# การประเมินความเป็นพิษของไกลโฟเสทที่ส่งผลต่ออัตราการตาย และการเปลี่ยนแปลงของเนื้อเยื่อที่พบในปูนา

# Toxicity Evaluation of Glyphosate on Mortality Rate and Histological Alteration in Black Rice Crab (*Sayarmia* sp.)

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## บทคัดย่อ

งานวิจัยนี้วัตถุประสงค์ เพื่อศึกษาความเป็นพิษของไกลโฟเสทที่มีต่อปูนา (*Sayarmia* spp.) โดยประเมินจากอัตราการตายและการเปลี่ยนแปลงของเนื้อเยื่อเหงือกและตับอ่อน ปูนารับสัมผัสกับไกลโฟเสทที่ระดับความเข้มข้น 0.02, 0.2, 2 และ 20 มิลลิลิตรต่อน้ำ 1 ลิตรผสมกับดินตะกอนจากนาข้าว 1 กิโลกรัม หลังจากที่ได้รับสัมผัสกับไกลโฟเสทปูนามีอัตราการตายคิดเป็น 20, 80 และ 100% เมื่อเวลาผ่านไป 96 ชั่วโมงที่ระดับความเข้มข้นของไกลโฟเสท 0.02, 2 และ 20 มิลลิลิตรต่อน้ำ 1 ลิตร ตามลำดับ ค่า LC50 ของปูนาหลังจากที่ได้รับสัมผัสกับไกลโฟเสทที่ระยะเวลา 24, 48, 72 และ 96 ชั่วโมง คือ 25.98, 12.55, 3.65 and 0.97 มิลลิลิตรต่อน้ำ 1 ลิตร ตามลำดับ การเปลี่ยนแปลงที่พบในเนื้อเยื่อเหงือกคือ เกิดการบวมและการคั่งของเลือด และการเปลี่ยนแปลงของเนื้อเยื่อตับอ่อนที่ตรวจพบ คือเกิดการขยายตัวของท่ออิพิทีเลียม เซลล์ถูกทำลาย เกิดแวคคิวโอล และเกิดการตายของเซลล์ โดยการเปลี่ยนแปลงของเนื้อเยื่อที่ตรวจพบนั้นขึ้นอยู่กับระยะเวลาและความเข้มข้นของไกลโฟเสทที่ปูนาได้รับสัมผัส จากผลการศึกษาครั้งนี้สรุปได้ว่าไกลโฟเสทส่งผลกระทบเชิงลบต่อปูนาทั้งต่ออัตราการตายและการเปลี่ยนแปลงของเนื้อเยื่อ ดังนั้นปูนาจึงสามารถนำมาใช้ในการติดตามการปนเปื้อนของไกลโฟเสทในแหล่งน้ำได้

**คำสำคัญ** : ค่า LC50 การวิเคราะห์โพรบิท ไกลโฟเสท ปูนา เนื้อเยื่อ

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## Abstract

This research aimed to study the toxicity of glyphosate on black rice crab (*Sayarmia* spp.) by assessing the mortality rates and alterations in gill tissue and hepatopancreas. The crab was exposed to glyphosate in different concentrations: 0.02, 0.2, 2 and 20 ml $L^{-1}$ of water mixed within 1 kg of clay from rice paddy. After exposed, mortality rates at 96 h were 20, 80 and 100% at the concentrations of 0.2, 2 and 20 ml $L^{-1}$, consecutively. The LC50 values of black rice crab at 24, 48, 72 and 96 h after exposure were 25.98, 12.55, 3.65 and 0.97 ml $L^{-1}$, respectively. The alterations in gill tissue occurred were edema and infiltration of haemocytes; in addition, there were distended lumen, damaged myoepithelial layer, large vacuole and necrosis in hepatopancreas. These alterations depended on exposure time and glyphosate concentration. Based on the results, it concluded that glyphosate adversely affected on black rice crab in both mortality rate and tissue alteration; thus, the black rice crab can be used as biomonitor for glyphosate contamination in the aquatic organism.


Keywords : LC50, probit analysis, glyphosate, black rice crab, histology

### Introduction

Crab is an important living organism playing the role in maintaining, modifying and regulating the environment by influencing both abiotic and biotic components. Their environmental niches are both predator and prey allowing them to locate at different trophic levels in each ecosystem (Siddon & Witman, 2004). Many reports revealed that these macro invertebrates can be used as an indicator species in aquatic habitat for changes in both abiotic and biotic factors (Maharajan *et al*., 2015). Tungare & Sawant (2000) and Maharajan *et al*. (2015) reported that pesticides have been extensively using in agricultural fields that they have physiological effects on pests such as inhibitory effects on growth, food intake, metabolism, enzyme activity and general development. Moreover, they can also affect to aquatic animals such as catfish (*Clariepinus gariepinus*), crab (*Paratelphusa jacquemontii*), and Asian sea bass (*Lates calcarifer*) (Ayanda & Egbamuno, 2012; Maharajan *et al*., 2015; Thanomsit *et al*., 2016a). These pesticides find their way as fate to surface waters through runoff and leaching (Konstantinou *et al*., 2006; Borggaard & Gimsing, 2008).

The Office of Agricultural Economics, Thailand reported that pesticide application has increased four-fold in 10 years, with importation of more than 100,000 tons (OAE, 2010; OAR, 2010). Herbicides represent the highest import followed by insecticides, fungicides, plant growth regulators, and other pesticides (Panuwet *et al*., 2012).

Roundup Transorb (RT) is a glyphosate-based herbicide widely used around the world; however, there has been few studies comparing the effects of the active ingredient and the formulated product (Moreno *et al*., 2014). Although WHO (1994) and Zouaoui *et al*. (2013) reported that pure glyphosate has not direct effect on human, it is in fact applied in agricultural field by mixing with surfactants such as polyoxyethykeneamine and alkylpolyphosphate amine. In 2000, Giesy *et al*. (2000) reported that the Roundup product comprising glyphosate and surfactant can pass through the cell membrane and resulting in the organism being exposed to and accumulate higher concentration of glyphosate. After the human consumptions of aquatic organisms collected from glyphosate contaminated areas, it can cause an adverse effect (Walker *et al*., 2006; Tiamkao, 2014).

In Thailand, black rice crab causes serious damage in rice paddies every farming season. However, local people in Northeastern Thailand cook that crab as local food grilling, black rice crab curry, and to process as pickled or salted crab papaya salad. In addition, it can be pounded and mixed with bran to give as animals feeding. In present, the natural habitat of crab has been decreased thus the farmers breed it for commerce (Pachanawan & Comepuch, 2010). However, there are some problems about breeding information. Thus, most crabs collected from natural habitat have a risk to accumulate some pesticides including glyphosate.

This study aimed to evaluate toxicity level of glyphosate and histological alteration occurred in black rice crab at different exposure times and concentrations. The achievement can be applied as preliminary information in monitoring glyphosate contamination in black rice crab and natural water quality management.

## Methods

### 1. Chemicals

Glyphosate used was the commercial type (N- (phosphonomethyl) glycine, isopropylamine salt 16% W/L SV. Dyes used were Hematoxylin and Eosin which they were purchased from Thermo SCIENCETIFIC, Thailand. Other chemicals were analytical grade from Ajax Finechem Pty Ltd, Thailand.

### 2. Animal acclimatization and treatment

Black rice crab (*Sayarmia* sp.) was purchased from local market in Surin province, Thailand. Their carapace length was 5.2±1.2 cm and weight was 32±1.1 g. They were fed and acclimated in laboratory room in Department of Fisheries, Faculty of Agriculture and Technology, Rajamangala University of Technology Isan Surin Campus, Surin province. After 5 days of acclimatization, the crab was applied to test toxicity level of glyphosate at the concentrations of 0, 0.02, 0.2, 2 and 20 ml $L^{-1}$. For the experiment, two liters of freshwater were prepared and then

mixed with 1 kg of clay in 20 L of glass tank (n=20 ) simulating in the paddy field condition. The study was divided to 5 treatments: (1) control group 1 L of distilled water, (2) 0.02 ml $L^{-1}$ glyphosate added, (3) 0.2 ml $L^{-1}$ of glyphosate, (4) 2 ml $L^{-1}$ of glyphosate, and (5) 20 ml $L^{-1}$ of glyphosate. The crab in each treatment was collected every 24 h for 4 days (n=3) in order to study physiological condition and their gill and hepatopancreas tissue was taken to study histological alteration (Fig. 1).

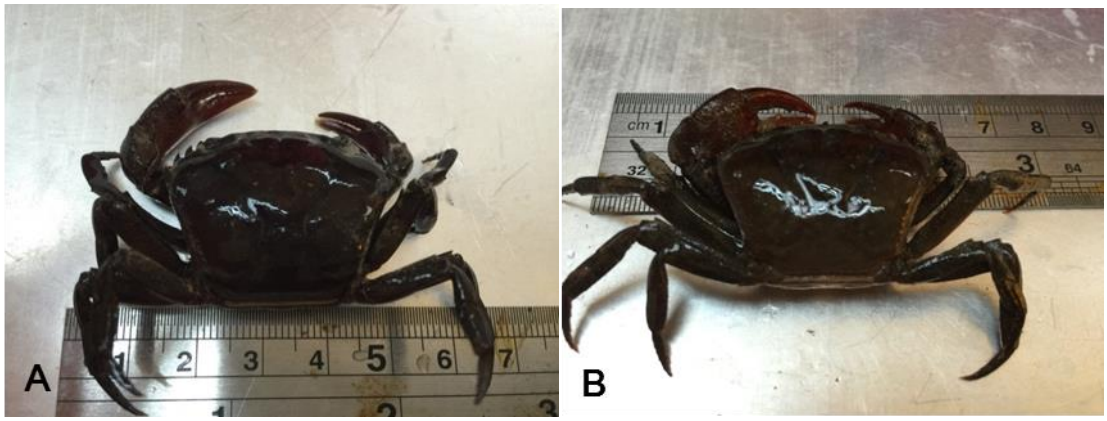


***Figure 1*** External characteristic of studied crab: (A) non-exposed (B) exposed to glyphosate for 96 h

**3. Toxicity testing**

The LC50 value was calculated by using probit analysis. The MS Excel version 2010 was used to find regression equation (Y = mortality; X = concentrations), and the LC50 derived from the best-fit line was achieved.

**4. Histological study**

After the crab tissue was excised and then fixed in aqueous Bouins fluid for 24 h, gill and hepatopancreas tissues, comprising 3 crabs of each group from 3 replicates, were processed for studying histological alteration. The procedure was slightly modified from the method of Chourpagar & Kulkarni (2013), that tissues were firstly dehydrated through 30-100% different alcohol grades and then washed using xylene. The tissues were embedded by cold and hot impregnations in paraffin wax. Serial sections were performed by cutting as 6 μm using rotary microtome that the sectioned gill and hepatopancreas were stained using hematoxylin and eosin as counter stain. The alteration in tissues was recorded and compared to the control. The alterations were classified based on the damage of tissues. The morphological alteration was classified in four severity factors, i.e., unchanged (-), mild occurrence (+), moderate occurrence (++) and severe occurrence (+++).

**Results and Discussion**

1. Cumulative mortality rate and LC50

After exposure of the crab to glyphosate, the mortality was firstly noticed at 24 h at the mixed concentrations of 2 and 20 ml $L^{-1}$ of 20 and 30% mortalities, respectively. Then, the mortality rate increased with time dependence. It was 40 and 50% mortalities at 48 and 72 h. Glyphosate in mixed concentration of 0.2 ml $L^{-1}$ caused 10% mortality. In the concentrations of 2 and 20 ml $L^{-1}$, mortality rates were 60% and 70% at 72 h. In the end of experiment (96 h), accumulative mortalities were 20, 80 and 100% at the mixed concentrations of 0.02, 2 and 20 ml $L^{-1}$, respectively as shown in Fig. 2.

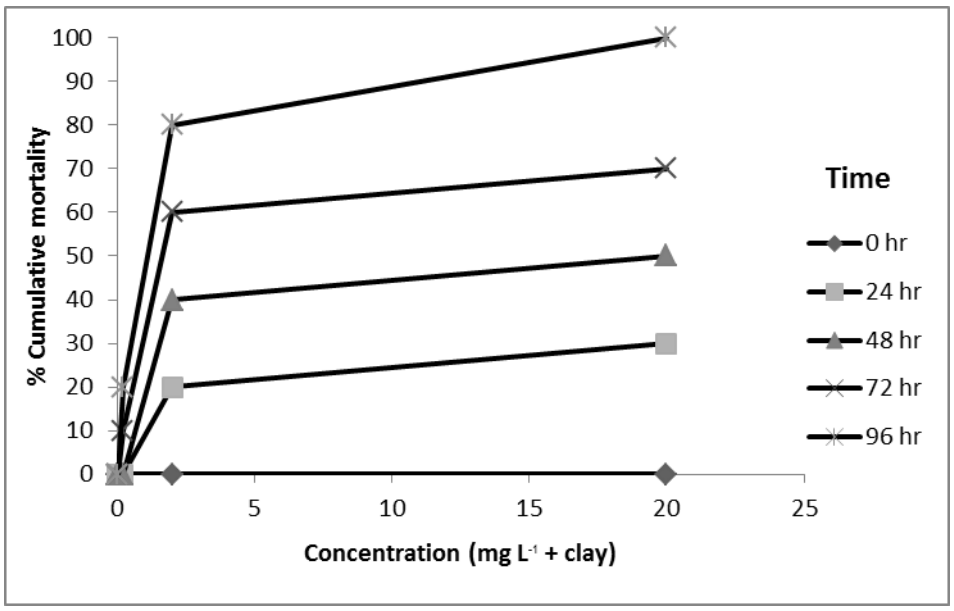


***Figure 2*** Cumulative mortality percentages in the black rice crab after glyphosate exposure at 0, 0.02, 0.2, 2 and 20 ml $L^{-1}$ for 24, 28, 72 and 96 h

The LC50 was calculated, by converting cumulative mortality percentage at 50% after 24, 48, 72 and 96 h of exposure to probit unit. Glyphosate concentration in water was also converted to log scale and then plotted linearly ( Fig. 3A-3D) . After a linear relationship was calculated and then substituted in cumulative mortality percentage at 50% in the form of a log scale. Next, the log value was converted to LC50 of glyphosate. The LC50 values obtained from probit analysis were 25.98, 12.55, 3.65 and 0.97 ml $L^{-1}$ at 24, 48, 72 and 96 h after exposure. The linear equations used in calculation were y=1.76X+2.5102, y=1.975X+2.8305, y=1.809X+3.9824 and y=2.595X+5.038, respectively (Table 1).

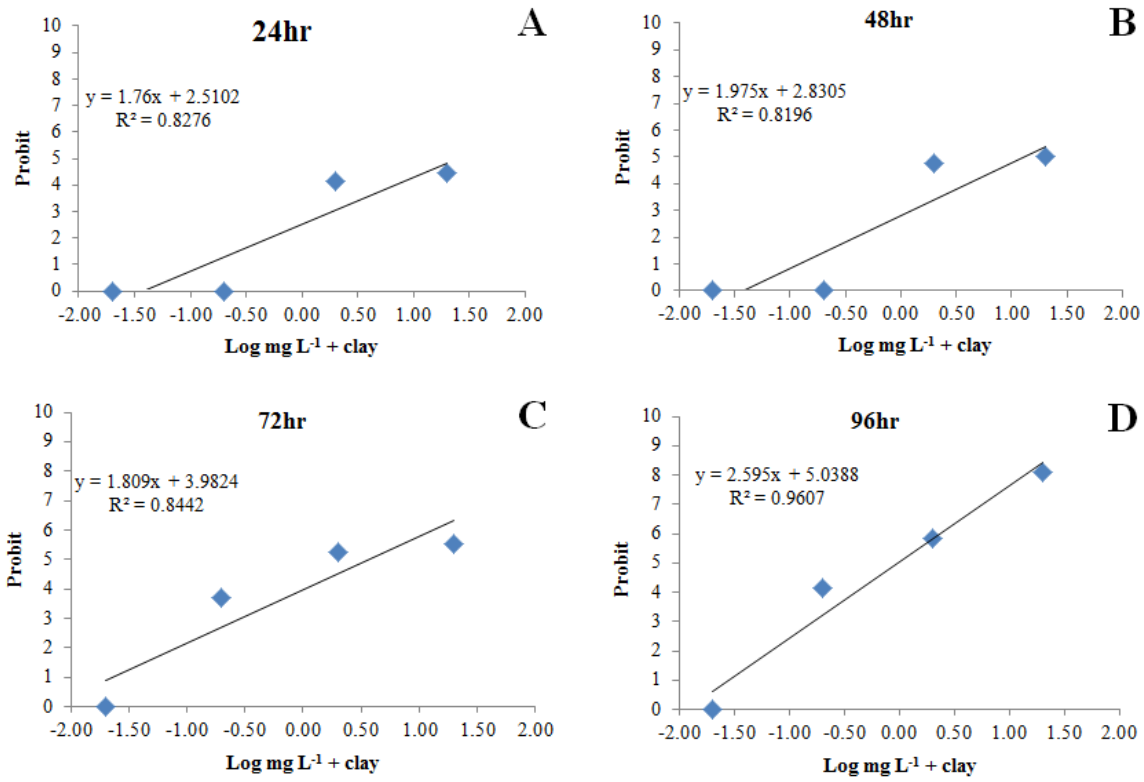


*Figure 3* The linear used to calculate LC50 values at 24, 48, 72 and 96 h after exposure

*Table 1* Linear equation applied to calculate LC50 and LC50 values in exposed crab after 24, 48, 72 and 96 h

| Time (h) | Equations | Log mg $L^{-1}$ | LC50 (mg $L^{-1}$ + clay) |
|---|---|---|---|
| 24 | y=1.76X+2.5102 | 1.41 | 25.98 |
| 48 | y=1.975X+2.8305 | 1.10 | 12.55 |
| 72 | y=1.809X+3.9824 | 0.56 | 3.65 |
| 96 | y=2.595X+5.038 | -0.01 | 0.97 |

2. Physiology and histology alterations

After opening the carapace of black rice crab of both exposed and non-exposed, it was found that the gill of exposed crab was pale and swollen. Moreover, their hepatopancreas color was a lighter pale compared to the non-exposed as shown in Fig. 4.

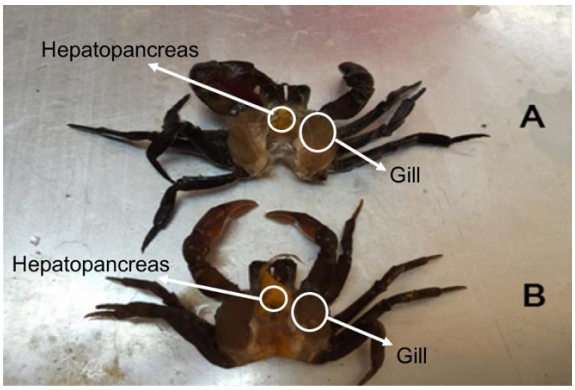


***Figure 4*** The condition of black rice crab with exposed to glyphosate for 96 h (A) and non-exposed (B)

After histological alterations in non-exposed crab was studied with comparing to the exposed, it was found that the non-exposed gill, the normal structure of gill were phyllobrachiate type consisting of central stem (axis, raphe) bore serially for the paired plate or leaf like lamellae. It found that the central axis had afferent and efferent haemal channels in each site. Whereas the connective tissue was located in the gill stem, the branched arthrocytes were in the stem. Besides, the proximal lamellae and lamellar cells were in the epithelium. The outer cuticular layer was liked to a single layer of gill epithelial cells in each plate and the gill epithelium illustrated deep pink of hematoxylin and eosin. The central axis comprised large amount of dark stained haemocytes (Fig. 5A). In the exposed crab, histological changes were as initially recorded after 24 h at the glyphosate concentration of 20 ml $L^{-1}$. The highest alterations found at 96 h were edema and infiltration of haemocytes (Fig. 5B-5E). For hepatopancreas, the changes were firstly found at 24 h at the concentration of 20 ml $L^{-1}$ as same as in the gill. The alterations were distended lumen and damaged myoepithelial cells compared to the structure in the non-exposed ones (Fig. 6A), that myoepithelial cells were destroyed with cell-tear characteristic causing cell abnormality. The alteration increased with an increasing in exposure times and glyphosate concentrations. The highest alteration found at 96 h with 20 ml $L^{-1}$of glyphosate which showed large vacuole and necrosis (Fig. 6B-6E and Table 2).

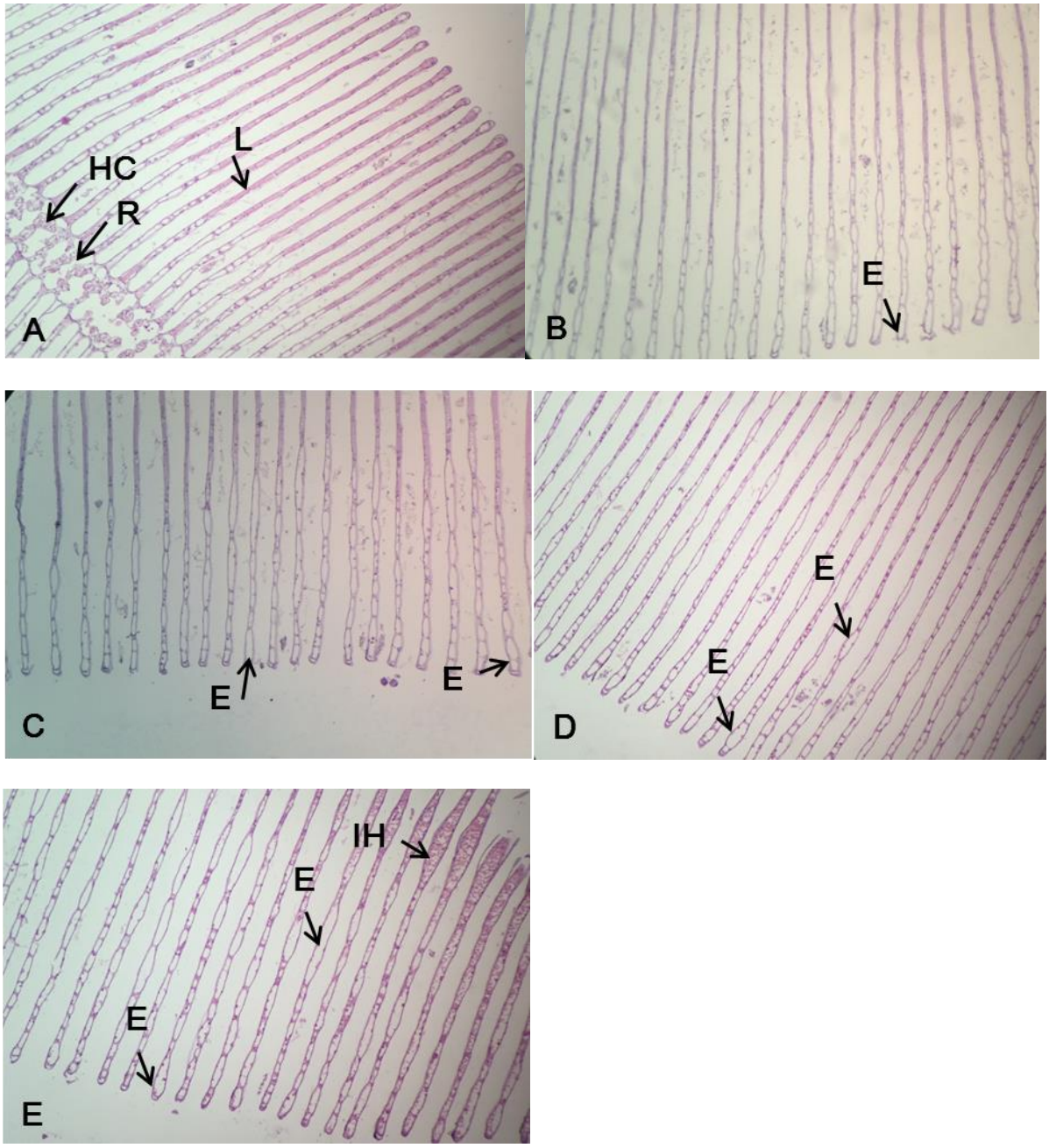


***Figure 5*** Histological alterations in gill of non-exposed black rice crab: (A) and the crab after exposed to glyphosate at the concentration of 20 ml $L^{-1}$ at 24 h (B), 48 h (C), 72 h (D) and 96 h (E); where L: lamella, HC: haemocytes, R: Raphe (Axis), E: edema, IH: infiltration of haemocytes (H & E, x10)

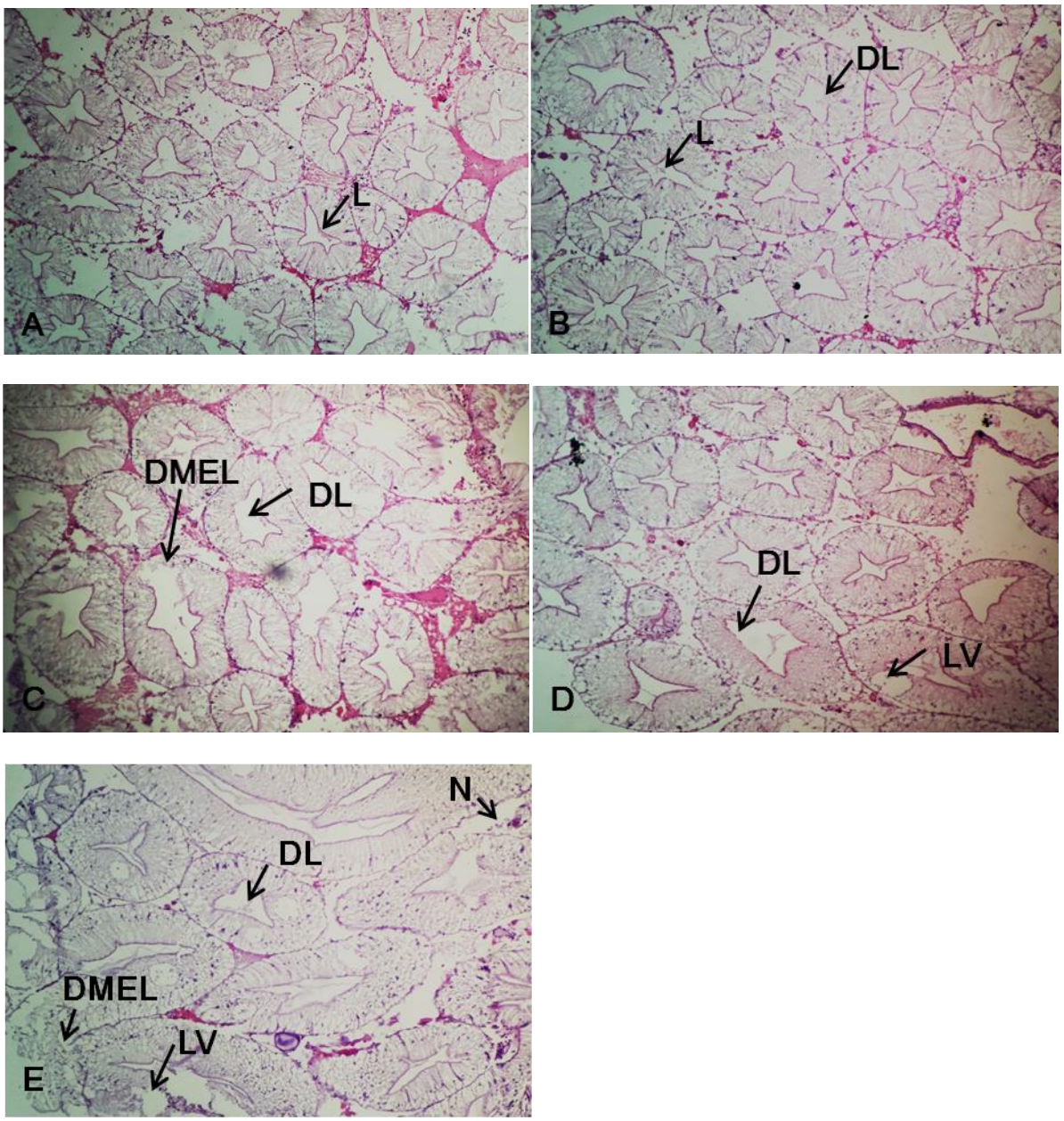


*Figure 6* Histological alterations in hepatopancreas of non-exposed black rice crab: (A) and the crab after exposed to glyphosate at the concentration of 20 ml $L^{-1}$ at 24 h (B), 48 h (C), 72 h (D) and 96 h (E); where L: lumen, DL: distended lumen, DMEL: damaged myoepithelial layer, LV: large vacuole, N: Necrosis (H & E, x10)

***Table 2*** Histological alterations in black rice crab after exposed to glyphosate at the concentrations of 0.02, 0.2, 2 and 20 ml $L^{-1}$ at 24, 48, 72 and 96 h

| Histological alterations | 24 h | | | | 48 h | | | | 72 h | | | | 96 h | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.02 $mlL^{-1}$ | 0.2 $mlL^{-1}$ | 2 $mlL^{-1}$ | 20 $mlL^{-1}$ | 0.02 $mlL^{-1}$ | 0.2 $mlL^{-1}$ | 2 $mlL^{-1}$ | 20 $mlL^{-1}$ | 0.02 $mlL^{-1}$ | 0.2 $mlL^{-1}$ | 2 $mlL^{-1}$ | 20 $mlL^{-1}$ | 0.02 $mlL^{-1}$ | 0.2 $mlL^{-1}$ | 2 $mlL^{-1}$ | 20 $mlL^{-1}$ |
| Gill | | | | | | | | | | | | | | | | |
| - Edema | - | - | - | - | - | - | - | + | - | - | - | ++ | - | - | ++ | +++ |
| - Infiltration of haemocytes | - | - | - | - | - | - | - | - | - | - | - | + | - | - | + | ++ |
| Hepatopancreas | | | | | | | | | | | | | | | | |
| - Distended lumen | - | - | - | - | - | - | - | + | - | - | + | ++ | - | - | ++ | +++ |
| - Damaged myoepithelial | - | - | - | - | - | - | - | + | - | - | + | ++ | - | - | ++ | +++ |
| -Large Vacuole | - | - | - | - | - | - | - | - | - | - | + | ++ | - | - | + | ++ |
| - Necrosis | - | - | - | - | - | - | - | - | - | - | + | + | - | - | + | ++ |

Remark: unchanged (-): changing less than 10%, mild occurrence (+): changing 10-30%, moderate occurrence (++): changing 31-70%, and severe occurrence (+++): changing 71-100%.

**Discussion**

Chemicals polluting the aquatic environment expressed a high environmental concern not only decreasing water quality but affecting living organisms. Invertebrates illustrate a significant potentiality to be used as sentinel organisms to monitor contamination. They are generally small, abundant and sessile and can tolerate to the contaminants. When exposed to the contaminants in both acute and chronic, their cascade-like stress responses are activated. For instance, histopathological lesions and presence of infected organisms are eventually reflected to their status and these sites (Stentiford & Feist, 2005).

Currently, many pesticides are extensively used in agricultural operations. These pesticides have various physiological effects on the pests, such as inhibitory effects on growth, food intake, metabolism, enzyme activity and general development (Tungare & Sawant, 2000) and adverse effects on aquatic animals. Many reports showed that glyphosate is extensively used in Thailand. There were many studies indicating that glyphosate contamination could be occurred and then affected to plants, animals, and human. Harayashiki *et al.* (2013) found that glyphosate could decrease the production and quality of sperm in guppies fish (*Poecilia vivipara*) after exposed to glyphosate in the concentrations of 130 and 700 µg L-1 (commercial form). Moreover, it could cause DNA alteration and mortality as in the study of Braz – Mota *et al*. ( 2015) which reported that freshwater fish

Amazon fish (*Colossoma macroponum*), showed the alteration in gill tissue and amount and type of blood cell. Based on these reports, it showed that this agrochemical had adverse effects on living aquatic organisms. Thus, we studied the toxicity of glyphosate by applying black rice crab as a bio-indicator because they are in widespread in paddy field in Thailand. Moreover, their habitats make them at risk to the glyphosate used by the farmer. In this study, glyphosate could cause mortality in black rice crab while it showed different concentrations compared to other chemicals. In addition, we also studied the mortality rate in different concentrations of glyphosate and exposure times that the crab exposed. The lowest concentration we found mortality was 2 ml L-1 mixed with 1 kg of clay particle. And, LC50 found were 25.98, 12.55, 3.65 and 0.97 ml $L^{-1}$ at 24, 48, 72 and 96 h, respectively. For external and internal morphological conditions, we found that external morphological condition could not be used as indicator compared to the internal especially gill and hepatopancreas.

Histological changes can be applied to evaluate pathological conditions by assessing abnormalities and tissue damages are found after exposed to the toxicants (Sprague, 1971; Andhale *et al*., 2011). Hence, the changes are considered indicator but also a useful data of cell and tissue damages (Shaikh *et al*., 2010). Many reports indicate that histopathology can be applied to evaluate the toxicity of heavy metal added pesticides (Jaykumar, 2002); however; its mode of action is still not-entirely understood. The alteration occurred differently in exposed and non-exposed conditions should be studied critically. In this study, we examined the effect of glyphosate on the crab in 2 tissues; gill and hepatopancreas.

Gill is an organ in crabs playing important role in excretion, acid base balance and ion regulation. Thus, after expose to the pollutants, all of these vital functions are adversely affected and significantly damage their health (Kumar & Tembhre, 2010). So, its gills can be used as an efficient tool to monitor potential impacts (Oliveira Ribeiro *et al*., 2005; Arellano *et al*., 2004; Vigliano *et al*., 2006). Maharajan *et al*. ( 2015) reported that after freshwater crab (*Paratelphusa jacquemontii*) exposed to chlopyrifos and cypermetrin, its gill tissues showed epithelial lifting, edema, necrosis, fusion of secondary lamellae and disappearance of haemocytes. It was in agreement with findings in exposed black rice crab. The gill alterations were hyperplasia, edema and infiltration of haemocytes. Moreover, our results were in agreement with found in crabs treated with lethal concentration of copper sulphate. In their gills, it was found vacuolization in the gill stem, ruptured gill lamellae, damaged connective tissue cells in the stem, destructed and congested haemocytes in the gill lamellae. In addition, the pillar cells were also damaged. The thin connective fluid band was found in between two gill lamellae

(Chourpagar & Kulkarni, 2013). However, the toxicity level was influenced by types and properties of toxicant, concentrations and animal species (Walker *et al*., 2006).

Hepatopancreas is not only digestive organ playing the role in absorption, digestion, storage and secretion but also where biotransformation and detoxification occurs in crustaceans. In the present study, the alterations found in hepatopancreas were distended lumen, damaged myoepithelial layer, large vacuolation and necrosis which increased with an increasing in exposure time and glyphosate concentration. Our findings were in agreement with many previous reports. Freshwater crab (*Paratelphusa jacquemontii*) exposed to chlopyrifos and cypermetrin, showed infiltration, formation of larger lumen size and disappearance of haemocytes in the hepatocytes (Maharajan *et al*., 2015). These histopathological alterations may be caused by the accumulation of the pesticide due to this organ providing the centre of storage, metabolism and detoxification. The rupture of basal laminae found in the hepatopancreatic indicated that tissue integrity occurred after exposed to chlorpyrifos and cypermethrin. The abnormal infiltration of haemocytes in the interstitial sinuses found in the hepatopancreas of the exposed animals indicated that the mechanism of cellular/host defense playing an important role to neutralize the tissue damage caused by chlorpyrifos and cypermethrin beacuse hemocytes are the most important cellular defense in crustaceans (Bodhipaksha & Weeks-Perkins, 1994; Maharajan *et al*., 2015).

All achieved results in this study could be used as preliminary information to evaluate toxicity of glyphosate on black rice crab in both mortality and internal morphological alteration; gill, pancreas and tissues. After literature review performed, we could not find the study on glyphosate toxicity in black rice crab in Thailand. Almost study focused in fish such as nile tilapia (*Oreochomis niloticus*) and Asian sea bass (*Lates calcarifer*) (Jiruangkoorskul *et al*., 2002; Thanomsit *et al*., 2016b). We found the research performed in freshwater crab (*Paratelphusa jacquemontii*) exposing to chorphyrifos and cypermetrin which are insecticides applied in India (Maharajan *et al*., 2015). Some researches focused in heavy metal toxicity such as cadmium and copper (Chourpagar & Kulkarni, 2013). However, the toxicity of glyphosate should be further studied because this study only focused in acute toxicity. It should be deeply studied in sub lethal or chronic toxicity.

## Conclusions

The black rice crabs are found in most part of Thailand especially in paddy field. Thus, they are in risk to expose with glyphosate which is an agro-chemical. The toxicity of glyphosate evaluation on this crab were done. The mortality rate and histological alteration caused by this chemical was depended on exposure time and

concentration. The gill alteration found were edema, hyperplasia and infiltration of haemocytes while there were distended lumen, damaged myoepithelial layer, large vacuolation and necrosis in hepatopancreas. Thus, black rice crab could be applied as bio-indicator for assessing and monitoring glyphosate contamination in the aquatic environment.

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