment with submerged plant *Vallisneria spiraslis* L. was conducted to investigate the effects of different hydraulic conditions on physiological responses when exposed to water polluted with copper (Cu) and nitrogen (N). Plants were divided into two groups and grown for 120 h in hydrodynamic and hydrostatic flumes, respectively, using the same nutrient solution with fixed concentrations of 0.64 mg l^{-1} CuSO$_4$ and 4.0 mg l^{-1} total N. Combined pollution with Cu and N resulted in rapid increase in chlorophyll in the first hour and relative growth rate of plants in hydrodynamic water increased from 0 to 0.073 in the first 8 h. Meanwhile, peroxidase (POD) activity in shoots sharply increased to alleviate supraoptimal oxidative stress. And proteolytic degradation in shoots was observed to be catalyzed as protein contents in shoots decreased. Persistent decline in total chlorophyll and relative growth rate was noticed in longer duration. In roots, POD activity increased only at 8 h and decreased after 24 h. Malonaldehyde (MDA) content in roots also increased only at 8 h and MDA content was lower ($P < 0.01$), while protein content was higher ($P < 0.01$) after prolonged exposure than those at 0 h. Comparison of different hydraulic groups indicated that hydrodynamic condition induced higher POD activity in shoots in the first hour to alleviate the toxicity resulted by Cu and N. Thus, relative growth rate of plants was significantly higher than that in hydrostatic group ($P < 0.05$). While roots in hydrodynamic condition were less influenced by water flow, its protein contents in hydrodynamic group were significantly higher ($P < 0.01$) than those in hydrostatic group at all durations. These results suggested that hydrodynamic condition alleviated combined toxicity of Cu and N in *V. spiraslis* L shoots and enhanced the accumulation of protein in roots.

Key words: Physiological response, hydrodynamic, copper, nitrogen, *Vallisneria spiraslis* L.

INTRODUCTION

Exposure to heavy metals is an increasing problem worldwide (Croudace et al., 1995; Ahsan et al., 2007; Meng et al., 2008). Meanwhile, nutrient enrichment of shallow coastal areas has led to a decline in environmental quality (Lillebø et al., 2005; Kowalkowsk, 2009). Heavy metal pollution and eutrophication has occurred simultaneously in much urban river water and shallow lakes (Cheung et al., 2003). Such combined pollution in water affects growth of aquatic plants and their potential for phytoremediation. Research on using hydrophytes to reduce levels of metals and nutrients was mainly by hydrostatic simulations. In fact, the characteristics of many eutrophicated shallow lakes are greatly affected by dynamic factors, such as wind, waves and the velocity of entering water. Hydrodynamic conditions play an important role in transportation of pollutants and influence growth of aquatic plants through water flows and shearing forces (Wang et al., 2008a; Maassen and Balla, 2010; Aldous et al., 2005). This indicates that the need for phytoremediation studies focused on hydrodynamic simulations.

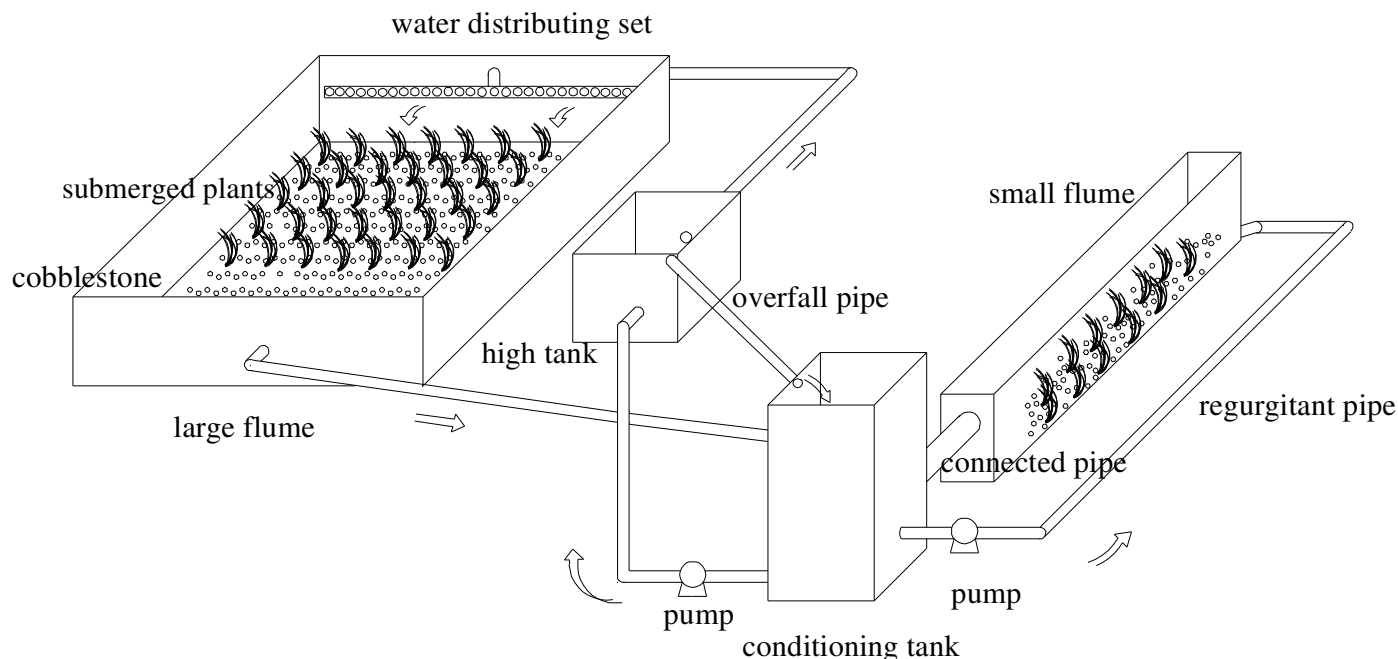*Corresponding author. E-mail: cwang@hhu.edu.cn. Tel: +86-(025)-83786028.

Figure 1. Experimental facility for hydrodynamic and hydrostatic treatments.

Copper (Cu) is released into the environment by human activities (Yruela, 2005). In aquaculture systems, Cu is regularly used in the form of $CuSO_4$ as an agent to control algal blooms and aquatic macrophyte infestations at concentrations of 0.5 to 2.0 mg L^{-1} (Boyd and Massaut, 1999). And, Cu is an essential micronutrient for plants and acts as a structural constituent in regulatory proteins, participates in photosynthetic electron transport (Yruela, 2005) and is involved in catalyzing redox reactions in mitochondria and chloroplasts (Marschner, 1995). However, plant accumulation of Cu to 25 to 50 mg kg^{-1} dry foliage is phytotoxic (Chaney, 1989). Excess Cu causes a broad range of deleterious effects such as inhibition of photosynthesis and pigment synthesis, disintegration of membrane and other metabolic disturbance (Lanaras et al., 1993). On the other hand, nitrogen (N) is an important element for most living organisms; however, it can be a toxic pollutant at high concentrations. Especially, excess ammonia can have a range of negative effects on plants at organism and cellular levels and thus, contribute to macrophyte decline (Cao et al., 2007; Wang et al., 2008).

Vallisneria spiraslis L. is a dominant submerged plant distributed in Taihu Lake and river courses in the downstream Yangtze River (Li, 2006). Nutrient removal and plant uptake by *V. spiraslis* have been studied (Bao et al., 2006; Wang et al., 2008b). Vajpayee et al. (2005) reported possible Cu-induced inhibition of nitrate reductase activity in *V. spiraslis*. Yang (2008) investigated the effects on protein and total chlorophyll when exposed for 10 days to different Cu and N concentrations under hydrostatic conditions; however, no data were collected under different hydraulic conditions.

To study the effects of hydraulic conditions on physical response of riparian plants, *V. spiraslis* L. were selected to be exposed in the combined pollutants with Cu and N under hydrodynamic and hydrostatic conditions in two flume experimental sets (Figure 1). Based on preparatory experiments, we used a time course of 120 h for this study. Relative growth rate, photosynthetic pigments and protein content of the plants were measured and MDA content was monitored to indicate membrane damage, while the level of POD was used to indicate the capacity of *V. spiraslis* L to withstand oxidative stress.

MATERIALS AND METHODS

Experiment facility

The experimental facility used (Figure 1) was described by Wang et al. (2008a). Water in the small flume was pumped from the conditioning tank through a regurgitant water pipe to form a circulation. Meanwhile, water in the conditioning tank was pumped to the high tank and then flowed by gravity through pipe to the large flume, where steady water flow was formed using water distribution set and finally, was discharged back to the conditioning tank circlingly. The nutrients and pollutants were vigorously mixed in the conditioning tank to ensure homogeneous water quality.

The flow velocities at the middle level of the water column were measured by an acoustic doppler velocimeter (ADV). The average velocities of five transects were 2.67 and 0.08 cm s^{-1} for the small and large flumes, respectively. The significant difference (P < 0.01, n = 5) in velocity was due to the great disparity in size of the two flumes and power of the two pumps, respectively. Thus, the small flume was considered as the hydrodynamic flume and the large flume was assumed to be the hydrostatic flume.

Table 1. External environment parameters collected at 14:00 pm everyday from hydrodynamic (D) and hydrostatic (S) flumes.

Date	2008-4-13		2008-4-12		2008-4-11		2008-4-10		2008-4-9	
Treatments	S	D	S	D	S	D	S	D	S	D
pH	7.31 ± 0.01	7.32 ± 0.02	7.88 ± 0.01	7.84 ± 0.01	7.97 ± 0.03	7.84 ± 0.02	7.37 ± 0.02	7.35 ± 0.01	7.34 ± 0.02	7.29 ± 0.02
DO (mg l^{-1})	8.1 ± 0.3	8.0 ± 0.3	8.4 ± 0.1	8.5 ± 0.2	8.6 ± 0.1	8.6 ± 0	8.4 ± 0.1	8.3 ± 0.3	8.6 ± 0.2	8.7 ± 0.2
EC (μm s^{-1})	328 ± 5	328 ± 3	324 ± 6	324 ± 2	324 ± 3	323 ± 4	324 ± 6	324 ± 3	323 ± 7	325 ± 5
ORP (mv)	504±2	506±2	473±1	461±7	478±6	463±4	500±5	500±2	489±6	539±7
Velocity (cm s^{-1})	0.08 ± 0.01	2.67 ± 0.02	0.08 ± 0.01	2.67 ± 0.02	0.08 ± 0.01	2.67 ± 0.02	0.08 ± 0.01	2.67 ± 0.02	0.08 ± 0.01	2.67 ± 0.02
Temperature (°C)	18.6 ± 0.3	18.7 ± 0.2	18.8 ± 0.1	19.2 ± 0.1	18.0 ± 0.1	18.4 ± 0.1	17.5 ± 0.4	17.1 ± 0.1	16.6 ± 0.2	17.0 ± 0.2
Illumination (lux)	9390 ± 16	8630 ± 19	10600 ± 26	9290 ± 32	1010 ± 10	998 ± 6	803 ± 5	778 ± 4	2110 ± 8	1810 ± 12
NH$_4^+$-N (mg l^{-1})	2.13 ± 0.08	2.04 ± 0.06	2.16 ± 0.07	2.28 ± 0.10	2.03 ± 0.03	2.05 ± 0.01	2.04 ± 0.02	2.17 ± 0.02	2.05 ± 0.03	2.08 ± 0.04
NO$_3^-$-N (mg l^{-1})	2.12 ± 0.04	2.11 ± 0.03	2.19 ± 0.11	2.12 ± 0.02	2.10 ± 0.05	2.04 ± 0.02	2.17 ± 0.06	2.19 ± 0.05	2.26 ± 0.09	2.32 ± 0.10

Treatment condition and plant material

To provide relatively invariable conditions of nutrition and pollutants for the entire experiment, half of the water in the system was renewed daily. Water temperature, pH, dissolved oxygen concentration and other conditions were measured daily. The test results indicated that the different hydraulic groups shared the same external environmental conditions except for flow velocity (Table 1).

The *V. spiraslis* L. were collected from Meiliang Bay of Taihu Lake. Before experiments, plants were cultivated with soil in laboratories for two weeks. And the plants with healthy growth and uniform size were chosen and then recultivated for one week in solution containing 1/80 Hoagland's trace elements (Hoagland and Arnon, 1950) and macro-nutrients including 0.05 mM CaCl$_2$, 0.007 mM KH$_2$PO$_4$, 0.2 mM MgSO$_4$ and 0.7 mM K$_2$SO$_4$. In the experiments, Cu and N were prepared using 0.64 mg L^{-1} CuSO$_4$ according to the experiment of Yang (2008) and N was prepared with KNO$_3$ and (NH$_4$)$_2$SO$_4$ at the ratio of 1:1 to obtain the total N concentration of 4.0 mg L^{-1} simulating conditions of Taihu Lake based on the report of Tian et al. (2007). Harvested plants were rinsed thoroughly with demineralized water and then blotted.

Plant growth parameters

Plant fresh biomass was weighed immediately after harvest and relative growth rate (*R*) was calculated as:

$$R = \frac{B' - B_0}{B_0}$$
(1)

Where, B_0 is the initial plant biomass (g) weighted at the beginning of the experiment and B' is the plant biomass (g) after exposure to pollution solution for a certain time.

A 0.3 g sample of shoot material was ground in liquid N$_2$ and then extracted in 96% ethanol for 24 h in darkness. After centrifugation at 10,000 × *g* for 10 min at 4°C, absorbance of the supernatant was measured at 649 and 665 nm. The chlorophyll concentration was calculated as described by Van Dijk and Roelofs (1988).

Determination of lipid peroxidation

The level of lipid peroxidation in plants was estimated by MDA content according to the method of Heath and Packer (1968). Material of 0.3 g fresh sample was ground in mortars with 5 ml of 1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 × *g* for 10 min and 1 ml of the supernatant was added into 2 ml reactive solution of 0.5% thiobarbituric acid in 20% TCA. After incubation in water for 30 min at 95°C, the mixture was refrigerated immediately to stop reaction. Sample was centrifuged at 10,000 × *g* for 5 min and the supernatant was measured by UV1900 spectrophotometer. Absorbance at 532 and 600 nm was recorded and the amount of MDA calculated by subtracting non-specific absorbance at 600 nm from absorbance at 532 nm, using the extinction coefficient (ε) = 155 mM^{-1} cm^{-1}.

Extraction and protein determination

Samples of 0.8 g of shoot and 0.4 g of root were separately ground in liquid N$_2$ and homogenized with 5 ml of 50 mM chilled potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1% polyvinyl pyrrolidone (w/v). The supernatant for measurement was obtained after centrifugation of homogenate at 4°C.

Protein content was estimated spectrophotometrically at 595 nm (Bradford, 1976), with bovine serum albumin (BSA) as the standard.

Assay of antioxidant enzyme POD

POD activity was tested by estimating the oxidation of guaiacol caused by H$_2$O$_2$ (Upadhyaya et al., 1985). The reaction mixture was composed of 2 ml of 50 mM potassium phosphate buffer (pH 5.5), 100 μl of 2% H$_2$O$_2$, 1 ml of 0.05 mM guaiacol and 100 μl of enzyme extract. Activity was determined by monitoring the increase of absorbance at 420 nm, using ε = 26.6 mM^{-1} cm^{-1}. One unit of POD activity was defined as the amount required to decompose 1 μmol guaiacol min^{-1} mg^{-1} protein.
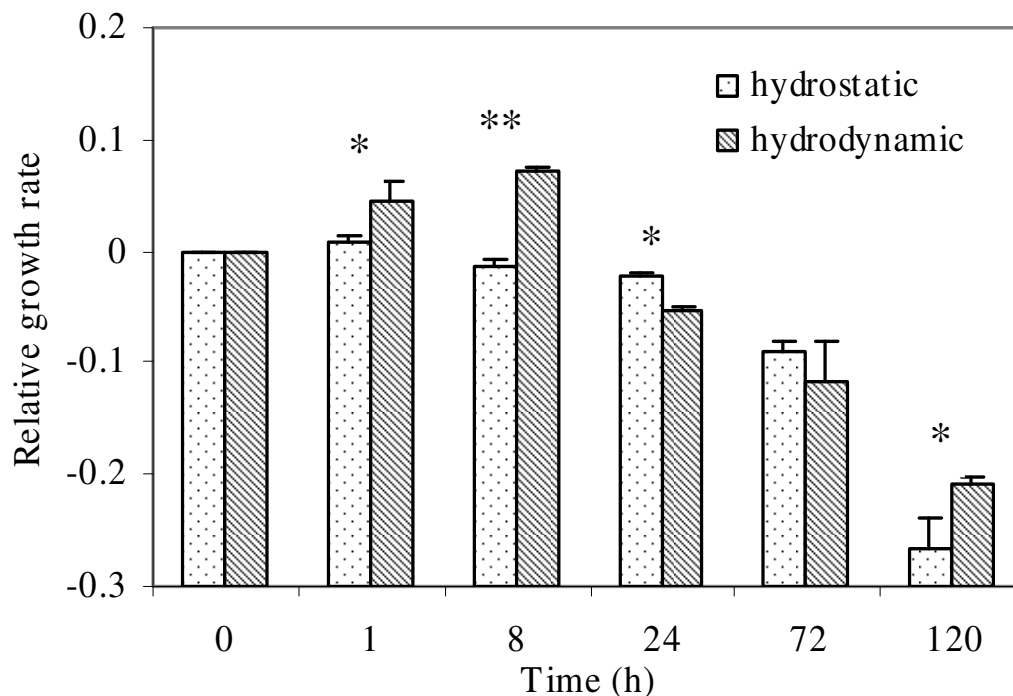
Figure 2. Effect on relative growth rate of *V. spiraslis* L. induced by different hydraulic conditions when exposed to combined Cu and N pollution. All values are means ± SD (n = 3). Significant differences between hydrodynamic and hydrostatic treatments are shown: * as P < 0.05 and ** as P < 0.01.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Data from different hydraulic treatments were compared by a two-way analysis of variance (ANOVA) and statistically different treatments were identified by t test. Statistical analyses were performed using the SPSS statistical package (ver. 11.5, SPSS Co., Chicago, USA). The star plots were derived using Microsoft Excel 2003 program. The flume experimental facility for hydrodynamic and hydrostatic treatments was processed with CAD 2004.

RESULTS

Effects on plant growth

Plants exposed to 0.64 mg l^{-1} Cu and 4.0 mg l^{-1} N showed significant effects on growth rate. Firstly, there were visual changes after 72 h of exposure, such as weak chlorosis of shoots and slight rot of roots. And the relative growth rate of plants in hydrodynamic condition increased slightly in the first 8 h followed by a rapid decrease after 24 h (Figure 2), while, they only increase slightly in the first 1 h in hydrostatic flume. In comparison to hydrostatic, significant higher growth rates were found in hydrodynamic group in short duration. Compared with 0 h, relative growth rate of plants decreased significantly with maximum decline of 21 and 27% at 120 h in hydrodynamic and hydrostatic flumes, respectively, (Figure 2).

Effects on photosynthetic pigments

Effects of different hydraulic conditions on the contents of photosynthetic pigments (total chlorophyll and chlorophyll a and b) in the shoots of *V. spiraslis* L were studied over time. Photosynthetic pigments increased slightly in the first hour and decreased after 24 h (Figure 3). There were significant effects on total chlorophyll and chlorophyll b caused by different hydraulic conditions at 1, 8 and 120 h (Figure 3b, c). However, the contents of total chlorophyll a rose abruptly at 1 h but there were no significant declines till 72 h in the hydrodynamic condition (Figure 3a). The ratio of chlorophyll a to chlorophyll b, which reflects the loss of pigments, fluctuated in the hydrodynamic condition; however, it gradually declined after 8 h in the hydrostatic group (Figure 3a, b).

Effects on MDA content

MDA content was measured to assess the level of lipid POD imposed by combined factors (Cu, N and hydro-dynamic conditions). Hydrodynamic treatment resulted in lower MDA levels in shoots than in the hydrostatic treatment. However, significant differences only happened at 8 and 120 h. Compared with 0 h, MDA in shoots increased to 0.0085 µmol g^{-1} at 8 h and followed by a slight decrease to 0.0061 µmol g^{-1} at 72 h in the
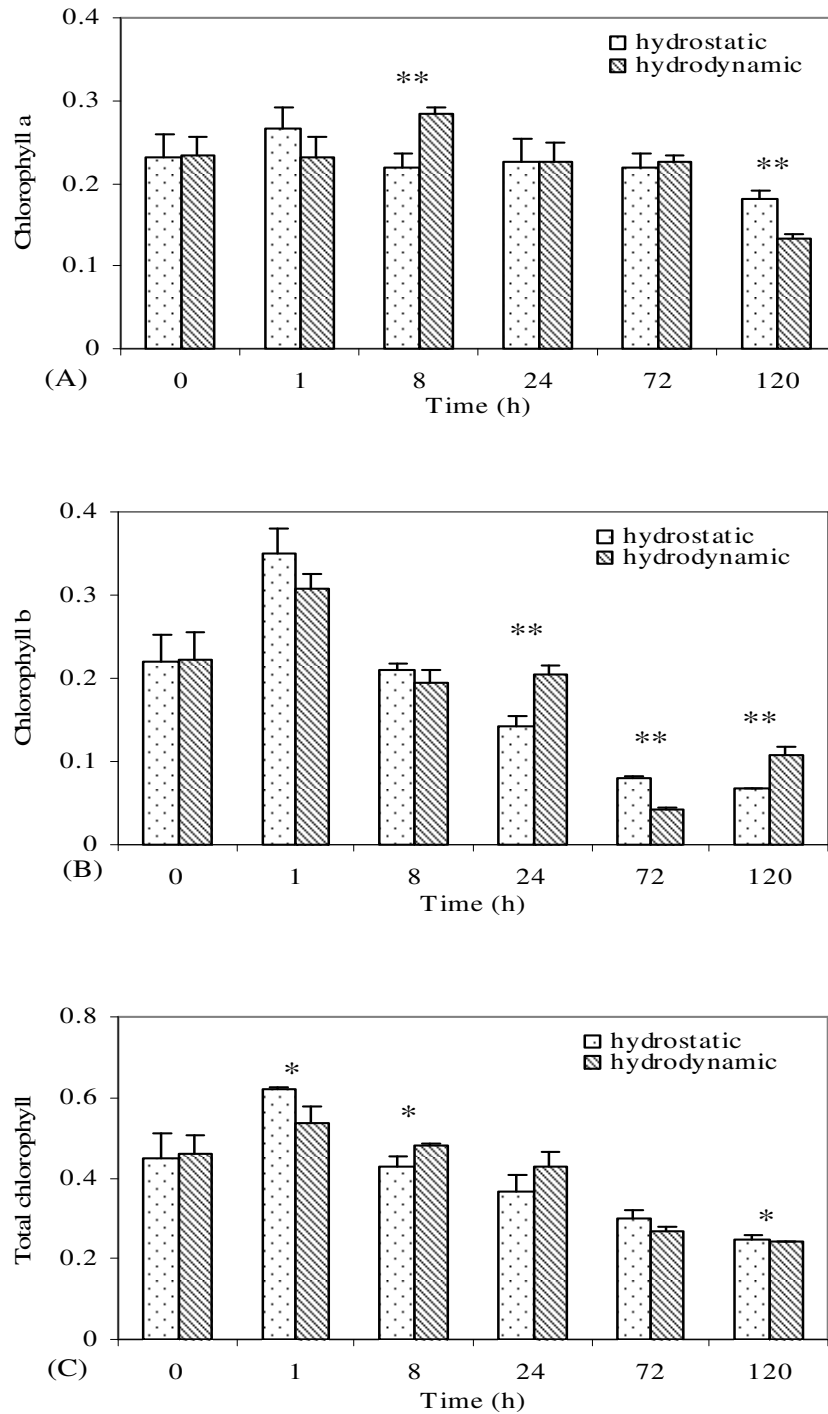
Figure 3. Effect on chlorophyll a (A) chlorophyll b (B) and total chlorophyll (C) contents (µmol g^{-1} fresh weight) of *V. spiraslis* L induced by different hydraulic conditions when exposed to combined Cu and N pollution. All the values are means ± SD (n = 3). Significant differences between hydrodynamic and hydrostatic treatments are shown: * as P < 0.05 and ** as P < 0.01.

hydrostatic case (Figure 4a). After prolonged exposure, the values of MDA content in shoots were similar to the level as they were at 0 h. In roots, there were significant differences between different hydraulic groups at 24 and

72 h. There were similar responses in roots and in shoots in hydrodynamic flume. Compared with 0 h, there were consistently significant decreases of MDA content in roots after 24 h, a decrease of 47 and 50% for hydrodynamic
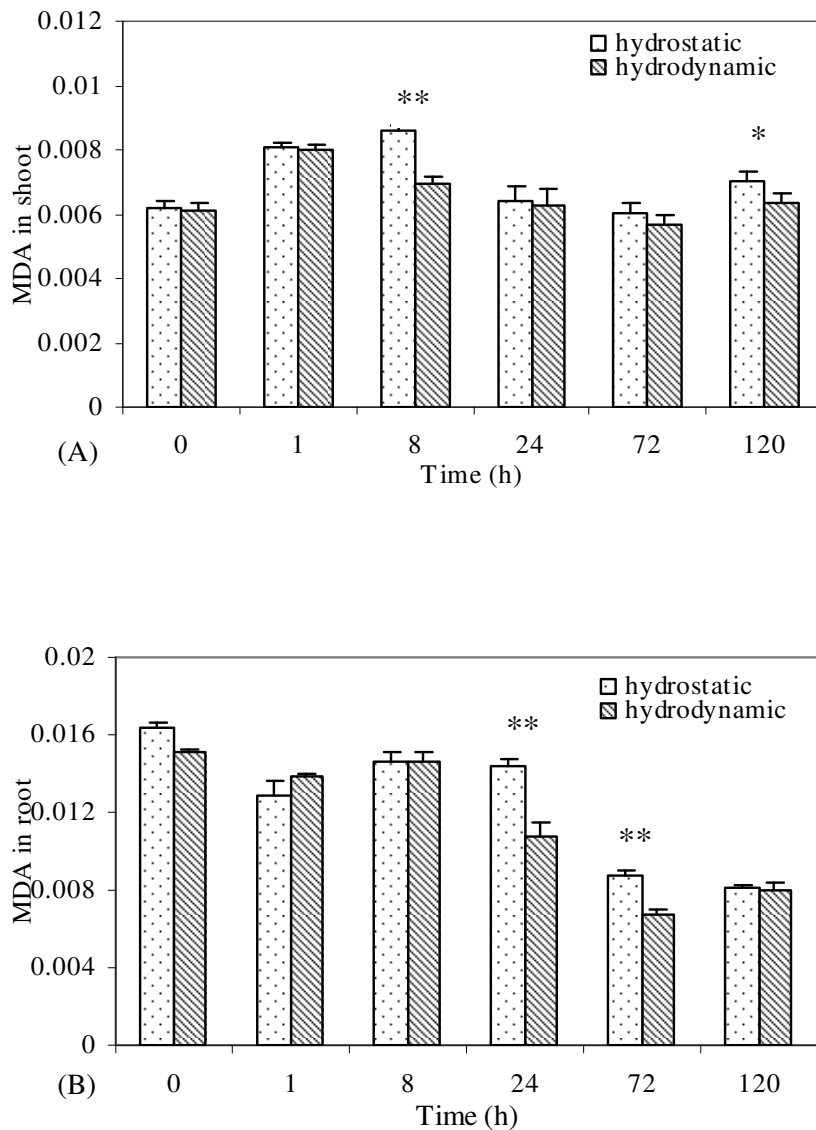
Figure 4. Effect on MDA content (µmol g^{-1} fresh weight) in shoots (A) and roots (B) of *V. spiraslis* L induced by different hydraulic conditions when exposed to combined Cu and N pollution. All the values are means ± SD (n = 3). Significant differences between hydrodynamic and hydrostatic treatments are shown: * as $P < 0.05$ and ** as $P < 0.01$.

and hydrostatic groups, respectively (Figure 4b).

Effects on protein content

Protein contents in both shoots and roots were examined (Figure 5). For shoots of the hydrodynamic group, the protein level increased in the first 24 h and decreased later. In comparison to hydrostatic, the hydrodynamic treatment resulted in significantly lower protein levels in shoots after 24 h of exposure (Figure 5a). Maximum protein contents were 1.06 and 1.35 mg g^{-1} fresh weight for the hydrodynamic and hydrostatic groups at 24 and 1

h, respectively. There were significant higher levels of protein in roots in the hydrodynamic than the hydrostatic group after 8 h. Levels of protein in roots increased con-sistently up to 72 h and then slightly decreased (Figure 5b). The maximum protein level increased 4.53 fold in roots treated in hydrodynamic conditions after 72 h.

Effects on POD activity

POD activity in shoots increased slightly in the first 8 h and sharply after 72 h of exposure. For the hydrodynamic group, the highest value of POD activity in shoots was
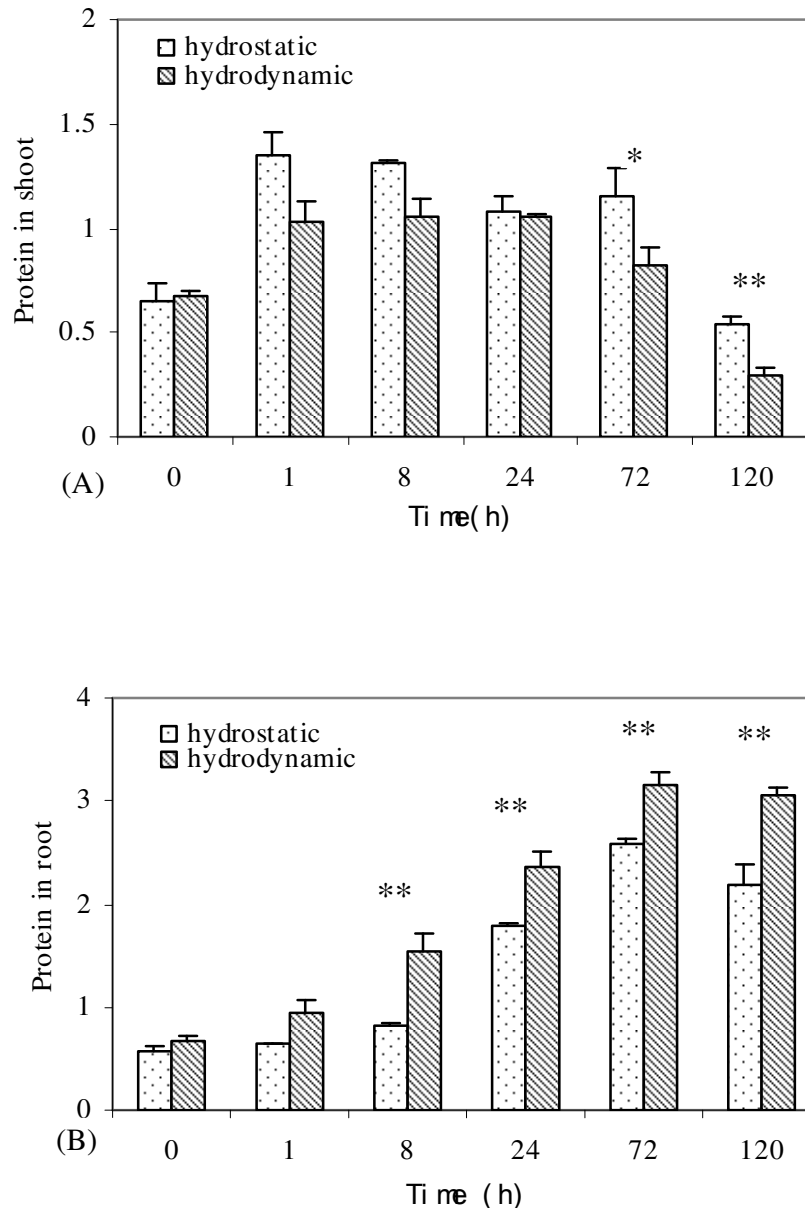
Figure 5. Effect on protein content (mg g^{-1} fresh weight) in shoots (A) and roots (B) of *V. spiraslis* L induced by different hydraulic conditions when exposed to combined Cu and N pollution. All the values are means ± SD (n = 3). Significant differences between hydrodynamic and hydrostatic treatments are shown: * as P < 0.05 and ** as P < 0.01.

1.78 units mg^{-1} protein at 120 h, it is about 5.4 times of the value at 0 h. In roots, POD activity in plants in the hydrostatic flume was significantly higher than the hydrodynamic group at 1 and 8 h. For the hydrostatic group, the maximum POD activity increased to 0.06579 units mg^{-1} protein within 1 h and declined to 0.00057 units mg^{-1} protein at 120 h. But, there was a continuous decrease of POD activity in roots over the time course for the hydrodynamic group which was remarkably different from that for dydrostatic group. And the declines were

significant after 24 h (Figure 6b).

DISCUSSION

Nutrient uptake and biomass change

Hydrodynamic conditions may change the external environment of aquatic plants and consequently affect plant growth and nutrient absorption (Xu and Qian, 2004).
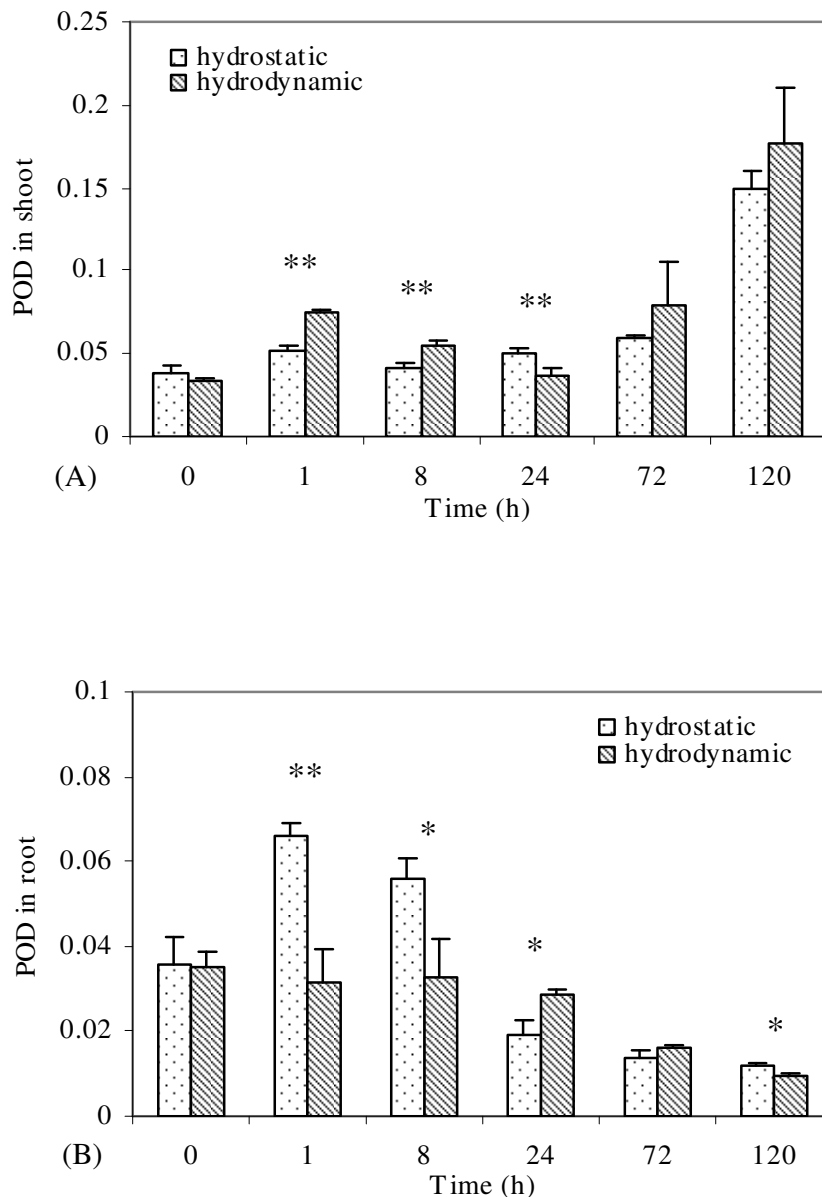
Figure 6. Effect on POD activity (unit mg^{-1} protein) in shoots (A) and roots (B) of *V. spiraslis* L induced by different hydraulic conditions when exposed to combined Cu and N pollution. All the values are means ± SD (n = 3). Significant differences between hydrodynamic and hydrostatic treatments are shown: * as $P < 0.05$ and ** as $P < 0.01$.

In this study, relative growth rate of plants between hydrodynamic and hydrostatic treatments were significantly different over the entire time course excluding at 72 h. Compared with the hydrostatic, the hydrodynamic condition resulted in a gradual increase of biomass in the first 8 h followed by a sharp decrease. This may be due to a hormesis effect of the hydrodynamic conditions. A hormesis effect is defined as any stimulatory or beneficial effect induced by a low dose of toxicant that cannot be predicted by extrapolation of detrimental or lethal effects induced by high doses of the same toxicant (Calabrese

and Baldwin, 2003). Relative growth rate of *V. spiraslis* L. during 120 h exposure indicated that hydrodynamic condition enhanced growth rate in short duration and mitigated the loss of biomass in longer duration. Also, Wang et al. (2008a) has reported that hydrodynamic condition resulted in more accumulation of nitrogen in *V. spiraslis* L. in comparison to the hydrostatic after 1 day N exposure. Because of the toxicity of high doses of Cu, the ability of absorption and transportation of macronutrients and micronutrients by *V. spiraslis* L. decreased and then the biomass of *V. spiraslis* L. decreased correspondingly.

Water flow can take away waste substances produced by plant metabolism and supply more opportunities for plant-nutrient contact. Thus, Xu and Qian (2004) found that N uptake of asparagus at water flow rates of 160 to 175 L h^{-1} was 18.3 to 24.3% higher than in hydrostatic water within 12 h. Due to the effect of water flow, it is possible that more nutrients were absorbed by plants in the hydrodynamic flume, which finally mitigated the decreasing trend of relative growth rate in this study.

Chlorophyll concentration in leaves of *V. spiraslis* L.

Effects of Cu were reportedly more noticeable when plants were grown on NO_3^- (Kumar et al., 1990). Separate N treatments and both N and Cu combined treatments were studied by Yang et al. (2008), results showed that the combination of Cu and N led to higher levels of total chlorophyll in *V. spiraslis* L. leaves compared with a separate N treatment after 1 day exposure. However, in the next 5 days, the total chlorophyll gradually decreased for the combined group but maintained a high level for the separate N group. Similar results were observed in this study. Combination of Cu and N increased chlorophyll concentration in the short term and decreased it in the long term. The increase of total chlorophyll in the first hour is probably due to the plenty supply of plastocyanin under Cu stress on *V. spiraslis* L. Degradation of total chlorophyll and chlorophyll b after 1 h exposure was probably due to distortion of chlorophyll structure and inhibition of photosynthetic pigment synthesis (Baryla et al., 2001) and the photoxidative mechanism of Cu-induced damage (Prasad et al., 2001). Damage of *V. spiraslis* L., including biomass reduction and photo-synthetic apparatus might reduce the redox and meta-bolic energy (Mosulén et al., 2003) and impair the uptake of essential elements such as manganese and iron (Somashekaraiah et al., 1992). In this study, the reduction in biomass and photosynthetic pigments were signifi-cantly positively correlated (R = 0.766 for hydrodynamic, R = 0.937 for hydrostatic group; both P < 0.05). In addition, total chlorophyll of *V. spiraslis* L. in hydrodynamic flume was significantly different from that in hydrostatic flume at 1 and 8 h in this study. Similarly, Wheel et al. (1980) found that photosynthesis of *Macrocystis pyrifera* leaves increased over 300% as the water flow velocity increased from 0 to 4 cm s^{-1}. However, Wang et al. (2008 a) concluded that the content of pigments in *V. spiraslis* leaves was lower in hydro-namic condition compared with the hydrostatic, while it is exposed to high concentration of nitrogen (60 mg l^{-1}). Such differences might be due to the different concen-trations of nutrition and contaminations used in these studies.

MDA contents in leaves of *V. spiraslis* L.

Generally, lipid peroxidation is led by highly reactive oxidant hydroxyl radicals (OH$^-$) through degradation of polyunsaturated fatty acids (Apel and Hirt, 2004). Excess Cu can lead to an effective reduction of lipid content (Ouariti, 1997) as well as to strong changes in fatty acid composition (De Vos et al., 1991). MDA is a secondary end product of polyunsaturated fatty acid oxidation. Srivastava et al. (2006) showed significant increases in MDA in *Hydrilla verticillata* leaves exposed to 25 μM Cu. In contrast, Gupta et al. (1996) demonstrated decreased MDA content in *H. verticillata* plants stressed by excess Cu. In this study, MDA content in shoots increased sharply in the initial exposure period, followed by a gradual decrease during prolonged exposure and finally, main-tained a level similar to that at 0 h. Meanwhile, MDA content in roots decreased over the whole experiment period. The reason for lipid peroxidation alleviation in roots was speculated to be the increased antioxidative response and translocation of Cu from root to shoot (Welsh and Denny, 1980). MDA content in shoots was more pronounced in the hydrostatic than the hydro-dynamic case during almost all of the experiment. However, in roots, there were differences between hydraulic treatments only at 24 and 72 h. The alleviated lipid peroxidation in hydrodynamic conditions may be due to the relatively thin boundary layer around shoots resulting in greater contaminants transportation. A boun-dary layer is a thin layer of fluid through which momen-tum, heat and mass are transported from the fluid stream to the plant surface. The greatest resistance to transport for submerged hydrophytes is through the boundary layer, while increasing the water motion around plant shoots decreases boundary layer thickness (Dromgoole, 1978; Lehman, 1978), thus, a thicker boundary layer around shoots under hydrostatic conditions led to nutrient deficiency, accumulation of other toxic substrates accu-mulation which finally resulted in more lipid peroxidation in shoots of plants in hydrostatic conditions.

Protein content in *V. spiraslis* L.

Total protein content in shoots increased continuously in the initial 24 h, followed by a decrease with prolonged exposure. In roots, protein content increased remarkably in 72 h and showed slightly declined trend. Protein stress resulted in antioxidant enzymes involving GSH synthesis. It was speculated to the initial increases in protein content in shoots (Toppi and Gabbrielli, 1999). Production of stress proteins alleviated lipid peroxidation in *V. spiraslis* L., so, MDA content and protein content were negatively correlated (R^2 = -0.956 for hydrodynamic group and R^2 = -0.813 for hydrostatic group in roots in the experiment). However, after long-term exposure, plants eventually failed to keep homeostasis and developed stress symptoms to decrease protein content as suggested by Seth et al. (2007). Suzuki et al. (2001) found that protein denaturation is an important component of responses related to metal toxicity. In our

study, proteolytic degradation in shoots was observed in longer duration. Decrease of proteins may be due to oxidative modification and increased proteolytic activity induced by free radicals (Romero-Puertas et al., 2002). Protein content in shoots in hydrodynamic conditions was significantly lower than under hydrostatic conditions after 72 h. Conversely, protein content in roots of the hydrodynamic group was significantly greater than the hydrostatic group after 1 h exposure in the combined pollution of Cu and N. The results indicated that the hydrodynamic condition induced a hormesis effect on roots due to the influence of water flow. In our study, plants of *V. spiraslis* L. were fixed in flumes with cobblestones around roots. Hydrodynamic parameters, like bottom shear stress and the rate of energy dissipation, were correlated with the diffusion characters of the pebbles layer thickness (Hearn et al., 2001) and then affected the pollutants stress on roots.

Antioxidant enzymes activity in *V. spiraslis* L.

Various stresses can induce reactive oxygen species (ROS) (Mittler et al., 2004) and excessive ROS can cause oxidative damage to bio-molecules such as lipids, proteins and nucleic acids, leading to cell membrane peroxidation, protein hydrolysis and even DNA strand breakage. To minimize this oxidative damage, plants have developed a complex defense anti-oxidative system, including non-enzymatic anti-oxidants as well as anti-oxidative enzymes such as peroxide superoxide dismutase (SOD, EC 1.15.1.1), catalase (EC1.111.1.6) and peroxidases (PODs) like guaiacol peroxidase (EC 1.11.1.7). Many studies have investigated several defense strategies in plants induced by metal concentrations. Phytochelatins (PCs) is part of the first protective responses and play an important role in the chelation of metals (Steffens, 1990), but not for Cu in plants. Schat et al. (2002) reported that PC-deficient *Arabidopsis* species were not Cu-hypersensitive. Cuypers et al. (2005) demonstrated no increase in level of PCs in 10-day-old bean seedlings treated with Cu.

To mitigate the oxidative damage initiated by ROS, plants have developed a complex defense anti-oxidative system, including low-molecular mass anti-oxidants as well as anti-oxidative enzymes (Guo et al., 2007). Super-oxide dismutase (SOD) and POD are essential components of the anti-oxidative defense system. SOD catalyzes the conversion of the superoxide anion to H_2O_2, while POD utilizes H_2O_2 in the oxidation of various inorganic and organic substrates. POD is located in the cytosol, cell wall and vacuolar and extracellular spaces (Mishra et al., 2006). Elevated POD activity is considered a stress marker enzyme with high affinity for H_2O_2 (Andrew et al., 2002). The results showed that POD activity in shoots increased slightly in the initial period, followed by a sharp increase after 72 h, with a rapid

decrease of protein content accordingly. This is assumed to be due to the fact that H_2O_2 was accumulated under the stress of Cu and N over the prolonged period. The formation of ROS during the oxidative burst could cause overload of anti-oxidants substrate and then lead to the inhibition of anti-oxidant enzymes. However, an oxidative modification of proteins, which can result in a higher proteolytic degradation, has been reported after heavy metal exposure (Romero-Puertas et al., 2002). Compared with hydrostatic, the significantly higher POD activity in shoots under hydrodynamic conditions occur-red at 1 and 8 h. POD activity in the hydrodynamic roots declined gradually over the whole time course. This implied that less stress was imposed on roots in the hydrodynamic flume and so did not increase POD activity. This may be attributed to the fact that roots were buried in the cobblestone layer in the flume bottom and the exchange of anti-oxidants substrates by water flow and oxidants stress were weakened on roots among the spaces of cobblestones. However, further investigation of this behavior is needed to determine the mechanism.

In summary, the biomass of *V. spiraslis* L. increased in short duration under the combined pollution with Cu and N, but finally decreased after 24 h exposure. Although, antioxidant enzyme POD responded to the oxidative stress induced by Cu and N, proteolytic degradation in shoots was not well restrained. Comparison of different hydraulic groups suggested that hydrodynamic condition alleviated the toxicity on plants thus, increased relative growth rate in hydrodynamic group. Turbulent hydro-dynamic condition enhanced exchanges of nutrients between water and plants which partially increased the accumulation of protein in the roots of *V. spiraslis* L.

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