www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 17: 821-832 (2017) DOI: 10.4194/1303-2712-v17_4_18

PROOF RESEARCH PAPER

Organ Specific Histopathology and Brain Acetylcholinesterase Inhibition in Rohu, *Labeo rohita* and Silver Barb, *Barbonymus gonionotus*: Effects of Three Widely Used Organophosphate Pesticides

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Received 09 November 2016
Accepted 24 January 2017

Abstract

Agricultural pesticides, eventually find their ways to aquatic ecosystems by different routes adversely affect the aquatic biota. To determine its potential hazards, acute toxicity tests (LC_{50}) of three commonly used organophosphate pesticides, Envoy 50SC, Samcup 50EC and Dursban 20EC on *Labeo rohita* and *Barbonymus gonionotus* were performed. The LC_{50} ($P<0.05$) of these pesticides were estimated at 0.110 (0.060-0.199), 0.217 (0.204-0.231) and 0.079 (0.073-0.084) ppm for *L. rohita* and 0.471 (0.440-0.500), 0.789 (0.754-0.824) and 0.273 (0.260-0.286) ppm for *B. gonionotus*, respectively. Pesticides abruptly altered the normal structures in various fish organs like gills, kidney and liver. The major alterations included missing gill lamellae, gill clubbing, fungal granuloma, fatty degeneration, lipid droplet formation, degenerating glomeruli and kidney tubules, hyperplasia, hemorrhage, pyknosis, increased number of vacuoles, and necrosis. Envoy 50SC, Samcup 20EC and Dursban 20EC showed significant inhibition on *L. rohita* AChE activity at 216.7±11.0, 207.3±5.0 and 146.7±5.5 nmol/min/mg protein, respectively. In *B. gonionotus*, Samcup 20EC and Dursban 20EC showed significant inhibition ($P<0.05$), which were recorded as 242.0±6.6 and 221.7±60.3 nmol/min/mg protein, respectively. Furthermore, pesticide treated *L. rohita* showed higher enzymatic inhibition (51.49%) than *B. gonionotus* (19.60%).

Keywords: Acute toxicity; gills; kidney and liver.

Introduction

The rapid growths of the human population and extensive habitat modification have imposed an increasing threat in several aquatic ecosystems. These result in drastic modification in normal physiological activities of different aquatic organisms, and making them endangered in the wild. Although there are large scale studies going all over the world to save endangered fish species (Rakhi, Reza, Hossen, & Hossain, 2015; Hossen, Reza, Rakhi, Takahashi, & Hossain, 2014; Reza, Rakhi, Hossen, Takahashi, & Hossain, 2013), it is imperative to estimate the causes of deterioration of these valuable aquatic resources. To support the growing human population and meet the constant demand for stable crop production, massive use of pesticides has turned out to be the constant practice in many countries. However, the benefits of pesticides are not without consequences. Pesticides also have adverse health effects on human by interfering into the food chain (Scholz *et al*., 2003; Storrs & Kiesiecker, 2004; Kannan, Ridal, & Struger, 2006). It is estimated that over 98% of sprayed insecticides and 95% of herbicides reach a destination other than their target species (Miller, 2004). If initiatives are not taken immediately, it could be a major alarming issue for the ecological community in near future.

Among four pesticide groups, the use of organochlorine pesticides have been replaced by organophosphate (OP) and carbamate (C) because of the former's longer persistence and accumulation in the environment (Colovic,´ Krstic,´ Ušc´umlic,´ & Vasic,´ 2011). Since last 50 years, using of OP and C has been increased to control a variety of insects in agriculture and the household environment (Cox, 1992). These pesticides, used in agricultural field eventually find their ways in aquatic ecosystems by different routes. Especially, aquatic habitats near low lands and organisms cultured in integrated farming systems are much more prone to pesticide exposure. Though insecticides are considered necessary to control pests in several commercial crops, they pose toxic threats to non-target organisms in different ecosystems (Bretaud, Toutant, & Saglio, 2000). Therefore, fish species used in integrated farming system are thought to be the target of pesticide toxicity.

Histopathology of different organs and acetylcholinesterase (AChE) activity have been used

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as biomarkers in recent studies to detect biological effects of different toxic compounds on aquatic species (Capkin, Boran, & Altinok, 2014; Kavitha & Rao, 2008; Magni *et al*., 2006; Rakhi, Reza, Hossen, & Hossain, 2013). It is also used to determine early warning signs of disease and detect long-term injury in cells, tissues, or organs (Peuranen, 2000; Marchand, Van Dyk, Pieterse, Barnhoorn, & Bornman, 2009).

AChE is the enzyme found in cholinergic pathways in the central and peripheral nervous systems and is also used in synaptic transmission (Colović, Krstić, Lazarević-Pašti, Bondžić, & Vasić, 2013). It degrades the neurotransmitter acetylcholine, and produces choline and an acetate group in both vertebrates and invertebrates (Varo, Amat, & Navarro, 2008). AChE is blocked by inhibitors like organophosphate and carbamate, which results to the excessive acetylcholine accumulation in the synaptic cleft. This would eventually cause neuromuscular paralysis and may lead to death of the organism by asphyxiation (Nunes, Carvalho, & Guilhermino, 2005; Purves *et al*., 2008; Xuereb, Chaumot, Mons, Garric, & Geffard, 2009). In this study, the effects of OPs on *Labeo rohita* and *Barbonymus gonionotus* have been determined by assessing the AChE inhibition in the brain and histopathological abnormalities in different organs.

Materials and Methods

Determination of LC_{50}

To determine the effects of OP pesticides, three widely used OPs, Envoy 50SC, Samcup 50EC and Dursban 20EC were collected from authorized dealer at Mymensingh, Bangladesh. Agricultural recommended doses were calculated for each at 0.108, 0.218 and 0.087 ppm, respectively by considering general water level of 6 inch in paddy field. Properly cleaned glass aquaria in three replications were filled with 50 L of tap water. In every aquarium, 10 acclimated *L. rohita* and *B. gonionotus* (averaging 4.0±0.5 cm and 3.2±0.06 gm) were placed. *L. rohita* and *B. gonionotus* were treated separately with pesticides by maintaining a control for each. During the experiment, temperature, dissolved oxygen (DO) and pH were recorded as 27.0±3.0 °C, 7.5±1.0 ppm and 9.25±2.1, respectively. After pesticide exposure, dead fishes were removed and mortality was recorded at 6, 12, 24, 48, 72 and 96 h of exposure time. The LC_{50} value of three OPs for *L. rohita* and *B. gonionotus* were determined through acute toxicity tests.

Histopathological Study of OPs Treated Fish

L. rohita and *B. gonionotus* were exposed to Envoy 50SC, Samcup 50EC and Dursban 20EC at and below agricultural recommended doses in glass aquaria and maintained for seven days. Control groups were maintained in pesticide free water. Following exposure, fish were collected and dissected. Gills, liver and kidney were collected and preserved in 10% neutral buffered formalin for further analysis. The preserved samples were then dehydrated, cleaned and infiltrated in an automatic tissue processor (ThermoFisher Scientific, Waltham, MA, USA), embedded in melted paraffin wax, and sectioned (5 µm) using a microtome machine (Leica Junc 2035, Leica Microsystems Srl, Milan, Italy). The sections were then stained with hematoxylin and eosin (H and E) stains. After staining, the sections were mounted with Canada balsam and kept overnight for the permanent slide. Pictures were taken using a photomicroscope (OPTIKA B-350). The extent of alteration was scored as severe (+++), moderate (++), mild (+), and not found (−). When a pathology occurred in >50% cell or area in maximum investigated slides, it scored severe (+++), followed by >25% for moderate (++), and <25% for mild (+).

AChE Activity Measurement of OPs Treated Fish

L. rohita and *B. gonionotus* were exposed to three different pesticides treated water in glass aquaria at two different concentrations (Envoy 50SC:0.058 ppm and 0.108 ppm; Samcup 50EC:0.108 ppm and 0.215 ppm, Dursban 20EC: 0.043 ppm and 0.087 ppm) for 10 days. Fish exposed to pesticide-free water were kept as control. Following exposure, three fishes were taken from each aquarium (n=9) for each pesticide. As AChE is maximally distributed in the brain of teleost (Kopecka, Rybakowas, Baršiene, & Pempkowiak, 2004; Ferenczy, Szegletes, Bálint, Ábrahám, & Nemcsók, 1997), brain samples were used to measure AChE activity. Brains were dissected and were placed in ice-cold 0.1-M sodium phosphate buffer (pH 8.0). Tissues were then homogenized using a glass-Teflon homogenizer in a homogenization buffer (0.1-M sodium phosphate buffer, 0.1% Triton X-100, pH 8.0) to achieve the final concentration of 20 mg tissue/ml phosphate buffer. Tissue homogenate was centrifuged at 10,000×g for 15 min at 4°C, and the supernatant was removed. An aliquot of supernatant was then removed and measured for protein concentration according to the method of Lowry, Rosebrough, Farr, and Randall (1951) using bovine serum albumin as a standard.

AChE activity in the fish brain was measured according to the method of Ellman, Courtney, Andres, and Featherstone (1961), as optimized by Habig, Di Giulio, and Abou-Donia (1988) and Sandahl and Jenkins (2002). Tissue homogenate (50 µl) was added to 900 µl of cold sodium phosphate buffer (0.1 M containing 0.1% Triton X-100, pH 8.0) and 50 µl of 5,5-dithiobis (2-nitrobenzoic acid) (6 mM), then vortexed, and allowed to stand at room temperature for 10 min. Aliquots of 200 µl in triplicate were then placed into microtiter plate wells. The reaction was

started with the addition of 50 µl of acetylthiocholine iodide (15 mM) specific for fish (Jash, Chatterjee, & Bhattacharya, 1982). Changes in absorbance were measured with a microplate reader (SpectraMax 340PC384, Molecular Devices LLC, Sunnyvale, CA, USA) at 412 nm for 10 min at 12 s intervals. The rates were calculated as follows:

$$R=5.74\ (10^{-4})\ \Delta A/C_0$$

Where, R = rate in moles substrate hydrolyzed per min per g of tissue;

ΔA=change in absorbance per min;

C_0=original concentration of tissue.

AChE activity was calculated as nmol/min/mg protein.

Statistical analysis

Data obtained from the acute toxicity tests were evaluated using the Probit Analysis Statistical Method. The LC_{50} values (with 95% confidence limits) were calculated and the significance level between the LC_{50} values and the different exposure times was analyzed using a $\chi 2$ test. The statistical data analysis was carried out using SPSS (SPSS, Chicago, IL, USA) version 16.0. Data of enzymatic inhibition of AChE activity were analyzed using one way analysis of variance (ANOVA) and expressed as mean ± SD. A post hoc Waller Duncan multiple test range was performed considering a 5% significant level using SPSS ver. 11.5 computer software program.

Results

LC_{50} of three OPs for *L. rohita* and *B. gonionotus*

After 96 h exposure, LC_{50} of Envoy 50SC, Samcup 50EC, Dursban 20EC for *L. rohita* and *B. gonionotus* were calculated and presented in Table 1.

Histopathological Observations of *L. rohita* and *B. gonionotus* Exposed to Envoy 50SC

In *L. rohita*, moderate gill clubbing, hemorrhage, pyknosis were observed at 0.058 ppm (Figure 1a), whereas in *B. gonionotus* the gills were found in almost normal condition except some missing of secondary gill lamellae (Figure 2a). On the contrary, severe gill clubbing, hemorrhage, pyknosis, hyperplasia, missing of secondary gill lamellae were observed for *L. rohita* at dose of 0.108 ppm (Figure 1b), while moderate missing of secondary gill lamellae and pyknosis were found when *B. gonionotus* were treated with the same dose of Envoy 50SC (Figure 2b).

Mild changes in vacuole, hemorrhage, fatty degeneration were found in the liver tissues from *L. rohita* treated with 0.058 ppm Envoy 50SC (Figure 1c), whereas moderate hemorrhage, fatty degeneration, lipid droplets were observed for the same fish species at 0.108 ppm (Figure 1d). In *B. gonionotus*, mild hemorrhage and lipid droplets were

Table 1. LC_{50} of *L. rohita* and *B. gonionotus* exposed for 96 h to Envoy 50SC, Samcup 50EC, Dursban 20EC

Species	Envoy 50SC				Samcup 50EC				Dursban 20EC			
	Conc. (ppm)	No. of dead fish	LC_{50} (P<0.05)	$\chi 2$ value	Conc. (ppm)	No. of dead fish	LC_{50} (P<0.05)	$\chi 2$ value	Conc. (ppm)	No. of dead fish	LC_{50} (P<0.05)	$\chi 2$ value
	0.00	0			0.00	0			0.00	0		
	0.033	0			0.1	0			0.04	0		
	0.066	0			0.134	2			0.052	6		
	0.05	6			0.167	8			0.067	10		
L. rohita	0.133	18	0.110 (0.060-0.199)	21.382	0.2	12	0.217 (0.204-0.231)	4.188	0.081	13	0.079 (0.073-0.084)	6.997
(n=30)	0.167	22			0.234	15			0.094	19		
	0.204	30			0.267	21			0.107	25		
					0.3	25			0.125	27		
					0.334	28			0.134	30		
					0.367	30						
	0.00	0			0.00	0			0.00	0		
	0.251	00			0.501	0			0.167	0		
	0.334	5			0.585	3			0.2	5		
	0.418	10			0.668	9			0.235	10		
B. gonionotus	0.501	18	0.471 (0.440-0.500)	2.582	0.751	14	0.789 (0.754-0.824)	4.224	0.268	13	0.273 (0.260-0.286)	5.248
(n=30)	0.585	23			0.835	17			0.302	17		
	0.668	25			0.919	21			0.335	23		
	0.751	28			1.002	24			0.369	26		
	0.835	30			1.086	27			0.402	29		
					1.169	30			0.436	30		

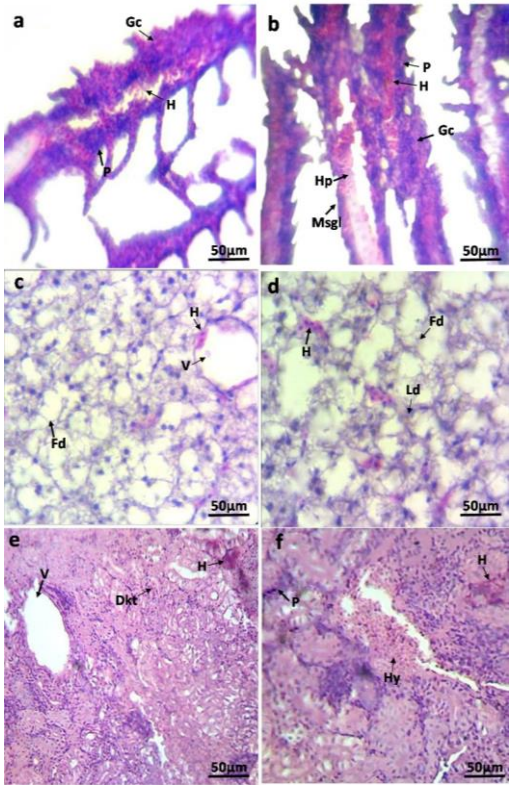

Figure 1. Photomicrograph of gills, liver and kidney of *L. rohita* after 7 days exposure to Envoy 50SC. (a) gill, (c) liver, and (e) kindey for exposure to 0.058 ppm Envoy 50SC. (b) gill, (d) liver, and (f) kindey for exposure to 0.108 ppm Envoy 50SC. Gc, gill clubbing; H, hemorrhage; P, pyknosis; Hp, hyperplasia; Msgl, missing of secondary gill lamellae; V, vacuole; Fd, fatty degeneration; Ld, lipid droplets; Dkt, degenerating kidney tubule; Hy, hyaline (H and E×430).

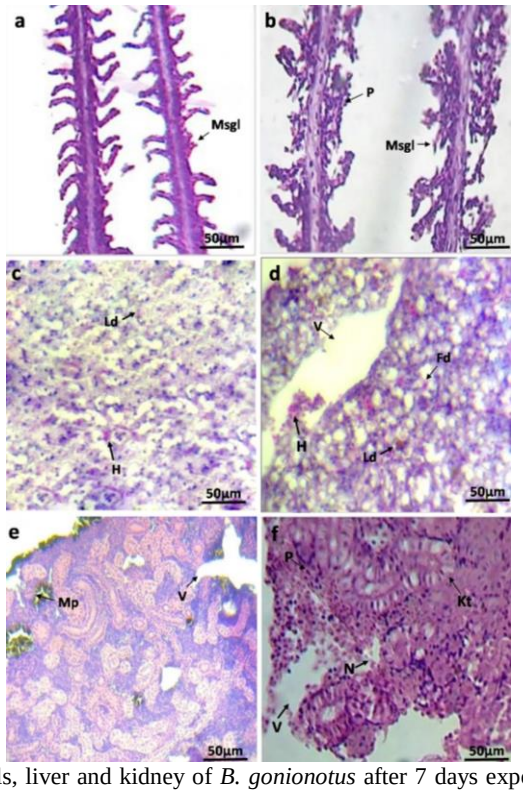

Figure 2. Photomicrograph of gills, liver and kidney of *B. gonionotus* after 7 days exposure to Envoy 50SC. (a) gill, (c) liver, and (e) kindey for exposure to 0.058 ppm Envoy 50SC. (b) gill, (d) liver, and (f) kindey for exposure to 0.108 ppm Envoy 50SC. Msgl, missing of secondary gill lamellae; P, pyknosis; H, hemorrhage; Ld, lipid droplets; Fd, fatty degeneration; V, vacuole; Mp, melanin pigment; N, necrosis (H and E×430).

observed at 0.058 ppm of Envoy 50SC (Figure 2c). Conversely, moderate alteration in hepatocytes including fatty degeneration, lipid droplets, vacuole and hemorrhage were assessed from fish exposed to 0.108 ppm Envoy 50SC (Figure 2d).

Histology of the kidney of *L. rohita* revealed pathological changes such as mild vacuole, degenerating kidney tubule and hemorrhage at 0.058 ppm (Figure 1e). At the same dose, the kidney tissues of *B. gonionotus* appeared normal but some melanin pigment and vacuoles were also seen in these tissue samples (Figure 2e). For the kidney of fishes treated with 0.108 ppm Envoy 50SC, pathology included moderate hemorrhage, pyknosis, hyaline for *L. rohita* (Figure 1f), and moderate vacuole, pyknosis and necrosis for *B. gonionotus* (Figure 2f).

Histopathological Observations of *L. rohita* and *B. gonionotus* Exposed to Samcup 50EC

Exposure of *L. rohita* at 0.108 ppm showed moderate missing of secondary gill lamellae, pyknosis, gill clubbing and necrosis (Figure 3a), but increasing the dose to 0.215 ppm, caused severe structural changes resulting in total missing of secondary gill lamellae, hemorrhage and fungal granuloma (Figure 3b). In *B. gonionotus*, exposure to 0.108 ppm of Samcup 50EC caused moderate hyperplasia, missing of secondary gill lamellae and hemorrhage (Figure 4a). However, severe structural alteration like hemorrhage, hyperplasia, missing of secondary gill lamellae, fungal granuloma, gill clubbing were assessed in the gills of *B. gonionotus* in the dose of 0.215 ppm (Figure 4b).

Increasing of dose from 0.108 ppm (Figure 3c) to 0.215 ppm (Figure 3d), caused the pathology of *L. rohita* liver to change towards severe pyknosis, fatty degeneration, hemorrhage, vacuole and lipid droplet. However, liver tissue from *B. gonionotus*, treated with 0.108 ppm Samcup 50EC showed moderate hemorrhage, pyknosis, vacuole, lipid droplets and fatty degeneration (Figure 4c). Fatty degeneration, hemorrhage, pyknosis, lipid droplets, necrosis and hyperplasia were recorded when *B. gonionotus* treated with 0.215 ppm Samcup 50EC (Figure 4d).

Severe structural changes of degenerating tubule, vacuole, necrosis and pyknosis were seen in *L. rohita* kidney (Figure 3f), at fishes exposed with 0.215 ppm Samcup 50EC, while moderate necrosis, degenerating kidney tubule, pyknosis were observed at the dose of 0.108 ppm (Figure 3e). In comparison to the *B. gonionotus*, exposed to 0.215 ppm (Figure 4f), fish exposed to 0.108 ppm revealed mild degeneration of kidney tubule and degenerating glomerular tubule (Figure 4e).

Histopathological Observations *L. rohita* and *B. gonionotus* Exposed to Dursban 20EC

L. rohita, exposed to 0.043 ppm of Dursban 20EC showed severe gill pathology with severe hyperplasia, secondary gill lamellae loss, hemorrhage, pyknosis, fungal granuloma and gill clubbing (Figure 5a). Moreover, severe hyperplasia, missing of secondary gill lamellae, hemorrhage, necrosis, and fungal granuloma were assessed at the dose of 0.087 ppm in *L. rohita* gill (Figure 5b). In case of *B. gonionotus*, 0.043 ppm of Dursban 20EC caused moderate missing of secondary gill lamellae, fungal granuloma and hyperplasia (Figure 6a), while severe gill clubbing, hemorrhage and fungal granuloma were appeared at the dose of 0.087 ppm (Figure 6b).

Moderate vacuole and hemorrhage were observed in liver of *L. rohita* exposed to 0.043 ppm Dursban 20EC (Figure 5c), whereas in 0.087 ppm exposure, severe hyperplasia, rupture of blood vessels resulting hemorrhagic area, vacuole, nuclear alteration, fatty degeneration and pyknosis were prominent (Figure 5d). Liver tissues from *B. gonionotus*, treated with 0.043 ppm Dursban 20EC possessed moderate hemorrhage, pyknosis and fatty degeneration (Figure 6c), whenever in dose of 0.087 ppm, severe alterations including pyknosis, hemorrhage, vacuole, nuclear alteration, fatty degeneration and necrosis were found (Figure 6d).

Remarkable structural changes of kidney of *L. rohita*, which included large vacuolation, hemorrhage, degenerating kidney tubule, pyknosis and degenerating glomerular tubule were assessed at the dose of 0.087 ppm (Figure 5f). In contrast, at the dose of 0.043 ppm, moderate vacuolation, hemorrhage, pyknosis, degenerating glomerular tubule were observed in the same fish species (Figure 5e). *B. gonionotus* kidney, exposed to 0.043 ppm Dursban 20EC showed comparatively mild degenerative changes, pyknosis and vacuolation (Figure 6e) than fishes exposed to 0.087 ppm, found with moderate hemorrhage, pyknosis and necrosis (Figure 6f). However, the whole observations are illustrated in Table 2.

AChE Activity of Fish Exposed to Three Pesticides

After 10 days exposure to Envoy 50SC, no significant inhibition of AChE activity (2.86%) were found in *B. gonionotus* (Figure 7a). The activities shown were 276.7±7.8 and 272.3±11.1 nmol/min/mg protein for 0.058 ppm and 0.108 ppm of Envoy 50SC, respectively. On the contrary, this organophosphate significantly affected the AChE activity of *L. rohita*, resulting to the inhibition of AChE activity at 15.28% for 0.058 ppm ($P<0.05$) and 28.24% for 0.108 ppm ($P<0.01$). In *L. rohita*, AChE activities were 255.7±8.7 and 216.7±11.0 nmol/min/mg protein at the dose of 0.058 ppm and 0.108 ppm, respectively (Figure 7a).

For the exposure to Samcup 50EC, the AChE activity of *L. rohita* showed significant inhibition at 0.108 ppm ($P<0.05$) and 0.215 ppm ($P<0.01$) of 255.7±8.7 nmol/min/mg and 216.7±11.0

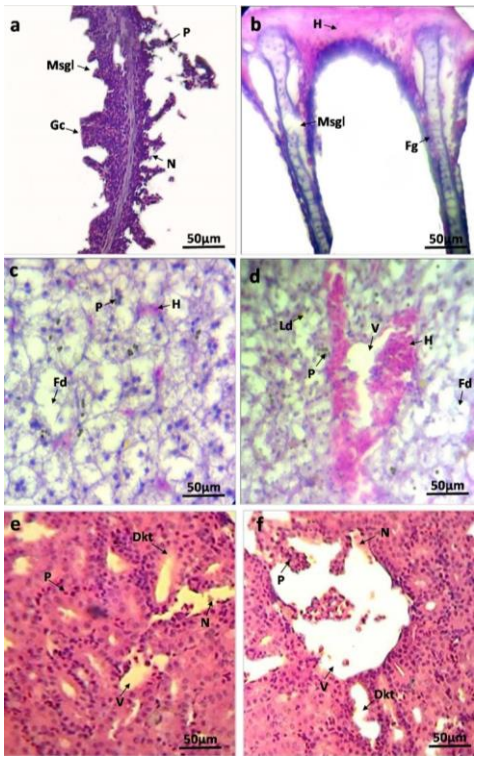

Figure 3. Photomicrograph of gills, liver and kidney of *L. rohita* after 7 days exposure to Samcup 50EC. (a) gill, (c) liver, and (e) kindey for exposure to 0.108 ppm Samcup 50EC. (b) gill, (d) liver, and (f) kindey for exposure to 0.215 ppm Samcup 50EC. Gc, gill clubbing; N, necrosis; P, pyknosis; Msgl, missing of secondary gill lamellae; H, hemorrhage; Fg, fungal granuloma; V, vacuole; Fd, fatty degeneration; Ld, lipid droplets; Dkt, degenerating kidney tubule (H and E×430).

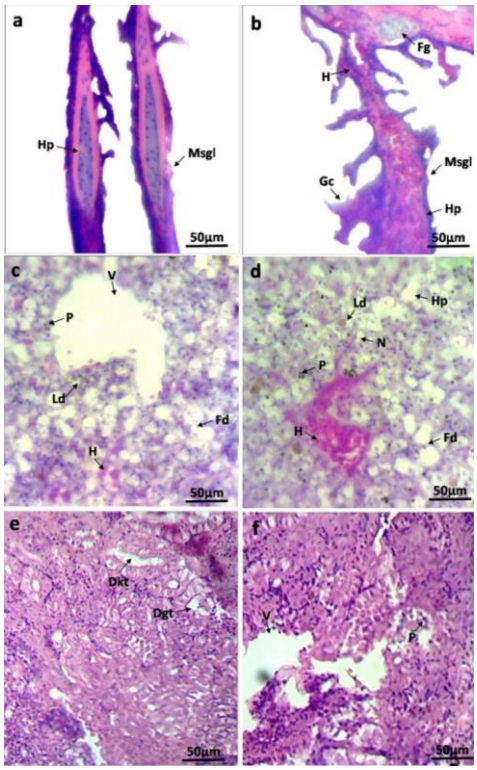

Figure 4. Photomicrograph of gills, liver and kidney of *B. gonionotus* after 7 days exposure to Samcup 50EC. (a) gill, (c) liver, and (e) kindey for exposure to 0.108 ppm Samcup 50EC. (b) gill, (d) liver, and (f) kindey for exposure to 0.215 ppm Samcup 50EC. Hp, hyperplasia; Gc, gill clubbing; N, necrosis; P, pyknosis; Msgl, missing of secondary gill lamellae; H, hemorrhage; Fg, fungal granuloma; V, vacuole; Fd, fatty degeneration; Ld, lipid droplets; Dkt, degenerating kidney tubule; Dgt, degenerating glomerular tubule (H and E×430).

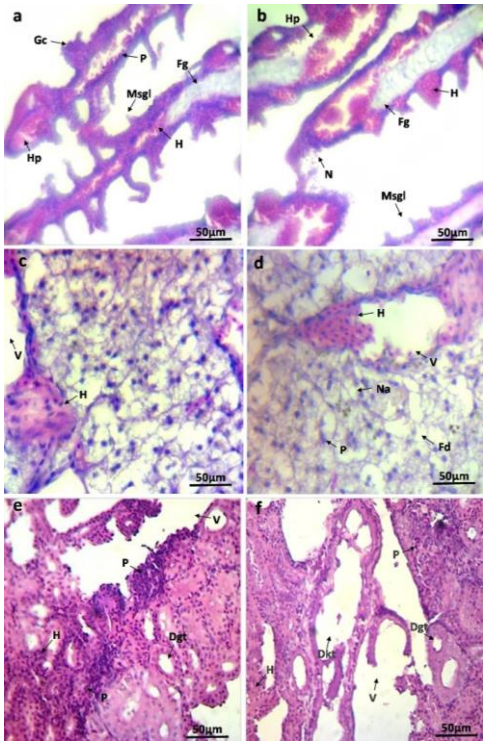

Figure 5. Photomicrograph of gills, liver and kidney of *L. rohita* after 7 days exposure to Dursban 20EC. (a) gill, (c) liver, and (e) kindey for exposure to 0.043 ppm Dursban 20EC. (b) gill, (d) liver, and (f) kindey for exposure to 0.087 ppm Dursban 20EC. Gc, gill clubbing; N, necrosis; P, pyknosis; Msgl, missing of secondary gill lamellae; H, hemorrhage; Fg, fungal granuloma; Hp, hyperplasia; V, vacuole; Na, nuclear alteration; Fd, fatty degeneration; Dgt, degenerating glomerular tubule; Dkt, degenerating kidney tubule (H and E×430).

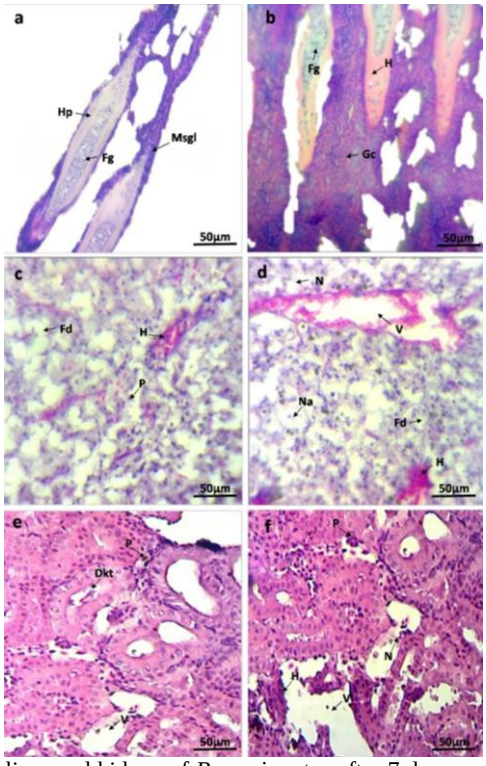

Figure 6. Photomicrograph of gills, liver and kidney of *B. gonionotus* after 7 days exposure to Dursban 20EC. (a) gill, (c) liver, and (e) kindey for exposure to 0.043 ppm Dursban 20EC. (b) gill, (d) liver, and (f) kindey for exposure to 0.087 ppm Dursban 20EC. Gc, gill clubbing; N, necrosis; P, pyknosis; Msgl, missing of secondary gill lamellae; H, hemorrhage; Fg, fungal granuloma; Hp, hyperplasia; V, vacuole; Na, nuclear alteration; Fd, fatty degeneration; Dkt, degenerating kidney tubule (H and E×430).

Table 2. Histopathological effects on *L. rohita* and *B. gonionotus* after 7 days exposure to Envoy 50 SC, Samcup 50EC, and Dursban 20EC

Pesticide	Organs	Pesticide (Conc.)	*L. Rohita*	Extent of alterations	*B. gonionotus*	Extent of alterations
Envoy 50SC	Gill	0.058	Gill clubbing, Hemorrhage, Pyknosis	++	Missing of secondary gill lamellae	+
		0.108	Gill clubbing, Hemorrhage, Pyknosis, Hyperplasia, Missing of secondary gill lamellae	+++	Missing of secondary gill lamellae, Pyknosis	++
	Liver	0.058	Vacuole, Hemorrhage, Fatty degeneration	+	Hemorrhage, Lipid droplets	+
		0.108	Hemorrhage, Fatty degeneration, Lipid droplets	++	Fatty degeneration, Lipid droplets, Vacuole, Hemorrhage	++
	Kidney	0.058	Vacuole, Degenerating kidney tubule, Hemorrhage	+	Vacuole, Melanin pigment	+
		0.108	Hemorrhage, Pyknosis, Hyaline	++	Vacuole, Pyknosis, Necrosis	++
Samcup 50EC	Gill	0.108	Missing of secondary gill lamellae, Pyknosis, Gill clubbing, Necrosis	++	Hyperplasia, Missing of secondary gill lamellae, Hemorrhage.	++
		0.215	Total missing of secondary gill lamellae, Hemorrhage, Fungal granuloma	+++	Hemorrhage, Hyperplasia, Missing of secondary gill lamellae, Fungal granuloma, Gill clubbing	+++
	Liver	0.108	Hemorrhage, Pyknosis, Fatty degeneration	++	Hemorrhage, Pyknosis, Vacuole, Lipid droplets, Fatty degeneration	++
		0.215	Pyknosis, Fatty degeneration, Hemorrhage, Vacuole, Lipid droplets	+++	Fatty degeneration, Hemorrhage, Pyknosis, Lipid droplets, Necrosis, Hyperplasia	+++
	Kidney	0.108	Degenerating kidney tubule, Necrosis, Pyknosis	++	Degenerating kidney tubule, Degenerating glomerular tubule	+
		0.215	Degenerating kidney tubule, Vacuole, Necrosis, Pyknosis	+++	Vacuole, Pyknosis	++
Dursban 20EC	Gill	0.043	Hyperplasia, Missing of secondary gill lamellae, Hemorrhage, Pyknosis, Fungal granuloma, Gill clubbing	+++	Missing of secondary gill lamellae, Fungal granuloma, Hyperplasia	++
		0.087	Hyperplasia, Missing of secondary gill lamellae, Hemorrhage, Necrosis, Fungal granuloma	+++	Gill clubbing, Hemorrhage, Fungal granuloma	+++
	Liver	0.043	Vacuole, Hemorrhage	++	Hemorrhage, Pyknosis, Fatty degeneration	++
		0.087	Hyperplasia, Hemorrhage, Vacuole, Nuclear alteration, Fatty degeneration, Pyknosis	+++	Pyknosis, Hemorrhage, Vacuole, Nuclear alteration, Fatty degeneration, Necrosis	+++
	Kidney	0.043	Vacuole, Hemorrhage, Pyknosis, Degenerating glomerular tubule	++	Vacuole, Pyknosis	+
		0.087	Vacuole, Hemorrhage, Degenerating kidney tubule Pyknosis, Degenerating glomerular tubule	+++	Hemorrhage, Pyknosis, Necrosis, Vacuole	++

Extent of alteration: Severe (+++), Moderate (++), Mild (+).

nmol/min/mg protein, respectively (Figure 7b), whereas for the same dose of same pesticide the AChE activity in the brain of *B. gonionotus* were calculated as 266.0±4.6 ($P<0.05$) and 242.0±6.6 nmol/min/mg ($P<0.001$) protein, respectively (Figure 7b). Significant decreases of enzymatic activities were found in both species indicating 31.2% and 12.62% reduction of that enzyme at agricultural recommended dose.

In case of Dursban 20EC, the AChE activities in the brain of *L. rohita* and *B. gonionotus* at the dose of 0.043 ppm calculated were 212.3±6.5 ($P<0.01$) and 258.0±5.6 nmol/min/mg ($P<0.001$) protein, respectively. While, at the dose of 0.087 ppm, the enzymatic activity were 146.7±5.5 and 221.7±60.3 nmol/min/mg protein, respectively. In both species

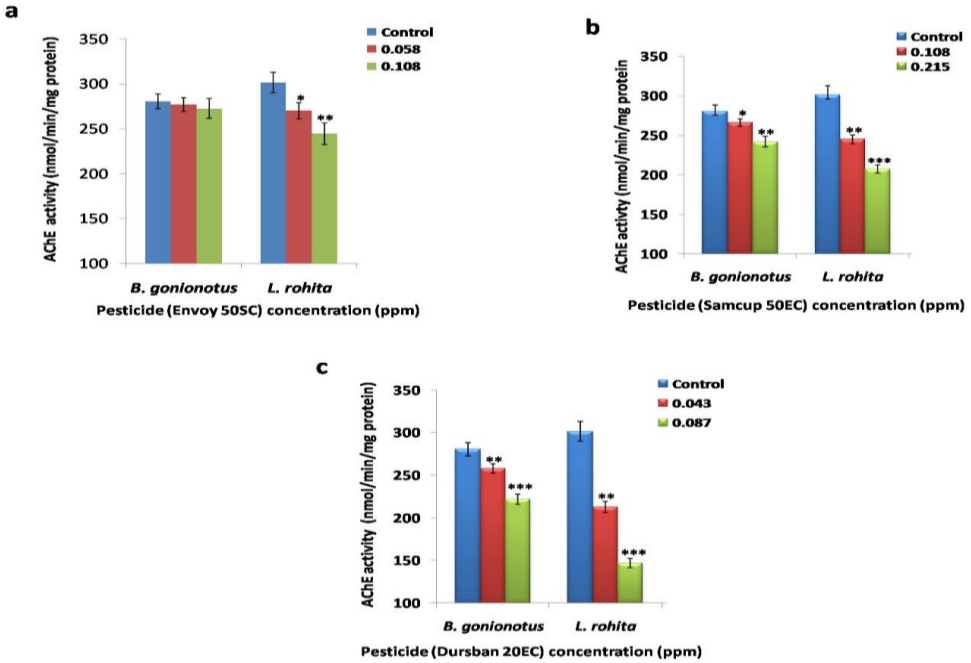

Figure 7. AChE activity (nmol/min/mg protein) measured in *L. rohita* and *B. gonionotus* exposed to Envoy 50SC (a), Samcup 50EC (b) and Dursban 20EC (c) for 10 days. Data presented as mean ±SD. *P<0.05, **P<0.01, and ***P<0.001.

brain AChE activity were reduced by 51.49% and 19.60% at agricultural recommended dose.

Discussion

LC_{50} of Three OPs for *L. rohita* and *B. gonionotus*

Determination of LC_{50} provides a view of deleterious effects of pesticides on test animals during a short time exposure under controlled condition. In this study, the LC_{50} values of Envoy 50SC were calculated as 0.110 (0.060-0.199) and 0.471 (0.440-0.500) ppm for *L. rohita* and *B. gonionotus*, respectively. The LC_{50} values of Samcup 50EC and Dursban 20EC were predicted as 0.217 (0.204-0.231) and 0.079 (0.073-0.084) ppm for *L. rohita* and 0.789 (0.754-0.824) and 0.273 (0.260-0.286) ppm for *B. gonionotus*, respectively. Lovely, Rahman, Hossain, and Mollah (2003) estimated the LC_{50} value of Dursban as 0.005 ppm for *Clarias gariepinus*, whereas Hossain, Halder, and Mollah (2000) found the LC_{50} value of Diazinon as 2.97 ppm for *L. rohita* at 96 h exposure. These revealed that different pesticides have different LC_{50} value, which is also species specific.

Histopathological Observation in OPs Treated Fish

Agricultural pesticides, especially OPs and C affect normal physiological functioning of aquatic living organisms at molecular and cellular level, and histopathological studies of different organs in this study clearly depicted their effect on fish species.

In the present study, two fish species *L. rohita* and *B. gonionotus* were exposed to three different organophosphates at two different concentrations for 7 days to assess in gills, liver and kidney changes. Mild to severe changes in gills were observed in case of both fish species treated with three pesticides. Pathologies appeared as gill clubbing, hemorrhage, pyknosis, missing of secondary gill lamellae, hyperplasia, necrosis, fungal granuloma etc. In all cases, the observed pathologies were prominent at higher dose of pesticides. The results were similar to other studies, where hypertrophy of gill lamellae and fusion of secondary lamellae of Zebrafish gills were also observed. Telangiectasis was also found at the tip of secondary gill lamellae in Nile tilapia after 96 h exposure to an organophosphate pesticide (Zodrow, Stegemanb, & Tanguay, 2004; Benli & Özkul, 2010).

In compare to the control, the hepatocytes and other tissues of liver showed ultra structural damage. Similar observations were made earlier by Hossain, Rahman, and Mollah (2002), from the liver of three fish species, *Anabas testudineus*, *Channa punctatus* and *Barbobes gonionotus* exposed to organophosphate pesticide. Appearance of lipidosis and hepatocyte hypertrophy were observed by Zodrow *et al*. (2004). Oropesa, Cambero, Gómez, Roncero, and Soler (2009) found necrotic foci and lipid droplets in liver of *Cyprinus carpio*, whereas histological analysis of silver catfish (*Rhamdia quelen*) showed vacuolation in the liver after exposure to the herbicide clomazone (Crestaniet *et al*., 2007). The pathologies of liver due to the exposure of pesticides were also supported by other studies, where additional glycogen depletion with lipidosis in the liver of Atlantic salmon (*Salmo salar*) and extensive histo-architectural changes in liver tissue of *Cyprinus*

carpio were observed after exposure to endosulfan and 4-tert-butylphenol, respectively (Glover *et al*., 2007; Barse, Chakrabartia, Ghosha, Palb, & Jadhao, 2006).

The haematopoetic and other tissues of kidney, however in the present study showed minor to severe structural damages compared to control. The result partially agrees with Hossain *et al*. (2002), where in more pathologies were found in *B. gonionotus*. This was due to the use of pesticides at sub-lethal concentrations, compare to the doses used in this study. More or less similar findings were also made by Oropesa *et al*. (2009). However, Zodrow *et al*. (2004) found no significant changes in kidney from control and 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) exposed zebrafish.

More structural damages in *L. rohita* compare to *B. gonionotus* indicated that the former species was much more susceptible to pesticides exposure. The study also suggested that Dursban 20EC and Samcup 50EC affect more on histological changes than Envoy 50SC.

AChE Activity in OPs Treated Fish

The use of AChE inhibition as a putative biomarker of neurotoxicity in pesticides exposed fish has been well established. In the present study, a significant ($P \leq 0.05$) elevated AChE inhibitions were observed in fish exposed to pesticides in a concentration depended manure. However, maximum inhibition in AChE activity (up to 51.49%) were reported from *L. rohita*, which was similar to the study conducted by Sancho, Ferrando, and Andreu (1998) on *Anguilla anguilla* exposed to 0.04 ppm fenitrothion (an organophosphate) which produced a 57% decline in AChE activity. A 51% decrease of AChE activity was also observed at fish exposed to 0.02 ppm. Chuiko (2000) made a comparative study of *in vitro* inhibition of brain and serum AChE activity by DDVP (Dichlorvos an organophosphate pesticide), conducted in 11 freshwater teleost species. Similar declining of ChE activities responsible for in vitro treatment with organophosphates has also been reported recently (Valbonesi, Brunelli, Mattioli, Rossi, & Fabbri, 2011; Rodrigues *et al*., 2011; Čolovic *et al*., 2011). Moreover, Pessoa *et al*. (2011) showed that pesticide mediated enzymatic inhibition affects behavioral pattern in *Oreochromis niloticus* while impaired oxygen consumption and ammonium excretion were suggested by Barbieri, Augusto, and Ferreira (2011).

Conclusion

The results of this study suggest that the use of pesticides even at recommended doses may cause serious harm to the *L. rohita* and *B. gonionotus*. Additional studies should be conducted to further evaluate the recovery of the fish by determining any histopathological changes and the presence of enzymatic inhibition in individuals subjected in OP treated water then transferred to uncontaminated water. Experiments on the residual effects of OP to the next progeny should also need to be conducted. Environmentally safe pesticides should be recommended by authorities for agricultural use.

Acknowledgments

This work is funded by Bangladesh Agricultural University Research System (BAURES). The authors are indebted to Professor Dr. Mohammad Shamsuddin of the Department of Surgery and Obstetrics, Faculty of Veterinary Science, Bangladesh Agricultural University for facilitating Field Fertility Clinic Laboratory.

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