



Toxicity of the herbicide flurochloridone to the aquatic plants *Ceratophyllum demersum* and *Lemna minor*

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Abstract

As a new and efficient selective pre-emergence herbicide, flurochloridone (FLC) has been widely promoted in recent years but readily results in residues in nature. As the primary producers and restorers of the water environment, aquatic plants are at risk of FLC exposure. In the present research, we studied the phytotoxicity of FLC in *Lemna minor* and *Ceratophyllum demersum*. The physiological and growth responses of these two aquatic plants exposed to different concentrations of FLC (0, 20, 100, 300, 1000, and 2000 µg/L) were measured. The results showed that FLC (≥ 20 µg/L) could cause serious photosynthesis pigment damage and bleaching in *C. demersum* and *L. minor*. Significant oxidative damage was observed in *L. minor* at 20 µg/L FLC, while there was no severe oxidative damage in *C. demersum*. At 100–300 µg/L FLC, peroxidase (POD) and superoxide dismutase (SOD) were activated to scavenge free radicals in *L. minor*, while POD acted as a protective enzyme in *C. demersum*. At higher concentrations of FLC (≥ 1000 –2000 µg/L), *L. minor* reached less than healthy stability through the regulation of the antioxidant enzyme system and the chlorophyll a/b value. POD, SOD, and protein content returned to normal levels, and the growth parameters increased. However, in *C. demersum*, the enzymes POD and SOD and soluble protein were damaged, and oxidative stress reached the highest level at 1000–2000 µg/L FLC. Taken together, our results suggested that when treated with FLC, *L. minor* was more sensitive at lower doses (20 µg/L) and more adaptive at higher doses (1000–2000 µg/L) than *C. demersum*.

Keywords Submerged plants · Floating plants · Photosynthesis pigments · Bleaching herbicide

Introduction

Herbicides are among the most widely applied agrochemicals worldwide, significantly increasing agricultural productivity and crop yields (Singh et al. 2019; Bolognesi 2003). The

application of herbicides to arable land is still the most effective and accepted method for plant protection from weeds, with the environment consequently and inevitably being exposed to these chemicals (Deniz et al. 2003). Flurochloridone (FLC) is a selective pre-emergence and persistent herbicide developed by Stauffer Chemical Co. in the late 1970s and was put into production in the mid-1980s (Shi et al. 2016; Wang et al. 2016). FLC, (3RS, 4RS; 3RS, 4RS)-3-chloro-4-chloromethyl-1-(α , α , α -trifluoro-m-tolyl)-2-pyrrolidone, has a solubility of 28 mg/L in water of 20 °C, and it is readily soluble in organic solvents such as acetone, chlorobenzene, and xylene. FLC is mainly used for cereals, sunflowers, potatoes, and other crops to control a variety of broadleaf weeds and some grass weeds that are difficult to control, such as *Portulaca oleracea* L. and *Veronica didyma* (Liu et al. 2015; Buhler 1988; Wang et al. 2016; Jursík et al. 2015). The most widely used FLC-based commercial formulations are called Racer, Rainbow, and Twin Gold Pack (Deniz et al. 2003; Soloneski et al. 2016). In the past 20 years, FLC has been widely used in Europe and the USA due to its good efficacy against weeds and crop safety (Shi et al. 2016; Liu et al. 2015).

Highlights

FLC was phytotoxic to aquatic plants *L. minor* and *C. demersum* in acute exposure experiments.
FLC caused serious bleaching of *L. minor* and *C. demersum* at 20 µg/L. Compared with *C. demersum*, *L. minor* was more sensitive at lower doses of FLC (20 µg/L) and more adaptive at higher doses (1000–2000 µg/L).

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However, its pervasive use in farming can result in residues in nature (EFSA 2018; Milanova and Grigorov 2010; Rouchaud et al. 1997). Three months after application (1.0 kg ha^{-1}), FLC residues can still be detected in the 0–10 cm layer soil. Moreover, under heavy rainfall conditions, the herbicide can even penetrate down to 25 cm in the soil profile (Milanova and Grigorov 2010).

Extensive use of this chemical is likely to cause environmental pollution, especially water environmental pollution, because aquatic ecosystems are more sensitive to pollutants than terrestrial ecosystems (Singh et al. 2006). As primary producers in aquatic ecosystems, aquatic plants are often initial indicators of water pollution. In addition, aquatic macrophytes are of great importance in enhancing the biodiversity and stability of water ecosystems (Mitsou et al. 2006). There are many reports about aquatic macrophytes playing important roles in water transparency, biomass accumulation, and nutrient cycling in aquatic environments (Singh et al. 1994; Garrido-Perez et al. 2008; Jonsson et al. 2009; Wang et al. 2011; Xu et al. 2013). However, recent reports have shown that water pollution is gradually degrading aquatic plants (Wu et al. 2003; Guo et al. 2016).

FLC is a monophenyl pyrrolidinone that has been called “bleachers” because it can cause bleaching of plant leaves through interference with carotenoid biosynthesis (Lay and Niland 1983; Ogawa et al. 2015). The target site of FLC in weeds is phytoene desaturase (PDS) (Celar and Kos 2016). As a key enzyme in the carotenoid synthesis process, PDS can catalyze the dehydrogenation of phytoene to further form carotenoids. FLC prevents the further reaction of phytoene by noncompetitive binding with PDS, resulting in the accumulation of phytoene and the reduction of carotenoid synthesis in plants (Lay and Niland 1983; Weinberg et al. 2003; Ogawa et al. 2015). Carotenoids are important photosynthetic pigments in aquatic plants (Montero 2011). They serve as accessory light-harvesting pigments in photosynthesis and act to protect the chlorophyll from photooxidative damage (Shi et al. 2016; Weinberg et al. 2003). Therefore, carotenoid damage may cause bleaching, oxidative damage, and even the death of plants.

In recent years, research on the toxicity of FLC has gradually increased. According to the European Food Safety Authority (EFSA), FLC has moderate acute toxicity to birds, fish, and phytoplankton and is highly toxic to algae (EFSA 2010). Phytotoxic studies on FLC have mostly focused on terrestrial plants. For example, Kaya and Yigit (2012) measured the glutathione content, glutathione *s*-transferase, and glutathione reductase activities in FLC-pretreated *Vicia sativa* L. and found that FLC exposure can cause harmful effects on the antioxidant system in *V. sativa*. Devlin et al. (2010) investigated the pigment content change in wheat and corn whose seed was preimbibed in FLC solutions. They found that wheat seedlings appeared almost completely bleached when treated

with $320 \mu\text{mol/L}$ of the herbicide. Few studies have focused on the toxicity of FLC to aquatic macrophytes. Due to the weed-killing mechanism of FLC, we speculate that it is highly likely to have serious phytotoxic effects on aquatic plants. In addition, an increasing number of studies in recent years have found that FLC is not always as safe as earlier studies have shown (Shi et al. 2016; Liu et al. 2015).

Reactive oxygen species (ROS) are byproducts produced during the normal metabolism of plants, including the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($\cdot\text{OH}$). ROS act as signaling molecules for regulating development and initiate the response to environmental stressors at low concentrations and can cause toxic effects on plants at high concentrations (Pitzschke et al. 2006). Studies have shown that herbicides such as FLC and glyphosate can cause enhanced generation of ROS (Liu et al. 2019; Zhong et al. 2017; Kaya and Yigit 2014; Kielak et al. 2011). Plants have antioxidant enzyme systems, including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), to scavenge ROS and protect cells from membrane lipid peroxidation (Kaya and Yigit 2014; Singh et al. 2006). The content of malondialdehyde (MDA), the final product of membrane lipid peroxidation, directly reflects the degree of oxidative damage of plants. In addition, as the important material basis for the structure and function of plant organs, the protein content is also an important indicator for assessing plant response under stress (Zhou et al. 2018).

In this study, the typical submerged plant *Ceratophyllum demersum* L. and floating plant *Lemna minor* L. were selected as research materials for aquatic macrophytes. Both *C. demersum* and *L. minor* are commonly found in freshwater ecosystems such as ponds, lakes, ditches, and quiet streams that may be threatened by herbicide residues around the world (Keskinan et al. 2004; Axtell et al. 2003). The aim of our research was to investigate the toxicity of FLC to *L. minor* and *C. demersum* and to compare the responses of these two aquatic plants exposed to FLC.

Method

Plant culture and treatments

L. minor and *C. demersum* used in this research were collected from the Hanjiang River ($106^\circ 12' \sim 114^\circ 14' \text{ E}$, $30^\circ 08' \sim 34^\circ 11' \text{ N}$), the largest branch of the Yangtze River, People's Republic of China. Prior to the experiment, *L. minor* and *C. demersum* plants were cultivated in several transparent plastic tanks for acclimatization and multiplication for approximately 1 week. During the experiment and acclimatization period, the plants were cultivated in 10% Hoagland's solution (Hoagland and Arnon, 1950) in a plant growth chamber (HP1000GS-B, Ruihua Instrument & Equipment Co., Ltd.,

Hubei, China). The water temperature was 24 ± 2 °C, the light intensity was 120 ± 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, and the photoperiod was 24 h of light.

The flurochloridone (FLC) used in these experiments was chemically pure ($\geq 95\%$, supplier: Widely Chemical Technology Co., Ltd., Hubei, China). Herbicide concentrations were set at 0 (control treatment), 20, 100, 300, 1000, and 2000 $\mu\text{g/L}$. Due to the lack of research on the toxicity of FLC to aquatic plants, the treatment concentrations were set according to the effective concentration of FLC to terrestrial plants or FLC analog to aquatic plants (Devlin et al. 2010; Kaya and Yigit 2012; Macdonald et al. 2008). Culture solution was changed every 24 h to ensure the stability of FLC concentrations. Experiments were performed in three replicates, and uniform *L. minor* and *C. demersum* plants were placed into 36 transparent plastic tanks (25 fronds per tank for *L. minor* and 3 plants per tank for *C. demersum*). All plants were harvested for determination of growth and physiological indexes after 7 days of exposure to FLC.

Growth parameter measurements

Harvested plants were rinsed with double distilled water, and plant surface moisture was dried gently with absorbent paper. The fresh weight of plants was determined with an analytical balance (OHAUS Corp., New Jersey, USA). For *L. minor*, the frond area was measured with a leaf area meter (Yaxin-1241, Yaxin Science instrument technology Co., Ltd., Beijing, China).

Photosynthetic pigment content measurement

Photosynthetic pigments were measured to evaluate the etiolation degree of plants. Plant leaves (0.2 g fresh weight) were cut into pieces, ground, and placed in 25 ml flasks. Then, 96% ethanol was added to fill the flasks to the 25 ml mark. The flasks were placed in the dark for 24 h, and a sample was then measured with a spectrophotometer at 470, 649, and 665 nm. The chlorophyll content values were calculated according to Lichtenthaler and Wellburn (1983).

Soluble protein measurement

Soluble protein contents were determined following the method of Bradford (1976). Fresh leaves weighing 0.1 g were homogenized with phosphate buffer (pH 7.8) and ground. The homogenate was subsequently centrifuged for 20 min at 4000 rpm. The supernatant was separated and used for the soluble protein content assay. A suitable aliquot of protein extract was thoroughly mixed with Coomassie brilliant blue reagent, and the absorbance of the mixture was measured with a spectrophotometer at 595 nm. A standard curve using different bovine serum albumin concentrations was used as a protein standard.

Antioxidant enzyme activity measurement

Fresh leaves weighing 0.5 g were homogenized with phosphate buffer solution (pH 7.8) containing 1% (w/v) polyvinyl pyrrolidone (PVPP), 0.1 mM ethylenediaminetetraacetic acid (EDTA), and 0.4 mM mercaptoethanol at 4 °C. The homogenate was centrifuged at 8000 rpm for 15 min at 4 °C. The supernatant was stored at 4 °C and used for the assay of enzyme activity. SOD, POD, and CAT activities in the leaf cells of plants were determined spectrophotometrically according to Elavarthi and Martin (2010).

For the SOD activity measurements, the reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 0.075 mM nitroblue tetrazolium (NBT), 0.01 mM EDTA, and 0.002 mM riboflavin, and every 3 ml mixture contained 0.05 ml enzyme extract. After 15 min of reaction in 4000 lx light, the absorbance of the reaction mixture was measured at 550 nm. The unit of SOD activity (U g^{-1}) was defined as the amount of enzyme that inhibited the initial rate of reaction by 50% in the absence of the enzyme. The reaction mixture of POD activity contained 100 mM potassium phosphate buffer (pH 7.0), 20 mM guaiacol, and 65 mM H_2O_2 . The absorbance change of the mixture was measured by adding 0.1 ml of the enzyme extract to 3 ml of the reaction solution. The increase in absorbance caused by the oxidation of guaiacol was monitored at 470 nm. One activity unit of POD (U g^{-1}) was defined as the amount of enzyme that caused an increase of 0.001 absorbance per minute. For the estimation of CAT activity, the reaction mixture comprised 50 mM phosphate buffer (pH 7.0), 20 mM hydrogen peroxide (H_2O_2), and a suitable aliquot of enzyme. The absorbance of the reaction solutions was measured at 240 nm. One unit of CAT activity (U g^{-1}) was defined as the amount of CAT that decomposed 1 mg H_2O_2 in 1 min at 37 °C.

MDA content measurement

The content of MDA in leaves was measured using the thiobarbituric acid (TBA) method ($\mu\text{mol/g FW}$) (Zhao et al. 1991). Fresh plant leaves (0.2 g) were homogenized with 2 ml 5% trichloroacetic acid (TCA) at 8000 rpm for 10 min at 4 °C. The supernatant was separated and mixed with 0.67% 2-thiobarbituric acid (TBA). The mixture was heated at 100 °C for 30 min and subsequently homogenized at 800 rpm at 4 °C. The absorbance of the supernatant was measured at 450 nm, 532 nm, and 600 nm.

Statistical analysis

All experimental results are expressed as the mean \pm standard error of the three replicates. One-way analysis of variance (ANOVA) was performed to assess the variability of data and validity of results. The least significant difference (LSD)

was estimated to determine a significant difference between treatments, and differences were considered significant at $P < 0.05$ (SPSS 10.0 for Windows).

Results

Effects of FLC on photosynthetic pigments

Bleaching always appeared in plant leaves in different concentrated solutions of FLC. In this experiment, the contents of chlorophyll a and b and carotenoids in *L. minor* and *C. demersum* under different concentrations of FLC were determined. Compared with the control group, the pigment content of all treatment groups of both plants was significantly reduced (Fig. 1).

Under the treatment of 20 to 2000 $\mu\text{g/L}$ FLC, the contents of chlorophyll a, total chlorophyll, and carotenoids of *L. minor* significantly decreased to approximately 20–30% of the control group, but there was no significant difference between the treatment groups. The content of chlorophyll b in *L. minor* reached a minimum at 20 $\mu\text{g/L}$ FLC, and the value was 23.4% of that measured in the control group. As the treatment concentration increased, the chlorophyll b contents in *L. minor* significantly increased but were still far lower than the control group, accounting for approximately 35–45% of the value measured in the control. All the pigment contents of *C. demersum* exposed to low concentrations of FLC (20–300 $\mu\text{g/L}$) were significantly lower than those of the control group, accounting for 29–41% of the value measured in the control group. Compared with the lower concentration, the pigment contents were further significantly decreased under the higher concentration of FLC treatment (1000–2000 $\mu\text{g/L}$), and the

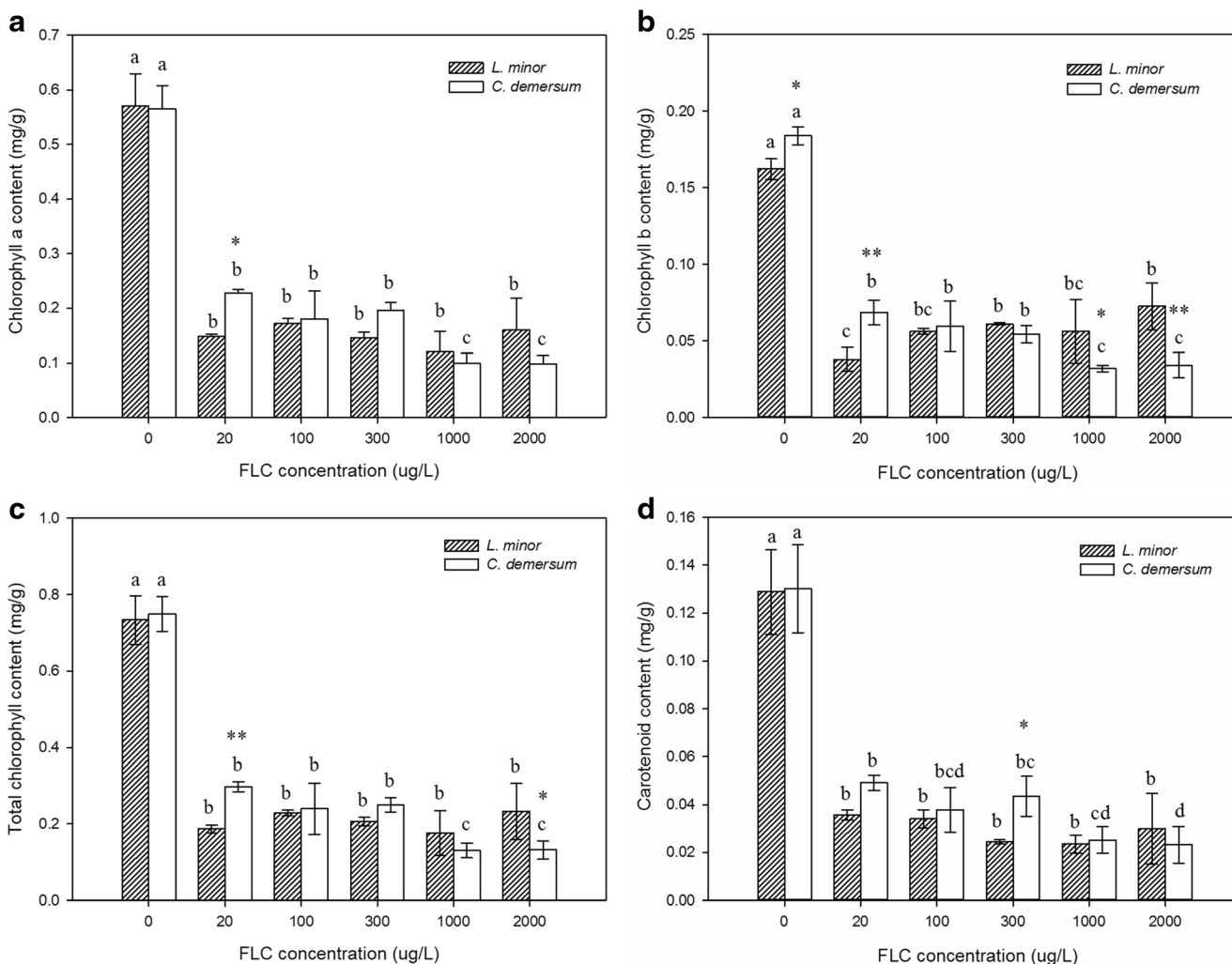


Fig. 1 Effect of FLC on the contents of chlorophyll a (a), chlorophyll b (b), total chlorophyll (c), and carotenoid (d) in the leaves of *L. minor* and *C. demersum*. All values represent the mean of three replicates \pm standard deviation. ANOVA significant at $p < 0.05$. Bars with different letters are

significantly different among different exposure concentrations ($p < 0.05$, LSD test). Bars with stars are significantly different between two plant species under the same dose of FLC ($p < 0.05$, LSD test)

contents were 17% and 19%, respectively, of those measured in the control group.

A comparison of the pigment contents of *L. minor* and *C. demersum* indicated that the chlorophyll a and carotenoid contents of the two plants were consistent under FLC stress. However, the chlorophyll b content and total chlorophyll content of *C. demersum* were significantly higher than those of *L. minor* in the low-concentration FLC treatment group (20 µg/L) and were significantly lower than those of *L. minor* in the high-concentration FLC treatment group (1000, 2000 µg/L). The chlorophyll a/b value of *L. minor* decreased significantly at a treatment concentration above 300 µg/L, while the chlorophyll a/b value of *C. demersum* showed no significant change (Table 1).

Effects of FLC on antioxidant enzymes

Adding different doses of FLC to the plant culture media resulted in changes in enzyme activity (Fig. 2). For *L. minor*, a significant increase was observed in SOD activity in the 100 µg/L FLC treatment. There was no significant difference at other concentrations. In contrast to *L. minor*, the SOD activities in *C. demersum* presented a decreasing trend as the herbicide concentration increased. The SOD activities of *C. demersum* in the experimental group were significantly lower than that of the control group when the FLC concentration was higher than 100 µg/L. Under the treatment of 100–2000 µg/L FLC, the SOD activities in *L. minor* were significantly higher than those in *C. demersum*.

Changes in the activity of CAT are shown in Fig. 2b. The CAT activities in *L. minor* decreased significantly at 20 µg/L FLC and maintained the same level as the FLC concentration increased. The CAT activities of *C. demersum* showed a significant decrease in the 20–300 µg/L FLC treatment, and there was no significant difference compared with the control group under higher FLC concentrations (1000–2000 µg/L). A comparison of the differences between the two species indicated

Table 1 Influence of FLC on the chlorophyll a/b ratio of *L. minor* and *C. demersum*. Means with different letters in the same columns are significantly different among different exposure concentrations ($p < 0.05$, LSD test). Means with stars are significantly different between two plant species under the same dose of FLC ($p < 0.05$, LSD test)

FLC concentration (ug/L)	Chlorophyll a/b ratio	
	<i>L. minor</i>	<i>C. demersum</i>
0	3.52 ± 0.29 ^{ab}	3.07 ± 0.23 ^{ab}
20	4.01 ± 0.75 ^a	3.36 ± 0.34 ^{ab}
100	3.07 ± 0.27 ^b	3.03 ± 0.19 ^{ab}
300	2.38 ± 0.19 ^c	3.62 ± 0.32 ^{b**}
1000	2.17 ± 0.12 ^c	3.11 ± 0.63 ^{ab*}
2000	2.17 ± 0.32 ^c	2.90 ± 0.31 ^{a*}

that the CAT activity in the control group of *L. minor* was significantly higher than the CAT activity in *C. demersum*.

The POD activities in both *L. minor* and *C. demersum* presented an initial increase and a subsequent decreasing trend (Fig. 2c), and statistically significant differences were observed at 100 µg/L FLC. The POD activities in the leaves of *C. demersum* were significantly higher than those in the *L. minor*, and the enzyme activities in *C. demersum* were 5.4–7.8 times those measured in *L. minor*.

Effects of FLC on membrane peroxidation

There was a slight trend of an initial increase followed by a decrease in the MDA content of *L. minor* (Fig. 3). The maximum MDA content in *L. minor* was observed at 20 µg/L, and the minimum at 2000 µg/L. Based on analysis of variance, MDA contents in *L. minor* showed a significant difference only between the treatment groups, and the treatment groups did not differ significantly from the control. For *C. demersum*, significant differences compared with the control were noted when the FLC concentration was above 1000 µg/L.

Effects of FLC on soluble protein

As shown in Fig. 4, the soluble protein content in *L. minor* showed a significant decrease under exposure to a lower concentration of FLC (≤ 300 µg/L). There was no significant change compared to the control group at higher treatment concentrations. The content of soluble protein in *C. demersum* decreased with increasing FLC concentration, and a significant decrease was observed after 300–2000 µg/L FLC treatment. The soluble protein contents in *L. minor* were significantly higher than those in *C. demersum*.

Effects of FLC on growth

Table 2 shows the change in individual growth indicators in *L. minor*. Both the frond area and fresh weight showed a significant decrease at 20–300 µg/L FLC concentration. Under the high-concentration treatment (1000–2000 µg/L), the leaf area increased but was still significantly lower than that observed in the control, and the fresh weight of the plants increased to a level that was not significantly different from the control group.

As shown in Table 3, the fresh weight of *C. demersum* decreased significantly at 20–300 µg/L and reached its minimum at 300 µg/L FLC. When exposed to higher doses (1000–2000 µg/L), the fresh weight increased but remained significantly lower than that in the control group. There was a significant increase in length in the 1000–2000 µg/L FLC treatment (Table 3).

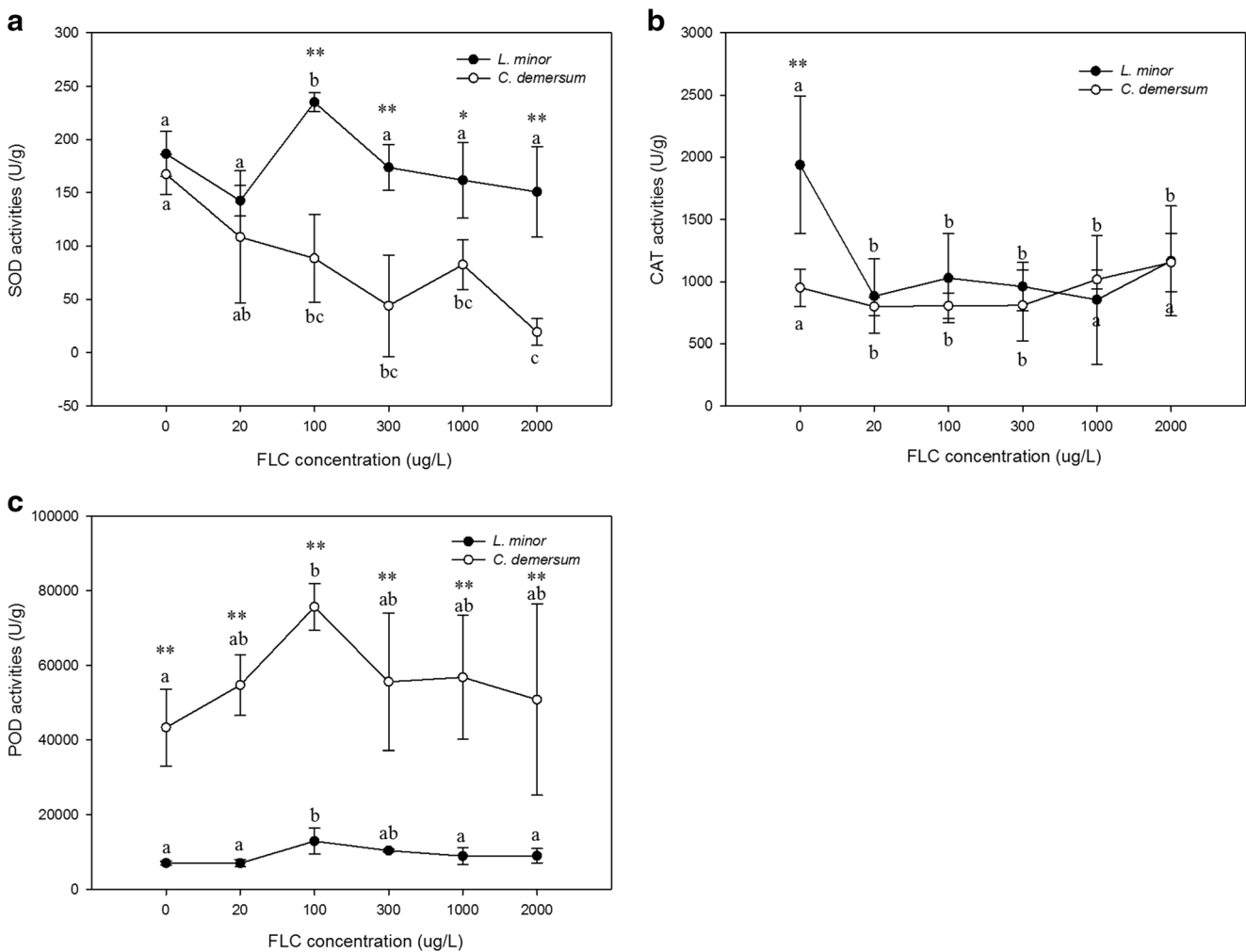


Fig. 2 Effect of FLC on activities of the antioxidant enzyme SOD (a), CAT (b), and POD (c) in the leaves of *L. minor* and *C. demersum*. All values represent the mean of three replicates \pm standard deviation. ANOVA significant at $p < 0.05$. Bars with different letters are

significantly different among different exposure concentrations ($p < 0.05$, LSD test). Bars with stars are significantly different between two plant species under the same dose of FLC ($p < 0.05$, LSD test)

Discussion

Studies have shown that FLC can interfere with carotenoid biosynthesis by inhibiting the activity of PDS (Lay and Niland 1983; Shi et al. 2016; Weinberg et al. 2003). In this study, the carotenoid synthesis of the floating plant *L. minor* was significantly reduced at 20 $\mu\text{g/L}$ FLC (Fig. 1c). It is worth noting that when the concentration of FLC exceeded 20 $\mu\text{g/L}$, the carotenoid content in *L. minor* leaves did not continue to decrease but was stable to a certain extent. This is most likely because a concentration of 20 $\mu\text{g/L}$ resulted in saturation FLC binding with the PDS in *L. minor* leaves. Thus, higher concentrations of FLC did not cause further damage to the carotenoids. FLC also showed significant inhibition of the pigment content of *C. demersum* at 20 $\mu\text{g/L}$ (Fig. 1). However, unlike *L. minor*, stronger inhibition was observed in *C. demersum* at higher exposure to FLC (1000–2000 $\mu\text{g/L}$). One of the reasons for this difference may be that *C. demersum* has a larger

specific leaf area than *L. minor*, resulting in a relatively larger area of contact with toxicity (Liu 1999; Macdonald et al. 2008; Zhang 2017). Therefore, *C. demersum* was relatively more stressed than *L. minor* when exposed to higher concentrations of FLC. Many studies have shown that the two species have different resistances to herbicides (Rice et al. 1997; Fairchild et al. 1998; Cedergreen et al. 2004; Coutris et al. 2011).

Studies have shown that chlorophyll is more unstable in cells than carotenoids and is easily oxidatively decomposed by singlet oxygen produced during photosynthesis (Weinberg et al. 2003). Carotenoids can rapidly accept the triplet chlorophyll excitation energy, thereby preventing them from forming a singlet oxygen and protecting the chlorophyll from oxidative decomposition (Weinberg et al. 2003; Ogawa et al. 2015; BoÈger 1996). Moreover, carotenoids also protect the action of thylakoid membrane lipids. Therefore, in the leaves of treated plants in this study, the chlorophyll was rapidly

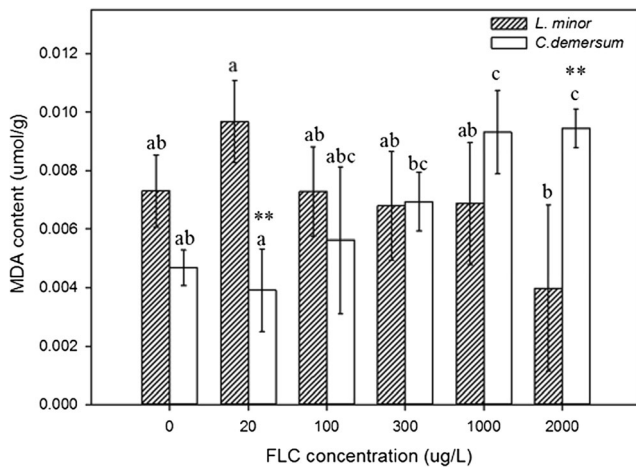


Fig. 3 Effect of FLC on MDA contents in the leaves of *L. minor* and *C. demersum*. All values represent the mean of three replicates ± standard deviation. ANOVA significant at $p < 0.05$. Bars with different letters are significantly different among different exposure concentrations ($p < 0.05$, LSD test). Bars with stars are significantly different between two plant species under the same dose of FLC ($p < 0.05$, LSD test)

decomposed without the protection of carotenoids. Moreover, the destruction of the thylakoid membrane would inhibit photosynthesis, which further hinders chlorophyll synthesis. As shown in Fig. 1, the decreasing trend of the chlorophyll content was consistent with that of carotenoids, in both *L. minor* and *C. demersum*, indicating that chlorophyll damage in plants was probably entirely caused by decreased carotenoid synthesis. At this point, our research is consistent with a study on terrestrial plants, i.e., wheat and corn (Devlin et al. 2010).

Chlorophyll a and b in plants can be transformed into each other in the chlorophyll cycle. Chlorophyll a can be converted to chlorophyll b under the catalysis of oxygenase, and chlorophyll b can be deoxidized to chlorophyll a (Zhou et al. 2007).

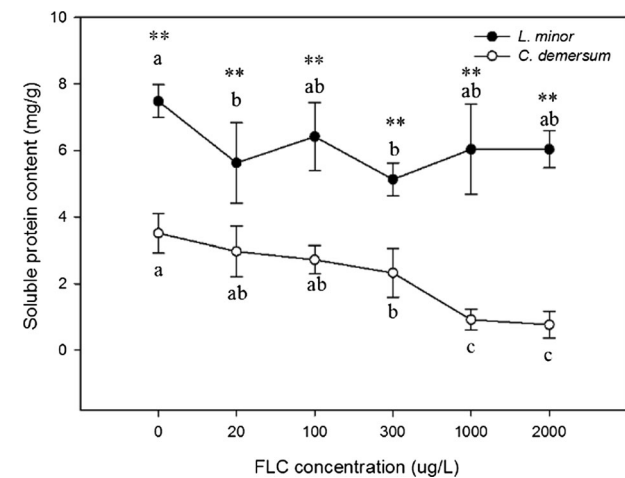


Fig. 4 Effect of FLC on soluble protein contents in the leaves of *L. minor* and *C. demersum*. All values represent the mean of three replicates ± standard deviation. ANOVA significant at $p < 0.05$. Bars with different letters are significantly different among different exposure concentrations ($p < 0.05$, LSD test). Bars with stars are significantly different between two plant species under the same dose of FLC ($p < 0.05$, LSD test)

Table 2 Influence of FLC on frond area and fresh weight of *L. minor*. Means with different letters in the same columns are significantly different among different exposure concentrations ($p < 0.05$, LSD test)

FLC concentration ($\mu\text{g/L}$)	Frond area (mm^2)	Single frond fresh weight (mg)
0	9.24 ± 0.32^a	8.96 ± 0.93^a
20	6.67 ± 0.55^d	6.04 ± 0.76^c
100	6.29 ± 0.28^d	6.22 ± 0.16^c
300	7.04 ± 0.23^{cd}	6.80 ± 0.47^{bc}
1000	7.67 ± 0.12^{bc}	8.25 ± 0.26^a
2000	8.09 ± 0.84^b	8.08 ± 0.27^{ab}

In this study, the chlorophyll a/b value of *L. minor* showed a significant decrease in the 300–2000 $\mu\text{g/L}$ FLC treatment (Table 1). This indicated that more chlorophyll a was converted into chlorophyll b in *L. minor* under higher doses of FLC. It has been reported that the conversion of chlorophyll b to chlorophyll a is a marker of leaf senescence and is the first step of the decomposition of chlorophyll b (Zhou et al. 2007; Pruzinska 2005). Studies have also shown that the a/b value is significantly negatively correlated with plant drought resistance, and chlorophyll a is more sensitive to reactive oxygen species than chlorophyll b and is also more susceptible to damage (Zhang and Tan 2001). Therefore, we speculate that the transformation of chlorophyll b to chlorophyll a in the leaves of *L. minor* in this study was a manifestation of plant stress resistance. In addition, the decrease in the a/b value in this study may also be related to the regulation of plant light absorption. In higher plant leaves, chlorophyll mainly absorbs red light and blue light, and carotenoids mainly absorb blue light and green light (Xu 2013a, b). In the aquatic environment dominated by blue-green light, plant carotenoids play an important role in absorbing light energy. Studies have shown that chlorophyll b has a stronger capacity to absorb blue-violet light than chlorophyll a. Thus, a lower chlorophyll a/b ratio indicates a stronger light capture ability under low light conditions (Xu 2013a, b; Martin and Warner 1984). In this

Table 3 Influence of FLC on plant length and fresh weight of *C. demersum*.

FLC concentration ($\mu\text{g/L}$)	Length (cm)	Fresh weight (mg)
0	17.38 ± 0.49^a	3225.33 ± 70.23^a
20	17.18 ± 0.53^a	3000.67 ± 264.61^a
100	16.07 ± 0.37^a	2102.67 ± 145.96^c
300	16.71 ± 0.07^a	1656 ± 208.91^d
1000	19.27 ± 0.98^b	2483.67 ± 43.54^b
2000	18.72 ± 1.28^b	2312 ± 207.88^{bc}

Means with different letters in the same columns are significantly different among different exposure concentrations ($p < 0.05$, LSD test)

research, when carotenoids were severely damaged in *L. minor*, the decline in the a/b value may have enhanced the light capturing ability of plants in the water environment, helping to maintain photosynthesis in plants. As shown in the results, the a/b value of *C. demersum* did not change significantly, indicating that the photosynthesis system of *L. minor* was more tolerant than that of *C. demersum* when exposed to FLC levels higher than 300 $\mu\text{g/L}$.

The reduction of carotenoids and the decomposition of chlorophyll caused by FLC can increase the ROS level in plants (Xu 2013a, b). Excess ROS can oxidize biofilms and change the fluidity and permeability of cell membranes (Pitzschke et al. 2006). As the final product of membrane lipid peroxidation, the MDA content indicates the level of ROS stress in plants. The MDA content results indicate that the oxidative stress in *L. minor* was significantly high at 20 $\mu\text{g/L}$ FLC. The CAT activities in *L. minor* were significantly decreased when the FLC concentration exceeded 20 $\mu\text{g/L}$, which means that CAT was severely damaged by ROS. However, excess ROS at 20 $\mu\text{g/L}$ FLC acted as a signal molecule to activate antioxidant systems. The enzymes SOD and POD were activated at 100 $\mu\text{g/L}$ FLC and acted as ROS scavengers to protect the plants from oxidative damage. When the FLC concentration exceeded 300 $\mu\text{g/L}$, the SOD and POD activities and the MDA content all decreased to normal levels. The reason why the oxidative stress decreased in *L. minor* at higher FLC concentrations (300–2000 $\mu\text{g/L}$) is that the decrease in the chlorophyll a/b value enhanced photosynthesis and increased the resistance of plants. In contrast to *L. minor*, no obvious membrane lipid peroxidation under lower (20–300 $\mu\text{g/L}$) FLC levels was observed in *C. demersum*. The significant decrease in SOD activities when the FLC treatment concentration exceeded 100 $\mu\text{g/L}$ can be explained by the damage caused by excess free radicals. In addition, the inactivation of SOD exacerbated ROS accumulation, which adversely affects plant cells. POD was activated at 100 $\mu\text{g/L}$ FLC and acted as a protective enzyme. The excess ROS was decomposed by POD, and the MDA content in plant cells was maintained at a relatively normal level. However, the decrease in POD activities at higher FLC concentrations (≥ 300 $\mu\text{g/L}$) may be due to the inactivation of the enzyme by accumulated ROS. The enzyme CAT in *C. demersum* was sensitive to FLC stress and was slightly damaged at 20–300 $\mu\text{g/L}$ FLC. When the ROS stress significantly increased at 1000–2000 $\mu\text{g/L}$ FLC, CAT was activated and acted as a scavenger of harmful free radicals. However, due to the enzyme damage caused by ROS at lower concentrations of FLC, the level of CAT activity did not increase to the level that could completely eliminate harmful free radicals in plants; thus, *C. demersum* still experienced high oxidative stress in the 1000–2000 $\mu\text{g/L}$ treatment.

Soluble proteins are important osmotic adjustment substances and the material basis for plant organs and are often used as indicators of plant stress resistance (Zhou et al. 2018).

The decomposition of soluble protein in *L. minor* under lower FLC levels may be due to the highly oxidative stress induced by the herbicide. The slight increase in protein content at FLC levels higher than 100 $\mu\text{g/L}$ can be explained by the increase in SOD, POD, and other antioxidant enzymes. When the treatment concentration exceeded 300 $\mu\text{g/L}$, the soluble protein content returned to normal as the oxidative stress decreased. In *C. demersum*, increasing ROS accumulation resulted in a marked decrease in the protein content when the treatment concentration was above 300 $\mu\text{g/L}$.

Based on a combination of the physiological and growth parameters, we explain the toxicity of FLC to *L. minor* as follows: FLC caused serious and irreversible damage to the photosynthesis pigments of the plants at low doses (20 $\mu\text{g/L}$) and thus induced high oxidative stress in the plants. Studies have shown that ROS can obviously inhibit the growth of aquatic plants (Mishra et al. 2006; Zhou et al. 2018). The plants exhibited initial oxidative damage symptoms, and plant growth was also affected and decreased significantly at lower levels of FLC (20–300 $\mu\text{g/L}$). At 1000–2000 $\mu\text{g/L}$ FLC, the biomass of *L. minor* increased due to the relief of oxidative stress, but the growth parameters were still lower than those in the control because of the irreversible damage to the pigments and some enzymes. With respect to *C. demersum*, the decline in biomass at 20–300 $\mu\text{g/L}$ FLC was mainly due to the decline in photosynthesis. It is noteworthy that the plant biomass was significantly lower but plant length was higher than in the control group when FLC doses exceeded 1000 $\mu\text{g/L}$. This phenomenon of stem elongation with biomass decline has occurred in aquatic plants under low light conditions (Zeng et al. 2009). Because the photosynthesis system was highly stressed at higher FLC concentrations, the unexpected elongation of *C. demersum* stems is probably related to the adaptation of the decline in photosynthesis. The increase in biomass at 1000–2000 $\mu\text{g/L}$ was partly due to CAT activation and partly due to the increase in plant length.

In general, 20 $\mu\text{g/L}$ FLC had a significant inhibitory effect on the chlorophyll content of the submerged plant *C. demersum* and the floating plant *L. minor*, which seriously affected photosynthesis and caused visible bleaching of the plants. This may be related to the mode of transport of FLC in plants. Studies have shown that FLC transport in plants is mainly through apoplastic pathways, and this transport mode allows rapid spread, so even low concentrations of FLC can quickly affect plants (Weinberg et al. 2003). At 20 $\mu\text{g/L}$ FLC, *L. minor* plants were under relatively stronger oxidative stress, while *C. demersum* plants showed no oxidative damage, which means that *L. minor* is more sensitive to FLC than *C. demersum* at lower treatment concentrations. When exposed to higher concentrations of FLC (100–2000 $\mu\text{g/L}$), *L. minor* showed some level of adaptability, reaching less than healthy stability, through the regulation of its own antioxidant enzyme system and the chlorophyll a/b value. *C. demersum*

suffered irreversible oxidative damage, and POD and SOD were inactivated under higher exposure concentrations. In addition to species differences, another reason for this difference is that the exposure degrees of these two species in the water environment were different. *C. demersum* is a submerged plant whose organs are completely underwater, while *L. minor* is a floating plant whose organs are exposed to the air rather than directly in contact with contaminated water. In addition, *C. demersum* has a higher specific leaf area, which means higher resource turnover and higher exposure degree to the pollutants in the water environment (Liu 1999). Moreover, the leaf area of *L. minor* showed a dose-dependent increase when exposed to FLC concentration, which also enhanced photosynthesis to a certain extent and enhanced its adaptability. Therefore, *L. minor* was more sensitive to low doses of FLC (20 µg/L) than *C. demersum*. Moreover, compared with *C. demersum*, *L. minor* was more adaptable under higher concentrations of FLC (1000–2000 µg/L).

Studies have shown that single-species toxicity tests can be extrapolated to predict the impact at the community level (McGregor et al. 2008; Coutris et al. 2011). Therefore, it can be inferred from the results of this experiment that low concentrations of FLC can have toxic effects on *L. minor* and *C. demersum* communities in nature. The amount of FLC applied should be controlled, and the FLC emission concentration of agricultural sewage should be less than 20 µg/L.

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Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

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