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Original Research Paper

Morphological and Physiological Responses of *Lemna minor* to Aniline

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ABSTRACT

The ecotoxicological effects of aniline on *Lemna minor* have been evaluated based on both morphological and physiological responses in this paper. The results showed a significant inhibition to frond number and area, growth index, and biomass of *L. minor*. The contents of both chlorophyll *a* and *b* went down significantly after exposure to aniline. Aniline shows an acute toxic effect on the vegetative growth of *L. minor* and the effect is correlated with its concentration. Aniline might impact the growth of *L. minor* through destroying its photosynthesis. The activities of CAT, POD and SOD shown different responses to aniline at different times, but higher concentration of aniline and persistence would be more harmful to *L. minor* according to the changes of the activities of antioxidant enzymes. *L. minor* could be used as an indicator to monitor the existence of aniline in water bodies.

INTRODUCTION

Urbanization and industrialization are considered as two vital factors contributing to increasing pollution (Sim & Balamurugan 1991, Harvey et al. 2002, Wang et al. 2008, Dhote & Dixit 2009, Zhang et al. 2011). Wastes resulting from urbanization and industrialization often contain contaminants, such as heavy metals and organic chemicals, which are typically hazardous to environment (Sengupta & Venkatachalam 1994, Kucukmehmetoglu & Geymen 2008, Su et al. 2010, Cumar & Nagaraja 2011). Water bodies around urban areas are often acting as important receivers of many pollutants (Kucukmehmetoglu & Geymen 2008, Dhote & Dixit 2009). Therefore, increasing studies have paid great attention on the ecological effects of pollutants on water ecosystems in recent years (Teisseire et al. 1998, Mitsou et al. 2006, Olette et al. 2008, Dhote & Dixit 2009, Gorzerino et al. 2009). Because of their important roles in monitoring and removal of pollutants, plants are attracting a growing number of researchers' interests.

Aniline is a main industrial chemical for producing numerous products, such as pigments and herbicides (EPA 1994). It is also commonly used as a raw material for rubber industry (EPA 1994). In both the developing and developed worlds aniline is a very important industrial chemical, both historically and presently (Uter et al. 2007). For instance, La (2010) reported that about 1,735,000 tons of aniline produced every year in China. In United States, the estimated total production capacity of aniline was 1,380 million pounds only in 1992 (Mannsville 1992). Aniline release happens easily in the production of polymers, pesticides, pharmaceuticals and dyes (EPA 1985). It is reported that about 1.6 million pounds was released to environments in 1992 and 16 thousand pounds of them was released directly to surface water (TRI92 1994). When it is released into our surroundings, it could lead to toxic symptoms such as cyanosis, dyspnea, fatigue to human beings and also some adverse effects appeared in rats and other animals (EPA 1994). However, little is known about its environmental influences, especially its influences on aquatic ecosystem (Ammann & Terry 1985). Although some studies have shown that aniline shows great toxicity to animals (EPA 1994, Bhunia et al. 2003), it is not enough for us to completely understand its environmental effects.

As a model aquatic plant, *Lemna minor* has been widely used to test toxicity of contaminants for its small size, structural simplicity, rapid growth, easy cultivation and widespread occurrence (Wang & Williams 1990, Wang 1991, Moody & Miller 2005). In this paper, the effect of aniline on the vegetative growth and physiological responses of *L. minor* were investigated.

MATERIALS AND METHODS

Plant material: *L. minor* L., an aquatic floating plant, has been used to test the toxic effect of aniline on aquatic plants in this experiment. It is widely distributed in still or slightly flowing waters except in Arctic and Antarctic regions (Landolt 1986), and it is also among the most standardized test organisms in ecotoxicological assessment (OECD 2002, ISO 2004, Naumann et al. 2007, Radiae et al. 2010). In this study, *L. minor* was collected from a pond in the outskirts of

Yangzhou city of Jiangsu Province, China and cultivated for accommodation in our greenhouse (the temperature was 24±2°C, the light was provided by metal halide bulbs for 14h/d) for a week before treatment by aniline.

Toxicity test: The toxicity testing method developed by Organization for Economic Cooperation and Development (OECD 2002) was adopted after little modification. All plants were cultivated separately in plastic containers (10 cm high, 20 cm in diameter) containing 1000 mL of Hoagland medium with different aniline concentration diluted in the nutrient medium. In each container, ten plants (30 fronds) with similar sizes ($p>0.05$) were carefully placed. And another 30 fronds were kept to determine their fresh and dry weights. The modified Hoagland solution (Table 1) was added periodically into the containers during the whole experiment. Aniline concentrations selected for this study ranged from 0.1 to 1.0 g/L according to the pre-treatment experiment results. The concentration gradients of aniline were prepared as follows: 0.0 g/L (CL), 0.1 g/L (G1), 0.2 g/L (G2), 0.4 g/L (G3), 0.6 g/L (G4), 0.8 g/L (G5), 1.0 g/L (G6). The dose-response tests conditions were the same during the experiment. In each group, fifteen and six replicates were designed for evaluating morphological and physiological responses of *L. minor* to aniline, respectively.

Determination of frond number, leaf area and biomass: The frond number of *L. minor* in each groups was recorded every day from beginning (day 0) to the end of the experiment (day 7). The fresh and dry weights of *L. minor* at the beginning of the experiment were determined by the thirty individuals untreated by aniline. Meanwhile, frond area was measured (n=70) only on day 0 and 7 respectively by LI-3000C Portable Area Meter (LI-3000C Transparent Belt Conveyer Accessory) from LI-COR, Inc.

During the experiment, other changes of morphological characteristics, such as decolour of fronds, root fracture, and new unmatured fronds produced from mother fronds were also recorded.

Determination of photosynthetic pigments: Approximately 50 mg of fresh frond material in each group at different times was stored in fridge with -70°C for determining chlorophyll and carotenoid contents of *L. minor* after the treatment by aniline. The method used by Lichtenthaler (1987) was applied to measure and calculate the chlorophyll and carotenoid concentrations. The iced material was first homogenized in 3 mL 80% (v/v) buffered acetone (800 mL acetone, 195 mL water, 5 mL ammonia (25% w/v) and then centrifuged at 4000rmp for 15 min. Absorbance was measured in a spectrophotometer at 663 nm, 646 nm, and 470 nm.

Determination of antioxidant enzyme activities: Approximately 100 mg of fresh fronds was homogenized in 5 mL cold sodium phosphate buffer (0.1 M, pH 7.8, within 1% polyvinyl pyrrolidone) to obtain CAT enzyme extract. Then the homogenate was centrifuged at 12000rpm (4°C) for 15 min. The supernatant was used as the enzyme extract which was saved for analysis. All the work for preparation of enzyme extract was carried out at 4°C according to Zou (2000). POD and SOD enzyme extracts were the same as the CAT extract except for the sodium phosphate buffer (0.05 M, pH 7.0, within 1% polyvinyl pyrrolidone).

POD activity was determined spectrophotometrically by measuring the increase in absorbance at 470 nm after 20 min incubation at room temperature. The reaction mixture contained sodium phosphate buffer (50 mM, pH 7.0, 2 mL), H_2O_2 (10mM, 0.8 mL), guaiacol (1%, v/v, 0.8 mL) and enzyme extract (0.2 mL) (Razinger et al. 2007). The reaction started by adding H_2O_2.

CAT activity was evaluated spectrophotometrically by measuring the consumption of H_2O_2 at 240 nm (Aebi 1984). Where the testing medium contained in final volume of sodium phosphate buffer (200 mM, pH 7.8, 1.5 mL), H_2O_2 (100 mM, 0.1 mL), and enzyme extract (0.2 mL) in a final volume of 3 mL.

SOD was determined according to Zou (2000). SOD activity was measured spectrophotometrically by measuring the increase in absorbance at 560 nm after exposure of light 25 min incubation at 30°C. The reaction mixture contained sodium phosphate buffer (50 mM, pH 7.0, 1.5 mL), Met (130mM ,0.3mL), NBT (750 μM, 0.3 mL), EDTA-Na_2 (100μM, 0.3 mL), riboflavin (20μM, 0.3mL) distilled water (0.5 mL) and enzyme extract (0.1 mL).

Determination of MDA activity: MDA activity was determined to indicate the level of lipid peroxidation of fronds as described by Zhao (2000). Enzyme extract (2 mL) and thiobarbituric acid (0.5%, v/v, 2 mL) were boiled for 15 min and then centrifuged at 4000 rpm for 15 min. The supernatant was measured spectrophotometrically at wavelength of 532 nm, 600 nm and 450 nm.

Data analysis: Growth index (GI) was calculated as follows (Khellaf & Zerdaoui 2009):

$$\text{Growth index}=\frac{\text{biomass(t=7days)}}{\text{biomass(t=0days)}}$$

Comparison between control and treatments was statistically analysed by one-way ANOVA using SPSS 17.0 packages. The differences were statistically significant at $p < 0.05$.

RESULTS

The effect of aniline on the fronds of *L. minor*: The frond number of *L. minor*, whether in control or in treatment

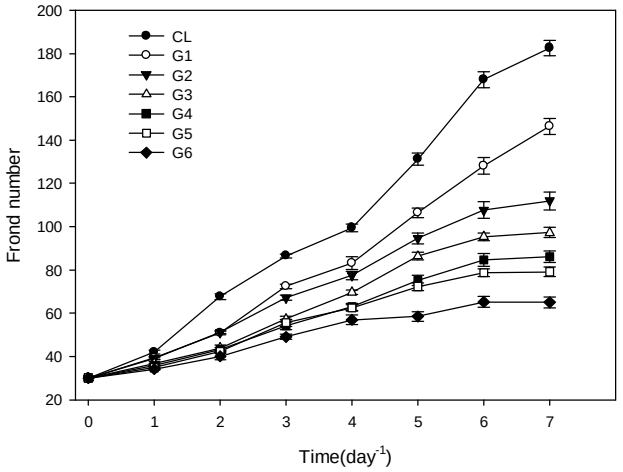

Fig. 1: Influence of aniline on frond number of *Lemna minor* L. during the experiment (n = 15, mean ± SE).

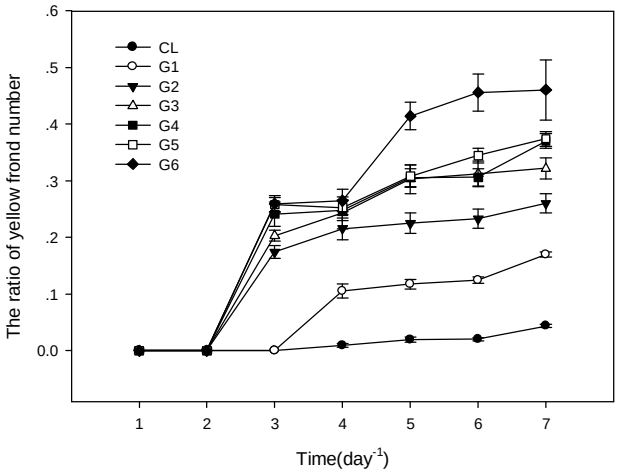

Fig. 2: The changes of the ratios of yellow frond number/the total frond number with treatment time.

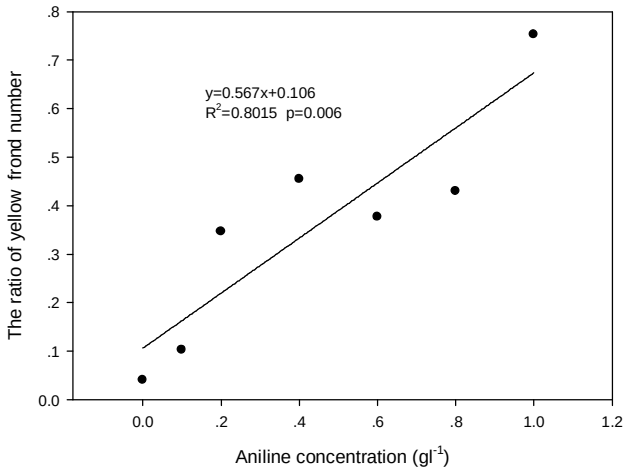

Fig. 3: The regression between the ratio of yellow frond number/the total frond number and aniline concentration.

groups, tended to increase steadily during the experiment (Fig. 1). However, the increase of the frond numbers in the treatment groups was inhibited significantly compared with control at the end of the experiment (df = 98, 6, F = 181.56, $p < 0.001$). There were also significant differences of the frond numbers among the treatment groups at the end of the experiment (df = 89, 5, F = 90.75, $p < 0.001$). This result suggested that the higher the concentration of aniline was, the greater its inhabitation on the frond number of *L. minor* would be.

Aniline could accelerate the fronds of *L. minor* turning yellow. At the end of the experiment, there was significant difference in the ratios of the yellow frond number/the total frond number among different groups (F = 73.973, $p < 0.001$). Apart from G1, the ratios in the other experiment groups were significantly greater than that in control (Fig. 2). The effect of aniline on the fronds tuning yellow was correlated significantly with its concentration (x) (y = 0.567x+0.106, $R^2 = 0.8015$, $p = 0.006$) (Fig. 3).

There was a significant check to the growth of frond area during the experiment. As shown in Fig. 4, the frond areas in the treatment groups decreased significantly compared with control, and there was a significant negative linear relationship between the frond area (y) and aniline concentration (x) at the end of the experiment (y = -4.557x+9.831, $R^2 = 0.8788$, $p = 0.0018$).

The effect of aniline on biomass accumulation and growth index of *L. minor*: Compared with control, the dry weight of *L. minor* in each experiment group decreased significantly ($p < 0.001$). Additionally, there was a significant negative correlation between the biomass and the aniline concentration (y = -0.0058x+0.0064, $R^2 = 0.6740$, $p = 0.0236$).

In all experiment groups, growth index was significantly smaller than that in control (Fig. 5). The inhibitory effect of aniline on growth index of *L. minor* was negatively correlated to its concentration (y = -3.0573x+3.1866, $R^2 = 0.6147$, $p = 0.0369$).

Effects of aniline on chlorophyll *a* and *b*: After exposure to aniline for 24 hours, the contents of both chlorophyll *a* and *b* had no difference in G1 and G2, but went down significantly in group G3, G4, G5 and G6 compared with control (Fig. 6). It suggested that aniline had an acute toxic effect on chlorophyll *a* and *b* contents in *L. minor* when its concentration was equal to or above 0.4 g/L.

Effects of aniline on CAT, POD and SOD: Table 2 showed the results of activities of CAT, POD and SOD in *L. minor* after exposure to aniline for 24 h, 72 h, and 120 h. The activities of three anti-oxidative enzymes had different responses to aniline at different times.

Table 1: The composition of modified Hoagland medium.

Substance	Concentration (mg/L)	Substance	Concentration (mg/L)
KNO_3	505.5	H_3BO_3	2.68
$Ca(NO_3)_2$	816.025	$CuSO_4 \cdot 5H_2O$	0.08
$MgSO_4$	567.341	$ZnSO_4 \cdot 7H_2O$	0.22
KH_2PO_4	136.09	$MnCl_2 \cdot 4H_2O$	1.81
K_2SO_4	174.25	H_2MoO4	0.093
Na_2EDTA	7.45	$FeSO_4 \cdot 7H_2O$	5.57

Table 2: The activities of CAT, POD and SOD in *L. minor* at different times after treated with aniline (n = 6, mean ± SE, *p < 0.05 and **p < 0.01 compared with control).

	CAT (U gFW^{-1} min^{-1})			POD (U $mgFW^{-1}$ min^{-1})			SOD (U gFW^{-1} min^{-1})		
	24	72	120	24	72	120	24	72	120
Control	156.6±10.0	193.5±12.8	327.7±7.0	21.6±1.3	15.5±0.9	13.9±1.1	316.4±50.6	204.1±11.3	510.8±21.9
G1	151.6±10.4	284.1±14.0**	334.2±36.6	24.0±1.6	14.9±0.5	17.9±1.1*	427.6±38.1	251.3±16.0	687.2±69.9*
G2	143.2±6.7	215.0±8.0	322.7±15.0	23.2±1.5	18.1±0.8*	19.8±1.2**	400.1±43.7	223.7±10.5	558.9±42.6
G3	145.8±8.1	198.5±10.2	328.4±10.1	24.3±1.6	19.2±1.0**	22.8±1.5**	335.4±54.8	419.1±73.4**	646.2±32.1
G4	137.8±6.5	190.5±16.0	305.9±8.8	21.1±0.8	16.0±0.6	17.8±1.0*	323.3±45.7	389.3±61.9**	688.5±101.3*
G5	140.8±12.4	158.1±17.8	330.7±20.1	18.7±1.1	12.2±0.5**	22.0±2.0**	160.5±11.9*	497.7±16.2**	581.9±33.3
G6	113.0±9.4**	132.5±5.6**	271.0±14.0**	16.3±0.6**	22.9±1.4**	13.0±0.5	82.7±26.0**	444.9±64.3**	441.1±40.7

After treatment of 24 h with aniline, only the activity of CAT in G6 was significantly lower than that in control (df = 5, t = 4.642, p = 0.006), but no differences were observed between that in other experiment groups and the control. Similar results were also observed after exposure to aniline at 72h and 120 h except G1 at 72 h (df = 5, t = -8.713, p = 0.0003) (Table 2). Although the activities of CAT in experiment groups showed a similar tendency with control during the treatment, the activity of CAT would be significantly inhibited when the concentration of aniline ≥ 1.0g/L (Table 2).

The variation of the activities of POD during the treatment under aniline was more complex compared with CAT (Table 2). After treated for 24 h, 72 h and 120 h, the activities of POD in control decreased steadily, while that in the experiment groups experienced a process of descending and then ascending except G6. The activity of POD after treated for 24 h was significantly lower than that in control and significantly higher at 72 h. After 120 h, there was no difference of the activities of POD between G6 and control (Table 2). Compared with control, the activities of POD at 120 h in the experiment groups expect G6 were significantly greater. The results suggested that aniline would increase the POD activities of *L. minor* when its concentration was under 1.0 g/L, while it would inhibit the POD activities when its concentration was ≥1.0g/L and exposing time was over 120 h.

After exposed to aniline for 24 hours, the activities of SOD in G5 and G6 were significantly lower than that in control (Table 2). After treated for 72 h by aniline, the activities of SOD in G3, G4, G5 and G6 were significantly higher than that in control, but only two groups (G1 and G4) were significantly bigger than that in control after 120 h.

Effects of aniline on MDA: Compared with the control, the MDA content of *L. minor* in each experiment group after 3 and 7 days decreased significantly (p<0.001)(Fig. 7). Additionally, there was a significantly positive correlation between the MDA content and the aniline concentration (day 3: y = 8.2509x+5.8000, R^2 = 0.7317, p = 0.0141; day 7: y= 4.1374x+10.8434, R^2 = 0.7723, p = 0.0092).

DISCUSSION AND CONCLUSION

The effects of aniline on vegetative growth of *L. minor*: Although mortality of organisms caused by contaminants is paid much attention in a range of researches (Umeki 2002, Maddocks et al. 2009, Wu et al. 2011, Goodrich & Jacobi 2012), there are also a variety of other consequences rather than mortality (Ammann & Terry 1985), such as inhibition of vegetative growth. For plants which rely on asexual propagation to increase their offspring numbers, inhibition of vegetative growth often implies that their propagation to be inhibited. In our study, although mortality of *L. minor* lead by aniline is not obvious, aniline shows a significant inhibition to frond number and area, growth index and biomass of *L. minor*. There was a significantly negative relationship between the dry weight of *L. minor* and the aniline concentration. In addition, visible damages in *L. minor* such as chlorosis, necrosis and frond disconnection were also observed at or above the concentration of aniline (0.1g/L) after 72 hours. Aniline could also accelerate the fronds of *L. minor*

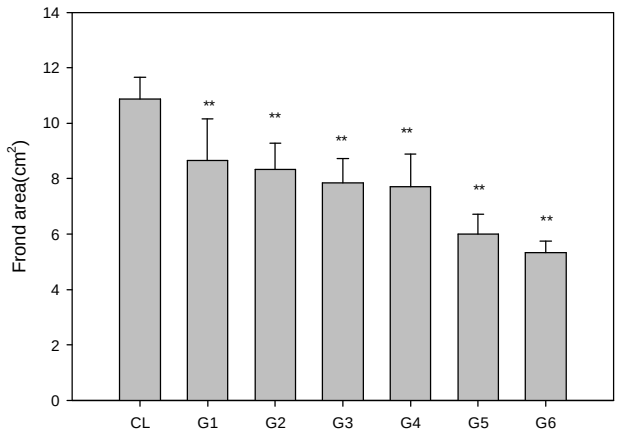

Fig. 4: The frond area of different experimental groups at day 7 (n = 6, mean ± SE).

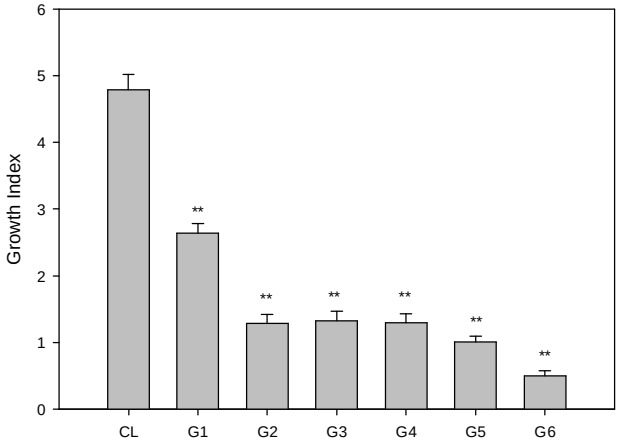

Fig. 5: The growth index of different experimental groups at the end of the experiment (day 7) (n = 15, mean ± SE, **p < 0.01 compared with control).

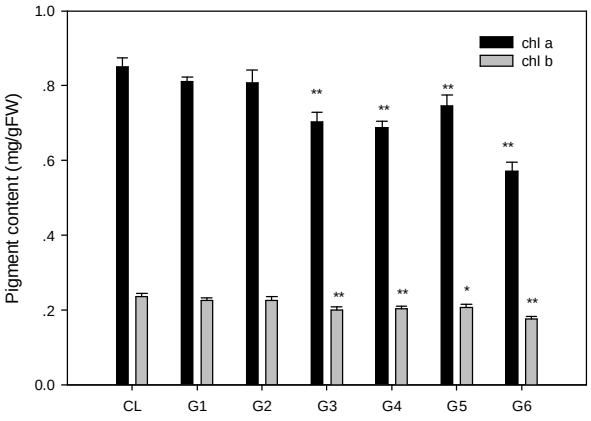

Fig. 6: The pigment contents of different experimental groups exposed to aniline after 24 hours (n = 6, mean ± SE, *p < 0.05 and **p < 0.01 compared with control).

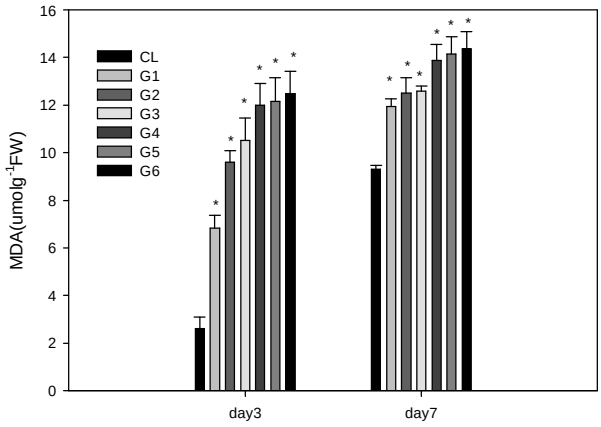

Fig. 7: Effects of aniline on MDA content in *Lemna minor* on day 3 and 7 (n = 6, mean ± SE, 6 repetition, *p < 0.05 and **p < 0.01 compared with control).

turning yellow. After exposure to aniline for 3 days, yellow fronds were appeared in all treatment groups, and the ratios of the yellow frond number/the total frond number increased with the aniline concentration and the treatment time. The contents of both chlorophyll *a* and *b* went down significantly in treatment groups G3, G4, G5 and G6 compared with control (Fig. 6) after exposure to aniline for 24 hours. Aniline shows an acute toxic effect on the vegetative growth of *L. minor* and the effect is correlated with its concentration. The similar results have been reported by Ammann & Terry (1985) that cell growth of *Chlorella vulgaris* declined significantly when it was exposed to concentrations of aniline above 183.9 ppm.

Many researches reported that persistent organic pollutants and heavy metals influenced the vegetative growth of species from the family of Lemnaceae (Teisseire et al. 1998, Mkandawire & Dudel 2007, Khellaf & Zerdaoui 2010, Kim et al. 2010). Mitsou et al. (2006) discovered that duckweed could degrade propanil to 3,4-DCA or 3,4-dichloroacetanilide, the latter is more toxic to *L. minor* in contrast to propanil and causes the decrease of the growth rate in *L. minor*. De Prado et al. (2000) demonstrated that atrazine could reduce the fresh weight of plants. Both propanil and isoproturon inhibited the photosynthetic electron transport of Photosystem II (PS-II) in chloroplasts (Devine et al. 1993, Bottcher & Schroll 2007). Plants' protein D1 (a key polypeptide of the PS-II reactive centre) could be damaged by exposure to toxic pollutants, which would disrupt photosynthesis and limit growth of plants (Fuerst & Norman 1991). Based on our experimental results, we could presume that aniline might destroy photosynthetic pigments, especially chlorophyll *a* and *b* at first and then inhibit its photosynthesis. Inhibited photosynthesis would further impact cell division

and new frond formation. Frankart et al. (2003) found that flazasulfuron acts indirectly on photosynthesis and as an inhibitor of protein synthesis it may result in a disorder in the assembly of chlorophyll-protein complexes, which confirmed our hypothesis. Meanwhile, the decrease of pigment content and the increase of MDA content occurred on day 1 and day 3/7, respectively. Chlorophyll destruction due to the lipid peroxidation (Heath & Packer 1968) and MDA is the decomposition product of polyunsaturated fatty acids in biomembranes. Dhindsa et al. (1981) concluded that the decline of chlorophyll content was because of the lipid peroxidation in chloroplast membranes and similar results were observed by Hou et al. (2007).

The physiologic responses of *L. minor* to aniline: Apart from photosynthetic pigments, antioxidant enzymes of *L. minor* showed an obvious variation after exposure to aniline in our experiment. Antioxidant enzymes of plants play an important role in scavenging the excess reactive oxygen species (ROS) which were accumulated during plants are under a range of environmental stress conditions (e.g., heavy metals, organic pollutants, cold, water deficit, etc.) (Mittler 2002). CAT, localized in glyoxysomes and peroxisomes, scavenges most of the hydrogen peroxide (Smith et al. 2009). It is important to keep the balance of H_2O_2 level in cell and as special enzymes to catalyse H_2O_2 into water and oxygen. POD presents in chloroplastic (stroma and thylakoid-bound), peroxisomal, cytoplasmic and mitochondrial inter-membrane space and belongs to enzymes involved in regulation of growth and H_2O_2 level, development and senescence processes of plants, defence mechanisms, etc. (Cosio & Dunand 2009, Maksimov et al. 2011). SOD is ubiquitous in all aerobic organisms and in all sub-cellular compartments (chloroplast, mitochondria, peroxisomes, cytosol, etc.), and prone to ROS mediated oxidative stress, namely quench one O_2^- becoming H_2O_2 and another oxidized to O_2(Gill & Tuteja 2010).

In our experiment, the activities of three anti-oxidative enzymes showed different responses to aniline at different times (Table 2). Only when the concentration of aniline ≥1.0g/L, was the activity of CAT significantly inhibited. Aniline could increase POD activities of *L. minor* at lower aniline concentration (<1.0g/l) but inhibit them at higher concentrations (≥1.0g/L). This is one of the typical characteristics for plants response to stressed factors (Teisseire et al. 1998, Hou et al. 2007). The changes of SOD activities were closely correlated with the concentration of aniline and exposure time, but SOD in almost all treatment groups was inhibited significantly. It is commonly accepted that plants will pass through different physiological states from resistance to exhaustion when they are exposed to a long-term stress (Lichtenthaler 1996). The activities of antioxidant enzymes are often closely related to the tolerance capabilities of aquatic macrophytes (Roy et al. 1992). All these results suggested that higher concentration of aniline and persistence would be more harmful to *L. minor*.

Aromatic compounds, such as aniline and isoproturon, are hard to be degraded by organisms (Bottcher & Schroll 2007). For example, after exposure to 10 mg/L aniline for 60 hours, the degradation rates of sterile fronds and nature fronds was about 20% (8 mg/L in nutrient medium) and 100% (0 mg/L in nutrient medium), respectively (Hoang et al. 2010). Hoang et al. (2010) and Toyama et al. (2006) discovered that *Spirodela polyrrhiza* could accumulate some bacteria, organic compounds and secrets enzymes (peroxidase and laccase) to accelerate removal of recalcitrant compounds in the tissue of roots. Sensitivity to a pollutant is often positively correlated to plant root systems (Lewis 1995). These results mean that roots of *S. polyrrhiza* play an important role in absorbing and depredating some pollutants in water bodies. However, almost all roots of *L. minor* detached from fronds after exposure to aniline over 72 hours in our experiment. It indicates that *L. minor* is less effective in the removal of aniline. Many morphological parameters of *L. minor* are sensitive to aniline. As a widely distributed aquatic plant, it could be used as an indicator to monitor the existence of aniline in water bodies.

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