Development of an antibody technique for acetylcholinesterase expression detection in the gill of Nile tilapia (*Oreochromis niloticus*) as a glyphosate-based herbicide biomarker

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ABSTRACT

Widely used glyphosate-based herbicides can remain in agricultural fields or be spread into the surrounding environment. This study aimed to develop an antibody technique for assessing acetylcholinesterase (AChE) expression in Nile tilapia (*Oreochromis niloticus*) after glyphosate exposure in the assessed tissues consisting of plasma, muscle, gills, and liver. Results showed that the cumulative mortality of fish exposed to glyphosate increased with exposure time and glyphosate concentration. The LC50 was evaluated using probit analysis. A sub-lethal concentration of 2 µL L⁻¹ glyphosate-based herbicide altered behavioural and physiological appearances. AChE expression decreased compared to that in the control group with increasing glyphosate exposure time. The 71 kDa AChE was consecutively expressed in plasma, muscle, gill, and liver under laboratory and field conditions, as detected by dot blot and Western blot analysis. Furthermore, laboratory and field studies of the gills showed positive immunohistochemical results. Although this study could detect AChE expression in many tissues, using gills to assess AChE exposure allowed the fish not to be sacrificed compared with other organ studies, and this technique can be used in both laboratory and field conditions. In conclusion, the antibody technique can be applied to measure AChE expression in the gill tissue to assess glyphosate-based herbicide exposure.

1. Introduction

Fish are commonly susceptible to toxicants, including glyphosate-based herbicides, released into the aquatic environment, causing synaptic transmission blocks. Acetylcholinesterase (AChE) is an enzyme found in the central and peripheral nervous systems. It functions in cholinergic pathways and synaptic transmission [1] in both vertebrates and invertebrates through the degradation of acetylcholine (ACh), a neurotransmitter, into choline and acetate groups [2]. AChE inhibition by organophosphate and carbamate causes ACh accumulation in the synaptic cleft, leading to neuromuscular paralysis and death by asphyxiation [3-5]. Thus, AChE concentration can be used as a biomarker of pesticide and herbicide exposure.

AChE is used as a biomarker to indicate glyphosate-based exposure by detecting enzyme activity using spectrophotometry. Several studies have shown that AChE can be used to test toxicity and evaluate water quality. However, the direct measurement of AChE is limited by time owing to fast degradation, and it is not easy to test for AChE activity. Direct measurement of AChE using spectrophotometry as an immunological technique with antigen-antibody specificity is a promising method owing to its simplicity, high precision, and low cost and can be used with several tissue samples. Hence, this immunological technique is useful for AChE detection because of its high sensitivity and specificity [6].

The objectives of this study were: (1) to evaluate the toxicity of glyphosate on the behaviour of Nile tilapia by investigating its...
histological effects and (2) to investigate the histological changes in the gills, liver, kidneys, and brain over 4 days of exposure. A field test was conducted in the Huai-Saneng Reservoir, Mueang District, Surin Province. The results of AChE expression can be used to monitor pesticide and/or glyphosate herbicide exposure and advise policy-maker level management concerned with consumer health risks.

2. Materials and method

2.1. Chemicals

Glyphosate® (N-phosphomethyl glycine isopropyl ammonium 48 % (W/V) SL) was purchased from Thai Agriculture (Thailand) in commercial form. The glyphosate used in this study was used as a solution because of its convenience. Other reagents for enzymatic activity and AChE extraction from Nile tilapia were purchased from GE Healthcare (Thailand). The reagents used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), dot blot, and Western blot (Bio-Rad (Thailand)); polyclonal antibody specific to AChE (PAb-electric eel AChE, Raybiotech©) and Goat Anti-Rabbit Horseradish Peroxidase conjugate (GAR-HRP, # ab 6741, Abcam Co. Ltd.); and all other chemicals used were of analytical grade.

2.2. Animal husbandry

Adult Nile tilapia (Oreochromis niloticus) (116.88 ± 21.69 g, 22.39 ± 1.40 cm, mean ± SD) were used as the test organism and were obtained from the Department of Fisheries, Faculty of Agriculture and Technology, Rajamangala University of Technology Isan, Faculty of Agriculture and Technology Animal Handling Standards and were approved by the Committee for Biological Experimentation on Animals from the Department of Fisheries Science, Rajamangala University of Technology Isan Surin Campus. The animal use licence number is UI-03405–2559.

2.3. Bioassays

Acute toxicity bioassays were conducted at 24, 48, 72, and 96 h to determine the LC10, LC50, and LC90 values for the commercial formulation of glyphosate following the Organization for Economic Co-operation and Development (OECD) guidelines for testing of chemicals [7]. Three replicates of 10 fish specimens were exposed to four selected glyphosate-based herbicide concentrations, i.e., 10, 20, 30, and 40 μL L−1. Mock treatment without the test chemical was administered to the control group. The experiment was conducted in a concrete pond (80 cm × 80 cm × 60 cm) containing 150 L of water. Behaviour and other external changes in the fish were observed daily. The mortality of the fish after glyphosate exposure was recorded from 24 to 96 h and analysed using MATLAB (MATLAB 2010, © 1984–2009 The MathWorks, Inc.). The LC90 values were determined with Probit analysis every 24 h.

2.4. Fish behaviour and morphology

A sublethal concentration of glyphosate was used to examine behavioural responses according to OECD [6] guidelines in correlation with the AChE expression in brain tissue.

2.5. Extraction of AChE and protein determination

The fish were anaesthetised with eugenol/ethanol 50 % (v/v) solution, the caudal peduncle was cut, and the blood was collected from the caudal vein. Blood samples were pooled for the AChE expression analysis. Also, gill, liver, and muscle were excised, preserved in ice-cold 0.02 M phosphate buffer at pH 7.4 (containing 0.1 % Triton X-100, 0.05 M NaCl), and then homogenised in an ice bath using an Ultra-Turrax homogeniser. Tissue homogenate (0.4 g mL−1) was used for protein extraction. Protein concentration levels in plasma, muscle, liver, and gills were determined using the Bradford technique [8]. At a 1:100 dilution, the protein concentration was measured following the protocol described by Thanomsit et al. [9].

2.6. Sensitivity, specificity, and cross-reactivity of AChE analysed by dot blot and Western blot techniques

The primary antibody, PAb-rabbit anti-fish AChE (PAb-electric eel AChE, Raybiotech©), at a dilution of 1:200 was used for sensitivity and cross-reactivity testing of AChE in Nile tilapia. Protein samples from the brains of Nile tilapia were diluted with Phosphate-buffered Saline (PBS) to final concentrations of 10, 5, 2.5, 1.25, 0.65, 0.31, 0.15, 0.08, 0.04, and 0.02 μg mL−1. The method described by Thanomsit et al. [9] was followed to spot and dry 1 μL of each sample. The specificity and cross-reactivity of AChE were investigated using the PAb-electric eel AChE. Proteins of interest extracted from Nile tilapia were separated using 10 % SDS-PAGE, then blotted with Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad) according to Prasatkaew et al. [10].

2.7. Enzyme activity

Enzyme activities were tested in triplicate using the colorimetric method. Liver, muscle, and gill tissues were prepared and homogenised in Tris-HCl buffer (0.02 M, pH 7.4) containing 0.1 % Triton X-100 and 0.05 M NaCl using a Potter-Elvejem glass/Teflon homogeniser. All tissue homogenates were centrifuged for 65 min at 3,500 g at 5 °C, and the enzyme activity of AChE from the supernatant was measured as described by Tham et al. [11]. In contrast, the plasma was centrifuged for 5 min at 3,500 g at 5 °C. AChE activity was measured using a colorimetric microplate reader. The specific activity was determined as μmol hydrolysed per min mg−1 of protein or U mg−1 of protein and was calculated based on an extinction coefficient of 13.6 mM/cm [12].

2.8. Immunohistochemistry of Nile tilapia

For immunolocalization of AChE expression, 6 μm of gill tissue sections were deparaffinized and probed with 1:200 PAb-electric eel AChE at 37 °C for 3 h. After washing the excess PAb-electric eel AChE out with PBS, the secondary antibody (goat anti-rabbit IgG, conjugated with peroxidase; 1:1000 dilution) was applied, and the chromogenic substrate solution (0.03 % 3,3′-diaminobenzidine tetrahydrochloride, 0.06 % H2O2, and 0.05 % CoCl2 in 0.15 M PBS, pH 7.4) was added. Next, the gill tissue was stained with H&E, paraffinized, and mounted with Permount.

2.9. Statistical analysis

All parameters were analysed using descriptive statistics (mean and standard deviation). Differences in enzyme activity in response to exposure time and tissue type were investigated by analysis of variance (ANOVA) and Tukey’s pairwise comparison using Minitab® 17 software (entitlement i.d.: 2ec6–c33). Differences were considered statistically significant at p < 0.05.

2.10. Detection of AChE expression in field conditions using immunoperoxidase

To study AChE expression in Nile tilapia under field conditions, fish were collected from the Huai-Saneang Reservoir, Surin Province (Latitude: 14.8024507 and Longitude: 103.5294035) over two seasons: rainy (wet) and summer (dry). The sampling stations in these fields cultivate
crops twice yearly (usually alternating between rice and other crop species). In each season, five fish were collected (n = 5) and kept cool during transport to the laboratory. The gills were dissected and collected. Protein was extracted, and its concentration was measured before use in Western blot, dot blot, and immunohistochemistry as per the laboratory study.

3. Results

3.1. Toxicity testing and morphological studies

After Nile tilapia exposure to dissolved glyphosate at different concentrations of 0, 10, 20, 30, and 40 µL L⁻¹, the morphological changes and cumulative mortality were examined at 24, 48, 72, and 96 h. Morphological changes included the loss of some scales, dark-coloured scales, torn tails and fins, and increased skin mucus. When the operculum was opened, bleeding was observed in the gills. At 72 and 96 h, we found that the fish began to move to the upper surface of the pond more than the control fish or those from earlier time points did. Gill area impairment was found, which was more abnormal than before, and it had started to tear. The gill teeth were also disordered (data not shown). Cumulative mortality increased with exposure time and glyphosate concentration, as shown in Fig. 1.

Minitab software was used to evaluate the LC₅₀ values using probit analysis. Each sample group contained 10 Nile tilapia, and each treatment was performed in triplicate. The LC₅₀ values at 24, 48, 72, and 96 h were 66.5614 (61.4033–73.2088), 56.7568 (53.1164–61.2718), 48.6190 (46.0765–51.6533), and 40.0988 (38.5289–41.8802) µL L⁻¹, respectively.

To study the AChE expression after exposure to sub-lethal concentrations of glyphosate, a glyphosate concentration of 2 µL L⁻¹ was selected for the toxicity test. The effects of glyphosate on Nile tilapia were evaluated at 0, 3, 6, 12, and 24 h after exposure to sub-lethal concentrations. They were separated into five phases: swimming, loss of equilibrium, respiratory function, pigmentation, and other clinical signs, which were referenced from the OECD [7]. Acute changes in swimming behaviour were observed after 3 h of exposure. We observed tumbling and hyperexcitability and an increased tendency to surface, surrounding swimming at 6–12 h. Fish were later lethargic and ceased swimming at 12–24 h.

Abnormal movement in fish with an off-balance after glyphosate exposure was observed compared with that of the control. At 3 h after exposure, the fish began tumbling, with partial balance loss. Fish behaviour changes increased with increasing exposure time and reached a steady state at 12 h exposure time. In addition, rapid respiration, including hyperventilation, was observed, with skin hyperpigmentation.

![Fig. 1. Morphological changes and cumulative mortality percentage of Nile tilapia exposed to glyphosate. The red circles indicate changes in the external characteristics of the glyphosate-exposed Nile tilapia compared to that of the control group.](image-url)
3.2. Cross-reactivity of PAb-electric eel AChE

A cross-reactivity test was performed to verify that commercial PAb-electric eel AChE (Bio-Rad; # 0200-0042) was appropriately used. In this study, the cross-reactivity test was performed by dot blot (Fig. 2-A) and Western blot (Fig. 2-B and 2-C) in Nile tilapia, according to Tha-nomtis et al. [9]. AChE was collected from the brains of the fish. The purified protein was confirmed to be AChE using column chromatography. Purified AChE was detected in the hybrid catfish brain with purified protein was confirmed to be AChE using column chromatography. Purified AChE was detected in the hybrid catfish brain with purified AChE indicated by an AChE band at a molecular weight of 71 kDa (protein content of 8 \(\mu\)g \(\mu\)L \(^{-1}\)). However, the drawback of the AChE purification process is that it is complicated and time-consuming. Thus, crude protein extracted from the brains of Nile tilapia was investigated. The protein patterns from the brain tissue showed different protein types. Proteins were separated by SDS-PAGE, blotted, and stained with PAb-electric eel AChE. It was found that PAb-electric eel AChE was specifically bound to AChE in Nile tilapia, and the immunoreactive protein bound was at 71 kDa of molecular weight. The results suggest that a higher amount of protein was required (10 \(\mu\)g \(\mu\)L \(^{-1}\)), and this crude protein was subsequently used to detect AChE expression in the fish brain (Fig. 2-C).

3.3. Enzyme assays

After crude protein extraction from each organ was determined, enzyme activity was measured to assess AChE expression. We found that AChE activity was affected by organ type and glyphosate exposure time \((p < 0.05)\). Enzyme activities in each studied organ were significantly different \((p < 0.05)\), with the highest enzyme activity found in plasma at 94.27 ± 1.20 \(\mu\)mol min \(^{-1}\) \(mg\) \(^{-1}\) protein.

For muscle, the highest enzyme activity was 90.15 ± 0.32 \(\mu\)mol min \(^{-1}\) \(mg\) \(^{-1}\) protein at 72 h, significantly different from all other exposure times \((p < 0.05)\). There was no significant difference in the enzyme activity in the gills among the different exposure times.

However, activity increased slightly with longer exposure times. Enzyme activity was 87.50 ± 0.87–91.15 ± 1.46 \(\mu\)mol min \(^{-1}\) \(mg\) \(^{-1}\) protein.

In the liver, enzyme activity was dramatically affected by exposure time. The lowest activity found was 19.73 ± 6.20 \(\mu\)mol min \(^{-1}\) \(mg\) \(^{-1}\), significantly different from other exposure times \((p < 0.05)\). However, the enzyme activities at 24 and 48 h after exposure were not significantly different, at 92.19 ± 2.88 and 84.52 ± 5.91 \(\mu\)mol min \(^{-1}\) \(mg\) \(^{-1}\) protein, respectively (Fig. 3).

3.4. Development of antibody technique to evaluate AChE expression

After the enzyme activities in plasma, muscle, liver, and gill were studied, the cross-reactivity of PAb-electric eel AChE in plasma, muscle, liver, and gill of Nile tilapia exposed to glyphosate at sub-lethal concentrations was studied by Western blot to determine AChE specificity. A positive cross-reaction was presented as a brown immunoreactive band at a molecular weight of 71 kDa. Dot blot using PAb-electric eel AChE at a dilution of 1:200 and incubation for 12 h with crude protein extracted from plasma, gill, muscle, and liver could detect AChE expression over a range of antigen concentration levels: 10, 5, 2.5, 1.25, 0.65, 0.31, 0.15, 0.08, 0.04, and 0.02 \(\mu\)g \(\mu\)L \(^{-1}\). Detection limits were 0.04 \(\mu\)g \(\mu\)L \(^{-1}\) in plasma, gill, and muscle and that in liver was 0.16 \(\mu\)g \(\mu\)L \(^{-1}\) (Fig. 6-A).

Proteins extracted from the plasma, muscle, liver, and gill of Nile tilapia after glyphosate exposure for 24, 48, 72, and 96 h were investigated and compared with that in the control using SDS-PAGE. Proteins extracted from the plasma, gill, muscle, and liver were 3.5, 10, 12, and 10 \(\mu\)g \(\mu\)L \(^{-1}\), respectively. After the protein was stained with Coomassie brilliant blue, several protein bands were visible at various sizes, and AChE bands were present at a molecular weight of 71 kDa.

AChE expression decreased with increasing glyphosate exposure time (Fig. 4–1B to 4–1E). At 1:200 a dilution in Western blot and dot blot analysis, AChE clearly showed immunoreactive bands at a molecular weight of 71 kDa (Figs. 4–2B to 4E) that were consistent across the plasma, gill, muscle, and liver. Therefore, plasma, gill, muscle, and liver are viable alternative tissues for monitoring AChE expression by immunoassays under field conditions. We suggest that gills, having the benefits of easy extraction and sample preparation and no fat interference, are ideal for identifying protein patterns because they have a sharp and visible protein band by SDS-PAGE (Figs. 4–1D).

Immunohistochemistry was used to evaluate the gills of Nile tilapia exposed to glyphosate compared with that of the control group. At sublethal concentrations, AChE expression was observed in the gill tissue at all exposure times. In addition, the gill tissue was swollen with increased secretion, causing the accumulation of blood cells. This
evidence indicated that the gill lamella and gill filaments were damaged (Fig. 5).

3.5. Field study

Since AChE expression could be identified with dot blot, Western blot, and immunohistochemistry in the laboratory, the detection of AChE using antibody techniques under field conditions was investigated. We decided to use the gill as the tissue sample for field condition analysis because of its ease of extraction and as it is the primary contact tissue for aqueous contaminants. AChE expression and morphological changes were analysed to monitor glyphosate contamination in the field study. The study area was the Huai-Saneng Reservoir, Meuang District, Surin Province, which is an agricultural area cultivated every season throughout the year. Thus, AChE expression was examined in both wet and dry seasons. AChE expression between the two seasons is shown in Table 2 and Fig. 6. The degree of difference for physiological changes, i.e., gill tissues and AChE expression, was higher in the dry season than in the wet season, as assessed by dot blot, western blot, and immunohistochemistry.

4. Discussion

4.1. Bioassay and toxicity testing

Currently, many methods are available for evaluating glyphosate contamination. In this study, we examined the toxicity levels of glyphosate on Nile tilapia in the laboratory to identify various effects, including toxicity levels that cause mortality and behavioural and morphological alterations. The expression of AChE in various tissues, especially the gills, was assessed using antibody techniques under laboratory and field conditions.

Ani et al. [12] suggested that glyphosate is an herbicide with low toxicity formulated from iso-propylamine. Glyphosate can affect
juvenile fish by causing mortality, behavioural alterations, loss of equilibrium, air gulping, hyperactivity, decreased opercula movement, erratic swimming, and jerky movements. All symptoms can lower the survival rate of the exposed organisms. In this study, the cumulative mortality rate of Nile tilapia increased with the exposure time. The LC$_{50}$ mortality data differed from the results found in the study by Ani et al. [13], who examined the acute toxicity of glyphosate on juveniles of Clarias gariepinus. The acute toxicity bioassay was conducted to determine LC$_{50}$ values at 96 h following the probit analysis method. In comparison, the safe level of the test pesticide was estimated by multiplying the LC$_{50}$ values at 96 h with different application factors. The LC$_{50}$ values (with 95 % confidence limits) at 24, 48, 72, and 96 h, estimated by Probit analysis, were 34.72 (31.02–37.20), 31.90 (28.12–33.89), 27.40 (24.98–29.30), and 24.60 (21.95–26.54) mg L$^{-1}$, respectively. There were significant differences ($p < 0.05$) in LC$_{50}$ values obtained at different exposure times [13].

Moreover, the toxicity results for Nile tilapia after exposure to glyphosate were different from those of three sturgeon species (Huso huso, Acipenser stellatus, and A. persicus) under laboratory conditions. The results showed that increasing glyphosate exposure time, up to 168 h, led to a lower LC$_{50}$ concentration of glyphosate for H. huso, A. stellatus, and A. persicus at 26.4, 23.2, and 27.5 mg L$^{-1}$, respectively [14]. At 96 h post-exposure to at least 50 mg L$^{-1}$ glyphosate, mortality is observed in all the treatments. Clinical signs of the affected fish included increased mucous secretion, dark skin, and gasp dead [15]. Different glyphosate forms are used, including power and solution forms, which could affect the onset of action and clinical signs differently. However, our study used a solution form because it is commercially available in Thailand. All the evidence above shows differences in the toxicity of glyphosate for different fish species and different glyphosate forms used; however, they show a fairly consistent set of fish responses and a trend of increased lethality with exposure time, which is consistent with our findings.

### 4.2. Behaviour and morphology

Behavioural alterations in fish exposed to toxic herbicides have been reported, including loss of schooling behaviour, surfacing,
hyperactivity, erratic swimming, seizures, loss of buoyancy, high cough, mucus secretion in the gill, flaring of the gill arches, head shaking, and restlessness before death [16]. Therefore, evidence of changes in fish behaviour was examined to evaluate toxic substance exposure from contamination in aquatic environments. Yang et al. [17] reported that the last key parameter for assessing the effect of environmental stress is behaviour responsiveness in certain organisms.

Behavioural and morphological changes included dark and fallen scales, tail and fin damage, mucus soaking, and gill bleeding. At 72 h and 96 h, fish moved to the pond surface owing to gill impairment, consistent with the results of Thanomsit et al. [18], who reported the effects of glyphosate on Asian sea bass (*Lates calcarifer*), which performed erratically swam, lacked balance, and had faded body and gill colour.

In this study, the effects of exposure were measured according to the following five categories: swimming, loss of equilibrium, respiratory function, pigmentation, and other clinical signs. For acute toxicity, changes in behaviour first presented as altered swimming after a 3 h exposure time. At 6–12 h, tumbling and hyperexcitability with surfacing, surrounding, and erratic swimming were observed. After exposure for 12–24 h, the fish were lethargic, weak, and ceased swimming, in accordance with the findings of Ani [13]. A positive correlation was found between fish mortality and both glyphosate concentration and exposure time.

### 4.3. Enzyme activity and cross-reactivity of AChE in Laboratory

AChE is an important enzyme for controlling neuronal activity and is sensitive to organophosphate inhibition. AChE from fish brains was less sensitive to organophosphate inhibition than that from terrestrial animals. Although this sensitivity is known to vary among species, it has not been evaluated in fish [19]. Many forms of AChE molecules can anchor to the membrane, ensuring the immobilisation necessary for the enzyme activity found in synapses and the motor end-plate. AChE purification is an essential step to prepare carefully; therefore, the non-specific binding of esterase activity should affect false-positive result analysis [19]. Modesto and Martinez [20] reported a significant decrease in AChE enzyme activity at 96 h post-exposure to Round up® in the fish brain (*Prochilodus lineatus*) and at 32 h post-exposure in fish muscle. Therefore, AChE activity was used to evaluate glyphosate exposure in this
study. The AChE activity in Nile tilapia significantly differed among the tested organ types ($p = 0.05$). The highest AChE activity was found in plasma at $94.27 \pm 1.20 \mu$mol min$^{-1}$ mg$^{-1}$ protein.

Shankar [21] highlighted the importance of antibody-based techniques for evaluating health management measures, including diagnosis, identification, stereotyping, antigen characterisation, epidemiology, and vaccines. A previous study developed a conventional polyclonal antibody and antigen preparation process using rabbit specimens for disease diagnosis, stereotyping, and other biological applications. Therefore, to assess AChE expression, polyclonal antibodies were directly used for dot blot, Western blot, and immunohistochemistry in Nile tilapia gills, muscle, liver, and plasma. The detection limits were shown in different tissues by the dot blot analysis. The value for plasma, gill, and muscle was $0.04 \mu$g mL$^{-1}$, while it was $0.16 \mu$g mL$^{-1}$ for the liver. Western blot analysis showed that hybridisation from a commercial polyclonal antibody with Nile tilapia AChE was specific. As found from SDS-PAGE, the 71 kDa band of AChE was immunoreactive in different tissues, such as plasma, gill, muscle, and liver, similar to the results of AChE expression in Golden apple snail ( Pomacea caloculata ) [22].

In this study, the gills were selected to investigate AChE expression. Generally known as a multifunctional organ, fish gills are considered interesting for study by many researchers. Despite a significant amount of work done on the morphological examination of gills, this organ is relatively underused in health evaluation in fish [23]. Glyphosate-exposed Nile tilapia gills were swollen with increased secretion and accumulation of blood cells, with lesion severity increasing with exposure time to glyphosate. During microscopic localisation analysis, AChE expression was found in both the control and treated groups in the gill lamellae and filaments. The physical and chemical alterations of fish gills being sensitive to the prevalent surrounding conditions like temperature, pH, salinity, and heavy metals allow the gill tissue to be used as a biological indicator for tracking waterborne toxicants [24] relevant to our study in both laboratory and field conditions.

### 4.4. AChE in the field

The study of pesticide contamination in water sources has recently received increasing attention. The increased use of pesticides leads to the contamination of aquatic environments and accumulation in aquatic animals, which can pass up to humans as the highest consumer. Many researchers are interested in studying these problems. For example, Hansson [25] evaluated AChE activity in Perca fluviatilis collected from a contaminated area in Stockholm, Sweden. Fish samples were taken from 10 stations with AChE activity being highest at non-contaminated sites and lowest at the most contaminated sites. Anandhan et al. [26] reported that the highest activity was observed in the brain, followed by the muscle, gill, liver, and kidney in two freshwater bony fishes in India: Channa striatus and Orectochromis mossambicus. In this study, immunological techniques studied AChE expression and localisation in the Nile tilapia gills. We selected the Huai-Saneng Reservoir, Meuang District, Surin Province, for the field study. Thanomset et al. [8] identified that the Huai-Saneng Reservoir is very important to residents and nearby areas for both food security and the economy. Moreover, the fishery in that reservoir can be harvested year-round because the area was very fertile. Thus, AChE expression was examined in both wet and dry seasons. AChE expression was observed in both seasons, but the level of AChE expression in the dry season was higher than that in the wet season. The use of multiple techniques was effective in studying AChE expression in fish gills. Therefore, this study provides a useful array of antibody techniques for assessing pesticide exposure in Nile tilapia, particularly glyphosate, and perhaps in other fish species and water sources.

## 5. Conclusion

Glyphosate-based herbicides are highly toxic to Nile tilapia and exhibit a strong dose-effect relationship. In this study, the toxic effects of glyphosate were determined in terms of concentration and exposure time. Our results revealed that concentration and exposure time caused behavioural responses through AChE expression in Nile tilapia. A process to measure glyphosate exposure in live Nile tilapia was presented for sublethal conditions. The correlation analysis showed that toxicity levels, behavioural alterations, and AChE expression in the plasma detected by the antibody technique, gill, liver, and muscle of Nile tilapia were significantly correlated with glyphosate concentration and exposure time. Although all the studied tissues exhibited AChE expression, we decided to study the gills instead of other tissues because fish were not sacrificed, and gills could be used as a representative tissue for glyphosate exposure. Finally, antibody techniques comprising dot blot, western blot, and immunohistochemistry provided a useful method for the evaluation of AChE expression under both laboratory and field study conditions.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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