Effect of Chlorpyrifos Ethyl on Acetylcholinesterase Activity in Climbing Perch (*Anabas testudineus*, Bloch, 1972)

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Abstract The high use of pesticides in intensive rice farming in the Mekong Delta constitutes a potential hazard to the environment and to people's health. Chlorpyrifos ethyl (CPF) is a commonly used organophosphate (OP) insecticide, but information about its potential negative impacts on the aquatic environment in the Mekong Delta is scarce. Both acute and subacute toxicity tests were performed in a static nonrenewable system to investigate the effects of CPF on brain acetylcholinesterase (AChE) activity in native climbing perch fingerlings (*Anabas testudineus*, Bloch, 1972). Environmental parameters, such as dissolved oxygen, water temperature, and pH, were similar to field conditions in the Mekong Delta. In a 96-h lethal concentration (LC₅₀) test, fingerlings of climbing perch were randomly exposed to five levels of CPF ranging from 0.8 to 4.5 ppm. Five sublethal levels of CPF (1, 5, 10, 15, and 20 % of the 96-h LC₅₀ value) were tested to assess the sensitivity and recovery of the brain AChE activity in climbing perch fingerlings exposed to CPF. The results showed that CPF were moderately toxic to climbing perch with a 96-h median LC₅₀ of 1.73 ppm. CPF also caused long-term AChE inhibition with 70 % inhibition remaining after 96 h for the four highest test concentrations. The recovery of brain AChE activity in fish placed in CPF-free water was very slow, and after 7 days the brain AChE

activity was still significant lower in fish from the four highest concentrations compared with the control. The results from this study indicate that OP insecticides, such as CPF, can have long-lasting sublethal effects on aquatic species in the Mekong Delta.

The Mekong Delta occupies approximately 12 % of Vietnam's total area, but it contributes >50 % of the total national rice production (Renaud and Kuenzer 2012). Since the 1980s, there has been a steady increase in the production of rice, through increased areas of rice farming and application of more intensive farming methods, supported by a high use of agrochemicals (Renaud and Kuenzer 2012). Many farmers currently apply three crops per year, with a high use of both fertilisers and pesticides, to meet the expected increase in future rice yields (Cau 2011; Guong and Hoa 2012). Pesticide imports to Vietnam increased from 20,000 to 72,560 tons during the period 1991–2010 with a corresponding increase in number of active ingredients (a.i.) applied from 77 to 300 during the same period of time (Toan et al. 2013). The increased use of pesticides has been followed by an increased risk for negative effects on the environment and on people's health (Berg et al. 2012; Dasgupta et al. 2007; Sebesvari et al. 2012).

Chlorpyrifos ethyl (CPF) is a commonly used organophosphate (OP) insecticide in the Mekong Delta (Heong et al. 1998; Huan et al. 1999; Phong et al. 2010). Although OPs have a lower tendency to bioaccumulate in aquatic organisms compared with many organochlorines, they are often more toxic to aquatic organisms (Tilak et al. 2001). CPF is known to be highly toxic to freshwater fish and aquatic invertebrates because of its ability to inhibit the acetylcholinesterase (AChE) enzyme and cause

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malformation of the nervous system (O'Brien 1976; Peakall 1992). According to Peakall (1992), the inhibition of AChE results in abnormal respiration, swimming, feeding, and social interactions in aquatic organisms due to loss of coordination, tremors, muscle spasms, convulsions, and even death.

As shown by several studies, the sensitivity of brain AChE activity to different inhibitors can be used as a biomarker to indicate the degree of sublethal effects from OP and carbamate insecticides on aquatic organisms (Coppage et al. 1975; Sancho et al. 2000). However, as pointed out by Chuiko et al. (2003), the AChE response to different inhibitors varies substantially between different fish species. Considering the high use of both OPs and carbamates in the Mekong Delta, there is a need to provide more information on the response of the AChE activity in local fish species exposed to these pesticides in rice fields in the Mekong Delta. This is an important step to develop locally relevant biomarkers that can be used to identify long-lasting sublethal effects from pesticides on aquatic organisms under tropical conditions.

In this study, climbing perch (*Anabas testudineus*) was selected as a model organism because it is a native fish commonly found in rice fields, where it lives and reproduces, and thus is exposed to agrochemicals during all of its life stages (Rainboth 1996). In addition, it is an important food fish that is considered to be of high quality, thus contributing to local people's livelihoods and well-being in the Mekong Delta (Edwards et al. 1997; Herre 1952; Klemick and Lichtenberg 2008). Climbing perch has also been shown to be an important predator of rice pests, such as brown plant hoppers [*Nilaparvata lugens* (Stål)], and could potentially help to reduce the use of insecticides to control these pests (Nam et al. 2012).

Although there are no official reports about the status of climbing perch populations in the Mekong Delta, local farmers indicate that the amount of wild fish in rice fields has decreased during the last decades (Edwards et al. 1997). Acute and chronic toxicities of several pesticides have been investigated in climbing perch (Binoy et al. 2004; Choudhury et al. 1993; Cong and Linh 2010; Jilna and John 2011), but information regarding the effects of CPF is scarce. No information is available on the inhibition of AChE by CPF in climbing perch.

The aim of the present study was to investigate the effects on brain AChE activity in climbing perch fingerlings exposed to sublethal concentrations of CPF that were similar to the concentrations found in rice fields in the Mekong Delta. The study thus provides a basis for evaluating sublethal effects on fish by OP insecticides used in rice farming and for developing locally relevant and feasible biomarkers for the monitoring of sublethal effects by pesticides on fish from the Mekong Delta.

Materials and Methods

Fish

Climbing perch fingerlings (3–4 g) were bought from a hatchery in Co Do district, Can Tho city, Vietnam. The fingerlings were produced by a brood-stock caught from an area with no use of pesticides in the Mekong Delta to ensure that the fingerlings were not affected by any previous exposure of pesticides to the brood-stock. The fingerlings were acclimatized in a 600-L fiberglass tank for approximately 3 weeks. The fingerlings were fed red worms (*Tubifex* sp.) daily at approximately 5 % of the total fish fresh weight. The fiberglass tank was cleaned daily before feeding and again 4 h after feeding. To remove uneaten food and faeces, approximately 50 % of the water was changed on a daily basis. Feeding was stopped 1 day before the experiment was initiated.

Insecticides

Vitashield 40EC, an OP insecticide (containing 40 % CPF [*O,O*-diethyl *O*-(3, 5,6-trichloro-2-pyridinyl)-phosphorothioate; common name “chlorpyrifos”; inert ingredients including emulsifiers and solvents ≤100 %) was purchased from Thanh Son Hoa Nong Company (Binh Chanh district, Ho Chi Minh city, Vietnam).

Experimental Design

Ninety-Six-Hour LC₅₀ Toxicity Test

A toxicity range-finding experiment was performed to determine a CPF concentration that would kill 10–90 % of the climbing perch fingerlings in 96 h. The experiment was performed in 50-L fiberglass tanks containing 20 L of tap water with three simultaneous replicates of nine treatments containing 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, and 4.5 ppm of CPF. The commercial product (Vitashield 40EC, Thanh Son Hoa Nong Company, Vietnam) was diluted 500 times with distilled water to achieve the stock solution with the nominal concentration of 800 ppm of CPF, which is equivalent to 2000 ppm of Vitashield 40EC (Thanh Son Hoa Nong Company, Vietnam).

The accuracy of the nominal concentration was checked with gas chromatography, and the final CPF concentration of the stock solution was determined to 760 ppm. The stock solution was then added to the nine treatments to obtain the desired concentrations.

Within 5 min after preparing the different concentrations, 10 fish were randomly removed from the fiberglass stocking tank and added to each experimental tank. There were no feeding and water exchange during the experiment. Dead fish were removed from the tanks to minimize

any interference with the live fish. Mortality was recorded at 1, 3, 6, 9, 12, 24, 36, 48, 60, 72, 84, and 96 h. Dissolved oxygen (DO), pH, and temperature were measured daily in the morning (6:00–7:00 am) and in the afternoon (14:00–15:00 pm) using a pH meter (HANNA HI8314, Hanna Instruments, USA) and a DO meter (HANNA HI9146, Hanna Instruments, USA) throughout the experiment.

Based on the toxicity range-finding experiment, a 96-h LC₅₀ acute toxicity test was performed. The test was performed in 50-L fiberglass tanks containing 20 L of tap water (static nonrenewable) that had been aerated overnight. The experiment was performed with three simultaneous replicates of five treatments containing 0.8, 1.2, 1.9, 2.9, and 4.5 ppm of CPF and a control. The treatment concentrations were prepared as mentioned previously. Each replicate contained 10 fish that had been randomly taken from the fiberglass stocking tank and added to the different concentrations. The experiment tanks were regularly monitored during the whole experiment (96 h). Dead fish were removed from the tanks. Mortality was recorded at 1, 3, 6, 9, 12, 24, 36, 48, 60, 72, 84, and 96 h. DO, pH, and temperature were measured daily in the morning (6:00–7:00 am) and in the afternoon (14:00–15:00 pm) using a pH meter (HANNA HI8314, Hanna Instruments, USA) and a DO meter (HANNA HI9146, Hanna Instruments, USA) throughout the experiment.

AChE Sensitivity and Recovery Tests

The AChE sensitivity test was performed using 50-L fiberglass tanks containing 40 L of tap water that had been aerated overnight to eliminate residues of chlorine in the water. The experiment was performed with three simultaneous replicates including a control and five concentrations of CPF corresponding to 1, 5, 10, 15 and 20 % of the identified 96-h LC₅₀ concentration, which is equivalent to 17.3, 86.5, 173, 259.5, and 346 ppb of CPF. The treatment concentrations were prepared as mentioned previously. Each replicate contained 30 fish, which were randomly removed from the stocking tank. During the experiment (96 h), there was no feeding, aeration or water exchange. DO, pH, and temperature were measured daily at 6:00–7:00 am and at 14:00–15:00 pm. Two fish were removed after 1, 6, 12, 24, 48, 72, and 96 h from each experimental tank, killed on ice, and processed for AChE analysis within 12 h.

The recovery test was initiated after 96 h by replacing all of the water in the experimental tanks with CPF-free water. Afterward, 50 % of the water was replaced on a daily basis. DO, pH, and temperature were measured daily at 6:00–7:00 am and at 14:00–15:00 pm. To avoid any harmful build up of uneaten food during the experiment, the fish were fed *Tubifex* worms two times

per day at approximately 5 % of the fish fresh weight. After 3 h, the remaining food in each tank was siphoned out into glass containers using a separate system for each treatment concentration. The siphoned water was allowed to settle for 30 min before the supernatant was returned to the same fiberglass tank. At days 1, 3, 5 and 7, two fish were randomly caught from each tank, immediately killed on ice, and processed for AChE analysis.

AChE Assay

The brain was dissected out on ice and placed in a pre-weighed Eppendorf tube on ice before measurement of the brain weight. Each brain was then homogenized on ice in 6 mL of 0.1 M phosphate buffer (pH 7.4, prepared by mixing mono and dibasic sodium hydrogen phosphate) using a glass homogenizer. The homogenates were transferred into 10-mL glass tubes and centrifuged at 2000×g at 4 °C for 20 min (Centrifuge 4k15; Sigma, Osterode am Harz, Germany). Supernatant, 1.5 mL, was removed to an Eppendorf tube and kept on ice for AChE analysis within 12 h. The AChE activity was determined according to the method described by Ellman et al. (1961). All measurements were performed in an air-conditioned room at 25 °C. For each measurement, a cuvette was prepared containing 2.65 mL of 0.1 M phosphate buffer (pH 7.4) and 100 µL of 3 mM 5,5 dithio-bis (2-nitrobenzoic acid) (Sigma Aldrich Chemie, Steinheim, Germany). Immediately before measurements, 50 µL of 10 mM acetylthiocholine iodide (Sigma Aldrich Chemie, Steinheim, Germany) and 200 µL of supernatant were added, and the solution was mixed well. Blanks were prepared with 200 µL of buffer instead of the supernatant. Two blanks were used for each sample measurement. AChE enzyme activity was measured using an ultraviolet/visible spectrophotometer (model UV2 2000E; ATI Unicam, Cambridge, UK) for 10 cycles (3 min and 18 s) with an auto interval (22 s) at a wavelength of 412 nm when the increase in absorbance with time was linear. The results of these measurements were expressed as a rate (absorbance per minute), from which the AChE activity was calculated.

Statistical Analysis

The 96-h LC₅₀ value for CPF was calculated by probit analysis (Finney 1971) using the probit function in SPSS for Windows (SPSS Inc, Chicago, IL, USA). Differences in AChE activity between treatments were analyzed using one-way analysis of variance with Dunnett's test for post hoc comparisons after checking for normality and homogeneity of variance. SPSS for windows (version 17.0, SPSS Inc, Chicago, IL, USA) was used to analyse the data.

Results

Ninety-Six-Hour LC₅₀ Toxicity Test

DO, pH, and temperature during the acute toxicity test were similar to the field conditions in rice fields in the Mekong Delta. The temperature varied between 24.2 and 24.4 °C. The pH was stable throughout the experiment ranging from 6.7 to 6.9 (Table 1). There were no significant differences in pH and temperature between the different treatments at any sampling time. However, the DO level in the control was 3.5 mg/L, which was significant higher than the DO levels in the different treatments ($P < 0.05$). The DO level in the treatment containing 0.8 mg CPF/L was significant lower compared with the control, but it was significant higher compared with the other treatments ($P < 0.05$) (Table 1).

Nine hours after the experiment was initiated, fish mortalities were recorded in the two highest concentrations (4.5 and 2.9 ppm) (Table 2). In the following two lower concentrations (1.9 and 1.2 ppm), the first fish died after 12 h. In the lowest concentration (0.8 ppm), the first fish died after 60 h. By this time, the majority of the fish in the two highest concentrations had died. At the end of the experiment, only one fish had died in the control, whereas <50 % of the fish in the lower concentrations and approximately 90 % of the fish in the highest concentrations had died. The 24-, 36-, 48-, 60-, 72-, 84-, and 96-h LC₅₀ values of CPF for climbing perch fingerlings were determined to 2.5, 2.2, 2.0, 1.91, 1.82, 1.78, and 1.73 ppm, respectively (Table 2).

AChE Sensitivity Test

The temperatures varied between 26.7 and 26.8 °C, and the pH ranged between 6.9 and 7.0 during the experiment (Table 3). There were no significant differences in pH and temperature between the different treatments at any sampling time. However, with increasing CPF concentrations, the DO levels were significantly reduced ($P < 0.05$). The highest DO level was found in the control, and the lowest DO level was found in the

treatment containing the highest concentration of CPF (346 µg CPF/L) (Table 3).

There was a clear correlation between the CPF dose and inhibition of brain AChE activity. After 1 h, no signs of inhibition were observed in fish exposed to 1 and 5 % of the 96-h LC₅₀ concentration (Fig. 1). The brain AChE activities in the fish from these treatments were even higher than those in the fish from the control treatment ($P < 0.05$). However, the fish exposed to 10, 15, and 20 % of the 96-h LC₅₀ concentration showed brain AChE inhibition of 15.6, 8.2, and 17.7 %, respectively (Fig. 1). After 6 h, brain AChE activities had decreased in all treatments, and the inhibition levels ranged between 20 and 50 %, with the highest reduction in fish exposed the highest concentration. After 48 h, the inhibition peaked, with inhibition levels >50 % for all treatments and >75 % for the three highest concentrations. At the end of experiment (96 h), the inhibition levels of the fish exposed to 1, 5, 10, 15, and 20 % of the 96-h LC₅₀ concentration were 34.6, 43.5, 67, 64.1, and 69.8 %, respectively (Fig. 1). There were no clear signs of recovery among the exposed fish.

AChE Recovery Test

Temperature, DO, and pH were relatively constant during the experiment. The temperature varied between 27.4 and 27.6 °C, and DO levels varied between 2.5 and 2.7 mg/L. pH varied between 7.1 and 7.2 (Table 4). No significant differences were found between treatments at any time ($P > 0.05$).

One day after the removal of the water containing CPF, brain AChE levels had decreased to 23, 43, 52, 54, and 57 % for fish exposed to 1, 5, 10, 15, and 20 % of the 96-h LC₅₀ concentration, respectively (Fig. 2). At day 3, inhibition levels had decreased to 19, 34, 44, 50, and 53 %, but they were still significant higher than that of the control ($P < 0.05$). The fish exposed to the lowest concentration recovered fully after 5 days, whereas the inhibition levels in fish exposed to the higher concentrations remained significantly higher than the control after 7 days ($P < 0.05$) (Fig. 2).

Table 1 Water parameters in static 96-h LC₅₀ test on climbing perch fingerlings exposed to CPF

CPF (ppm)	0	0.8	1.2	1.9	2.9	4.5
T (°C)	24.4 ± 0.05	24.3 ± 0.04	24.3 ± 0.04	24.3 ± 0.02	24.2 ± 0.04	24.2 ± 0.05
pH	6.9 ± 0.07	6.8 ± 0.05	6.7 ± 0.05	6.7 ± 0.04	6.7 ± 0.04	6.8 ± 0.04
DO (mg/L)	3.5 ± 0.10 ^a	2.4 ± 0.20 ^b	2.2 ± 0.17 ^{bc}	1.7 ± 0.20 ^c	1.7 ± 0.20 ^c	1.8 ± 0.18 ^c

Data are expressed as mean ± SE of triplicate measurements. Values having different superscripts within the same row are significantly different ($P < 0.05$)

Table 2 Cumulative mortality (% of dead fish) of climbing perch fingerling exposed to CPF

Time (h)	CPF (ppm)						LC ₅₀ (ppm)
	Control	0.8	1.2	1.9	2.9	4.5	
0	0	0	0	0	0	0	
6	0	0	0	0	0	0	
9	0	0	0	0	6.67	16.67	
12	0	0	6.67	10.00	36.67	40.00	
24	0	0	13.33	20.00	63.33	70.00	2.50
36	0	0	16.67	30.00	63.33	80.00	2.20
48	0	0	26.67	33.33	70.00	80.00	2.00
60	0	10.00	26.67	36.67	73.33	83.33	1.91
72	0	10.00	26.67	46.67	76.67	86.67	1.82
84	0	13.33	33.33	50.00	76.67	86.67	1.78
96	3.33	13.33	33.33	50.00	86.67	90.00	1.73

Table 3 Water parameters in AChE sensitivity test on climbing perch fingerlings exposed to CPF

CPF (ppb)	0	17.3	86.5	173	259.5	346
T (°C)	26.8 ± 0.32	26.7 ± 0.29	26.7 ± 0.30	26.7 ± 0.30	26.7 ± 0.29	26.7 ± 0.31
pH	6.9 ± 0.05	6.9 ± 0.05	6.9 ± 0.03	7.0 ± 0.01	7.0 ± 0.01	7.0 ± 0.01
DO (mg/L)	2.7 ± 0.05 ^a	2.4 ± 0.06 ^b	2.4 ± 0.06 ^b	2.24 ± 0.06 ^b	1.77 ± 0.10 ^c	1.44 ± 0.15 ^d

Data are expressed as mean ± SE of triplicate measurements. Values having different superscripts within the same row are significantly different ($P < 0.05$)

Fig. 1 Brain acetylcholinesterase (AChE) inhibition in climbing perch (*Anabas testudineus*) fingerlings exposed to chlorpyrifos ethyl (Vitasield 40EC, Thanh Son Hoa Nong Company, Vietnam) during 96 h at concentrations of 1, 5, 10, 15 and 20 % of the 96 h LC₅₀ value for chlorpyrifos ethyl. The vertical bars show standard error. Points with different letters are significantly different ($P < 0.05$) from each other. Each data point corresponds to the mean of six samples

Table 4 Water parameters in AChE recovery test on climbing perch fingerlings exposed to CPF

CPF (ppb)	0	17.3	86.5	173	259.5	346
T (°C)	27.6 ± 0.22	27.5 ± 0.22	27.4 ± 0.22	27.4 ± 0.22	27.4 ± 0.22	27.4 ± 0.22
pH	7.2 ± 0.1	7.1 ± 0.01	7.2 ± 0.01	7.1 ± 0.02	7.1 ± 0.01	7.2 ± 0.01
DO (mg/L)	2.7 ± 0.07	2.6 ± 0.06	2.7 ± 0.06	2.5 ± 0.06	2.5 ± 0.06	2.5 ± 0.06

Data are expressed as mean ± SE of triplicate measurements. Values having different superscripts within the same row are significantly different ($P < 0.05$)

Fig. 2 Recovery from brain acetylcholinesterase (AChE) inhibition in climbing perch (*Anabas testudineus*) fingerlings exposed to different concentrations of chlorpyrifos ethyl for 96 h followed by 7 days recovery in chlorpyrifos ethyl free-water. The vertical bars show standard error. Points with different letters are significantly different ($P < 0.05$) from each other. Each data point corresponds to the mean of six samples

Discussion

In the Mekong Delta, rice fields are natural and important habitats for climbing perch, and this fish species is directly exposed to pesticide levels that can cause sublethal effects during all of its life stages (Stadlinger et al. 2015; Tam et al. 2015). Although no population survey on climbing perch has been published from the Mekong Delta, the perception among local people is that climbing perch population have undergone a decline during the last 25 years (Edwards et al. 1997; Klemick and Lichtenberg 2008), which partly could be due to the increased use of pesticides in the Delta. Climbing perch is a supplementary source of food and income for many households in the Mekong Delta, and declining yields of this species affect local people's livelihoods and well-being.

In this study, we found that CPF is moderately toxic to climbing perch ($1 < \text{LC}_{50} < 10$ mg/L) with a 96-h LC_{50} value of 1.7 mg/L. This indicates that CPF is more acute toxic to climbing perch than diazinon and fenobucarb, two other commonly used insecticides in the Mekong Delta, where the 96-h LC_{50} has been reported to be 6.55 mg/L (Cong et al. 2008b) and 11.37 mg/L (Lan 2004), respectively.

Climbing perch seems to be more tolerant to CPF than many other freshwater fish. The 96-h LC_{50} values of CPF for juvenile and adult Nile tilapia (*Tilapia niloticus*), common carp (*Cyprinus carpio*), mosquito fish (*Gambusia affinis*), and guppy (*Poecilia reticulata*) were 98.7 and 154.0 (Oruç 2010), 580 (Xing et al. 2012), 297 (Kavitha and Rao 2008), and 175.8 $\mu\text{g/L}$ (Sharbidre et al. 2011), respectively.

The comparatively high 96-h LC_{50} values of CPF found in this study could be due to some losses of CPF in the water through dissipation to the air or hydrolysis. The fairly high temperature (24 °C) probably had a positive influence on both the evaporation and hydrolysis processes, whereas the slightly acid water (pH 6.8) may have had a negative influence on the hydrolysis of CPF (Christensen et al. 2009). CPF's $\log K_{ow}$ (4.7) (Smith 1981) and K_{oc} values (7000–25,000 mL/g) (Racke 1992) also indicate that some CPF may have been adsorbed to the experimental tanks.

Climbing perch has a good ability to adapt to harsh environmental conditions, and the temperature, pH and DO levels recorded during the experiments were well within the range of field conditions reported for climbing perch in the delta (Albert 1914; Vromant et al. 2001a, b). Climbing perch is an obligate air-breathing species. Obligate air-breathing fish must breathe air periodically even if the DO levels in water are high; otherwise, they will suffocate. The ability to use oxygen directly from the atmosphere makes climbing perch less sensitive to low oxygen levels in water, and the low levels of DO during the experiments were not seen to affect the survival of the fish (Armbruster 1998). Many studies have shown that climbing perch can tolerate and adapt to extremely harsh environments such as low pH values, high temperatures, and especially low DO concentrations (Davenport and Abdulmatin 1990; Khoa and Huong 1993; Klepper et al. 1992).

The exposure to CPF resulted in significant inhibition of brain AChE activity in climbing perch for all tested concentrations, indicating that CPF causes significant sublethal effects in climbing perch at doses well below the 96-h LC_{50} value. The lower oxygen levels in treatments with high

CPF concentrations also indicated that the fish were stressed by the exposure to CPF and used an increased amount of energy (increased respiration) to counteract the negative effects from CPF. This results in less energy for growth, and reduced growth has been proposed to be a sensitive measure of chronic toxicity to CPF (Arunachalam et al. 1980; Arunachalam and Palanichamy 1982). Reduced growth was also found in a recent study on climbing perch exposed to CPF in rice fields in the Mekong Delta (Tam et al. 2015). Hegazi et al. (1898) found that sublethal concentrations of CPF not only inhibited brain AChE activity in *Clarias lazera* but also reduced muscle and liver glycogen and blood glucose levels.

Brain AChE activity was clearly influenced by the concentration and duration of the exposure to CPF. Levels of brain AChE inhibition increased rapidly during the first 48 h of exposure. Then the inhibition levels were slightly reduced, but they were still significant higher than those of the control. The reduced inhibition indicates an increase in protein synthesis by the fish to compensate for the blocked AChE enzymes by CPF (Gill et al. 1990; Ram et al. 2003).

The inhibition levels of all concentrations, except 1 % of the 96-h LC₅₀ value, remained >30 % for most of the experiment period. Aprea et al. (2002) noted that inhibition levels >30 % of brain AChE activity affected the normal behaviour of the fish and that death occurred at inhibition levels >70 % (Aprea et al. 2002; Fulton and Key 2001). Fish exposed to the three highest concentrations (10–20 % of the 96-h LC₅₀ value) reached >70 % AChE inhibition after 48 h, but no dead fish was recorded for these treatments, indicating a high tolerance by climbing perch. Several studies indicates that air-breathing fish increased their oxygen uptake from the atmosphere and reduced the uptake of DO from the water when exposed to pesticides (Cong et al. 2011; Natarajan 1981; Saligo and Trijasse 1998; Toan 2009). This could be a strategy for air-breathing fish, such as climbing perch, to reduce the uptake and toxic effects from pesticides in the water (Affonso and Rantin 2005; Armbruster 1998; Cong et al. 2011).

According to the pesticide label of the manufacturer, the highest recommended dose of Vitashield 40EC is 0.8 L/ha. With a water level in the rice field of 20 cm and assuming that 50 % (Cong et al. 2006) of the sprayed insecticide reaches the water, the concentration of CPF in water would be approximately 80 µg/L. This concentration is 4.6 % of the 96-h LC₅₀ value. Although this is only a rough estimate of the concentration in water, it still indicates that the recommended dose of Vitashield 40EC (Thanh Son Hoa Nong Company, Vietnam) may not directly kill climbing perch but may cause long-term sublethal effects on the health and normal development of the fish. A recent field study showed that the concentrations of CPF in rice field water, sprayed with 0.8 L Vitashield 40EC/ha, decreased

quickly during the first day. The concentrations were well below 1 % of the 96-h LC₅₀ values of CPF for climbing perch fingerlings, but it still resulted in reduced brain AChE activity, growth, and survival in climbing perch (Tam et al. 2015). Increased mortality was only seen several days (days 5–12) after the spraying, indicating that the acute 96-h LC₅₀ test may not fully capture the delayed effect from CPF. Thus, even if 80 µg/L is an overestimate of the expected concentration in water, much lower concentrations of CPF in water are likely to cause negative effects on fish in the rice fields, especially because many of these species are likely to be less tolerant than climbing perch. No dead fish was recorded in the AChE sensitivity experiment, although the concentration ranged between 17 and 346 µg/L, which could be due to a more controlled and less stressful environment in the laboratory compared with the rice fields but also because the test period was not long enough to capture the delayed effect caused by CPF.

In addition to the lowered brain AChE activity, increased oxygen consumption, and changed behaviour of the tested fish, such as lowered activity, erratic swimming, and rapid gill movements, also occurred indicating sublethal effects on the fish nervous system. These findings are consistent with those of several other researchers (Bálint et al. 1995; Hai et al. 1997; Levin et al. 2004; Pan and Dutta 1998; Richendrfer et al. 2012; Sancho et al. 2000) who observed changes in feeding capability, swimming activity, avoidance of predators, and spatial orientation due to brain AChE inhibition caused by OP insecticides.

The results from this study also show that the effects from CPF on climbing perch remain many days after the spraying of the pesticide has stopped. This is of a special concern in the Mekong Delta because many farmers in the Delta spray their fields several times per crop and often grow three crops per year (Berg 2001; Berg and Tam 2012). This means that aquatic organisms in the rice fields may continuously be exposed to, and stressed by, elevated levels of pesticides, which are often combined in different mixtures with potential synergistic effects.

Except for the two lowest concentrations (0.0173 and 0.0865 ppm), the inhibition of brain AChE activity in climbing perch remained >30 % after 7 days in CPF-free water. In a previous field study, inhibition of AChE activity remained at 10–20 % in climbing perch fingerlings 12 days after spraying rice fields with 0.8 L Vitashield 40EC/ha (Tam et al. 2015). In a study on snake-head fish (*Channa striata*) exposed to diazinon sprayed on rice fields in the Mekong Delta, the brain AChE activity dropped to a minimum value 3 days after spraying and only gradually recovered to 74 % of prespraying activity after 21 days (Cong et al. 2008a).

Rao (2004) found that the activity of brain AChE in *Oreochromis mossambicus* was fully recovered 22 days after exposure to monocrotophos (containing oxon group,

P=O), whereas it took 36 days to recover from the exposure to OPs containing a thiol group. Although the detoxifying ability depends on the physiochemical properties of the pesticide (De Bruijn et al. 1993) and also differs between fish species (Keizer et al. 1991), it is clear that OPs, like CPF, generate long-term sublethal effects on aquatic organisms. This could have implications for the overall production of fish and other aquatic organisms in rice fields because the use of pesticides may affect the both growth and survival rates of these organisms (Berg et al. 2012; Tam et al. 2015).

Conclusion

In conclusion, CPF was found to be moderately toxic to climbing perch fingerlings with a 96-h LC₅₀ value of 1.73 ppm. The brain AChE activity of climbing perch fingerlings was very sensitive to CPF. At the lowest concentration (1 of the % 96-h LC₅₀ value or 0.0173 ppm CPF), brain AChE activity was inhibited by 54.6 % after 48 h of exposure. The recovery of brain AChE activity was very slow, and at the termination of the experiment after 7 days, only the fish exposed to the lowest dose (1 % of the 96-h LC₅₀ value) was fully recovered. The results indicate that CPF concentrations used by rice farmers in the Mekong Delta may cause sublethal effects on the fish nervous system, which in turn could affect fish growth and yield. This should be further investigated through field studies on the effect of rice farmers' application of CPF in combination with other insecticides on climbing perch as well as other organisms in rice fields.

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