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Effects of three pesticides on superoxide dismutase and glutathione-S-transferase activities and reproduction of *Daphnia magna*

Yuzhi Song[1,2], Prof. Mindong Chen[1], Prof. Junying Zhou[3]

[1]Nanjing University of Information Science and Technology, China
Jiangsu Collaborative Innovation Centre of atmospheric environment and equipment technology (AEET)
[2]Nanjing University of Information Science and Technology, China
Jiangsu Key Laboratory of Agricultural Meteorology, College of Applied Meteorology
[3]Nanjing Institute of Environmental Sciences, China, MEP

*Corresponding author's e-mail: syz70@nuist.edu.cn

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Abstract: Applying pesticides to crops is one of the causes of water pollution by surface runoff, and chlorpyrifos, trifluralin and chlorothalonil are used respectively as insecticide, herbicide and fungicide for crop plants widely. To explore effects of three pesticides on aquatic organisms, superoxide dismutase (SOD) and glutathione S-transferase (GST) activities were determined after 24 h and 48 h exposure of *D. magna* with ages of 6–24 h to several low concentrations of chlorpyrifos (0.36, 0.72, 1.43, 2.86, 5.72 $\mu g \cdot L^{-1}$), trifluralin (0.17, 0.33, 0.66, 1.33, 2.65 $mg \cdot L^{-1}$) and chlorothalonil (0.09, 0.18, 0.36, 0.72, 1.43 $mg \cdot L^{-1}$) respectively. Main reproductive parameters including first pregnancy time , first brood time , the number of first brood and total fecundity after 21 d exposures at the same concentrations of pesticides as described above were also measured. The results showed that the activities of GST increased in lower concentrations and decreased in higher concentrations after 24 h exposure to three pesticides, respectively. The activities of SOD showed the same changes after 48 h exposure. With the time prolonged, the activities of GST decreased while the activities of SOD increased. After 21 d exposure, the first pregnancy time and first brood time were delayed, while the number of the first brood and total fecundity per female decreased with increasing concentrations. These results corroborated that GST activity was more sensitive to those pesticides than SOD activity, and there was a significant relationship between total fecundity and pesticides-dose($r>0.94$, $n=6$), GST activity after 48 h exposure and total fecundity after 21 d exposure ($r>0.92$, $n=6$).

Introduction

Pesticide residues in the environment are an important source of water pollution due to extensive use of pesticides in agricultural production (Zhou 2008, Palma et al. 2009, Smalling et al. 2013). According to statistics, the amounts of pesticides used have reached 50–60 thousand tons annually in China, about 80% of which entered the environment directly (Zhou 2008). Large amounts of pesticides have been gathered to water bodies by material recycles in natural ecological systems, causing the decline of water quality, furthermore, damaging the ecosystem and people's health to serious levels (Zhang 1987, Lin and Xue 1997, Hayasaka et al. 2012). At present, negative effects promoted by pesticides on aquatic ecology were prominent (Rodriguez-Mozaz et al. 2004, Bajet et al. 2012). Pesticides and their metabolites damaged aquatic organisms either directly or indirectly by food chains, which reduced biological diversity and natural ecosystem balance (Palma et al. 2009, Wiszniowski et al.2011, Hayasaka et al. 2012, Van der Oost et al. 2013). So the high-toxic and high--residual pesticides which were traditionally used have been forbidden to produce and applicate in China, certain pesticides such as chlorpyrifos (insecticide), chlorothalonil (fungicide) and trifluralin (herbicide) have been widely used due to their moderate or low toxicity and low residue instead. However chlorpyrifos, chlorothalonil and trifluralin are highly toxic to aquatic organisms, despite their rapid degradation in the environment according to the United States Environmental Protection Agency (US EPA 1996), and the concerned researches focus on acute toxic effects of the three pesticides (US EPA 1996, Wang et al. 2011), but few researches have been reported about the effects of chlorpyrifos, trifluralin and chlorothalonil on biochemical indicators of superoxide dismutase (SOD) and glutathione-S-transferase (GST) and chronic toxicity in aquatic invertebrates. To protect aquatic organisms' diversity, there is a growing need to understand the effects of three pesticides on biochemical indicators of SOD and GST and chronic toxicity in aquatic invertebrates.

In toxicological studies, *D. magna* is used widely as model bioassay organisms to measure toxicity of chemicals or contaminants dissolved in water. Fecundity and survivorship are traditionally endpoints in toxicity bioassays, while

recent studies have focused on other sub-lethal endpoints. Some researchers found that reproduction of *D. magna* was affected by its exposure to direagents and atrazine, and that the exercise capacity of *D. magna* was inhibited by organophosphorus pesticides and carbamate pesticides through inhibiting cholinesterase (Dodson et al. 1999, Barata et al. 2004, Ren et al. 2007). However various contaminants could affect the enzyme activities differently in *D. magna*. Li and Yang (1997) found that glutathione S-transferase (GST) activity was inhibited by deltamethrin and acetofenate, but it was not influenced by trichlorfon and carbaryl. Xu et al (2013) found that GST activity in *D. magna* was induced by difenoconazole, and that there was a correlation between the GST activity and application dosage. Peng et al (2012) found that the activities of SOD and GST were induced initially and inhibited afterwards with increasing exposure to concentration of BDE-47. Although the effects of some chemical pollutants in water on biochemical parameters of *D. magna* have been widely researched, there is still lack of a clear understanding about the effects of chlorpyrifos, trifluralin and chlorothalonil on biochemical indicators of SOD activity and GST activity and chronic toxicity in *D. magna*.

In this paper, we determined SOD and GST activities in *D. magna* after 24 h and 48 h exposure to the three commonly--use pesticides (chlorpyrifos, trifluralin and chlorothalonil); the first pregnancy time, first brood time, the number of first brood and total fecundity for 21 d exposure were also determined. The objectives of this study were 1) to perform the relationship between determining index and application dosage. 2) to compare the relative sensitivity of SOD and GST in *D. magna* to the three commonly-used pesticides, and 3) to assess the linkage of selected enzyme activities to fecundity under the sublethal concentration.

Materials and methods

Chemicals

Chemicals used for toxicity bioassays were chlorpyrifos 480 g/L EC (emulsifiable concentrate), chlorothalonil 75% WP (wettable powder) and trifluralin 48% EC. Chlorpyrifos 480g/L EC and chlorothalonil 75% WP were purchased from Dongguan City Ruidefeng Biology and Technology Co., Limited, and trifluralin 48% EC was obtained from Hebei Tianwei Biology and Technology Co., Limited. Individual pesticide stock solutions of all listed above were prepared in standard water for one day before experiments and stored at 0~4°C in the dark.

Animal cultures

D. magna was obtained from the Aquatic Organism Laboratory of Nanjing University. It is a monoclonal body with the same individual size and strong swimming ability. Cultures were maintained in 2 L glass beakers with 1 L of standard water (ISO 1989) under static renewal conditions. The culture water was renewed twice a week. The organisms were fed daily with algae (Scenedesmus oblquus) with a density of 2.0×10^5 cell mL^{-1}. $Daphnia^{-1}$. Animal density was 20 animals per 1 L. *D. magna* was kept under controlled temperature of 22±0.5°C and photoperiod of 16 h light: 8 h dark with light intensities ranging from 2000 to 3000 Lx. All experiments were carried out with *D. magna* with the ages of 6–24 h from a single clone derived from the healthy parent stock culture. The sensitivity of the daphnids was test in our lab according to the book of Toxicity Test Method for Aquatic Organisms (Zhou and Zhang 1989).

Chronic reproduction test

To evaluate the effects of chlorpyrifos, trifluralin and chlorothalonil on the reproduction of *D. magna*, tests were conducted for 21 days according to the "*D. magna* reproduction test" provided by test guideline 211 of the Organization for Economic Cooperation and Development (OECD 2008). The pesticide concentrations used in this bioassay were derived from the 48 h LC_{50} values for *D. magna* obtained from our previous studies (LC_{50} (48 h): 5.72 $\mu g\cdot L^{-1}$ chlorpyrifos, 2.65 $mg\cdot L^{-1}$ trifluralin, 1.43 $mg\cdot L^{-1}$ chlorothalonil). The specimens were exposed to 0.36, 0.72, 1.43, 2.86, 5.72 $\mu g\cdot L^{-1}$ of chlorpyrifos or 0.17, 0.33, 0.66, 1.33, 2.65 $mg\cdot L^{-1}$ of trifluralin or 0.09, 0.18, 0.36, 0.72, 1.43 $mg\cdot L^{-1}$ of chlorothalonil. Ten replicates were set up for each concentration including the control (standard water without pesticides).

Daphnids were fed daily with algae (*Scenedesmus oblquus*) with a density of 2.0×10^5 cell $mL^{-1}\cdot Daphnia^{-1}$. The exposure solutions were renewed once a day to keep pesticides concentrate over 80% of the initial concentration according to the results of our pervious test. The experiments were conducted in 100 mL beakers with 50 mL exposure solution. One *D. magna* at the age of 6–24 h from a single clone derived from the healthy parent stock culture was placed into the beaker. The beakers were then capped by parafilm, and they were maintained at 20 ± 1°C with a 12:12 h (light: dark) photoperiod, pH was 7.5–7.8, dissolved oxygen concentration was greater than 8.4 $mg\cdot L^{-1}$. During the test, observations were conducted every day by removing the daphnids from the containers, and recording the female daphnids first pregnancy, the first brood time and the number of first brood, as well as total fecundity.

SOD and GST activities analysis

The pesticide concentrations used in biochemical tests were the identical concentrations of each pesticide and control measure as used in the test for chronic reproduction. Twenty daphnids with the ages of 6–24 h were put in each beaker with the test solutions. Three replicates were set up for each concentration including the control. Biochemical tests were performed under static non-renewal conditions for 48 h without feeding. All the other experimental conditions were the same as described in the previous section. Animals were collected and were high-pressure homogenized in Phosphate buffer solution (PBS) at pH 7.8. The homogenate was centrifuged for 20 min at 10000 r/min. The supernatant was collected to serve as basic source of the enzyme and protein. All the above performances were conducted at 4°C. About 10 daphnids were used for preparation conducted after 24 h and 48 h exposure, respectively. The samples were assayed for enzyme activity and the protein content as described below.

The protein content was determined according to total protein quantitative assay kit. SOD activity was determined using photochemical assay based on the reduction of nitro blue tetrazolium (NBT) according to Total Superoxide Dismutase (T-SOD) assay kit (Hydroxylamine method). One unit of the enzyme activity was defined as the amount of enzyme required

for 50% inhibition of NBT reduction measured at 560 nm at 25°C. The GST activity was assayed by the spectrophotometric method using 1 mM GSH and 1 mM 1-chloro-2, 4-dinitrobenzene (CDNB) as substrates. One unit of glutathione S-transferase activity is the amount of enzyme catalyzing conjugate of 1 μmol CDNB and GSH at 37°C per minute.

Statistical analysis

All data were presented as mean±SE. The data on biochemical parameters and biological parameters of *D. magna* were analyzed using SPSS for Windows. The one-way ANOVA analysis was performed to determine the significant difference among different treatments. Significant differences between exposure groups and control groups were assessed by the Dunnet-t.

Results and discussion

The effects of three commonly used pesticides on SOD activity in D. magna

SOD plays a crucial role in the antioxidant system, intervening in the first transformation by dismuting the most reactive, dangerous free radicals into ions that are less reactive. So SOD assays were used to evaluate antioxidant activity (Shi et al. 2010). In this experiment, after 24 h exposure, SOD activities of *D. magna* did not change significantly in all treatments of pesticides and were nearly similar to those in the control group ($P<0.05$) (Fig. 1: A–C) .The results indicated that three commonly used pesticides had no effect on SOD activity of *D. magna* in a short time. However after 48 h of exposure, SOD activity showed a tendency to increase first and then decrease with the increases of the concentrations of pesticides in the water body (Fig. 1: A–C). More specifically, when the concentrations of pesticides did not exceed the values of C3 (0.72 μg·L^{-1} chlorpyrifos, 0.33 mg·L^{-1} trifluralin, 0.18 mg·L^{-1} chlorothaloni), SOD activities increased significantly as compared with those in their control groups respectively ($P<0.01$) and their peaks were 59.33 U·mg protein^{-1} in the treatments of chlorpyrifos, 55.23 U·mg protein^{-1} in the treatments of trifluralin and 62.73 U·mg protein^{-1} in the treatments of chlorothaloni, nearly 1.5 times greater than those in their control groups respectively (Fig. 1: A–C). After that, with the increase of exposure concentrations, SOD activity decreased gradually (Fig. 1: A–C). When the concentration of chlorpyrifos was 5.72 μg·L^{-1} and the concentrations of trifluralin and chlorothaloni were 1.33 and 0.72 mg·L^{-1} respectively, there was no significant difference between the treatment group and the control group ($P<0.05$) (Fig. 1: A–C). while the concentrations of trifluralin and chlorothalonil were 2.65 and 1.43 mg·L^{-1} respectively, SOD activities were inhibited significantly as compared with those in the control group ($P<0.01$). Peng et al (2012) found that SOD activity was increased after exposure to low concentrations of BDE-47 and inhibited after exposure to high concentration for *D. manga*. This is consistent with the writer's research findings. Low doses of pollutants were induced and promoted SOD activity. With increasing exposure concentrations, more toxicant could enter tissues, and cell-structure and function was damaged by a rapid ROS generation under the stress, which impeded SOD production, and SOD activity was inhibited (Calabrese 2005).

The effects of 3 commonly used pesticides on GST activity in D.magna

The effects of 3 commonly used pesticides on GST activity are presented in Fig. 2. After 24 h of exposure, GST activity showed a tendency to increase first and then decrease with

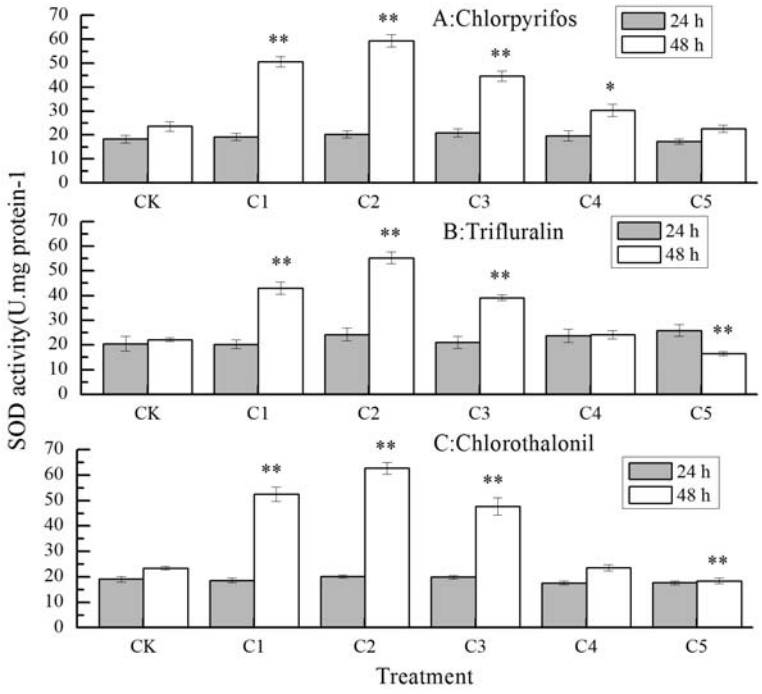

Fig. 1. SOD activity in *D. magna* (Data are presented as mean standard error. Asterisks indicate a significant difference to control (*: $p<0.05$, **: $p<0.01$). CK indicate control; C1, C2, C3, C4, C5 indicate pesticides concentrations (0.36, 0.72, 1.43, 2.86, 5.72 μg·L^{-1} of chlorpyrifos or 0.17, 0.33, 0.66, 1.33, 2.65 mg·L^{-1} of trifluralin or 0.09, 0.18, 0.36, 0.72, 1.43 mg·L^{-1} of chlorothalonil)

increases of concentrations of pesticides in the water body (Fig. 2: A–C). Low concentrations of three pesticides exposure increased GST activity, while high concentrations of three pesticides exposure inhibited GST activity, and when the concentration of pesticides was C1 (0.36 μg·L^{-1} chlorpyrifos, 0.17 mg·L^{-1} trifluralin, 0.09 mg·L^{-1} chlorothalonil), GST activities reached their peaks of 17.67, 18.47 and 40.94 U·mg protein^{-1} respectively (Fig. 2: A–C). After that, GST activity decreased with the increasing concentrations of chlorpyrifos (Fig. 2: A). When the concentration of chlorpyrifos was 1.43 μg·L^{-1} (C3), there was no significant difference between the treatment group and the control group ($P<0.05$), and when the concentrations of chlorpyrifos was over 2.86 μg·L^{-1} (C3), GST activity was inhibited significantly as compared with that in its control group ($P<0.01$) (Fig. 2: A). With increasing concentrations of trifluralin and chlorothalonil, GST activities showed the same change trend as that in the treatment of chlorpyrifos (Fig. 2: A–C), while the concentration of trifluralin was 0.66 mg·L^{-1} (C3), GST activity was already inhibited significantly as compared with that in its control group ($P<0.01$) (Fig. 2: B). After 48 h exposure to chlorpyrifos and trifluralin respectively, GST activity showed a clear decreasing trends with the increases of the concentrations and were lower significantly as compared to those in the control group ($P<0.01$) (Fig. 2: A-B). While under the same condition, after 48 h exposure to chlorothalonil, GST activity showed the same change as that after 24 h exposure, which was increasing first and then decreasing with the increases of the concentrations of chlorothaloni. Additionally, GST activities after 48 h exposure of three pesticides were significantly lower than those of 24 h exposure respectively. This showed that GST activity was inhibited with increasing of exposure concentrations and time for more toxicant to enter the body.

GST is soluble protein with low molecular weight in various cells and tissues. GSTs are a family of detoxification enzymes that catalyze the conjugation of glutathione (GSH) with electrophilic compounds, thus preventing toxicity. Some GST isoenzymes have antioxidantase activity to defense against oxidative damage and peroxidative products of DNA and lipids (Gadagbui and James 2000, Chen et al. 2006). The toxicity of many exogenous compounds can be modulated by induction of GSTs. So they might be playing an important role in detoxifcation metabolism. The GST activities in *D. magna* and *M. macrocopa* were induced at lower concentrations and inhibited at higher concentrations after 24 h exposure to BDE-47 and three phenol compounds (phenol, pyrocatechol or resorcinol), respectively (Zhang et al. 2009, Peng et al. 2012). Some research showed that the expression of GST is a crucial factor in determining the sensitivity of cells and organs in response to a variety of toxins in the aquatic organism, and dose-effect relationship (Van der Oost et al. 2003, Xu et al. 2013). It was also demonstrated that there was a significant dose-effect relevance between the concentration of pesticides and GST activity response of *D. magna* in this experiment (Fig. 2: A–C).

Further analysis found that GST activity changed significantly after 24 h exposure to three commonly-used

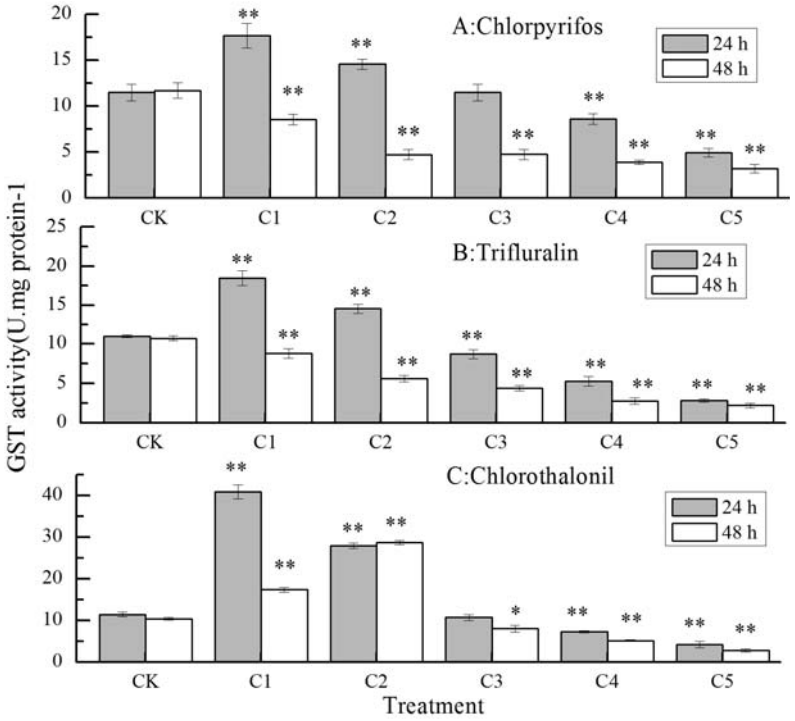

Fig. 2. GST activity in *D. magna* (Data are presented as mean standard error. Asterisks indicate a significant difference to control (*: $p<0.05$, **: $p<0.01$)). CK, C1, C2, C3, C4, C5 as in Fig. 1

The time at first pregnancy, first brood time, the number of first brood and 21d total fecundity and etc. were often used to evaluate the chronic toxicity in *D. magna* (OECD 2008). Some research found that the initiation of the pregnancy and spawning time were prolonged, and fecundity decreased under the stress of difenoconazle and bifenthrin (Ye et al. 2004, Xu et al. 2013). Xiong et al (2013) also found the initiation of the pregnancy and spawning time of D.magna were prolonged and fecundity decreased under the stress BDE-47. All previous research results were consistent with the results of this study.

pesticides respectively (Fig. 2), while SOD activity did not significantly change after 24 h exposure until after 48 h exposure (Fig. 1). Maximum activity of GST can be obtained at the concentrations of C1 (0.36 μg·L^{-1} chlorpyrifos, 0.17 mg·L^{-1} trifluralin, 0.09 mg·L^{-1} chlorothalonil), and maximum activity of SOD at the concentrations of C2 (0.72 μg·L^{-1} chlorpyrifos, 0.33 mg·L^{-1} trifluralin, 0.18 mg·L^{-1} chlorothalonil). This indicated that GST activities were more sensitive to pesticides than SOD activities.

The effects of three commonly used pesticides on reproduction of D. magna

The first pregnancy time and first brood time of D. magna were all prolonged with increasing concentrations of three commonly used pesticides respectively (Tab. 1). When concentrations of chlorpyrifos and trifluralin were 0.72 μg·L^{-1}, 0.33 mg·L^{-1} respectively, the times of first pregnancy were significantly prolonged as compared with those in the control group ($P<0.05$), while the first brood time reached significant level at concentrations of 1.43 μg·L^{-1} chlorpyrifos and 0.66 mg·L^{-1} trifluralin as compared with those in the control group ($P<0.05$) respectively. When concentration of chlorothalonil was 0.72 mg·L^{-1}, the first pregnancy time and first brood time of daphnia were all prolonged significantly as compared with those in the control group ($P<0.01$).

The number of first brood per female decreased gradually with the increase of exposing concentrations, as chronic toxicity index. When the concentration of chlorpyrifos was 2.86 μg·L^{-1}, the number of first brood was significantly lower as compared with those in the control group ($P<0.05$), while after exposure of 0.33 mg·L^{-1} trifluralin and 0.18 mg·L^{-1} chlorothalonil, there were significant differences between the treatment group and the control group ($P<0.05$), respectively. Total fecundity per female also decreased gradually with the increase of exposing concentrations. When concentrations of chlorpyrifos and trifluralin and chlorothalonil were 0.72 μg·L^{-1} and 0.33 mg·L^{-1} and 0.18 mg·L^{-1} respectively, there were extremely significant differences between the treatment group and the control group ($P<0.01$). Thus, total fecundity of 21 d per female was more sensitive to pesticides as comparing with other reproductive index.

Further analysis found that, in this experiment, fecundity was more sensitive to three commonly-used pesticides than the other reproduction index tested, and there was a significant relationship between total fecundity and pesticides-dose (Tab. 3), and GST activities were related to total fecundity significantly (Tab. 3). The GST might be a more suitable biomarker than SOD to evaluate the chronic toxicity of three commonly-used pesticides in a short time.

Conclusions

- Effects of three pesticides on SOD and GST activity of *Daphnia magna* were significant, both SOD activity and GST activity were induced at low concentration and inhibited at high concentration, while GST activity was more sensitive to three commonly-used pesticides than SOD activity.
- Influences of three pesticides on chronic toxicity index such as first pregnancy time, first brood time, the number of first brood and 21 d total fecundity per female was increased with increasing concentrations of pesticides in the water phases respectively and fecundity was more

Table 1. First pregnancy time, first brood time of *D. magna* after exposure of three commonly used pesticides. CK ,C1, C2, C3, C4, C5 as in Fig.1 (*: *P<0. 05*; **: *p<0.01*)

	First pregnancy time/d			First brood time/d		
	Chlorpyrifos (μg/L)	Trifluralin (mg/L)	Chlorothalonil (mg/L)	Chlorpyrifos (μg/L)	Trifluralin (mg/L)	Chlorothalonil (mg/L)
CK	6.68±0.35	6.72±0.23	6.66±0.33	8.18±0.55	8.08±0.35	8.11±0.36
C1	6.99±0.42	6.83±0.46	6.73±0.44	8.31±0.69	8.21±0.44	8.18±0.64
C2	7.01±0.28*	7.01±0.38*	6.89±0.41	8.61±0.92	8.39±0.42	8.29±0.32
C3	7.09±0.31**	7.12±0.41*	7.08±0.63	8.89±0.67*	8.68±0.47**	8.43±0.46
C4	7.14±0.29**	7.18±0.35**	7.19±0.47**	9.27±0.88**	8.82±0.65**	8.74±0.48**
C5	7.22±0.46**	7.31±0.56**	7.27±0.57**	9.44±0.81**	9.20±0.61**	9.03±0.40**

Table 2. First brood and 21d total fecundity of *D. magna* after exposure of three commonly used pesticides. CK, C1, C2, C3, C4, C5 as in Fig.1 (*: *P<0. 05*; **: *p<0.01*)

	Number of first brood/n			21d total fecundity/n		
	Chlorpyrifos (μg/L)	Trifluralin (mg/L)	Chlorothalonil (mg/L)	Chlorpyrifos (μg/L)	Trifluralin (mg/L)	Chlorothalonil (mg/L)
CK	5.60±0.70	5.80±0.55	5.70±0.62	100.30±2.41	101.40±3.45	103.40±3.45
C1	5.40±0.70	5.40±0.33	5.70±0.83	97.40±4.62	98.60±4.42	101.30±3.57
C2	5.30±0.67	5.20±0.77*	5.30±0.57*	87.00±4.32**	92.20±6.42**	97.30±5.78**
C3	5.10±0.74	5.00±0.44**	5.00±0.16**	78.60±5.70**	84.30±4.90**	96.40±4.90**
C4	4.80±0.92*	4.80±0.62**	4.70±0.32**	68.00±6.13**	79.50±6.03**	88.40±6.46**
C5	4.20±0.79**	4.60±0.79**	4.30±0.49**	57.30±5.54**	72.40±5.38**	75.60±7.37**

Table 3. Relationship between concentration of pesticide and total fecundity, GST activity and total fecundity (n=6) (*: *P<0. 05*; **: *p<0.01*)

Types of pesticides	Regress equation (x:Concentration of pesticides; y:total fecundity)	r	Regress equation (x:GSTactivity; y:total fecundity)	r
Chlorpyrifos	$y = 95.69e^{-0.098x}$	0.968**	$y = -1.0292x^2 + 19.774x + 8.229$	0.925**
Trifluralin	$y = 97.225e^{-0.124x}$	0.944**	$y = -0.3501x^2 + 7.711x + 58.609$	0.982**
Chlorothalonil	$y = 107.17e^{-0.4x}$	0.968**	$y = .0079x^3-0.6119x^2+13.998x+10.94$	0.967**

sensitive to three commonly-used pesticides than the other reproduction indices.

- There was a significant relationship between total fecundity and pesticides-dose, GST activity after 48 h exposure and total fecundity after 21 d exposure. GST was a more sensitive biomarker than SOD to evaluate the chronic toxicity of three commonly-used pesticides in a short time.

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