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Evaluation of Alachlor Herbicide Impacts on Nile Tilapia (*Oreochromis niloticus*) using Biochemical Biomarkers

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Herbicide application as a part of agricultural processes may have impacts on nontarget organisms, especially aquatic lives and their environment. Often as a component of sustainable agriculture in tropical countries, fish farming is practiced on farmlands. Thus, to supplement risk assessment studies, it is important to obtain information on the effects these xenobiotics have on local species.

Alachlor (2-chloro-*N*-[2,6-diethyphenyl]-*N*-[methoxymethyl)acetamide]) is one of the most widely used chloroacetanilide herbicides for the control of annual grasses and many broadleaf weeds. Its extensive use and its persistence in ground and surface water have posed potential risks for human exposure, not only to professional applicators and farmers, but also to general populations. There have been numerous studies on alachlor, but much less investigation of its effects on fish and aquatic species. Alachlor has been listed as carcinogenic and highly toxic to humans despite slight acute toxicity (Orme and Kegley, 2005). Thus, many countries have banned alachlor from their markets.

The main objective of this study was to evaluate the effects of alachlor herbicides on relative activities of acetylcholinesterase (AChE) and glutathione-S-transferase (GST) in various tissues of Nile tilapia (*Oreochromis niloticus*). Nile tilapia, a local fish species of high economic importance, is a benthic omnivorous cichlid commonly found in fish ponds and streams of tropical countries, including Thailand. The effects of short-term contaminant exposure, measured as the biochemical and physiologic responses in Nile tilapia, may be used to assess and monitor alachlor contamination in agricultural settings.

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Materials and Methods

Healthy 3-month-old Nile tilapia (*O. niloticus*) weighing 10 to 12 g were purchased from a commercial hatchery in Bangkok, Thailand. Acclimatization of the fish to laboratory conditions for 2 weeks was allowed using dechlorinated tap water that had the following physicochemical characteristics: temperature (26 ± 2°C), pH (7.2–8.0), total hardness (50–70 mg/L as calcium carbonate [$CaCO_3$]), alkalinity (60–65 mg/L), and conductivity (190–210 μmhos/cm). Chlorine residual and ammonia were below detection limits. These parameters were measured according to the experimental procedures described in the Standard Methods for the Examination of Water and Wastewater (APHA, 1998). The fish were fed twice daily with 38% protein commercial fish food (CP-Pet; Pokphand Animal Food Co. Ltd., Bangkok, Thailand). The quantity of food was 2% of the initial body weight per day.

Alachlor (48% W/V EC) was purchased from Monsanto, Bangkok, Thailand. The commercial formulation of alachlor (48%W/V EC) was used in this experiment to simulate a field condition because it is the commercial preparation of alachlor used in agriculture. The alachlor preparation was added to the aqueous test solution at a concentration of 381.9 μg/L alachlor (96-h LC_{50}) for acute toxicity testing, and at a concentration 175 μg/L alachlor (50% maximum acceptable toxicant concentration, MATC) for subacute toxicity testing (Peebua, 2005). During the exposure period, for both acute and subacute toxicity tests, the test solution

was renewed every 2 days, and the presence of alachlor in the test water was checked with gas chromatography using an electron capture detector (GC/ECD; Agilent 6890/7683, Agilent Technologies, Santa Clara, CA, USA).

In the acute toxicity test, Nile tilapia were exposed to a constant concentration of 381.9 µg/L alachlor (96-h LC_{50}) for 96 h. Dead fish were collected and discarded daily, and only the living fish were sampled for subsequent enzyme assays. Nine fish were collected from the treatment tank after 24, 48, 72, and 96 h of pesticide exposure. The fish then were rinsed with distilled water and anesthetized in MS222 solution. Serum and tissues of the fish (e.g., brain, liver, kidney, gills, and muscle) were dissected quickly on a cold plate, weighed, and stored frozen (–80°C) for subsequent analysis.

After exposure to 381.9 µg/L alachlor for 96 h, a recovery period was allowed as the fish were transferred to clean water. At 24, 48, 72, and 96 h in a recovery period, nine fish were removed for tissue collection as described earlier.

In subacute toxicity testing, the fish were exposed to a constant concentration of 175 µg/L alachlor (50% MATC) for 1 month. At 2 and 4 weeks, 20 fish were removed. Fish serum and tissues were obtained and stored at –80°C for subsequent chemical and biochemical analysis. The alachlor in the samples was analyzed according to US EPA RCRA SW-846 method 3620 and 8080A (US EPA, 1987).

Relative AChE enzymatic activity was determined according to the method previously described (Ellman et al. 1961), with an adaptation to a microplate system using 25 μL of sample homogenate, 75 μL of phosphate buffer (0.1 M, pH 7.5), 25 μL of 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) (0.01 M), and 25 μL of acetylthiocholine iodine (0.075 M). The reaction was initiated by adding the substrate at 27 ± 1°C, and the color development was recorded continuously for 5 min at 412 nm in a spectrophotometer (Bio-Rad Model 3550 plate reader, Bio-Rad Laboratories, Hercules, CA, USA). The relative AChE activity then was calculated as µmol/mg of protein/min.

Relative GST enzymatic activity was determined according to the method previously described (Habig et al. 1974), with an adaptation to a microplate system using 30 μL of sample homogenate, 30 μL of 1-chloro-2,4-dinitrobenzene (CDNB) (0.1 mM), 30 μL of glutathione (GSH) (0.1 mM), and 300 μL of phosphate buffer (1 M, pH 6.5). The plate was preincubated for 3 min at 30°C and read at 340 nm (Bio-Rad Model 3550 plate reader). The assay was run at 30°C, and the activity was monitored for 5 min. Relative GST activity was expressed as µmol/mg of protein/min.

Protein concentration was spectrophotometrically estimated with concentrated Coomassie Brilliant Blue (G-250) according to the method previously described (Bradford, 1976). Then the relative protein concentration was measured at the 595 nm.

All data were presented as means ± standard deviation. Variations in AChE and GST activities were tested by one-way analysis of variance (ANOVA), followed by Tukey-Honest Significant Difference (HSD) as a post hoc comparison of means post hoc Tukey–Kramer test. The level of significance was set at 0.01 and 0.05.

Results and Discussion

Biomarkers have been used increasingly not only as effective tools for environmental monitoring, but also as early warning sentinels in ecologic risk assessment (Adams et al. 2000). Biomarkers are useful indicators of pollution because they are sensitive and usually show detectable signs of sublethal stress response in an organism exposed to a toxic substance (Chambers and Boone 2002). Two biomarkers, AChE and GST, have been used extensively in several organisms because they are induced when the organisms are exposed to toxic substances or environmental contaminants (El-Merhibi et al. 2004; Roche et al. 2003; Zhang et al. 2004).

In the acute toxicity study, the relative activities of both AChE and GST in serum, brain, kidney, and gills of *O. niloticus* exposed to 381.9 µg/L of alachlor were not significantly different from those of fish from the control. However, fluctuations of AChE and GST were observed in liver and muscle (Table 1). A significant increase in AChE activity occurred within the first 24 h after the exposure ($p < 0.01$). During the induction period, the level of increment was not altered with the exposure time. However, at the end of the experiment (96 h after exposure to alachlor), the activity level resumed to the standard level and remained unchanged for the rest of the experiment, including the recovery period ($p > 0.01$).

During the recovery period (Table 1), the fish exposed to a constant concentration (381.9 µg/L of alachlor) for 96 h and transferred to clean water resumed the AChE activity in all the sampled tissues to the control level. This normalization event could be the result of an induced biotransformation of alachlor in fish, which still needs detailed investigation. The literature contains little information on the recovery of AChE activity after alachlor herbicide intoxication. *In vivo* studies have indicated a period of 1 week for recovery of brain AChE activity in the fish *Sarotherodon mossambicus* after thiobencarb intoxication (Babu et al. 1989). It is known that the time required for recovery is a function of toxicant concentration and exposure time.

On the other hand, when the fish were exposed to a lower concentration of the toxicant (175 µg/L of alachlor), but with a longer exposure time, substantial changes in the AChE level in blood serum, brain, kidney, and gills were observed, but not in liver and muscle (Table 2). Thus, a

Table 1 Relative acetylcholinesterase (AChE) and glutathione-S-transferase (GST) activities (μmol/min/mg protein) in tissues of Nile tilapia (*Oreochromis niloticus*) exposed to 381.91 μg/L of alachlor

Time (h)		AChE activity		GST activity	
		Liver	Muscle	Liver	Muscle
Exposure period	0	0.42 ± 0.23	0.30 ± 0.09	2.15 ± 1.05	1.07 ± 0.28
	24	0.35 ± 0.10	0.45 ± 0.12[a]	2.15 ± 0.58	0.89 ± 0.18
	48	0.67 ± 0.39	0.44 ± 0.10[a]	3.13 ± 0.68	0.87 ± 0.14
	72	0.70 ± 0.27	0.42 ± 0.12[a]	3.50 ± 1.24[a]	0.81 ± 0.10[a]
	96	0.72 ± 0.35	0.31 ± 0.05	3.75 ± 1.46[a]	0.68 ± 0.15[a]
Recovery period	24	0.69 ± 0.17	0.38 ± 0.07	2.95 ± 1.51	0.69 ± 0.16[a]
	48	0.62 ± 0.07	0.34 ± 0.15	3.83 ± 1.49[a]	0.77 ± 0.25[a]
	72	0.59 ± 0.17	0.31 ± 0.10	3.48 ± 0.17[a]	0.74 ± 0.19[a]
	96	0.48 ± 0.25	0.32 ± 0.07	3.15 ± 0.09	0.83 ± 0.22

Note: Values are expressed as means ± standard deviation (n = 9)

[a] Values significantly different from controls by one-way analysis of variance (ANOVA) (Tukey-HSD) ($p < 0.01$)

Table 2 Relative acetylcholinesterase (AChE) and glutathione-S-transferase (GST) activities (μmol/min/mg protein) in tissues of Nile tilapia (*Oreochromis niloticus*) exposed to 175 μg/L of alachlor

Tissues	AChE activity				GST activity			
	2 Weeks		4 Weeks		2 Weeks		4 Weeks	
	Control	Exposed	Control	Exposed	Control	Exposed	Control	Exposed
Serum	0.34 ± 0.09	0.19 ± 0.03[a]	0.36 ± 0.17	0.46 ± 0.11[a,b]	1.05 ± 0.19	1.12 ± 0.17[a]	1.06 ± 0.04	1.69 ± 0.17[a,b]
Brain	0.19 ± 0.04	0.25 ± 0.04[a]	0.20 ± 0.06	0.27 ± 0.12[a,b]	0.84 ± 0.13	0.88 ± 0.18[a]	0.84 ± 0.01	0.98 ± 0.19[a]
Liver	0.43 ± 0.16	0.43 ± 0.22	0.43 ± 0.10	0.45 ± 0.22	2.15 ± 0.14	2.56 ± 0.13[a]	2.18 ± 0.15	3.08 ± 0.05[a,b]
Kidney	0.29 ± 0.12	0.34 ± 0.13[a]	0.29 ± 0.14	0.34 ± 0.25[a]	0.87 ± 0.14	1.34 ± 0.41[a]	0.91 ± 0.17	1.36 ± 0.08[a]
Gill	0.28 ± 0.06	0.35 ± 0.12[a]	0.29 ± 0.02	0.27 ± 0.07[b]	1.48 ± 0.17	2.55 ± 0.14[a]	1.52 ± 0.11	2.73 ± 0.08[a]
Muscle	0.30 ± 0.19	0.33 ± 0.11	0.30 ± 0.12	0.29 ± 0.11[b]	1.07 ± 0.19	1.01 ± 0.25[a]	1.12 ± 0.27	0.87 ± 0.17[a]

Note: Values are expressed as means ± standard deviation (n = 20)

[a] Value significantly different from controls by one-way analysis of variance (ANOVA) (Tukey-HSD) ($p < 0.05$)

[b] Significant difference between the relative activities measured 2 and 4 weeks after exposure to alachlor

physiologic study on the recovery of AChE activity after a long exposure to a subacute toxicity may be worth pursuing.

Relative GST activity in muscle after the fish had been exposed to 381.91 μg/L alachlor for 96 h was decreased ($p < 0.01$). The reduction of relative GST activity in muscle occurred 72 h after exposure to alachlor (Table 1). The reduction in the activity persisted even after the treated fish had been transferred to clean water. Then it appeared to diminish, with the activity resuming in 96 h of the recovery period.

On the other hand, the relative GST activities in liver were increased 72 h after exposure to alachlor because the liver is the major detoxification organ in the body. The induction of GST in the liver resumed to a control level in 96 h of the recovery period.

The relative AChE and GST activities in the subacute toxicity test (175 μg/L alachlor) are presented in Table 2. No mortality was observed. The AChE activities in the blood serum and brain of Nile tilapia 4 weeks after exposure to alachlor were significantly increased compared with the control level, and the values were measured 2 weeks after exposure ($p < 0.05$). The activities of AChE in muscle, liver, kidney, and gills remained largely unchanged at both 2 and 4 weeks after exposure to alachlor, as compared with the control level ($p > 0.05$). The GST activities in all tissue samples were affected 2 weeks after exposure. The activities in blood serum, brain, liver, kidney, and gills were significantly elevated, as compared with the control level ($p < 0.05$). Only the GST activity in the muscle significantly decreased ($p < 0.05$). At 4 weeks after exposure, the GST activities in blood serum, brain, liver, kidney, and gills remained elevated, as compared with the control level ($p < 0.05$), possibly because of detoxification processes related to GST activities in the treated fish.

In Nile tilapia, it seemed that a significant induction of AChE in the muscles was observed in the acute toxicity

study (Table 1). In blood serum, we observed no apparent acute toxicity of relative AChE activities in the Nile tilapia exposed to the sublethal concentration of alachlor. However, the subacute toxicity of alachlor appeared as an inhibition of AChE in serum observed during the first 2 weeks after exposure (Table 2). After that, the activity resumed and was substantially increased 4 weeks after exposure (Table 2). Thus, the exposure duration and the concentration of alachlor seemed to interact on the serum AChE level.

Differences in the rate of changes in GST activities may lead to the different degree of damages observed in the tissues of the treated fish. The activity of hepatic GST, which plays a major role in detoxifying xenobiotics, generally is higher in fish exposed to the higher toxicant concentrations than in those exposed to the lower concentration (Qian et al. 2004). As a result, the liver cell injury apparently is greater in fish exposed to a low concentration of the toxicant during a prolonged period because a correlation between liver GST activity and levels of xenobiotic substance have been shown in other species (Petrivalksy et al. 1997).

There possibly is a mismatch in the kinetics of induction in phase 1 biotransformation and phase 2 (conjugation/detoxification) systems. Different reactions were observed possibly because of the different enzymatic biotransformations within each tissue (Stroomberg et al. 2004). Certain biotransformation phase 1 and 2 metabolic enzymes, such as cytochrome P-450, glutathione, and GST, are under the regulatory control of steroid hormones in some fish. Phase 1 is the predominant biotransformation pathway. It generally involves the addition or exposure of functional groups to the xenobiotic, for example, by oxidation or hydrolysis.

The most extensively examined system, from the viewpoint of biomarkers, is the oxidase system, which involves oxidation by varied isoenzymes of cytochrome P-450. The phase 2 reaction is a biosynthetic reaction, in which the foreign compound or a phase 1–derived metabolite is covalently linked to an endogenous molecule, depending on the organism species, environmental conditions, and substrate (Sipes and Gandolfi 1991). According to this study, therefore, the observed induction of GST activities may be related to the elevated activity of AChE because alachlor has been known to modify the fatty acid profile of the exposed organism.

In conclusion, the relative activities of AChE and GST in Nile tilapia (*O. niloticus*) exposed to alachlor could be used in combination as a biomonitoring tool to assess the hazardous effects of alachlor on an aquatic nontarget organism. This study also may contribute to an understanding about the stress physiology of fish exposed to xenobiotics in the aquatic environment of agricultural settings.

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