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Acute Toxicity and Neurotoxicity of Chlorpyrifos in Black Tiger Shrimp, *Penaeus monodon*

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Abstract

Acute toxicity and neurotoxicity of chlorpyrifos were determined in black tiger shrimp, *P. monodon*. LC_{50} values after 24 to 96 h of exposure were between 149.55 and 59.16 nmol/L. To determine the neurotoxicity of chlorpyrifos, the inhibition of acetylcholinesterase was monitored in the gill of the shrimps exposed to lethal (0.019, 0.194, and 1.942 µmol/L) and sub-lethal (0.019, 0.194, and 1.942 nmol/L) concentrations of chlorpyrifos. In lethal dose exposure, the AChE activities observed in shrimp exposed to 0.194, and 1.942 µmol/L of chlorpyrifos were significantly lower (1.7 and 3.3 times) than that of control shrimp after 30 min of exposure ($p<0.05$). In sub-lethal exposure tests, the AChE activity of shrimp was significantly lower (1.9 times) than that of control shrimp after exposure to 1.942 nmol/L of chlorpyrifos for 72 h ($p<0.05$). The sensitive reduction of AChE activity at the sub-lethal concentration, which was 30 times lower than 96 h LC_{50} value found in this study, indicates the potential use as a biomarker of chlorpyrifos exposure.

Keywords: *Penaeus monodon*; acetylcholinesterase activity; acute toxicity test; chlorpyrifos; organophosphate insecticide

1. Introduction

Chlorpyrifos is a widely used organophosphate insecticide in agricultural and urban pest control products throughout the world. Like most organophosphate pesticides, chlorpyrifos is a neurotoxin. Animals are killed or paralyzed by exposing to chlorpyrifos due to the irreversible inhibition of acetylcholinesterase (AChE), resulting in the disruption of nervous system. Studies on the inhibition of AChE have been reported on various numbers of non-target animals exposed to chlorpyrifos (Devi *et al*., 2005; Rao *et al*., 2003; Colombo *et al*., 2005; Wheelock *et al*., 2005; Howard *et al*., 2005). The sensitivity varied depending on life stages and species (Bocquene *et al*., 1993; Payne *et al*., 1996; Stien *et al*., 1998; Kirby *et al*., 2000). Currently, the measurement of the inhibition of the AChE has become one of the biomarkers most frequently used for detecting exposure to organophosphate compounds (Sarkar *et al*., 2006).

In Thailand, hundreds of formulated compounds containing chlorpyrifos as active ingredient have been imported and registered to the office of agricultural regulation for agricultural purposes for more than 10 years (Toxic Substance Information Center, 2002). Water sampled from surface water in agricultural areas was contaminated with chlorpyrifos (Ciglasch, 2003; Ballarin, 2004). Many native crustacean species can be adversely affected by the contamination of this pesticide.

The black tiger shrimp, *Penaeus monodon*, one of the native crustaceans, has become one of the most important species in Thailand from an economical point of view. The increasing number of shrimp farms in agricultural areas where potentially damaging compounds such as chlorpyrifos are heavily used has become a great concern. Therefore, in this study, *P. monodon*, was used as test organism to determine the adverse effects of chlorpyrifos. The study includes acute toxicity tests to establish the baseline toxicity

of chlorpyrifos on the shrimp and the measurement of AChE activity in gill of the shrimp to evaluate the inhibition of AChE.

2. Materials and Methods

2.1. Chemicals

Chlorpyrifos (commercial grade 40% W/V) was purchased from local supplier. Desired stock solutions were prepared by diluting chlorpyrifos in methanol.

2.2. Quantification of chlorpyrifos

Gas chromatography (GC) analysis was performed to quantify 40% W/V chlorpyrifos stock solution and chlorpyrifos residue in experiment water. For this purpose we used an Agilent 6890 GC equipped with an AG6890 Series autosampler and split/splitless injector (Agilent Technologies, Inc. Wilmington, DE, USA). An Agilent US1653687H HP-5 fused-silica capillary column, 30 m x 0.32 mm i.d., 0.25 μm film thickness was used as analytical column. The detector Agilent micro-ECD served as detector. Calibration standards were prepared using 99.5% purity chlorpyrifos (Chem Service, Inc., USA). The dilutions, for both sample and standard, we used pesticide grade 95% n-hexane (RCI Labscan Limited, Thailand).

2.3. Animals

Juvenile *P. monodon* (18-20 g) were acclimatized to laboratory conditions and fed twice daily for 1 week prior to the experiment. Water quality parameters in the acute toxicity test tank were monitored during the experiment.

2.4. Acute toxicity test

Static acute-toxicity tests were conducted in basic accordance with procedures described by APHA, AWWA, WEF 1992. Exposures were conducted in 200-L tanks containing 100 L test solution. For the range finding test, shrimps were randomly assigned into 4 test tanks. Each tank contained 20 shrimps. Serial concentrations of chlorpyrifos, 0, 0.019, 0.194, and 1.942 μmol/L, respectively, were applied to the shrimps. The results of the range finding test were then used as criteria for the definitive test when the shrimps (20 shrimps for each concentration) were exposed to water at 5 concentrations of chlorpyrifos (0, 0.019, 0.039, 0.078, 0.155, and 0.194 μmol/L). Mortality was recorded until 96 h and the LC_{50} was estimated by the Probit method (EPA Probit Analysis Program, Version 1.5). The No-observed-effect-concentration (NOEC) was determined by Dunnett's multiple comparison procedure (USEPA, 1989) using Dunnett program (version 1.5). EC_{50} values were also determined to assess the insecticide level at which 50% inhibition of AChE activity would occur.

2.5. Acetylcholinesterase activity

The AChE activity was determined from the gill of the shrimp exposed to both lethal and sub-lethal concentrations of chlorpyrifos. For the lethal concentration test, shrimps were exposed to chlorpyrifos at the concentrations of 0, 0.002, 0.019, 0.194, and 1.942 μmol/L and the AChE activity was determined after 30 min of exposure. For the sub-lethal concentration test, shrimps were exposed to chlorpyrifos at concentrations of 0, 0.019, 0.194, and 1.942 nmol/L. Tested shrimps (*n=5*) were collected and subjected to the measurements of AChE activity at 24, 48, 72, and 96 h, respectively. AChE activity was determined according to Ellman *et al*. (1961) and Scaps *et al*. (1997) with modifications. Briefly, 10 μL of the supernatant of gill homogenate (Dissected gill was added with (1:4 W/V) ice-cold 0.01 M Tris-HCl, pH 8.0 and homogenized using glass mortar and pestle. Supernatant was separated from cell debris by centrifugation at 6000 g for 5 min, 4°C), 250 μl of 0.1 M phosphate buffer, pH 8.0, 20 μl of 0.01 M DTNB (5,5'-dithio-(2 nitrobenzoic acid)) in phosphate buffer, pH 7.0, and 20 μl of 0.075 M acetylthiocholine iodide in phosphate buffer, pH 8.0 were sequentially added. The kinetic activity of AChE was examined at 410 nm for 10 min. The amount of protein was determined according to Bradford (1976). The activity was expressed as amount of substrate (nmole) hydrolyzed per min per mg of protein. Normality and homogeneity of variances were tested using Shapiro-Wilk and Levene's test. Significant differences between groups of treatment were examined using post hoc Duncan's new multiple range test in SPSS (version 11.5). Statistically significant difference was considered at $P<0.05$.

3. Results and Discussion

3.1. Water quality

Water quality characteristics in the acute toxicity test tank were monitored (salinity: 10 ppt.; temperature: 28°C; pH: 8.02±0.22; total hardness as $CaCO_3$: 644.4±345.1 mg/L; alkalinity as $CaCO_3$: 140±12 mg/L; conductivity: 17.2±1.6 μS/cm; and dissolved oxygen: 6.8±0.4 mg/L). The result showed the qualities of the water during the experiment were still in the optimal limit of water for *P. monodon* aquaculture.

Table 1. Residue concentration of chlorpyrifos in treatment water

Initial Concentration (µmol/L)	Residue concentration (µmol/L)(%)		
	1 h	24 h	48 h
0	<LOD	<LOD	<LOD
0.019	0.006±0.002(29.64)	<LOD	<LOD
0.039	0.009±0.001(22.30)	0.0009±0.0007(2.30)	<LOD
0.078	0.022±0.011(28.55)	0.001±0.0007(1.76)	<LOD
0.155	0.035±0.005(22.70)	0.003±0.0005(1.98)	0.001±0.0003(0.97)
0.194	0.052±0.024(26.98)	0.004±0.0009(2.01)	0.002±0.0008(1.00)

Remark: Limit of detection (LOD)=0.0006 µmol/L

3.2. Quantification of chlorpyrifos

The result of GC analysis of commercial chlorpyrifos revealed that 6.81±0.09 mg/L (0.019±0.026 mmol/L) of chlorpyrifos was detected in 10 mg/L of stock commercial chlorpyrifos, indicating that commercial insecticide used in this study contained only 60% of the chlorpyrifos indicated on the product's label. Therefore, the concentration of chlorpyrifos presented in this study is used as the actual concentration detected by GC analysis.

Chlorpyrifos residues in water samples were quantified after 1, 24, and 48 h of chlorpyrifos spiked to treatment water. The results showed a rapid decrease of chlorpyrifos from the initial concentrations (Table 1).

Chlorpyrifos has relatively low hydrolysis (16–30 days) and low photolysis (11 days) half-life values (Liu *et al*., 2001). Mazanti *et al*. (2003) reported that chlorpyrifos disappeared rapidly (between 80% and 84% in the high and low insecticide treatments) within the first 10 days, while the second phase of the chlorpyrifos loss pattern was slower (18–20 days). The loss of chlorpyrifos in this study was rapid which was in agreement with those studies.

3.3. Acute toxicity test

Median lethal concentration at 24, 48, 72, and 96 h of chlorpyrifos to juvenile *P. monodon* were estimated to be 149.55, 80.46, 67.43, and 59.16 nmol/L, respectively.

The acute toxicity studies of chlorpyrifos in non-target aquatic organisms have been performed mostly on adult fish. Only a few investigations have been carried out with aquatic crustaceans and most of them were conducted on larval stages. The 96 h LC_{50} of chlorpyrifos for larvae of the mysid shrimp (*Neomysis integer*) and the grass shrimp (*Palaemonetes pugio*) were 3.71 and 4.28 nmol/L, respectively (Roast *et al*., 1999; Key and Fulton, 2006). In freshwater shrimp, *Paratya australiensis*, 96 h LC_{50} of chlorpyrifos is 1.79 nmol/L (Kumar *et al*., 2010). Thus, *P. monodon* appeared to be more tolerant to chlorpyrifos than these shrimp species at the very low levels of exposure. However, at the higher concentrations, *P. monodon* was more sensitive to the pesticide than some tested crustaceans. The 24 h LC_{50} value of chlorpyrifos for *P. monodon* (149.55 nmol/L) in our study was lower than that found in *Artemia salina* (9.09 µmol/L) (Varo *et al*., 2002) and *Litopenaeus stylirostris* larvae (6.44 µmol/L) (Reyes *et al*., 2002).

3.4. Inhibition of AChE activity

In lethal exposure tests, AChE activity from the gill of shrimp decreased in corresponce to the increased

Table 2. Inhibitory effects of chlorpyrifos on AChE (mean±S.D.) in gills of juvenile *P. monodon* at the lethal concentrations of chlorpyrifos (30 min post treatment).

Chlopyrifos concentration (µmol/L)	**AChE Activity (nmol/min/mg protein)(*N*=5)**
0	$4.33±1.51^{a}$
0.002	$3.56±0.71^{ab}$
0.019	$3.97±1.42^{ab}$
0.194	$2.46±0.82^{bc}$
1.942	$1.28±1.13^{c}$

Remark: The activity of AChE at 0 h = 3.45±0.93.

The same superscripts indicate that the AChE activity was not significantly different (*P>0.05*)

Table 3. Inhibitory effects of chlorpyrifos on AChE (mean±S.D.) in gills of juvenile *P. monodon* at the sub-lethal concentration of chlorpyrifos (24-96 h post treatment).

Chlopyrifos concentration (nmol/L)	**AChE Activity (nmol/min/mg protein)(*N=5*)**			
	24 h	**48 h**	**72 h**	**96 h**
0	6.66±1.32	3.25±0.79	4.15±1.17a	3.61±2.07
0.02	7.16±2.40	2.67±0.61	3.55±2.02ab	2.09±0.93
0.19	6.21±2.67	4.87±4.44	2.83±1.18ab	2.74±0.90
1.94	5.98±1.77	2.25±2.37	2.12±0.86b	1.94±0.21

Remark: The activity of AChE at 0 h = 6.79±3.51.
The same superscripts indicate that the AChE activity was not significantly different (*P>0.05*)

concentrations of chlorpyrifos (Table 2). This is in agreement with earlier results from various organisms, including grass shrimps, *P. pugio* (Key and Fulton, 2006) and worms, *Eisenia foetida*, (Rao *et al*., 2003). In grass shrimp larvae at 18-day-old, 50% inhibition of AChE activity was detected after 24-h exposure to chlorpyrifos at 7.70 (5.99-9.98) nmol/L. Shrimp mortality was also correlated with the inhibition of AChE activity (Key and Fulton, 2006). The inhibition of AChE activity in the lethal concentration-exposed worm, *Eisenia foetida*, increased from 62% to 91% after exposure to chlorpyrifos at 0.063 μg/cm^2 for 12 to 24 h (Rao *et al*., 2003).

In sub-lethal exposure tests, the AChE activity of shrimps was significantly lower than that of control shrimps after exposure to 1.94 nmol/L of chlorpyrifos for 72 h (*P<0.05*) (Table 3). Estimates of sub-chronic toxicity of chlorpyrifos on *P.monodon* juveniles including NOEC, PNEC, EC50 are shown in Table 4.

The AChE activity of *P. monodon* decreased by approximately 50% after exposure to chlorpyrifos at sub-lethal concentrations (1.94 nmol/L) within 72 h, when compared to control shrimp. The 72 h EC_{50} value for chlorpyrifos was estimated to be 0.14 nmol/L. This effective concentration was many times lower than the lethal levels, while most studies on other aquatic invertebrates indicated that the effect of chlorpyrifos can be observed when AChE inhibition is at near-lethal levels (Fulton and Key, 2001).

The NOEC value for mortality (387.92 nmole/L) was much higher than the contaminated levels observed in the field (between 0.57 to 4.28 nmol/L) (Nayavon, 1996; Ciglasch, 2003; Ballarin, 2004), while the NOEC for AChE inhibition (1.94 nmol/L) was considered to be in the same range of levels. From the result of the PNEC value estimated from mortality data (3.88 nmol/L), it is unlikely that chlorpyrifos at the levels present in the field could be harmful to *P.monodon*. However, the result based on AChE inhibition (0.020 nmol/L) indicates that there is potential risk for the shrimp. The sensitive reduction of AChE activity in *P. monodon* at the sub-lethal concentration (1.94 nmol/L) which was 30 times lower than 96 h LC_{50} (59.16 nmol/L) found in this study indicates the potential use as biomarker of chlorpyrifos exposure.

4. Conclusion

In current study, chlorpyrifos exhibited toxicity to *P.monodon* with a 96 h LC_{50} of 59.16 nmol/L, which is much higher than the average level of chlorpyrifos found in the surface water of Thailand (0.57 to 4.28 nmol/L). However, the result of AChE inhibition in the shrimp indicates the potential risk of chlorpyrifos at very low levels. The AChE activity of *P. monodon* appears to be significantly inhibited during sub-lethal exposure to chlorpyrifos. The estimated 72 h EC_{50} value for chlorpyrifos was 0.14 nmol/L, which was many times lower that the lethal levels, while that of other aquatic invertebrates are observed at near-lethal levels. This sensitive inhibition of AChE activity in *P. monodon* at the sub-lethal concentration indicates the potential use of AChE as biomarker of chlorpyrifos exposure. Currently, there are no water quality guidelines available in

Table 4. Estimates of sub-chronic toxicity of chlorpyrifos on *P.monodon* juveniles

Parameters	**NOEC (nmol/L)**	**LOEC (nmol/L)**	**PNEC (nmol/L)**	**EC_{50} (nmol/L)**
Mortality	387.92	776.98	3.88	54.05
AChE inhibition	1.94	2.85	0.20	0.14

Remark: NOEC, no observed effect concentration; LOEC, lowest observed effect concentration; PNEC, predicted no effect concentration; EC_{50}, median effective concentration

Thailand for pesticides such as chlorpyrifos. The results from this study provide the information on the toxicities of chlorpyrifos to *P. monodon* and will be further used for a criteria of pesticide used in aquaculture area or aquatic environment.

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