Adverse effects of chlorpyrifos and cypermethrin mixture on physiological alterations and cholinesterase expression on Nile tilapia (*Oreochromis niloticus)*

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ABSTRACT

The purpose of this study was to study the effects of chlorpyrifos mixed with cypermethrin insecticides on physiological changes and the application of cholinesterase (ChE) expression as a biomarker of exposure in Nile tilapia. Based on the results, it was found that the substances affected the physiology and behavior of exposed fish. Mortality rates increased with an increasing in concentration and exposure time. Enzyme activity in the fish brain decreased with an increasing in exposure time, which was significantly different from other organs ($P < 0.05$). From Western blot analysis, we found acetylcholinesterase (AChE), having a molecular weight of 71 kDa, which could be detected in the brain, muscle and gill. In contrast, butyrylcholinesterase (BuChE) could be detected in plasma at 85 kDa. The appropriate concentrations of ChE, causing cross–reactivity, were 12, 10 and 10 µg/µL in the brain, muscle and gill, respectively. In plasma, the appropriate concentration of protein applied was 3 µg/µL. The detection limits for ChE detection of dot blot technique were consecutively at 5, 2.5, 0.31 and 1.25 µg/µL in brain, muscle, plasma and gill. Based on this study, ChE could be used as a biomarker of exposure for chlorpyrifos mixed with cypermethrin insecticides by using antibody techniques, i.e., Western blot and dot blot techniques for detection.

Keywords: Acetylcholinesterase, cholinesterase, chlorpyrifos, cypermethrin, biomarker

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INTRODUCTION

In Thailand, chlorpyrifos (O, O–diethyl–O–3, 5, 6–trichlor–2–pyridyl phosphorothioate) is applied to varieties of pest insects because of its low price, availability and rapid degradation compared to other pesticides. It has been used worldwide for controlling pests in both agricultural and urban areas. It is not only the second largest selling organophosphate but also more toxic to fish than organochlorine compounds. The insecticides in this group affect the nervous system by inhibiting functions of acetylcholinesterase (AChE) enzyme, making acetylcholine (ACh) accumulation in the synapse. The fish exposed to pesticides exhibited quick body movement, restlessness, convulsions, excess mucous secretion, respiratory problems, loss of balance and change in color when exposed to different pesticides (Tawatsin *et al*., 2015).

Cholinesterases (ChEs) are a family of enzymes, used to catalyze the hydrolysis individually into choline and acetic acid, which is an essential process for restoration of the cholinergic neuron (Pohanka, 2011). ChEs are classified into two types which are acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BuChE; EC 3.1.1.8). It plays an important role in cholinergic neurotransmission by hydrolyzing ACh, which is expressed in nerve and blood cells. BuChE is not well understood for its importance. However, BuChE is known as plasmatic cholinesterase (ChE) or pseudocholinesterase. In the same way, AChE is called blood or erythrocyte cholinesterase, as its activity functions in the cell mass after blood centrifugation. The main biological function of AChE and BuChE is to regulate the transmission of nerve impulses through the neurotransmitter of ACh. The protein contents have been shown to decrease in the brain, gills, muscle, kidney and liver functions after pesticide stress (Ghazala *et al*., 2016).

The studies of ChE as a biomarker of pesticide exposure were proved in both laboratory and field study. For example, Mena *et al*. (2014) found ChE activity in brain and muscle of fish (*Astyanax aeneus*). Günther (1860) found that *Astyanax aeneus* exposed to ethoprophos in the laboratory expressed ChE activity even at the lowest concentration of tested ethoprophos. Besides, Chandrasekara and Pathiratne (2007) reported that chlorpyrifos and carbosulfan inhibited AChE activity in the brain of juvenile Nile tilapia (*Oreochromis niloticus*), which could be used as the biomonitoring of anticholinesterase pesticides.

In the case of long-term application of insecticides, especially organophosphate group such as chlorpyrifos, it can cause high adverse effects on the environment and exposed organisms such as insecticide resistance, new insect outbreaks, contamination in both vegetable products and aquatic environments and organisms (shrimp, shellfish and freshwater fish). Insecticides being accumulated in edible aquatic organisms are finally consumed by human being and then cause his health risk (Petchoy and Pung, 2017). Insecticides being accumulated in water sources by surface runoff result in water quality degradation and pose risk to the aquatic organism. In some cases, the contamination occurred in edible aquatic organisms, especially in the exported aquatic animals, which can cause the problem in international trading. For example, chlorpyrifos, which is in organophosphate group causing effects by both contact and ingestion, can result in adverse effects to hybrid catfish after being assimilated such as behavior and physiological changes. Moreover, it can cause mortality in high level (Mararam *et al*., 2016).

Most pesticides are presently improved their applications by mixing to be a new formula such as chlorpyrifos and cypermethrin (O, O–diethyl–O–3, 5, 6–trichlor–2–pyridyl phosphorothioate + (RS)–A–cyano–3–phenoxybenzyl (RS, 3RS, 1RS, 3RS)–3–(2, 2 dichlorovinyl)–2, 2 dimethylcyclopropanecarboxylate 50% + 5% w/v). This mixture has a better result in controlling pest insects. However, it causes negative effects after reaching aquatic environments. To our knowledge, there has been no study about the mixture of chlorpyrifos and cypermethrin in commercial forms.

In Thailand, Nile tilapia is an important economic fish, that can be found in both nature and aquaculture farm. In the case of fish being caught nearby the agricultural area, it might be

contaminated with that pesticide. Thus, this study aimed to investigate the effects of pesticide in the mixture of chlorpyrifos and cypermethrin (50%: 5% w/v) on Nile tilapia in different concentrations and exposure times. At first, the toxicity of insecticide solution of chlorpyrifos–cypermethrin mixture was evaluated by mortality rate at 10, 50 and 90% ($LC_{10,}$ LC_{50} and LC_{90}). Then, the physiological appearance was studied for setting useful guides of consumers to reduce risk in an exposure. Next, the antibody technique was developed to monitor alteration of ChE enzyme for using as bio–indicator of exposure. It can be used as preliminary information to further establish management and mitigation plans for insecticide contamination in aquatic organisms and environments.

MATERIALS AND METHODS

Chemicals

An insecticide solution of chlorpyrifos and cypermethrin (O, O–diethyl–O–3, 5, 6–trichlor–2–pyridyl phosphorothioate + (RS)–A–cyano–3–phenoxybenzyl (RS, 3RS, 1RS, 3RS)–3–(2, 2 dichlorovinyl)–2, 2 dimethylcyclopropanecarboxylate 50% + 5% w/v) was purchased from local market in Surin province. Acetylthiocholine iodide (ATCh) and 5, 5'–dithiobis (2–nitrobenzoic acid) (DTNB) were purchased from Sigma–Aldrich. Reagents for protein determination and immunoassay technique comprising Coomassie Brilliant Blue G–250 and Bovine Serum Albumin (BSA), nitrocellulose membrane, Bradford dye reagent and molecular weight protein were purchased from Bio–Rad. All compounds were analytical grade from Bang–Trading Co., Ltd., Thailand. The polyclonal antibody specific to AChE (catalog number 0200–0042, molecular weight 71 kDa) and BuChE (UniProt number p06278, molecular weight 85 kDa) were obtained from Bio–Rad and Sigma–Aldrich.

Animal Husbandry

In this study, the adult of Nile tilapia was used as a test animal. The average weight of fishes was 168.81 ± 11.36 g. Their width and length were 10.12 ± 1.82 cm and 19.09 ± 2.31 cm, respectively. Before experiments, fishes were acclimatized for 7 days. The experiment was divided to 2 parts; 1) to study the levels of chlorpyrifos mixed with cypermethrin, affecting on physiological changes and mortality rate of Nile tilapia, and 2) to study the sublethal concentration for further evaluating possibility in applying as bio–indicator of exposure using ChE as a biomarker of exposure.

Study on Physiological Characteristic of Nile tilapia

The physiological characteristic of Nile tilapia was studied in external by comparing the fish with and without exposure to the chlorpyrifos mixed with cypermethrin. Then, the changes were recorded.

Lethal Concentration (LC) of Chlorpyrifos Mixed with Cypermethrin in Nile tilapia

After acclimatization, ten fishes were transferred into a 500 L of cement tank. After that, the mixture of insecticide solutions of chlorpyrifos and cypermethrin were filled to reach final concentrations of 12.5, 15 and 20 ppb compared to the control without filling. The experiment was performed triplicately. The behavior changes were noted at 0, 24, 48, 72 and 96 h. The fish mortality rate was also recorded at different exposure times and concentrations for further calculating of lethal concentrations. In this study, the experiment was performed as static bioassay by early adding the chemical by once following the standard method of OECD (2014). The lethal concentrations of chlorpyrifos mixed with cypermethrin of Nile tilapia at 10 (LC_{10}), 50 (LC_{50}) and 90% (LC_{90}) for 24, 48, 72 and 96 h were calculated using probit analysis as described by Finney (1947).

Sample Extraction for Study AChE Expression

For sample preparation to study AChE expression in Nile tilapia, AChE was extracted from the tissue of brain, muscle and gill. The expression was evaluated by comparing AChE expression among the control group and the exposure groups (exposure concentrations in the sublethal level of 2.5, 5 and 10 ppb at exposure times at 0, 24, 48,

72 and 96 h). In brief, fish was obliterated before being dissected to collect organs. Then, fish organs were mashed by using homogenizer with adding Tris–HCl buffer (0.02 M, pH 7.4), containing 0.1% Triton X–100 and 0.05 M NaCl. The ration of brain for extraction was 1 g of tissue to 1 mL of buffer. Next, it was centrifuged at 5,000 rpm for 60 min, then the supernatant was kept at –20ºC for further study of protein form and AChE expression.

Moreover, the expression of BuChE in plasma of Nile tilapia was also studied. Tricaine methanesulfonate (TMS or MS–222, Sigma, USA) concentration of 150 mg/L was used as anesthesia. Then, after the fish was weighed and measured, the penetrating blood about 0.5 ml was collected at the caudal vein, located at the base of the fish's tail by using a 1 ml plastic syringe with the needle number 26G. EDTA was then filled to protect blood clotting. Next, it was centrifuged at 5,000 rpm for 5 min. The supernatant was kept at –20ºC for further performing as same as the other organs.

Protein Determination

Protein concentrations in plasma, brain, gill and muscle were determined and calculated before applying into sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), Western blot and dot blot technique. The extracted samples were melted at room temperature and then were dissolved in distilled water in the ration of 1:100. The sample was placed in two holes in a microplate (10 µl/hole) for replication. The standard protein of Bovine Serum Albumin (BSA; purchased from Bio–Rad company) with a concentration of 1.4 mg/mL was diluted as same as the samples to reach the concentrations of 0.7, 0.35, 0.175 and 0.0875 mg/mL. Then, a 200 µL of Bradford dye reagent was added in every hole and left for 5 min at room temperature. The 96 well plate was applied in a microplate reader and its absorbance was read at a wavelength of 595 nm. The absorbance value was used to set standard curve (absorbance value against BSA standard protein concentration) by x–axis is protein concentration (mg/ml) and y–axis is absorbance value. This relation was expressed by using linear regression (y = ax + b) and then applied to calculate protein quantity for further study of protein form and AChE expression.

Acetylcholinesterase Purification

AChE from the brain of Nile tilapia was purified by the column chromatography technique. The applied protein was eluted by 0.2, 0.4 and 1.2 M of sodium phosphate buffer (pH 7.0). Every 1.5 mL eluted fraction (total 120 fractions) was kept to measure absorbance at 280 nm. The amount of AChE in each fraction was calculated compared to the standard solution. The molecular weight of AChE was identified by gel electrophoresis technique.

Enzyme Assay

At the end of the exposure period (control, 24, 48, 72 and 96 h), the blood of five surviving Nile tilapia from each treatment was collected. Then, all fishes were killed and placed on ice. The tissues (brain, muscle and gill) were removed and were frozen in liquid nitrogen, then stored at –20°C until AChE assay. All enzyme tests were made in triplicate. Plasma, brain, muscle and gill tissues were weighed and homogenized in Tris–HCl buffer (0.02 M, pH 7.4) containing 0.1% Triton X–100 and 0.05 M NaCl using a Potter–Elvejhem glass/teflon homogenizer. The homogenates were centrifuged for 15 min at 3,500 rpm at 5°C. Then, the supernatant was used as the enzyme source. The activities of AChE and BuChE were measured as described by Ellman *et al*. (1961) and modified by Villescas *et al*. (1981). Aliquots of the supernatant (2 mL) from brain, muscle and gill tissues were incubated at 25°C for 2 min with 0.1 M phosphate buffer (pH 7.5) and 1 mM DTNB as the chromogen. After 2 min, the reaction was initiated by the addition of 0.08 M acetylthiocholine (AcSCh) as a substrate for the reaction mixture. The final volume was 2.0 mL. The absorbance was determined at 412 nm for 2 min. Enzyme activity was expressed as mmoL of AcSCh hydrolyzed per minute and per gram of protein. Each sample was assayed in triplicate. The BuChE (from plasma) assay was similar to the method used for the *in vitro* AChE.

Study on Protein Pattern Form in Nile tilapia by SDS–PAGE

The sample with a protein concentration of 10 µg/µL was prepared. Then, the separating gel was prepared from 10% of acrylamide solution (H_20 4.85 mL, 30% acrylamide mix 3.35 mL, 1.5 M Tris (pH 8.8) 1.65 mL, 10% SDS 100 µL, 10% APS 50 µL and TEMED 3.5 µL). The stacking gel 4% was prepared with H_20 3.05 mL, 30% acrylamide mix 670 µL, 0.5 M Tris (pH 8.8) 1.25 mL, 10% SDS 50 µL, 10% APS 60 µL and TEMED 5 µL. After the gel was polymerized, the sample was loaded and applied to electrophoresis set with 120 V for 1.30 h. Next, the gel was taken off and then stained with Coomassie Brilliant Blue R–250 for 1 h. After that, the gel was washed with destaining solution I and II until the protein band appeared.

Study on AChE and BuChE Expression in Nile tilapia by using Dot Blot Technique

The sample was prepared to final protein concentrations of 20, 10, 5, 2.5, 1.25, 0.75, 0.625 and 0.375 µg/µL. One µL of the sample was dropped onto the nitrocellulose membrane and left at room temperature until dried. Then, it was soaked in 5% of skimmed milk in PBS for 1 h. It was washed by PBS/0.5% Tween 20 for 5 min triplicately. Next, it was incubated in antibody specific to AChE and BuChE (1:50) for 12 h and then was incubated in Goat Anti–Rabbit Conjugated Peroxidase (GAR–HRP dilution 1:2,000) for 3 h. The positive result expressed as dark brown dot.

Study on AChE and BuChE Expression in Nile tilapia by using Western Blot Technique

The sample was prepared to a final protein concentration of 8 µg/µL. Then, it was mixed with sample buffer in the ratio of 1:1 to get antigen with a concentration of 4 µg/µL. It was put in boiled water for 5 min. The protein was separated by its molecular weight using SDS–PAGE technique. Then, the protein was transferred from gel into nitrocellulose membrane (fitted to gel plate). Nitrocellulose membrane and filter membrane were soaked in semi–dry transfer buffer. The nitrocellulose membrane was articulated with a gel plate and all bubbles were driven out.

After that, it was covered with filter membrane and placed on Trans–Blots SD semi–dry electrophoresis transfer cell by nitrocellulose membrane was on the lower side. The cell was connected with power supplied with electric current of 15 V for 15 min. Next, the nitrocellulose membrane was taken out and soaked in 5% skimmed milk in PBS for 1 h. It was washed with PBS/0.5% Tween 20 for 5 min triplicately. The nitrocellulose membrane was cut aligning each lane. Separated lane membrane was incubated in antibody specific to AChE at dilution of 1:50 for 12 h. The excessive was washed out by PBS/0.5% Tween 20 for 5 min triplicately. Then, it was soaked in Goat Anti–Rabbit Conjugated Peroxidase (GAR–HRP in dilution of 1:1,000) for 3 h. The excessive antibody was washed out by PBS (0.5% Tween 20) for 5 min triplicately. Next, it was incubated in substrate solution (0.03% DAB, 0.06% H_2O_2, 0.05% $COCl_2$ in PBS) to develop color expressing positive result. Nitrocellulose membrane was washed by distilled water and added with Clorox for inhibiting substrate interaction. The positive result expressed as a protein band with molecular weights of 71 kDa for AChE and 85 kDa for BuChE.

Statistics Analysis

The AChE and BuChE activity data were analyzed using one–way analysis of variance by completely randomized design (CRD) and comparing the difference in the mean of each experiment with Duncan's multiple range test which was expressed as mean ± standard deviation. The significant differences between treatments and controls were tested at $P < 0.05$.

RESULTS AND DISCUSSION

Biomarkers have been used extensively to provide the connection among external levels of contaminant exposure, internal levels of tissue contamination and early adverse effects in organisms. Assessing the impacts of contaminants on the health of aquatic organisms and ecosystems is challenging due to the presence of multiple stresses

and the complexity of ecosystems. For instance, they are considered as 'early warning' signals having the potential to detect an effect on target biota before one being observed at the population, community or ecosystem level. Hence, the use of biomarkers can be a critical line of evidence to understand relationships between stress and effects on coastal resources and to prevent detrimental impacts of contamination on ecosystem structure and function (Kroon *et al*., 2017). As generally known, the main biological function of AChE and BuChE is to regulate the transmission of nerve impulses through the neurotransmitter of ACh. The enzymatic activity showed the decreasing function of the brain, gill, muscle, kidney and liver after pesticide stress. Ghazala *et al*. (2016) suggested that the activity of ChE was significantly inhibited even at the lowest concentrations of triazophos in brain, blood, gills, muscle, kidneys and liver. As mentioned above, we, therefore, decided to investigate environmental relevant chlorpyrifos mixed cypermethrin concentrations affecting local fish, i.e., Nile tilapia which is a commercial and typical fish species inhabiting in local freshwater and being found nationwide. In this study, we evaluated its physical changes and mortality rates as well as applied cholinesterase as an exposure biomarker.

Toxicity Testing and Physiological Changes

According to the investigating the levels of toxicity causing cumulative death in Nile tilapia exposed to chlorpyrifos mixed with cypermethrin for 24, 48, 72 and 96 h with various concentrations of 12.5, 15 and 20 ppb, it was found that the mortality rate increased with the exposure duration and the concentration levels. Exposing Nile tilapia to chlorpyrifos mixed with cypermethrin for 24 h at the lowest concentration (12.5 ppb) caused 13.33 ± 5.77% mortality, but when exposed to them at higher concentrations, the mortality rate increased to 16.67 ± 5.77% at 15 ppb and to 23.33 ± 5.77% at 20 ppb. Comparing exposure for 24 h, the longer exposure duration (48, 72 and 96 h) caused an increasing mortality rate by about 10% at all concentrations (Figure 1A). Our study was conformed with Jidee *et al*. (2016) and Mararam *et al*. (2016) who found that pesticide toxicity causing fish death depended on the exposure time and pesticide concentrations.

Mararam *et al*. (2016) reported that commercial forms of chlorpyrifos (i.e., not mixed with cypermethrin) could directly affect the behavior and physiology of catfish and the level of change depended on the time and duration of exposure. Furthermore, Thanomsit *et al*. (2018) suggested that the effect of cypermethrin being widely applied in Thailand caused severe effects on aquatic organisms and water quality. After consuming contaminated fish, the contaminants may cause a health risk. In this study, the physiological change took place for 24 h. The change was examined as the wound around the fins, illustrating wounds and bleeding around the caudal fin and the end of the body and tail base (Figure 1B) corresponding to the results of Thanomsit *et al*. (2018) who found that Nile tilapia was changed in physiology and behavior when exposing to 17–beta estradiol and cypermethrin. For instance, their swimming was very fast, abnormal and directionless, Additionally, the operculum was always moving and there was a lesion around their mouth. Halappa and David (2009) explained that the caudal bending was the main morphological alteration during the exposure periods. The behavioral and morphological changes may be due to the inhibition of AChE activity. Inactivation of AChE activity results in excess accumulation of acetylcholine in cholinergic synapses leading to hyperstimulation and the cessation of neuronal transmission. Impaired behavioral responses and morphological deformities were observed even during recovery periods which may be a consequence due to the inhibition of AChE activity in brain and muscle by chlorpyrifos–oxon (CPO; the active metabolite of CPF) via biotransformation of bioaccumulated chlorpyrifos in the tissues. This study clearly illustrated that the exposure to chlorpyrifos mixed with cypermethrin affected the morphology of Nile tilapia. It could lead to physiological change that occurred over 24 h which could be examined from ulcers at the base of the fins and bleeding at the base of the caudal fin and the end of the body.

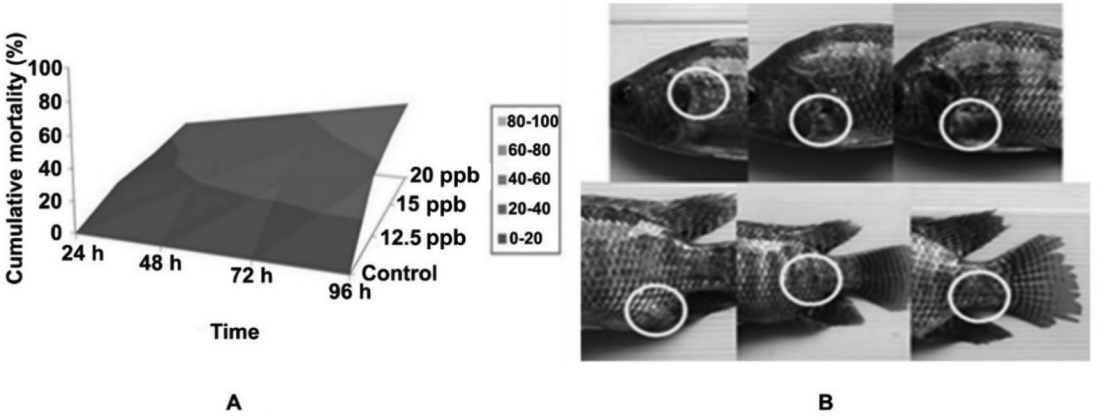

Figure 1 Toxicity levels affecting the death of Nile tilapia when exposed to chlorpyrifos mixed with cypermethrin at concentrations of 0, 12.5, 15 and 20 ppb for 24, 48, 72 and 96 h (A) and physiological changes (B). The yellow circle shows examples of physiological changes

Furthermore, Majumder and Kaviraj (2017) used bioassay conducted with technical grade and commercial formulation of cypermethrin in freshwater fish *Oreochromis niloticus* as the test fish. In this study, we used bioassay for toxicity testing of chlorpyrifos mixed with cypermethrin (commercial form) on Nile tilapia. The result showed high toxicity of this compound and the cumulative mortality percentage depended on the concentrations and exposure times. When the Nile tilapia was exposed to the substance, the toxicity levels that caused 10, 50 and 90% death were evaluated using Probit analysis. The results revealed that LC_{10} at 24, 48, 72 and 96 h were 9.019, 3.238, 0.352 and 0.063 ppb, respectively. The LC_{50} at 24, 48 and 72 h were consecutively 34.369, 28.047 and 22.543. While the LC_{50} at 96 h was 17.967 ppb that was different from the report of Majumder and Kaviraj (2017) who studied the toxicity of cypermethrin on *O. niloticus*. This may be due to the different forms and characteristics of the substance used. Finally, the LC_{90} at 24, 48, 72 and 96 h were 59.720, 52.856, 45.438 and 42.223 ppb, respectively (Table 1).

Table 1 Lethal concentrations of Nile tilapia

Time (h)	Lethal concentration (95% confidence)		
	LC_{10} (ppb)	LC_{50} (ppb)	LC_{90} (ppb)
24	9.019 (5.255–11.021)	34.369 (29.754–43.464)	59.720 (48.851–81.310)
48	3.238 (0.233–6.298)	28.047 (25.172–33.262)	52.856 (44.363–68.534)
72	0.352 (0.063–3.137)	22.543 (20.984–25.087)	45.438 (39.240–56.052)
96	0.063 (0.014–0.140)	17.967 (17.052–19.223)	42.223 (36.499–52.237)

Note: LC_{10} = lethal concentration causing 10% death, LC_{50} = lethal concentration causing 50% death, LC_{90} = lethal concentration causing 90% death

Majumder and Kaviraj (2017) also explained that even though the toxic substances used in the test are the same type of chemical but different grades would provide different effects to the fish. The commercial formulation was found to be more acutely toxic to *O. niloticus* (LC_{50} = 4.85 µg/L) than the technical grade of cypermethrin (LC_{50} = 9.74 µg/L) when exposed for 96 h. Exposure to sublethal concentrations (1.25 and 2.50 µg/L) of commercial cypermethrin for 96 h produced stress on the fish. However, the results from these experiments were different from the research of Nwani *et al*. (2013) who studied 96 h semi–static acute toxicity bioassay carried out to determine the LC_{50} value and behavioral responses of commercial formulation of chlorpyrifos (Termifos) on the freshwater fish *Clarias gariepinus*. The LC_{50} of different concentrations of Termifos in *C. gariepinus* were found to be 1.66, 1.30, 1.03 and 0.86 mg/L for 24, 48, 72 and 96 h exposure time, respectively, thus indicating that the pesticide is highly toxic to the fish.

In vitro Cholinesterase Enzyme Activity (AChE and BuChE)

As a specific biomarker for organophosphate such as carbamate pesticides and pyrethroids, the degree of the AChE inhibition with *in vivo* conditions is a good tool in continuous monitoring of insecticides, which may induce the nerve conduction disorders. Discharges of these insecticides into the environment, however, may cause unpredictable toxicity to human and numerous biological organisms. As a key enzyme that hydrolyzes the neurotransmitter acetylcholine in cholinergic synapses of both vertebrates and invertebrates, AChE is strongly inhibited by organophosphate, carbamate pesticides and pyrethroids at low concentrations (Zhu *et al*., 2015).

Nile tilapia is an economic fish, which was studied by many toxicologists. For instance, Pathiratne *et al*. (2009) reported the analysis of water pollutants in Sri Lankan waters using a suite of biomarkers in Nile tilapia (*Oreochromis niloticus*) residing in Bolgoda Lake which receives urban, industrial and domestic wastes from multiple sources. The results revealed that the ChE activities in brain and muscle of the fish from Bolgoda South Lake in April 2005 were depressed by 40% and 31% of the respective control levels. In October 2006, the ChE activities in brain and muscle of the fish from Bolgoda South Lake were reduced only by 25% and 22% of the respective controls.

In this study, the activity of ChE, including AChE and BuChE was examined after exposure for 24, 48, 72 and 96 h at concentrations of 0, 12.5, 15 and 20 ppb chlorpyrifos mixed with cypermethrin. The AChE activity was determined in the brain, muscle and gill. The study found that concentration and exposure time affected AChE enzyme activity. In the brain, the enzyme activity in control group (0 ppb) was significantly different from fish exposed at concentrations of 12.5, 15 and 20 ppb ($P < 0.05$). A similar result also found in all periods of exposure time (Figure 2A). In contrast, AChE enzyme activity in the muscle showed that more exposed substance and longer duration, especially at the time of 72 and 96 h, the AChE enzyme activity would likely decrease. However, the different results of AChE enzyme activity in muscle were observed when exposed for 24 and 48 h. The AChE enzyme activity was higher when exposed substance at concentrations of 15 and 20 ppb compared to control group (Figure 2B).

The AChE enzyme activity in the gill showed no difference in all concentrations studied at 24 h, with the amount of enzyme activity measured to be in the range of 9.45 ± 0.49 µmol min^{-1} g protein at 72 h and 96 h, which was significantly different from the control group ($P < 0.05$) at all concentrations (Figure 2C). The BuChE enzyme activity could be detected in the plasma at 48, 72 and 96 h. BuChE activity was significantly different from the control group ($P < 0.05$), with a tendency to decrease when exposed to a very long time (Figure 2D).

The AChE is strongly inhibited by organophosphate, carbamate pesticides and pyrethroids at low concentrations (De la Torre *et al*., 2002). Meanwhile, for this reason, this enzyme has widely been using as a specific biomarker for these compounds (Guilhermino *et al*., 2000). The compounds for depressing the AChE activity of fish and invertebrates, *in vitro* and/or *in vivo* conditions, have been demonstrated in several studies

(Guilhermino *et al*., 2000). However, the continuous detection of the AChE inhibition was hardly found in these insecticides with *in vivo* conditions (Zhu *et al*., 2015). On the other hand, BuChE is named according to its preference for the artificial substrate butyrylcholine. BuChE can split butyrylcholine with higher turnover number than AChE. It is also able to hydrolyze much slower than AChE, indole derivatives, adipoylcholine, benzoylcholine, acetylcholine/acetylthiocholine, butyrylcholine/butyrylthiocholine and propionylcholine/propionylthiocholine. However, BuChE is not able to split acetyl–β–methyl–thiocholine oracetyl–β–methyl–choline whereas AChE can (Pohanka, 2011).

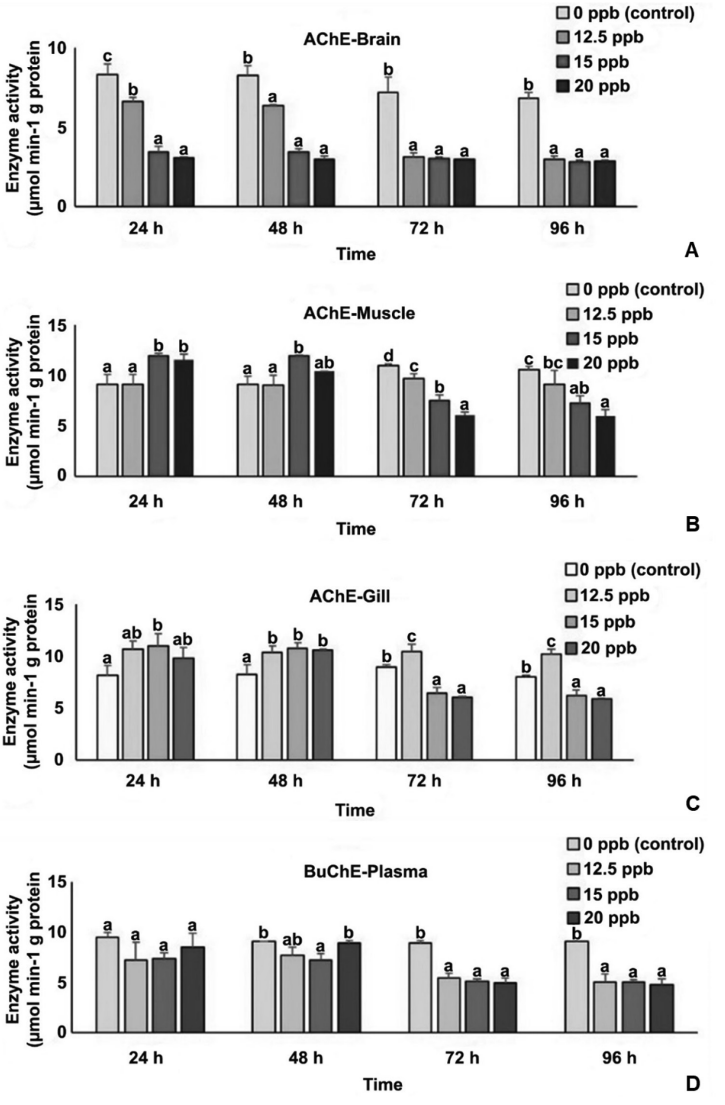

Figure 2 AChE enzyme activities in brain (A), muscle (B) and gill (C) and BuChE enzyme activities in plasma (D) of Nile tilapia that were exposed to chlorpyrifos mixed with cypermethrin at concentrations of 0, 12.5, 15 and 20 ppb for different experimental periods (24, 48, 72 and 96 h). Data are mean ± SD (n = 3). Different letters indicate statistically significant differences between group of concentrations at $P < 0.05$

Mahboob *et al*. (2014) suggested that the context of inhibition of AChE and BuChE in brain, blood, gills, kidneys and liver of *Labeo rohita* could be observed after exposure to insecticides. This enzyme inhibition is likely to affect the functions including metabolism and neurotransmission of all these organs. On Nile tilapia, when exposed to chlorpyrifos mixed with cypermethrin at concentrations of 0, 12.5, 15 and 20 ppb, the activities of cholinesterase, AChE and BuChE in the brain, muscle and gill were examined after exposure for 24, 48, 72 and 96 h. The result showed that concentration and exposure time affected AChE enzyme activity. In the brain, AChE enzyme activity in the control group was significantly different from the others (12.5, 15 and 20 ppb) at all exposure times. For AChE enzyme activity in muscle, when exposed to more substances at longer intervals especially in the periods of 72 and 96 h, AChE enzyme activity tended to decrease. But, in the gill at exposure times of 72 and 96 h, AChE enzyme activities of the exposed fishes at all concentrations were significantly different from the control group ($P < 0.05$). Our results on Nile tilapia corresponded with the study of Medaka (Zhu *et al*., 2015) who found that at the beginning of the exposure, the trend of AChE activity might increase and the AChE activity in dead individuals was lower than the live individuals. Through the synthesis of AChE in Medaka might help the AChE activity recover, the trends during the exposure in different treatments were downward, and it showed both exposure time and concentration dependently. Meanwhile, the higher temperature might cause the AChE inhibition earlier due to the higher metabolic rate. Therefore, as a specific biomarker for organophosphate, carbamate and pyrethroid, the level of the AChE inhibition with *in vivo* conditions is a good tool in continuous monitoring of insecticides, which may induce the nerve conduction disorders (Zhu *et al*., 2015). Walker *et al*. (2006) explained that the adverse effects of toxicant also depend upon the species, sex, time, concentration and route of exposure.

The activities of BuChE enzymes in plasma can be used as a relevant stress indicator (Velisek *et al*., 2006). However, the primary reason for the existence of BuChE is still unknown (Pohanka, 2011). Therefore, in this study, the expression of BuChE enzyme activity in the plasma of Nile tilapia was observed. At the exposure times of 72 and 96 h, the BuChE enzyme activity in plasma of the exposed fishes was significantly different from the control ($P < 0.05$) with a tendency to decrease when exposed to a long period. The BuChE is capable of detoxifying a large number of exogenous substances (e.g., procaine, succinylcholine, cocaine, heroin and acetylsalicylic acid) as well as protecting the body from the impact of organophosphorus AChE inhibitors (Pohanka, 2011).

Protein Expression on Nile tilapia

The SDS–PAGE was used to study the pattern of proteins in the brain, muscle, gill and plasma of Nile tilapia exposed to chlorpyrifos mixed with cypermethrin at various concentrations and exposure times. The pattern of protein was changed corresponding to the concentrations and exposure times as shown in Figure 3. In the brain, at all concentration levels, the AChE could be examined with a size of 71 kDa when exposed for 24 h, but as time passed to 48 h, there was a decrease in volume and it could not be observed at 96 h. The muscle was another tissue being used in the study, which was the side of the body. The protein pattern of AChE was found to be similar to that in the brain, which had a molecular weight of 71 kDa, was visible at all concentrations at 24 h of exposure. But when exposed for a longer period, the expression of the AChE was reduced. The 71 kDa protein bands became thinner, but smaller molecular proteins in the range 25–37 kDa were still found in all periods. The AChE in the gill tissue had a molecular size of 71 kDa, similar to that found in the brain and the muscle. At all levels of concentration and all time of exposure, the AChE showed clear expression.

But the tendency to find was decreasing according to the time of exposure. For BuChE, it was found only in plasma at 24 h of exposure with molecular weight as 85 kDa, which was larger than the AChE.

The AChE and BuChE expression in Nile tilapia were examined by using the antibody technique. For studying AChE expressions in the brain, muscle and gill of Nile tilapia, both Western blot and dot blot techniques were used. These techniques were also applied to investigate BuChE expression in plasma. For the study of AChE expression, partially purified AChE from the brain of Nile tilapia was used as a positive control (Figure 4A) by showing the molecular weight of AChE as 71 kDa when using Western blot probed with commercial polyclonal antibody specific to AChE (PAb–AChE) from electric eel at the dilution of 1:200 with the amount of 8 µg/µL, which is the best level used to investigate the protein form and the interaction with the antibody. Supernatant protein antigen content was extracted from the brain, muscle and gill of Nile tilapia that were exposed to chlorpyrifos mixed with cypermethrin at concentrations of 0, 12.5, 15 and 20 ppb for 24–96 h. It was found that AChE in the brain was expressed when the Nile tilapia was exposed to the substance only for 24 h at concentrations of 12.5 and 15 ppb and in only the control group.

The dot blot technique is convenient and easy as well as does not take time. Guillemin *et al*. (2009) reported that the dot blot technique has been developed since 1982 and was used to examine plentiful biological indicators caused by bacteria immune study and dermatological research that was affected by the reception of foreign bodies. For environmental applications, the dot blot is used to assess the exposure of pollutants and xenobiotic by examining the expression of various biological indicators, such as in the report of Nanthanawat *et al*. (2014) who suggested the dot blot technique for studying the monoclonal antibody specificity CYP1A.

The dot blot technique was used to evaluate the expression of AChE and BuChE as well as the Western blot technique by using the same dilution of the PAb–AChE antibody at 1:200, but different in the amount of the antigen. The AChE found in the supernatant was in the range of 0.02 –10 µg/µL (Figure 4B). When the Nile tilapia was exposed to chlorpyrifos mixed with cypermethrin at the level of concentrations of 0, 12.5, 15 and 20 ppb for 24, 28, 72 and 96 h, The lowest expression of AChE and BuChE were found when Nile tilapia was exposed to the substance at the highest concentration (20 ppb) for the longest time (96 h) of this study. The detection limits of dot blot for the expression cholinesterase in brain, muscle, gill and plasma were 5, 2.5, 1.25 and 0.31 µg/µL, respectively.

In the AChE protein in muscle, it could be measured when Nile tilapia was exposed for 24 and 48 h at all concentrations (Figure 4C) but could not be found in Nile tilapia that was exposed for 72 and 96 h (data not shown). The expression of AChE in the gill showed the expression at the same time in the muscle, which was found at 24 and 48 h, but at 48 h, the protein band with 71 kDa was lighter than those. The expression of BuChE enzyme could be detected in plasma only when Nile tilapia was exposed at all concentration levels for 24 h. The BuChE had a molecular weight of 85 kDa, which was larger than AChE (Figure 4C), the expression of AChE and BuChE corresponded as those reported in antibody sources. This study in Nile tilapia was consistent with the study of Ma *et al*. (2011) that reported 66.35 kDa of molecular weight of purified AChE protein in *Paradise estrogen* L. Koch by using the same technique of inspection with this study. Similar to Kuthyer *et al*. (2018), the molecules of AChE1a and AChE1b were approximately 71.72 kDa and 69.48 kDa, respectively, when extracted from Salmon louse (*Lepeophtheirus salmonis*) that was exposed to organophosphate pesticides.

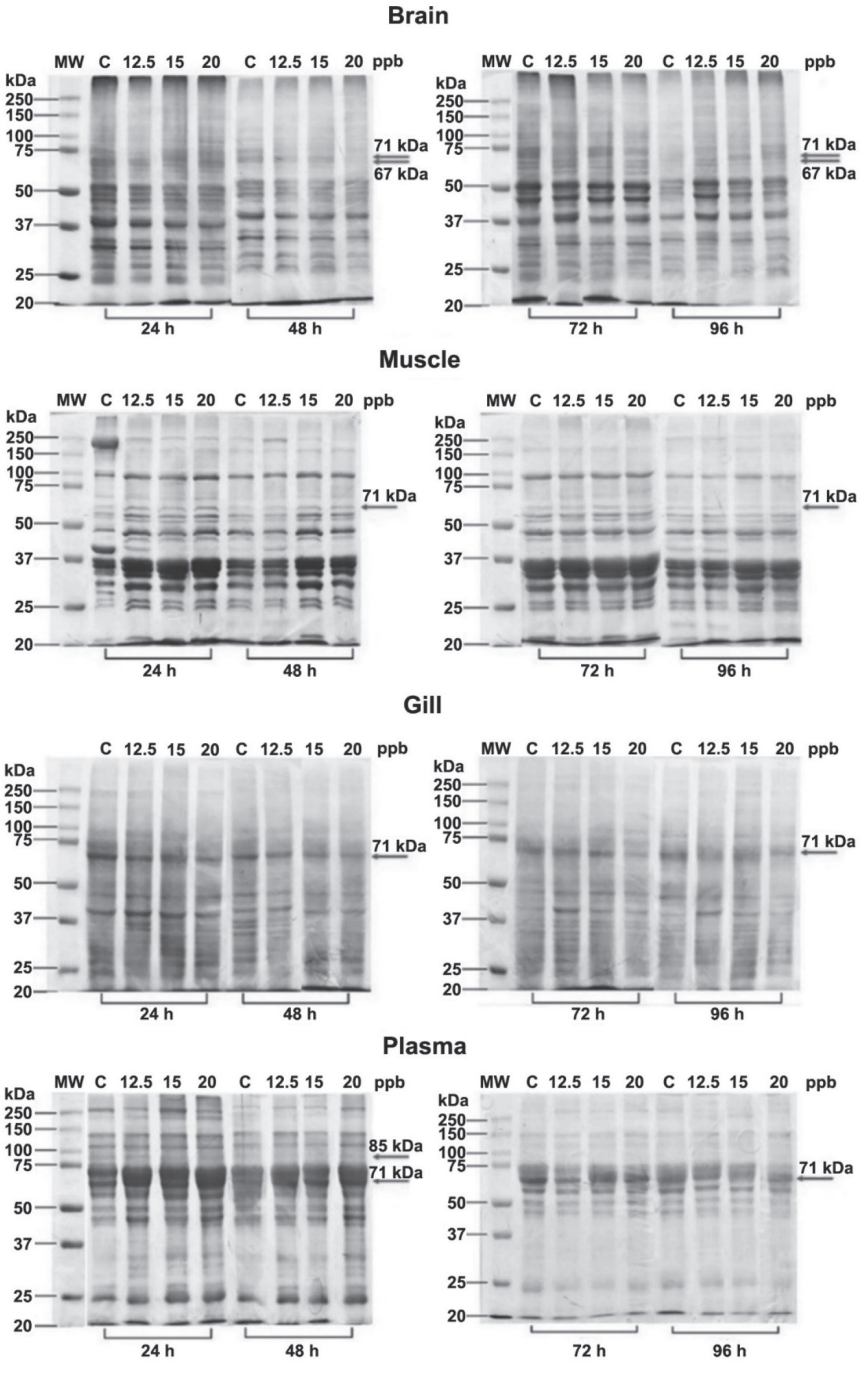

Figure 3 SDS–PAGE (10%) shows the protein profile from the brain, muscle, gill and plasma at concentrations of 0, 12.5, 15 and 20 ppb at 24, 48, 72 and 96 h. The protein band (8 µg/µL) was detected by Coomassie Brilliant Blue R–250. Arrow indicates molecular weight of AChE (71 kDa) and BuChE (85 kDa)

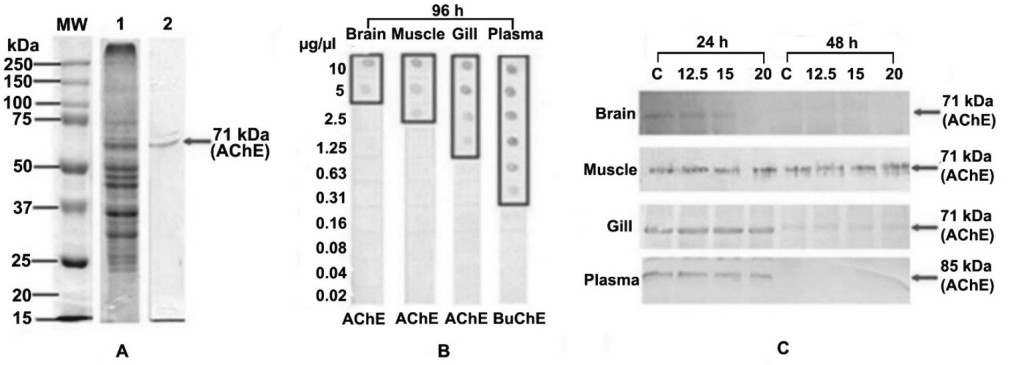

Figure 4 Dot blot analysis of AChE expression (A), lane 1 shows partial purification of AChE isolated from the brain of Nile tilapia using as the positive control, lane 2 shows a protein isolated from the brain of Nile tilapia blotted onto nitrocellulose membrane (0.45 µm) and incubated with PAb–AChE (1:200). Detection limit of dot blot technique when using PAb–AChE (1:200) to evaluate the expression of AChE in the brain, muscle and gill and detection limit when using PAb–BuChE (1:100; commercial antibody for human) to examine the expression of BuChE in plasma of Nile tilapia that was exposed for 96 h (B). Western blot analysis of AChE expression in the brain, muscles, gill and plasma using the PAb–AChE and PAb–BuChE (C)

CONCLUSIONS

Insecticides (chlorpyrifos mixed with cypermethrin) caused toxicity and cumulative mortality in Nile tilapia. The mortality rate increased according to the time and concentration levels that were exposed. Moreover, these chemicals caused changes in the behavior and morphology of Nile tilapia. The AChE and BuChE activities of mixture insecticides were observed in Nile tilapia *in vivo* and expressed the strongest inhibition according to both exposure time and concentration. Antibody technique based–immunoassay can be used to determine the expression of AChE. Western blot analysis showed the size of AChE, being a measure of exposure, that was 71 kDa found in the brain, gill and muscle. The molecular weight of BuChE in plasma was also 85 kDa for all tissues. The detection limits of the dot blot technique were 5, 2.5, 1.25 and 0.31 µg/µL for the brain, muscle, gill and plasma, respectively. In conclusion, immunoassay techniques (dot blot and Western blot) were developed to monitor alterations of cholinesterase enzyme for using as biomarker of exposure. This preliminary information was important for further management establishing and mitigation plan setting for insecticide contamination in aquatic organism and the environment.

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REFERENCES

Chandrasekara, L.W.H.U. and A. Pathiratne. 2007. Body size–related differences in the inhibition of brain acetylcholinesterase activity in juvenile Nile tilapia (*Oreochromis niloticus*) by chlorpyrifos and carbosulfan. Ecotoxicol. Environ. Saf. 67(1): 109–119.

De la Torre, F.R., L. Ferrari and A. Salibián. 2002. Freshwater pollution biomarker: response of brain acetylcholinesterase activity in two fish species. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 131(3): 271–280.

Ellman, G.L., D. Courtney, V.J. Andres and R.M. Featherstone. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7(2): 88–90.

Finney, D.J. 1947. Probit Analysis: A Statistical Treatment of the Sigmoid Response Curve. Macmillan.

Ghazala, S. Mahboob, K.A. Al–Ghanim, S. Sultana, H.F. Alkahem Al–Balawi, T. Sultana, F. Al–Misned, L. Ahmed and Z. Ahmed. 2016. Effect of triazophos on esterase activity and protein contents of liver, kidney, brain, blood and muscles of *Catla catla*, *Labeo rohita* and *Cirrhinus mrigala*. Pakistan J. Zool. 48(2): 513–518.

Guilhermino, L., M. Lacerda, A. Nogueira and A. Soares. 2000. *In vitro* and *in vivo* inhibition of *Daphnia magna* acetylcholinesterase by surfactant agents: possible implications for contamination biomonitoring. Sci. Total Environ. 247: 137–141.

Guillemin, N., B. Meunier, C. Jurie, I. Cassar-Malek, J.F. Hocquette, H. Levézeil and B. Picard. 2009. Validation of a dot blot quantitative technique for large scale analysis of beef tenderness biomarkers. J. Physiol. Pharmacol. 60(3): 91–97.

Halappa, R. and M. David. 2009. Behavioural responses of the freshwater fish, *Cyprinus carpio* (Linnaeus) following sublethal exposure to chlorpyrifos. Turk. J. Fish. Aquat. Sci. 9: 233–238.

Jidee, S., U. Jitgla, C. Netpra, S. Udduang and C. Thanomsit. 2016. Effect of glyphosate on fish behavior and mortality rate in Climbing Perch (*Anabas testudineus*, Bloch), pp. A193–A203. *In*: Proceeding of 8th Rajamangala Surin Conference, Rajamangala University of Technology Isan, Surin campus, Surin.

Kroon, F., C. Streten and S. Harries. 2017. A protocol for identifying suitable biomarkers to assess fish health: a systematic review. PLoS One 12(4): 1–43.

Kuthyer, F., M. Kocabaş, M. Erişir and F. Benzer. 2018. Cypermethrin–induced *in vitro* alterations on oxidative stress and quality of *Salmo coruhensis* spermatozoa. Indian J. Pharm. Educ. Res. 52(4): 71–76.

Ma, M., B. Zhang and S. Li. 2011. Purification and partial characterization of acetylcholinesterase from *Pardosa astrigera* L. Koch. J. Cell Anim. Biol. 5(1): 11–16.

Mahboob, S., Ghazala, K.A. Al–Ghanim, S. Sultana, F. Al-Misned and Z. Ahmed. 2014. Fish cholinesterases as biomarkers of sublethal effects of organophosphorus and carbamates in tissues of *Labeo rohita*. Pakistan J. Zool. 46(1): 121–127.

Majumder, R. and A. Kaviraj. 2017. Cypermethrin induced stress and changes in growth of freshwater fish *Oreochromis niloticus*. Int. Aquat. Res. 9: 117–128.

Mararam, N., O. KhantIwong, C. Reangjob, A. Wattanakornsiri, K. Saowakoon and C. Thanomsit. 2016. Toxicity level, behavioral change and mortality rate in hybrid catfish exposed to insecticide (chlorpyrifos), pp. A204–A211. *In*: Proceeding of 8th Rajamangala Surin Conference, Rajamangala University of Technology Isan, Surin campus, Surin.

Mena, F., M. Azzopardi, S. Pfennig, C. Ruepert, M. Tedengren, L.E. Castillo and J.S. Gunnarsson. 2014. Use of cholinesterase activity as a biomarker of pesticide exposure used on Costa Rican banana plantations in the native tropical fish *Astyanax aeneus* (Günther, 1860). J. Environ. Biol. 35: 35–42.

Nanthanawat, P., N. Khongchareonporn, W. Prasatkaew and P. Kanchanopas–Barnette. 2014. Production and characterization of monoclonal antibody specific to cytochrome P4501A (CYP1A) in Asian sea bass (*Lates calcarifer* Bloch) exposed to benzo [a] pyrene. Burapha Science Journal 19(2): 1–13.

Nwani, C.D., N. Ivoke, D.O. Ugwu, C. Atama, G.C. Onyishi, P.C. Echi and S.A. Ogbonn. 2013. Investigation on acute toxicity and behavioral changes in a freshwater African catfish, *Clarias gariepinus* (Burchell, 1822), exposed to organophosphorous pesticide, Termifos®. Pak. J. Zool. 45(4): 959–965.

OECD (Organisation for Economic Co-operation and Development). 2014. OECD Test Guidelines for the Chemicals. OECD Publishing, Paris, France.

Pathiratne, A., L.W.H.U. Chandrasekera and K.A.S. Pathiratne. 2009. Use of biomarkers in Nile tilapia (*Oreochromis niloticus*) to assess the impacts of pollution in Bolgoda Lake, an urban water body in Sri Lanka. Environ. Monit. Assess. 156: 361–375.

Petchoy, T. and T. Pung. 2017. Effect of chlorpyrifos and dimethoate spraying doses on residue concentrations in Scallion (*Alliumcepa var. aggregatum*). Agricultural Sci. J. 48(1): 108–117.

Pohanka, M. 2011. Cholinesterase, a target of pharmacology and toxicology. Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech Repub. 155(3): 219–230.

Tawatsin, A., U. Thavara and P. Siriyasatien. 2015. Pesticide used in Thailand and toxic effects to human health. Med. Res. Arch. 3: 1–10.

Thanomsit, C., S. Saowakoon, A. Wattanakarnsiri, J. Nanuam, W. Prasatkaew and P. Nanthanawat. 2018. Effect of 17 beta–estradiol hormone and cypermethrin insecticide on Nile tilapia. NUJST. 26(4): 119–131.

Veliseki, J., T. Wlasow, P. Gomulka, Z. Svobodova, R. Dobsikova, L. Novotny and M. Dudzik. 2006. Effects of cypermethrin on rainbow trout (*Oncorhynchus mykiss*). Vet. Med. (Praha) 51(10): 469–476.

Villescas, R., R. Oswald and H. Marimoto. 1981. Effects of neonatal undernutrition and cold stress on behavior and biochemical brain parameters in rats. J. Nutr. 111(6): 1103–1110.

Walker, C.H., S.P. Hopkin, R.M. Sibly and D.B. Peakall. 2006. Principle of Ecotoxicology. Taylor & Francis Inc., Bosa Roca, USA.

Zhu, J., C. Huan, G. Si, H. Yang, L. Yin, Q. Ren, B. Ren, R. Fu, M. Miao and Z. Ren. 2015. The acetylcholinesterase (AChE) inhibition analysis of medaka (*Oryzias latipes*) in the exposure of three insecticides. Pak. J. Pham. Sci. 28(2): 671–674.