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Toxic Effects of Pentachlorophenol on *Lemna minor*

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Pentachlorophenol (PCP), a major industrial chemical, has been extensively used throughout the world as a pesticide and general biocide in agriculture and industry. Pentachlorophenol (PCP) has been detected at part per million levels in human urine, adipose tissue and breast milk (Hattemer-Frey and Travis 1989). The high toxicity of chlorophenols to aquatic organisms is widely recognized and the lethal dose of chlorophenols for zooplankton has been determined in a number of experiments (Gokeen 1998).

Duckweed is small aquatic vascular floating plant. It is cultured easily in the laboratory and widely used as a test organism to study chemical toxicity. The toxicity of chlorinated phenols, from phenol to pentachlorophenol, to *Lemna gibba* tended to increase as the number of chlorine substituents on the phenol ring increased (Sharma et al. 1997). This paper evaluates the effect of PCP on duckweed (*Lemna minor*) growth and enzyme activities.

METHODS AND MATERIALS

Duckweed (*Lemna minor*) was collected from the Weijin River in Tianjin City. No PCP was detected from this river. Duckweed was cultured in hoagland solution in the laboratory for three weeks. One plant was selected for culture in Hoagland solution and grew more plants. Since the amount of duckweed produced by this plant was enough, these healthy plants were used in the test (Xiaohua and Zhonghan 1983).

IC_{50} (median inhibitory concentration) tests were conducted in a series of 100 mL beakers. A control and six concentrations of PCP (0.00, 0.10, 0.25, 0.50, 1.00, 2.50, 5.00 mg/L in Hoagland solution) were used as experimental treatments. Each concentration was replicated three times. Each beaker contained 80 mL of test water. The test solution in each beaker was renewed every two days. Illumination (24 hr light) was carried out at 25 $\pm$1℃. Tests lasted eight days.

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The number of fronds in each beaker was counted every two days.

Bioaccumulation tests were conducted in 250 mL beakers. Each beaker contained 200 mL of test water. The concentrations of PCP (0.2 and 0.5 mg/L in Hoagland solution) and a control were used, each replicated three times. The number of fronds and nitrate reductase activity in each beaker was determined every two days. The test solution in each beaker was renewed every two days. Illumination (24 hr light) was carried out at 25 $\pm$1℃. The test lasted ten days. At the end of the tests, PCP concentrations in each beaker and in the plants, the chlorophyll content, sugar content, chloroplast activity and peroxidase activity were determined.

The methods of determining chlorophyll content and IC_{50} were published (Song and Tianyi 1998). At the end of the bioaccumulation test, 2 mL of solution from each test beaker was used to determining nitrate reductase activity; 5, 10 or more fronds in each beaker were selected to determine the chlorophyll content; and separate sets of 20, 30 or more fronds in each beaker were selected to determine sugar content; peroxidase activity and chloroplast activity. The methods of determining sugar content, nitrate reductase activity, peroxidase activity and chloroplast activity were published (Song and Huang 2001).

The t-test statistical method was used to determine statistical significance between treatments with $p \leqslant 0.5$ (Zhenyu and Shuzhen 1993).

Every two days, 100 mL of each test solution, 5 ml n-hexane and 2 ml HCl were added into a 250 ml separating funnel. The funnel was shaken 300 times to extract PCP and was allowed to stand for 30 min. The organic phase was separated into a color comparison tube. The organic solution was purged with pure nitrogen to near dryness and stored in a refrigerator at 4℃to determine PCP content.

At the end of the tests, 50, 70 or more fronds were weighed and homogenized with water and digested in a 10% TMAH (tetramethylammonium-hydroxide) solution at 60℃for 1 hr. The digested solution was treated as above to extract the PCP from the duckweed.

A Waters Associates high-pressure liquid chromatograph system (HPLC Model 244), a Model 481 variable-wavelength UV detector (measurement set at 280 nm), a Model 680 system flow controller, and a Model 730 data station were used for PCP analysis. The analysis was performed by isocratic elution with a binary mixture of 85% methanol and 15% acetic acid buffer solution (pH 4.0) at a flow rate of 1.0 $ml.min^{-1}$. The analytical column was a 4.6mm$\times$250mm, 10μm Irregular-H C_{18} column (Tianjin Second Reagent Manufactory) and was

maintained at 25±0.5 ℃. A PHS-3C precision pH meter supplied by the Shanghai Electromagnetic Instrumentation Manufactory was used to measure the pH of the aqueous solutions. The detection limit was lower than 0.1 mg/L PCP.

At the end of the tests, 10 duckweed fronds each from the control and 1.0 mg/L PCP test concentration were seperately put into 5 ml of 2.5% glutaraldehyde aqueous solution; this solution was kept in a refrigerator at 4℃ for 35 hr to immobilize the fronds. Then the fronds were put into a 20% alcohol solution and kept in the refrigerator for further dehydration. The dehydration was continued using an increasing gradient (%) of alcohol solution. Dehydration with each alcohol concentration was conducted for 30 min. Finally, dehydration was carried out twice with absolute (anhydrous) alcohol. The fronds were kept in the refrigerator for use. Before observation by SEM (Scanning Electron Microscope, Model Hitachi x-650), the fronds were soaked in 1ml xylene for 5 min and then coated with gold. The fronds were then ready for observation by SEM to study the cell microstructure (Dai and Zhang 1992).

RESULTS AND DISCUSSION

The tests results are shown in Figures 1 –9 and Table 1.

From Figure 1, the 8 day IC_{50} of PCP to *Lemna minor* was 2.37mg/L. Figure 1 shows that at low PCP concentrations (0.10, 0.25mg/L), growth rate of duckweed is greater than that of the control. The growth rate of duckweed decreases with increasing PCP concentrations. From Figure 2, it can be seen that at 0.20 mg/L PCP, the number of duckweed fronds is larger than that of control. At 0.50 mg/L PCP, duckweed growth rate is slightly lower than that of control. These indicate that at low concentrations, PCP stimulates duckweed growth and at higher concentrations, PCP inhibits duckweed growth.

The data (Table 1) show that sugar content increased with 0.2 mg/L PCP and sugar content decreased with 0.5 mg/L PCP. Sugar content shows the anabolism activity of duckweed. This suggests that PCP may enhance duckweed anabolism at low concentration and inhibit duckweed anabolism at higher concentrations. PCP disturbs duckweed metabolism, which will change the duckweed rate or other physiological processes.

Figure 3 shows the effect of PCP on *Lemna minor* nitrate reductase activity. At 0.2 and 0.5 mg/L, PCP stimulates nitrate reductase activity. At 0.5 mg/L PCP, the nitrate reductase activity of duckweed was 69.2 times control. At 0.2 mg/L PCP, the nitrate reductase activity of duckweed was from 20 to 40 times that of the control during the experimental period. Over time, the relative nitrate reductase

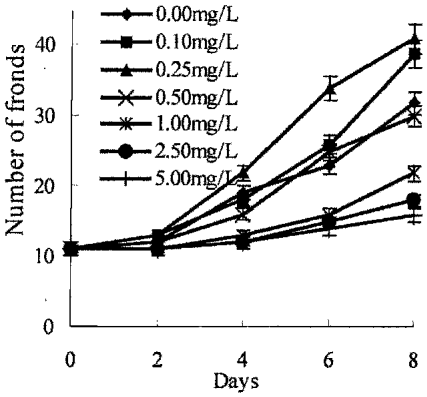

Figure 1. Effect of PCP on growth rate of *Lemna minor.*

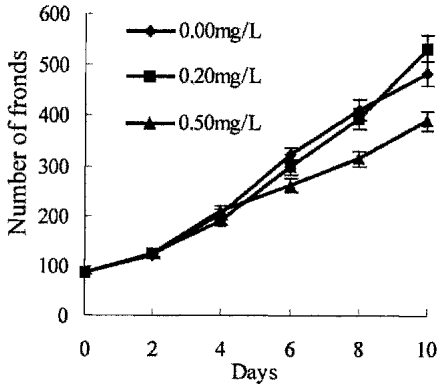

Figure 2. Effect of low PCP concentration on growth rate of*Lemna minor.*

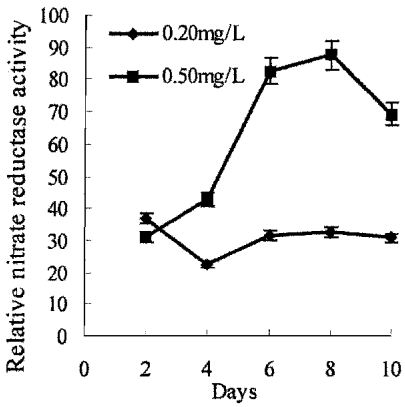

Figure 3. Effect of PCP on nitrate reductase activity of *Lemna minor.*

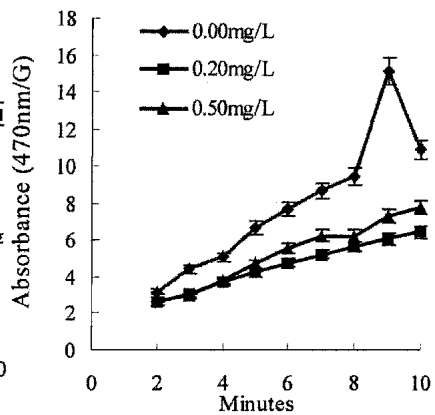

Figure 4. Effect of PCP on peroxidase activity of *Lemna minor.*

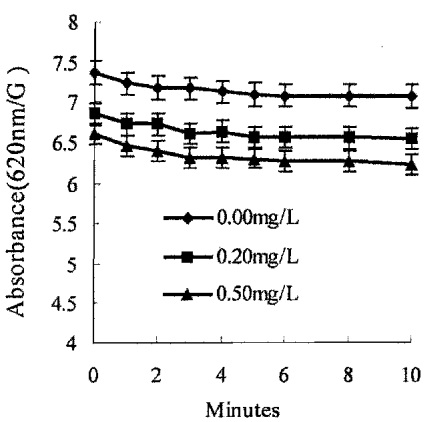

Figure 5. Effect of PCP on chloroplast activity of *Lemna minor*

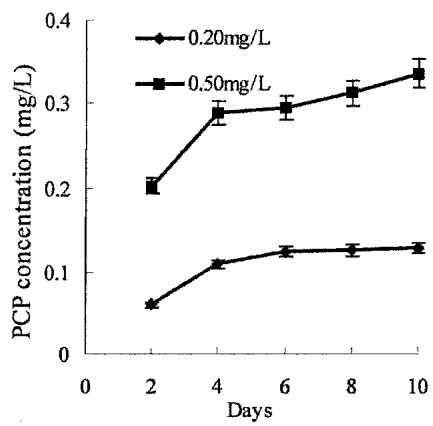

Figure 6. PCP concentration in test water.

Table 1. The effect of PCP on *Lemna minor* at the end of tests (10th day).

PCP concentration (mg/L)	Chlorophyll content (mg/g*)	Sugar content (mg/g*) **	PCP content in fronds (mg/g*)	Relative nitrate reductase activity**	Peroxidase activity (ΔA_{470}/g*)	Chloroplast activity (ΔA_{620} / mg chlorophyll)
0.00	1.31	5.14	-	1	1.34	0.042
0.20	2.12	5.69	0.035	30.6	0.85	0.027
0.50	2.05	4.77	0.058	69.2	0.84	0.029

* fresh weight **significant differences between treatments ($p \leq 0.5$)

activity was steady. This suggests that at 0.2 mg/L PCP, nitrogen metabolism of duckweed was maintained a high level. For 0.5 mg/L PCP, at the beginning of test, relative nitrate reductase activity in duckweed increased. However at the end of the test, duckweed relative nitrate reductase activity decreased, showing that PCP can stimulate duckweed nitrate reductase activity. At the end of the test, the toxic effect of PCP on duckweed reduces the stimulating effect of PCP on duckweed nitrate reductase activity.

From Figure 4, it can be seen that at 0.2 and 0.5 mg/L PCP, the peroxidase activity was 63.4 and 62.8 percent of the control, respectively. This indicates that the effects of PCP at 0.2 and 0.5mg/L showed little difference. The mechanism of PCP inhibition of *Lemna minor* peroxidase should be further investigated. Peroxidase acts as a detoxicating factor, so reduced peroxidase activity leads to reduced ability of duckweed to resist pollutants.

The chloroplast is needed for photosynthesis. Decreasing chloroplast activity causes low photosynthetic efficiency. At 0.2 mg/L and 0.5 mg/L, PCP inhibited duckweed chloroplast activity (Table 1), with activities being 64.3 and 71.2 percent of the control, respectively. Chlorophyll content was 162 and 157 percent of the control, respectively. This suggests that at a low level, PCP stimulates the duckweed chlorophyll content. However, the chloroplast activity of *Lemna minor* is reduced at 0.2 and 0.5 mg/L PCP. A similar result appeared in the study of triphenyltin toxicity to *Lemna minor* (Song and Huang 2001). This means that chloroplast activity and chlorophyll content may show different responses when duckweed plants are exposed to pollutants. Such test results imply that chloroplast activity cannot be substituted for chlorophyll content in a toxicity test. Chloroplast activity may be more useful than chlorophyll content for indicating a toxic effect of pollutants.

From Figure 6, PCP concentrations in the test water increased over time. The PCP absorbance in duckweed decreased on the 4th day. At the end of test, PCP

concentrations were nearly steady. The bioaccumulation factors (BCFs) of PCP in duckweed were 274.2 and 172.9 at 0.2 mg/L and 0.5 mg/L PCP on the 10th day, respectively. This suggests that PCP be more harmful at 0.5 mg/L than at 0.2 mg/L to *Lemna minor* by reducing absorb PCP efficiency .

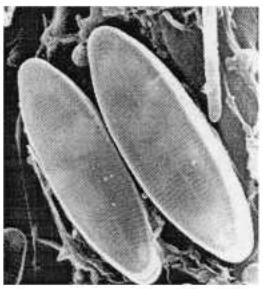

Figure 7. SEM picture of *Lemna minor* normal cell (4000×)

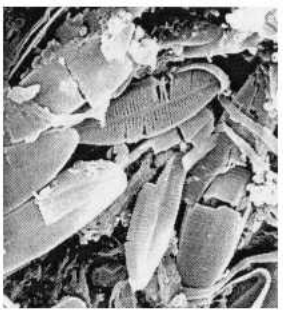

Figure 8. SEM picture of *Lemna minor* cell with 1.0 mg/L PCP (3000×)

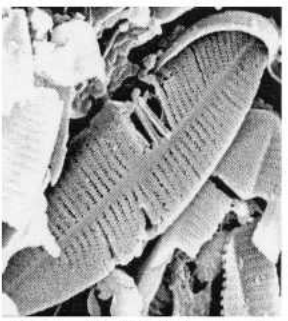

Figure 9. SEM picture of *Lemna minor* cell with 1.0 mg/L PCP (7000×)

The toxic effect inhibits the absorbability of duckweed to PCP at 0.5 mg/L. The BCF of *Hyalella azteca* for PCP was 132 (Nuutinen et al. 2003). The BCF values show that PCP can be absorbed and accumulated in organisms in an aquatic environment. This status implies that PCP could be enriched in organisms through a food chain involving duckweed.

Figures 7, 8 and 9 show normal duckweed and affected duckweed cells at 1.0 mg/L PCP. About 10% per cent of cells were affected by PCP in SEM observations. From Figures 8 and 9, it can be seen that the duckweed cells break leading to inclusion outflow from cell.

The 8 day IC_{50} of PCP to *Spirulina subsalsa* was 4.26 mg/L, and PCP stimulated the nitrate reductase activity of *Spirulina subsalsa* (Zhihui et al. 2000). These results are similar to this test data. The 48 hr LC_{50} of PCP to *Lumbriculus variegates* and *Chironomus riparius* were 143 μg/L and 898 μg/L (Kukkonen 2002). These data show that *Lemna minor* is less sensitive to PCP than an oligochaete worm, and more sensitive than bluegreen algae. PCP stimulates the mutation frequency of hypoxanthine phosphoribosyl transferase (Yoon et al. 1997). PCP causes nitrate reductase activity of soil microalgae to be decreased (Megharaj et al. 1998). These results, together with this study, suggest that PCP has different effects on various enzyme systems, not only in animals, but also in plants.

There are few reports on toxic effect of PCP on plant enzymes. The results of this study demonstrate that PCP affects various physiological functions of the plant.

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