
Acute Toxic and Hepatopancreas Syndrome Caused by Chlopyrifos Ethyl to Black Tiger Shrimp (*Penaeus Monodon*) and White Shrimp (*Litopenaeus vannamei*) in Mekong River Delta of Vietnam

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Abstract Acute toxicity (96h) and chronic toxic (60 days early life stage) tests were conducted in laboratory to determine the LC₅₀ and hepatopancreas syndrome of liver and gill of Chlopyrifos ethyl, an organophosphorus pesticide commonly used in Vietnam, to two major brackish water shrimps *Penaeus monodon* and *Litopenaeus vannamei* of Mekong River Delta to ensure the relevance of Chlopyrifos ethyl use with early mortality symptom (EMS) of shrimps in Asia countries and the safety standard for water environment. The results indicate that the acute median lethal concentration (LC₅₀) of Chlopyrifos ethyl to adults of *Penaeus monodon* and *Litopenaeus vannamei*, young age of *Litopenaeus vannamei* and *Penaeus monodon* is 0.1412; 0.1197; 0.0042 and 0.0041 mg l⁻¹ respectively. It means adults of both shrimps are more tolerant to Chlopyrifos ethyl than young ones (about 30 times) while the difference of tolerance between tested species are not clearly recognized. Surgical histopathological assessment of hepatopancreas syndrome necrotizing pancreatitis in Chlopyrifos ethyl showed exposure at low concentrations, shrimp in hepatopancreas samples collected at 10 and 20 days after treatment. Expression of the transformation is phenomenal concentration of blood cells around the liver pancreas and some changes in the structure of the hepatopancreas tube. When exposed at higher concentrations (80% or higher value of LC), the majority of experimental shrimp died within 10 days after exposure.

Keywords: Chlopyrifos ethyl; acute toxic; syndrome hepatopancreas, *Penaeus monodon*; *Litopenaeus vannamei*

Introduction

The Symptom of early mortality (EMS) enclosed with histopathology of liver and gill of two brackish water shrimps *Penaeus monodon* and *Litopenaeus vannamei* is threatening shrimp culture in Vietnam and Asia countries as well. Up to date, there has been no reason determined precisely, but the contamination of pesticide is considered as one of the direct or indirect potential hazards.

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Chlorpyrifos ethyl, a high toxic insecticide ranked to 2nd toxic group by WHO standard is commonly used in Vietnam to control various insect pests in paddy rice and upland crops. Due to high effectiveness against a wide range of arthropods and insect pests including Coleoptera, Diptera, Homoptera and Lepidoptera species, Chlorpyrifos is highly toxic to the nervous system and is also transformed inside animals to chlorpyrifos-oxon and 3, 5, 6-trichloro-2-pyridinol (TCP) both of which are many times more toxic to the nervous system than chlorpyrifos itself (Chambers *et al.*, 1989). Due to its lipophilic nature, fish are able to absorb and bioconcentrate chlorpyrifos to moderate or high levels and variable bioconcentration factors (BCF) between 100-5100 have been reported (Racke, 1993). They enter water bodies through intentional application, run-off from farms, aerial drift and accidental and illegal release (Zelikoff, 1994). In Vietnam, there were 164 registered products containing Chlorpyrifos (Vietnam, 2014).

Due to high dose and frequency usage, the contamination of Chlorpyrifos ethyl to water source is considered closely relevant. Meanwhile, the shortage of specific study on level of acute toxic and chronic symptom caused by Chlorpyrifos ethyl to the above shrimp species is constrained preventing the examination and treating measurement of EMS. This research aims to provide scientific data to ensure the potential relevance of Chlorpyrifos ethyl to EMS and to assist promulgating country's standard for MRL of Chlorpyrifos ethyl in shrimp culture water.

Materials and methods

Materials

Young *Penaeus monodon* and *Litopenaeus vannamei* (at post 15 and 12 respectively) supplied by local breeding agency were transported to the laboratory of Cantho University in a portable well-aerated white polythene bag containing 2% salt water from the breeding pool and transferred into composite tank 2m³ in 3-5 days. They were health checked before chemical treatment to confirm zero infection of liver pancreatic necrosis and then switched to the other tank containing salty water at 2% concentration (measured by refractometer), using Na₂CO₃ with volume 40gm⁻³ to raise the pH to about 10-20 mg l⁻¹ and maintaining continuous aeration. Temperature stability culture 26-28^oC. Water pH, temperature and salinity remain stable during the experiment time to ensure deviation of temperature less than 1^oC and salt concentrations less than 0.2% within 24 hours. During this period, the shrimps were fed with pelleted diet containing 35% crude protein twice per day at 5% body weight. Also, the water in the glass aquaria was changed once every two days (Reish, D.L. and Oshida, P.S., 1987).

Tests were undertaken with both young and adult shrimps grown up from young one (at 30 days age after rearing from post 15 and post 12 as above).

- Water: the sea brine is removed from the salt production base, adjust pH to 8.0 and salinity to 2%.
- Chemical: Technical grade containing 97% ai

Material and methods

Acute bio assay: LC₅₀ 96-h static bioassays were conducted in the laboratory following the methods of Spragne, (1975) and APHA; AWWA and WEF (1992). The acute concentrations for chlorpyrifos-ethyl were determined by two steps:

- Step 1: preliminary test: conducted with 8 treatment, including 7 concentrations of Chlorpyrifos ethyl and untreated check (UTC). Tested concentrations were determined by selecting one reference concentration which is an average of LC₅₀ value of Chlorpyrifos ethyl published for aquatic animals by Canada water quality guidelines for the protection of aquatic life (about 0.0268) plus with 3 higher and 3 lower levels of 10, 100 and 1000 times. Hence, the concentration range was 0.0000268; 0.000268; 0.00268; 0.0268; 0.268; 2.68; 26.8 and UTC.
- Step 2: Final test: basing on preliminary results, the lowest concentration of Chlorpyrifos ethyl which provided approaching 50% lethal will be selected for starting concentration of final test. Six higher concentration next to starting concentration were developed by x2; x4; x8; x 16; x32 and x64 times, and UTC.

Each concentration was replicated three times. Post stocking density was 30 individuals/ 5lit glass tank and adult stocking density was 20 units/ tank. The shrimp stock was moved to 25L of de-chlorinated tap water in the glass aquaria and stand for 30 minutes before introducing to test.

Survival and mortality were recorded from 1 to 6, 8, 16, 24, 72 and 96 hours. Shrimps were considered dead when the opercular movement ceased and there was no response to gentle probing. Identify LC₅₀ values by using Probit software BioStat denotes the relationship between pesticide concentration and mortality rate.

Sub-lethal bioassay and histological procedures

Based on 96-h LC₅₀ value, the concentrations for chronic study was 10%, 20%; 40%; 60%, 80%, 100%, 120% LC₅₀ as USEPA guideline (Oladimeji, A.A and Ologunmeta, R.T., 1987) and UTC. Each treatment was replicated by 3. Number of shrimps for each replicate was maintained as the same acute test. Test solutions were changed every 12hrs to ensure stable initial concentration less than 10% degradation during testing time (Flores, V., Galan, M., and Sales, D., 1980). Swollen abdomen were sampled at every 10 days within 60 days after pesticide handling. Each tank surgery three individuals. For histopathological analysis, shrimp sampled

were injected with AFA Davidson's fixative, processed, and stained with hematoxylin and eosinphloxine (H&E) using routine histological methods described by Lightner (1996). The histological sections were analyzed by light microscopy (x10, x40 and x100 for typical lesions of acute hepatopancreatic necrosis syndrome or any anormal lesions in the HP magnifications.

Results and Discussion

Determination of LC₅₀ – 96h values

Preliminary experiments: Preliminary results indicated that adults of *Litopenaeus vannamei* were killed at the starting concentration (0.0000268mg^l⁻¹) of chlorpyrifos but at low mortality. When raising tested concentration to 0.000268 and 0.00268mg^l⁻¹, the effect of chemical to that target shrimp was not significantly increased. Where as *Penaeus monodon* at both ages and young stage of *Litopenaeus vannamei* were began to die at the concentrtration of 0.00268mg^l⁻¹ (Table 1).

Table 1. Mortality of *Penaeus monodon* and *Litopenaeus vannamei* caused by Chlorpyrifos ethyl in priliminary tests (Can Tho University Lab., 2013)

Treatment ID	Concentration (mg ^l ⁻¹)	P/ P15	Mortality in 96h (%)		
			P/ adult	L/ P12	L/ adult
T1	0.0000268	0.00	0.00	0.00	4.44
T2	0.000268	0.00	0.00	0.00	3.33
T3	0.00268	21.59	3.57	22.62	8.89
T4	0.0268	100.00	25.00	70.24	88.00
T5	0.268	100.00	100.00	100.00	91.11
T6	2.68	100.00	100.00	100.00	96.67
T7	26.8	100.00	100.00	100.00	100
T8	UTC	0	0	0	0

Legent: T: treatment; P: *Penaeus monodon*; L: *Litopenaeus vannamei*; P12 and P15: post 12 and post 15; UTC: Untreated check

Preliminary experiments: Based on datas of priliminary test, 0.00268mg^l⁻¹ was selected as the starting concentration for final test. Six of next concnetrations were determined by multiple this concentration by 2; 4; 8; 16; 32 and 64 times. It was indicated that both *Penaeus monodon* and *Litopenaeus vannamei* at all tested ages were bagan to die at the first concentration of final test. Young shimps showed more sensitive with chlorpyrifos at the same concentration than adult ones. When raising concentration by 8 times of starting one (means 0.02144mg liter⁻¹), young shrimps of both species were completely died when chemical killed only

haft of adults at that concentration. From the concentration of 0,04288 liter⁻¹ and further, almost adult shrimps were killed (Table 2).

Table 2. Mortality of *Penaeus monodon* and *Litopenaeus vannamei* caused by Chlorpyrifos ethyl in priliminary tests (Can Tho University Lab., 2013)

Treatment ID	Concentration (mg l ⁻¹)	Mortality in 96h (%)			
		P/ P15	P/ adult	L/ P12	L/ adult
T1	0.00268	17.78	6.67	21.11	6.67
T2	0.00536	63.33	9.94	61.1	13.33
T3	0.01072	92.22	20.00	100	33.33
T4	0.02144	100	55.23	100	66.67
T5	0.04288	100	96.67	100	100
T6	0.08576	100	100	100	100
T7	0.17152	100	100	100	100
T8	UTC	0	1.67	0	0

Legent: T: treatment; P: *Penaeus monodon*; L: *Litopenaeus vannamei*; P12 and P15: post 12 and post 15; UTC: Untreated check

Determiration of LC50

With datas collected from final test, the probit lines are developed by Biostat software in Figure 1, 2, 3 and 4 to analyse the correlation between log of chemical concentration and mortality rate of young and adult of *Penaeus monodon* and *Litopenaeus vannamei* respectively, hence the LC50 value is automaticaly determined by the Biostat software (Figure 3). It is indicated that the highest LC50 value of Chlopyrifos ethyl is recorded with adults of *Penaeus monodon* (0.1412mg l⁻¹), next to adults of *Litopenaeus vannamei*, young age of *Litopenaeus vannamei* and *Penaeus monodon* with the values of 0.1197; 0.0042 and 0.0041 mg l⁻¹ respectively. It means adults of both shrimps are more tolerent to Chlopyrifos ethyl than young ones (about 30 times) while the difference of tolerent between two tested species are not clearly recognized. This study also indicated that both brackish water shrimp species currently cultivated in Mekong River Delta of Vietnam is more tolerent to Chlorpyrifos ethyl than other tested aquatic animals which can be died 50% at the concentration of 0.00268mg l⁻¹ in average only.

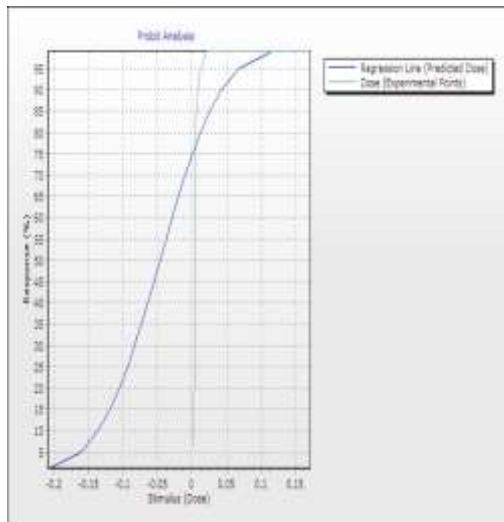


Figure 1. Probit line for determining LC_{50} of Chlopyrifos ethyl to young age of *Penaeus monodon*

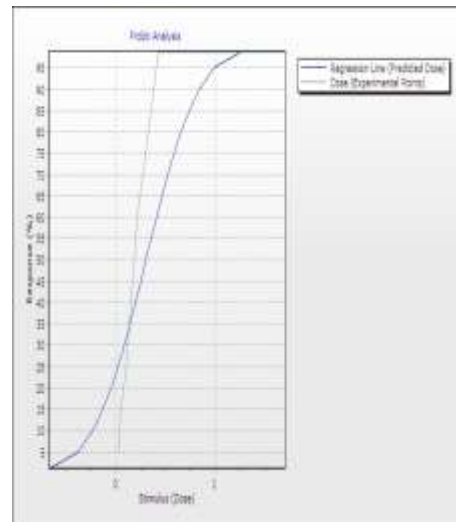


Figure 2. Probit line for determining LC_{50} of Chlopyrifos ethyl to adult of *Penaeus monodon*

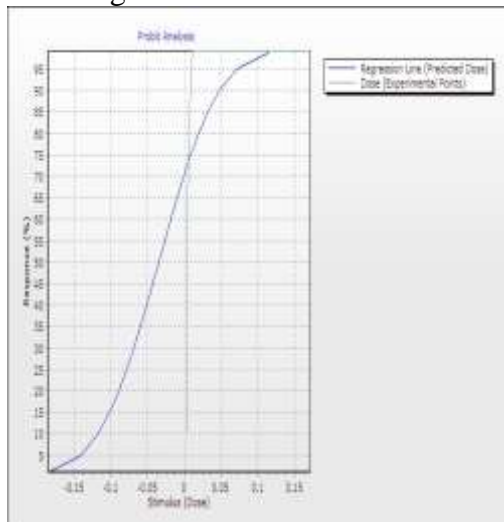


Figure 3. Probit line for determining LC_{50} of Chlopyrifos ethyl to young age of *Litopenaeus vannamei*

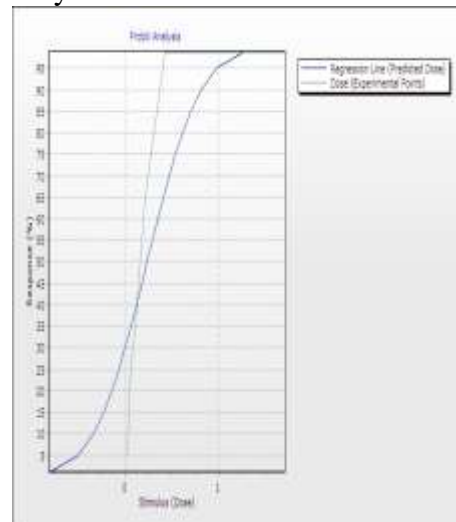


Figure 4. Probit line for determining LC_{50} of Chlopyrifos ethyl to adult of *Litopenaeus vannamei*

Table 3. The Biostat software analysis to determine LC50 value of Chlopyrifos ethyl to *Penaeus monodon* and *Litopenaeus vannamei*

Species/age	Determined mortality (%)	Probit (Y)	Log10[Dose (Stimulus)]	Dose (Stimulus)
<i>Penaeus monodon</i> / Young	50	5	-2.3876	0.0041
<i>Penaeus monodon</i> / Adult	50	5	-0.8501	0.1412
<i>Litopenaeus vannamei</i> / Young	50	5	-2.3773	0.0042
<i>Litopenaeus vannamei</i> / Adult	50	5	-0.9218	0.1197

Histology of gills and liver

The experiment was arranged with healthy shrimps (no liver disease pancreatic necrosis) tested by molecular biological methods. Sampling was conducted at 6 times, 10 days/ time from pesticide treatment. The samples obtained from the experiments were evaluated liver syndrome pancreatic necrosis by histopathological methods. Results are summarized in Table 4.

Table 4. Surgical histopathological assessment of hepatopancreas syndrome necrotizing pancreatitis in Chlorpyrifos ethyl

Sampling	1 st	2 nd	3 rd	4 th	5 th	6 th
Dose						
10% LC ₅₀	(-)	(-)	(-)	(-)	(-)	(-)
20% LC ₅₀	(+)	(-)	(-)	(-)	(-)	(-)
40% LC ₅₀	(-)	(-)	(-)	(-)	(-)	(-)
60% LC ₅₀	(+)	(-)	(-)	(-)	(-)	(-)
80% LC ₅₀	(+)	(-)	(-)	(-)	(-)	(-)
100% LC ₅₀	(*)					
120% LC ₅₀	(*)					
UTC	(-)	(-)	(-)	(-)	(-)	(-)

Ghi chú: (+) abnormalities sign on liver and gill; (-) No abnormalities sign, (*) no sample due to mortality

The histopathology specimens collected from UTC tank showed no signs of liver necrosis, as well as any abnormal expression through repeated sampling. Results are shown in Figure 5. Figure 5 showed a normal pancreas liver with the presence of pancreatic cell types B, R, F in hepatic duct, the normal division of E cells and no presence of the infectious pathogens (viruses, bacteria). The above results demonstrate the stability of experiment condition during layout.

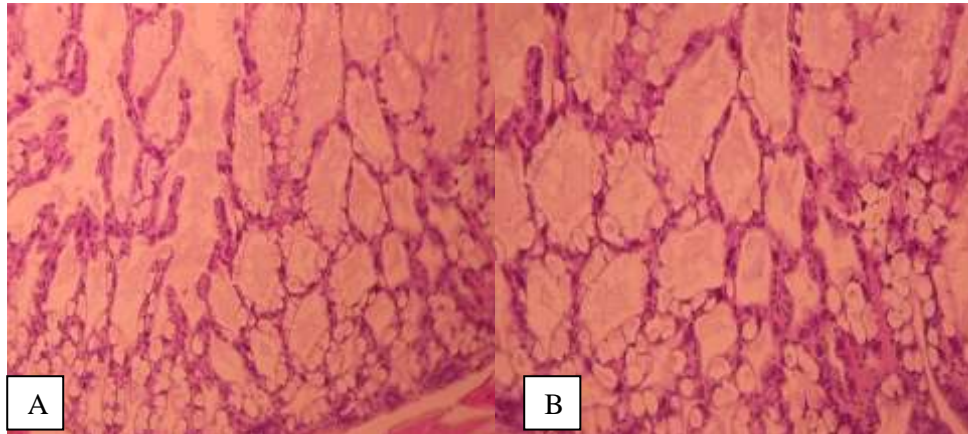


Figure 5. Histopathology shrimp hepatopancreas in the untreated groups (A: at 10days after treatment, B: at 40 days)

At the same time, it was also noted undetectable necrosis signs and any signs of liver disease when treated shrimps at the concentration equivalent with 10 and 40% LC_{50} value. However, several specimens collected from treatment 2 and 4 (20 and 60% LC_{50} value) can be detected some histopathological changes of the liver pancreas. Dynamic of histological treatments are shown in Figure 6, 7, 8, 9.

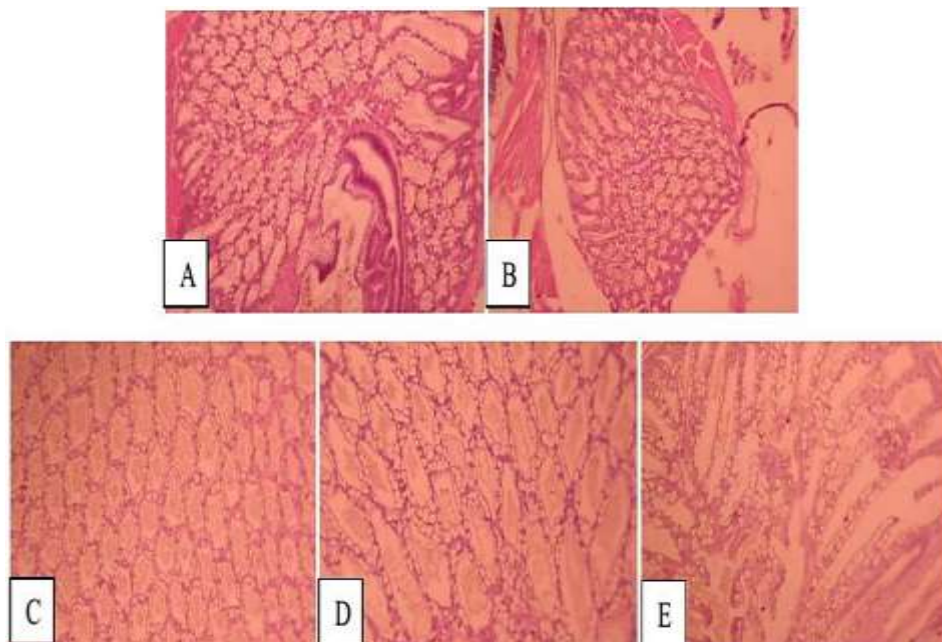


Figure 6. Histopathology shrimp hepatopancreas when treated with Chlopyrifos Ethyl at 10%LC₅₀ value sampled at 10 days (A); 20 days (B); 30 days (C); 40 days (D) and 50 days (E). No sign of abnormality recognized

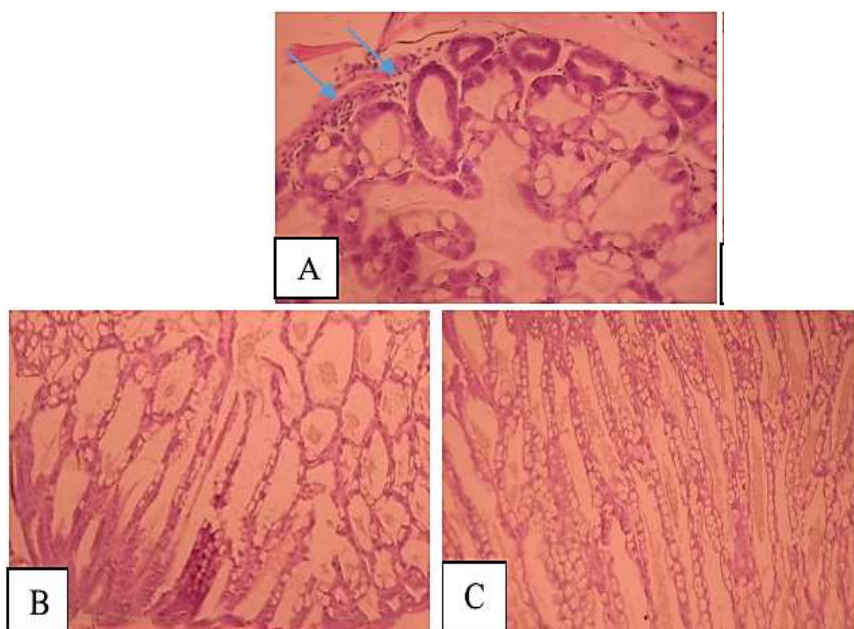


Figure 7. Histopathology shrimp hepatopancreas when treated with Chlopyrifos Ethyl at 20%LC₅₀ value sampled at 10 days (A); 50 days (B); 60 days (C). A showed the recognition of blood cell concentrated around the hepatopancreas (arrow)

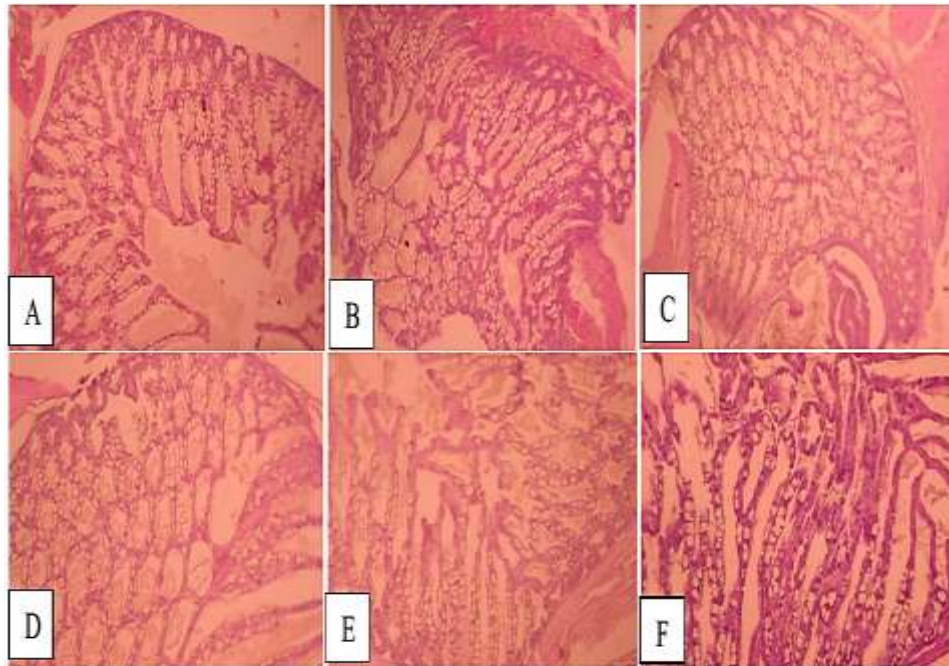


Figure 8. Histopathology shrimp hepatopancreas when treated with Chlopyrifos Ethyl at 40%LC₅₀ value sampled at 10 days (A); 20 days (B); 30 days (C); 40 days (D); 50 days (E) and 60 days (F). No sign of abnormality recognized

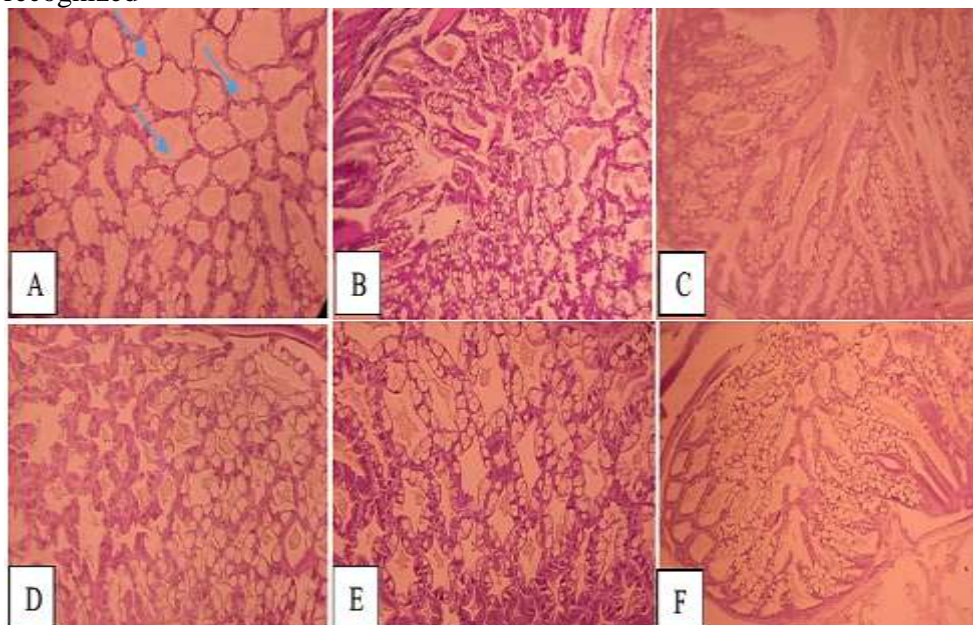


Figure 9. Histopathology shrimp hepatopancreas when treated with Chlopyrifos Ethyl at 60%LC₅₀ value sampled at 10 days (A); 20 days (B); 30 days (C); 40 days (D); 50 days (E) and 60 days (F). A showed hepatopancreatic tubes constricted, reducing B cells, R, F (arrows)

The abotion indicated that thought there were changes on the hepatopancreas of shrimp collected from treatments 2 (20% of LC₅₀ value) at 10days after treatment, only detecting the concentration of blood cells around liver pancreas (Figure 7), no change in the structure of the pancreatic duct liver. However, when observing the specimen collected from treatments 4 (60% of LC₅₀ value) at 10 days, there were some abnormal signs of hepatopancreatic tubes constricted enclosed with the decrease of number of cells B, R, F (Figure 9). Where as there was no change with other treaments (1 and 3) and other observation time. When contacting with the concentration of 100% and 120% of LC₅₀ value, almost shrimps died during 10days after treatment. No sample can be collected for surgery.

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