Endosulfan Toxicity to *Anabas testudineus* and Histopathological Changes on Vital Organs

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Abstract. The toxicity of endosulfan, an organochlorine type insecticide to a commonly consumed freshwater fish species, *A. testudineus* (40.68±9.03 g; 13.49±0.99 cm), was investigated under static conditions. The nominal endosulfan concentrations ranging from 10 to 80 µg/L subjected to the fish population results in 96-hour median lethal concentration, LC_{50}, of 35.2±3.99 µg/L. The toxicity is a function of both endosulfan concentration and exposure time ($p>0.05$). Histopathological analysis on vital organs exposed to sublethal concentrations indicates that structural changes started at sublethal dose and the effects aggravated with increasing endosulfan concentration. Gill was found to experience aneurism, hyperplasia in lamellar and autolysis of mast cell. Pyknotic nuclei and necrosis were observed in liver cell, while the lumen of renal tubule was found to narrow and haemorrhage was observed in cytoplasm cell. High LC_{50} compared to other fishes indicates that *A. testudineus* has high tolerant to endosulfan, however, endosulfan slowly alters the fish biochemistry and is potentially transferable to human

1 Introduction

Anabas testudineus, commonly known as climbing perch is a freshwater fish species widely populating irrigation canal and rice field of Southeast Asia countries. It is known as hardy fish as it can survive in mud and shallow water and for hours on land. The fish ability to endure harsh condition could lead to an increase in bioaccumulation of toxicants it is exposed to. On the other hand, endosulfan is widely known as toxic insecticide acting as a contact poison for variety of insects and mites. Various negative effects have been described as a result of fish exposure to endosulfan. Chronic effects on fish include oxidative damage [1], genotoxicity [2], damage to testes [3], changes in circulating thyroid hormones [4] and alteration of acetylcholinesterase activity in the brain [5]. Depressed levels of testosterone and estradiol have also been found in fish with elevated residues of endosulfan [6]. Other effects are reduced feeding behaviour [7], alterations in development

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[8], sexual and escape behaviour, and reproductive physiology [9-10]. Studies on endosulfan toxicity also showed that it is acutely toxic to most fishes with LC_{50} ranging from 0.1 to 41 µg/L [5, 11–22]. Although currently banned in many countries due to its adverse effects on human health and the environment, endosulfan is still continuously employed in several developing countries in Asia for high commercial value crops. In Malaysia, its residue was recently detected in a stream and tap water at a concentration of 0.112 µg/L endosulfan sulphate and 0.072 µg/L endosulfan II [23]. Bioaccumulation of endosulfan in the commonly consumed *A. testudineus* may lead to biomagnification of the toxicant from the non-targeted organism to consumer. A risk assessment on infants in Punjab, India [24] reported mean values of 90.69 ng g^{-1} β-endosulfan and 14.00 ng g^{-1} endosulfan sulphate excretion in breastmilk. Due to the high toxicity of endosulfan, this study aims to assess acute toxicity of endosulfan to juvenile *A. testudineus* when exposed to endosulfan-containing water samples. High median lethal concentration (LC_{50}) would be evidence for high survival. The toxic effects of endosulfan to the fish cellular was also investigated by histopathological analysis on several vital organs i.e. gill, liver and kidney.

2 Experimental

2.1 Acute toxicity and determination of LC_{50}

Juvenile *A. testudineus* of 40.68 g (±9.03 g) weight and 13.49 cm (±0.99 cm) length were obtained from local fish supplier in Perlis, Malaysia. Acclimatisation was done for 2 weeks in 200 gallons of dechlorinated tap water. The fishes were fed twice daily with commercial pellets during acclimatisation, however, food was not given for 48 h before and during the test to empty their guts. Endosulfan powder (GmbH, 99.5%, 64-67% α-endosulfan; 29-32% β-endosulfan) was mixed with 40 L water to produce desired stock solutions of 0.4 g/L. Acetone (Merck, 99.5%) was used at 0.005% (v/v) to aid in pesticide dissolution.

Five glass aquarium tanks were each filled with 40 L chlorine-free tap water and kept for 24 h prior to endosulfan addition. Water quality parameters in the aquarium tanks were measured daily. Average value of DO is 70±2%, temperature is 25±2ºC, total alkalinity is 80±3 mg/L $CaCO_3$, pH 6.9±0.2 and total hardness is 23±3 mg/L $CaCO_3$. Air compressor was used to maintain DO level in water. Static acute toxicity experiment was carried out based on Standard Methods [25]. Samples containing different nominal endosulfan concentrations (10, 20, 40, 60 and 80 µg/L) and two control samples (tap water) were used in the test. One of the control samples was added with acetone (0.005% v/v). Ten fishes were transferred in random into three replicate sample tanks to obtain a total of 30 fish population. Fish mortality was observed and dead fishes were immediately removed from the tank. None of the control fishes died throughout acclimatisation period and toxicity test. The LC_{50} was estimated with Probit Analysis, using Biostat Profesional Version 5.

2.2 Histopathology

Gill, liver and kidney were dissected and fixated in 10% neutral buffered formalin for 24 h at room temperature. Tissue processing started with dehydrating gill, liver and kidney samples through graded alcohol series, cleared in alcohol and embedded in paraffin. Paraffin sections of 5-10 µm thickness were trimmed using Jung Multicut RM2045 microtome and stained with haematoxylene-eosin. The tissues were then examined under light inverted microscope and photographed using Nikon photomicroscope.

3 Results and discussions

3.1 Acute toxicity and determination of LC_{50}

Fig. 1(a) and 1(b) show dose-response curves of fish mortality due to exposure to endosulfan, generated by Probit Analysis based on Finney Method (lognormal distribution) and Least Squares Method (normal distribution) respectively. The 96-h LC_{50} values are 32.39±4.32 µg/L (Finney) and 38.03±5.67µg/L (Least Squares) with no significance error ($p>0.05$). The average 96-h LC_{50} is 35.21±4.99 µg/L based on the two methods. This value is very high compared to those reported in studies on other fish species as shown in Table 1, except for European eel [22] at 41 µg/L, indicating its high survival.

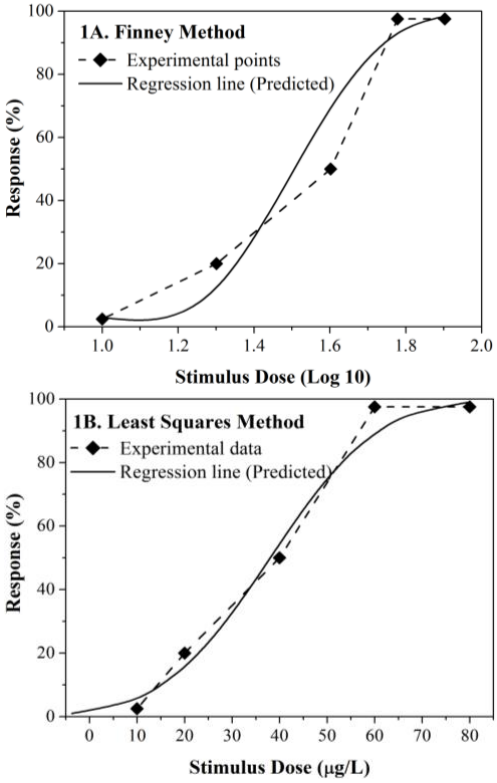

Fig. 1. Dose-response curve of fish mortality due to exposure to endosulfan by Probit Analysis based on (a) Finney Method (lognormal distribution) and (b) Least Squares Method (normal distribution).

Table 1. LC_{50} values for various fish species exposed to endosulfan.

Fish species	Weight/ Length	Loading	Endosulfan purity	Water quality	Test type	LC_{50} (96 h)	Ref.
Climbing perch (Puyu), *Anabas testudineus*	40.68±9.03 g/ 13.49±0.99 cm	10.17 g/L	99.5%	T: 25±2°C; pH: 6.9±0.2; DO: 70±2%; A: 80±3 mg/L $CaCO_3$; H: 23±3 mg/L $CaCO_3$	Static	35.2	This study
Florida flagfish, *Jordanella*	Larval fish (2-3 days)	10 fish/ 200 mL	99%	T: 25°C	Semi static	4.35	[11]

jordanella							
Spotted murrel, *Channa punctatus*	35.6±0.7 g	7.12 g/L	99.0%	T: 26-28°C; pH: 7.2-7.5; DO: 6.5-7.1 mg/L; A: 48-58 mg/L; H: 48-60 mg/L	Static	24.3	[12]
Teleostei, Perciformes, *Cichlasoma dimerus*	21.2±1.5 g/ 7.5±0.2 cm	3.18 g/L	94.9%	T: 25±1°C; pH: 7.3	Semi static	2.6	[13]
Tilapia, *Oreochromis mossambicus*	46.78 g	4.68 g/L	99%	T: 26.4-28.8°C	Static	3.609	[14]
Tilapia, *Oreochromis niloticus*	10.26±2.84 g/ 8.36±0.78 cm	10.26 g/L	99%	T: 24±1°C; pH: 7.5	Semi static	10.20	[15]
Nile tilapia, *Oreochromis niloticus*	150 g	37.5 g/L	35%	T: 28±2°C	Static	12.775	[16]
Tilapia, *Oreochromis mossambicus*	Fingerling	-	35%	-	-	1.42	[17]
European carp, *Cyprinus carpio*	5months/ 6-13 cm	5 fishes/ 20 L	-	T: 25±1°C; pH: 6.0-7.3	Static	2	[18]
Asian swamp eel, *Monopterus albus, Zuiew*	150-180 g/ 30-40 cm	49.5 g/L	33%	pH: 7.2±1°C; A: 80±1 mg/L $CaCO_3$	Static	0.42	[19]
Rainbow trout, *Onchorhynchus mykiss*	10.61 ± 1.69 g/ 10.0±0.4 cm	4.24 g/L	32.9%	T: 12.1±0.6°C; pH: 7.84±0.8; A: 16.5±0.8 mg/L $CaCO_3$	Semi static	1.75	[20]
Silver perch, *Bidyanus bidyanus*	50 mm	-	-	-	Semi Static	7.75	[21]
European carp, *Cyprinus carpio*	50 mm	-	96.2%	T: 25°C	Semi static	0.1	
Mosquito fish, *Gambusia affinis*	Age: 21 d/ 40 mm	-	96.2%	T: 25°C	Static	2.3	
Golden Perch, *Macquaria ambigua*	Age: 21 d/ 40 mm	-	96.2%	T: 25°C	Semi static	0.5	
Eastern rainbowfish, *Melanotaenia duboulayi*	Age: 48 d/ 40 mm	-	96.2%	T: 25°C	Static	11.4	
Bony bream, *Nematolosa erebi*	Age: 22 d/ 50 mm	-	96.2%	T: 26°C	Semi Static	0.2	
Rainbow trout, *Oncorhynchus mykiss*	Age: 24 d/ 55 mm	-	96.2%	T: 4°C	Static	1.6	
Harlequin fish, *Rasbora sp.*	30 mm	-	96.2%	T: 25°C	Flow through	0.2	
European eel, *Anguilla anguilla*	20-30 g/ 16-20 cm	-	96%	T: 20±2°C; pH: 7.9±0.2; A: 4.0±0.5	Static	41	[22]

	mmol/L; H: 240±10 ppm as $CaCO_3$

Note: T = temperature, DO = dissolved oxygen, A = alkalinity, H = hardness

However, it is important to note that LC_{50} is also dependent on various factors such as the type of acute toxicity test and the size of fish used in the toxicity tests. Sunderam et al. [21] and Capkin et al. [20] for example, obtained LC_{50} values of 1.6 and 1.75 µg/L for Rainbow trout using static and static renewal test respectively. On the other hand, Kumar et al. [14] and Kegley et al. [17] reported LC_{50} values of 3.609 and 1.42 µg/L for Tilapia of different sizes; i.e. 46.78-g fish and fingerlings respectively. Previous preliminary work on *A. testudineus* of bigger sizes [26] than the present study also produced higher LC_{50} (41.70 µg/L). Environmental factors including temperature, pH, alkalinity and turbidity were also found to correlate with endosulfan toxicity [27]. For instance, a pH of less than 5 would accelerate hydrolyzation of endosulfan to endosulfan sulphate, which is more toxic [28]. Capkin et al. [20] showed that LC_{50} of Rainbow trout did not only depend on fish size, but also the temperature and water quality including pH, alkalinity and hardness.

3.2 Histopathological Changes

Endosulfan toxicity at sublethal concentrations is also important as the fish biochemical reactions may be altered while being alive, leading to poisonous effect on the genotype and bioaccumulation. Therefore, histology analysis was done to investigate its effect on vital organs; gill, liver and kidney at sublethal phases. Fig. 2 shows gill cross sections of control fish (A) and fish exposed to 8.8 (B), 17.6 (C) and 35.2 (D) µg/L endosulfan under light microscope. Fig. 2A shows regular filament arrangement of primary lamellae with central cartilages, afferent and efferent arterioles in control fish. About 5 secondary lamellae or strata are attached to primary lamellae on basement membranes supported by pillar cells. Erythrocytes can be seen in spaces between lacunae and pillar cells.

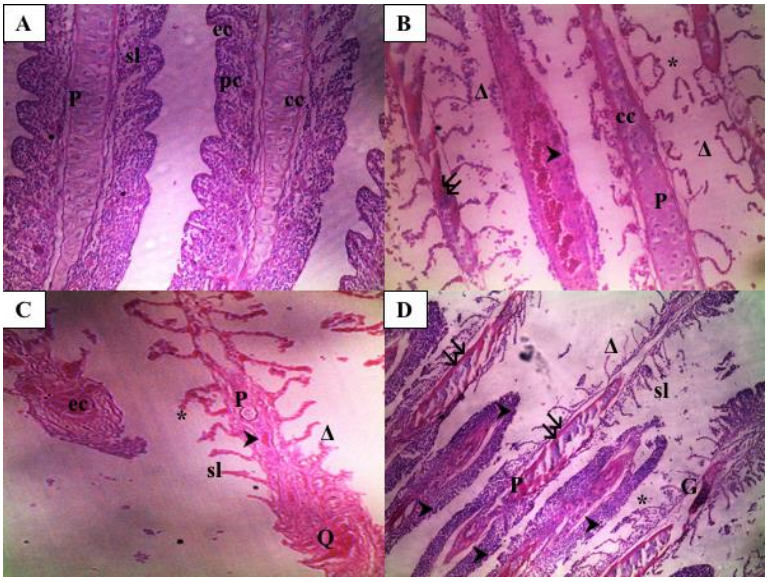

Fig. 2. Gill cross section of (A) control fish and fish exposed to (B) 8.8, (C) 17.6 and (D) 35.2 µg/L endosulfan under light microscope (H&E stain, x200 for ABC and x100 for D). Symbol: primary lamella (P); secondary lamella (sl); pillar cell (pc); erythrocytes (ec); chloride cell (cc); hyperplasia of

interlamellar epithelium (➤); epithelial lifting (Δ); fusion of secondary lamella (*); aneurism in primary lamella (↓); desquamation (Q); autolysis (G).

Fig. 2B shows that endosulfan causes hyperplasia of interlamellar epithelium, epithelial lifting, fusion of the secondary lamellae, aneurism in primary lamella and chloride cell lysis. Fig. 2C indicates that higher endosulfan concentration causes aneurism on secondary lamella and desquamation of the gill. In Fig. 2D, autolysis of mast cell and chloride cell on primary lamellar can be observed. Autolysis can lead to cellular necrosis, which may be indicative of cell injury or death. In previous studies, hyperplasia of the interlamellar epithelium was also observed on *Cichlasoma dimerus* [13], while Rainbow trout was reported to have lifting of lamellar epithelium, distal hyperplasia of lamellas and combination of lamellas [29] due to exposure to endosulfan.

Fig. 3 shows liver cross sections of control fish and fish exposed to 8.8, 17.6 and 35.2 µg/L endosulfan solutions under light microscope. Fig. 3A shows that liver tissue of control *A. testudineus* is composed of polyhedric hepatocytes with normal sinusoidal spaces. Kupffer cells can also be observed. On the other hand, Fig. 3B shows that cytoplasm of liver cell is high in vacuolated aspect and there is an increase in Kupffer cells. The structure of polygonal hepatocytes cannot be identified due to wide vacuolation, and the cell appears to become pyknotic, which may lead to fragmentation of nucleus in cytoplasm. Fig. 3C shows that the liver cell has swelled and ruptured indicating the presence of apoptosis, a biomarker of toxic exposure. The liver also experiences hyalinization of hepatocytes. In Fig. 3D, abnormal sinusoids can be observed with obstruction, while the accumulation of erythrocytes can be seen in the sinusoids probably due to inflammation in the blood vein or artery branch. Hepatocytes also become denser in the vacuolated cytoplasm of liver. Similar liver alteration such as cellular swelling, large vacuolation of cytoplasm and pyknotic nuclei of hepatocytes due to exposure to endosulfan were also reported by previous studies [30–31]. Comparative vacuolar distrophy and hypertrophy of hepatocytes were observed in Rainbow trout [29], while Onesided livebearer was found to experience hydropic degeneration, sinusoids dilation and necrosis in liver [32].

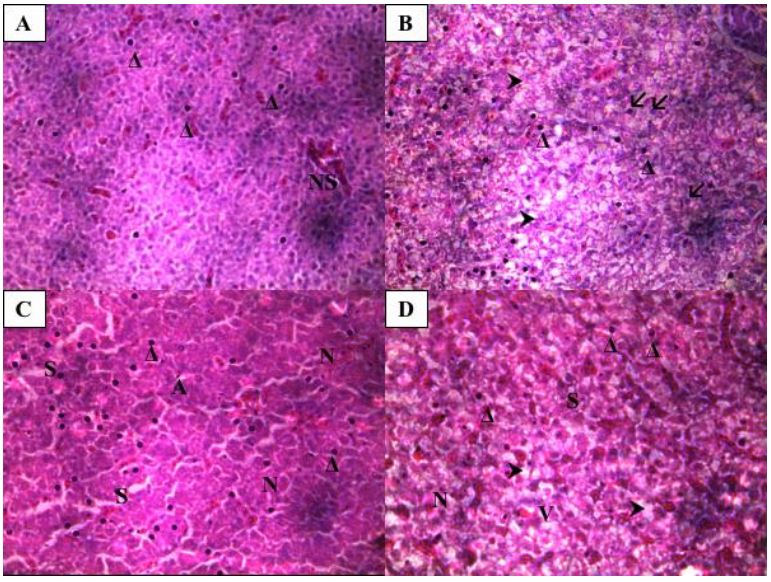

Fig. 3. Liver cross section of (A) control fish and fish exposed to (B) 8.8, (C) 17.6 and (D) 35.2 µg/L endosulfan under light microscope (H&E stain, x40). Symbol: normal sinusoids (NS); Kupffer cell (Δ); vacuolation of cytoplasm (➤); pyknotic nuclei (↓); sinusoid (S); necrosis (N); apoptosis (A).

Fig. 4 shows kidney cross sections of control fish and fish exposed to 8.8, 17.6 and 35.2 µg/L endosulfan under light microscope. The control fish in Fig. 4A is composed of distal convulated tubule, proximal convulated tubule, glomerulus and hematopoietic tissues, but the Bowman's space cannot be clearly observed. The peritubular capillaries can be observed with heavy nuclei of epithelial cells. Exposure to endosulfan causes inflammation and interstitial fluid mixing with blood as shown in Fig. 4B. Higher endosulfan concentration worsens the kidney condition, induces heavy bleeding and causes the lumen of the tubules to degenerate and become dilated (Figs. 4C and 4D). Other than that, intracytoplasmic vacuoles can be seen in epithelial cells of distal tubule, while haemorrhage occurs in hemopoietic cells. Previously, significant decrease in the dimension of Bowman's capsule and destruction of glomerulus shape due to precipitation of cytoplasm and karyolysis were also reported in *Channa punctatus* treated with endosulfan [33].

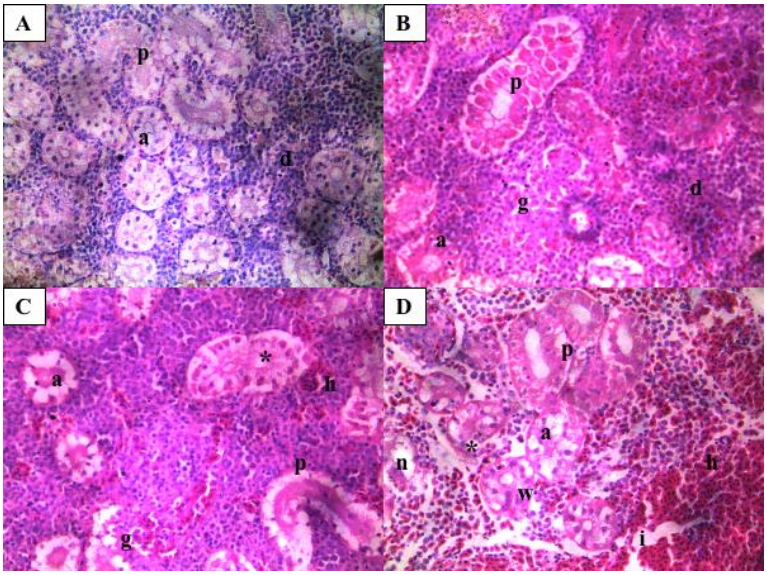

Fig. 4. Kidney cross section of (A) control fish and fish exposed to (B) 8.8, (C) 17.6 and (D) 35.2 µg/L under light microscope (H&E stain, x400). Symbol: renal tubule (a); hematopoietic tissue (d); proximal convoluted tubules (p); glomerulus (g); necrosis (n); haemorrhage (h); renal tubule degeneration (*), pyknotic (p); (D) intracytoplasmic (i) vacuoles; narrowing (w) of tubular lumen.

4 Conclusions

This work investigates the toxicity of endosulfan to juvenile *A. testudineus* using dose-response relationship and histopathological analysis. The toxicity was found to increase with endosulfan concentration. The high LC_{50} value obtained indicates that *A. testudineus* has high survival in contaminated surroundings. However, various structural changes were already induced on the morphology of the vital organs, i.e. gill, liver and kidney even with exposure to low, sublethal endosulfan concentration.

The authors acknowledge funding provided by Ministry of Higher Education Malaysia through Research Acculturation Collaborative Effort Grant (9017-00023).

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