ผลของไกลโฟเสทต่อพฤติกรรมและการเปลี่ยนแปลงของเนื้อเยื่อเหงือกในปลากะพงขาว (*Lates calcarifer*)

Effect of Glyphosate on Fish Behavior and Histological Alteration of Gills in Asian Sea Bass (*Lates calcarifer*)

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บทคัดย่อ

การศึกษาผลของไกลโฟเสท ที่มีต่อปลากะพงขาวหลังจากสัมผัสกับไกลโฟเสท ที่ระยะเวลา 24, 48, 72 และ 96 ชั่วโมง และระดับความเข้มข้น 0, 2.5, 5, 7.5 และ 10 มิลลิกรัมต่อลิตรตามลำดับ โดยการตรวจสอบการเปลี่ยนแปลงสรีรวิทยา อัตราการตายและการเปลี่ยนแปลงของเนื้อเยื่อเหงือก ในปลากะพงขาวที่ได้รับสัมผัสกับไกลโฟเสท ปลาจะว่ายน้ำผิดปกติ คือ ว่ายน้ำแบบควงสว่าน เสียการทรงตัว มีลำตัวสีซีดจางลง เหงือกมีสีซีดลง ท้องบวม และมีการตกเลือดตามส่วนต่าง ๆ ของร่างกาย อัตราการตายของปลากะพงขาวเพิ่มขึ้นตามระยะเวลาที่ได้รับสัมผัสไกลโฟเสท ค่า LC_{50} ที่คำนวณได้เมื่อปลากะพงขาวได้รับสัมผัสไกลโฟเสทคือ 5.57, 3.55, 2.5 และ 0.76 มิลลิกรัมต่อลิตรที่ระยะเวลาได้รับสัมผัส 24, 48, 72 และ 96 ชั่วโมงตามลำดับ การเปลี่ยนแปลงของเนื้อเยื่อเหงือกที่เกิดขึ้นพบทั้งหมด 3 ระยะ ระยะที่ 1 คือ การบวม การรวมตัวกันและการเปลี่ยนแปลงของเนื้อเยื่อบริเวณซี่เหงือกและเกิดการยกตัวของเนื้อเยื่อของอิพิทีเลียม ส่วนการเปลี่ยนแปลงที่เกิดขึ้นในระยะที่สอง คือ การคั่งของเลือด และระยะที่ 3 การเปลี่ยนแปลงที่พบมีความรุนแรงที่สุด คือเกิดการโป่งพองและมีการตายของซี่เหงือก จากผลการศึกษานี้ สรุปได้ว่าลักษณะการตอบสนองของปลากะพงขาวน่าจะนำมาใช้ในบ่งบอกให้มีการเฝ้าระวังการปนเปื้อนของไกลโฟเสทในแหล่งน้ำได้

คำสำคัญ : ปลากะพงขาว พฤติกรรม ไกลโฟเสท มิญชวิทยา ยากำจัดวัชพืช

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Abstract

Study on effects of glyphosate on Asian sea bass (*Lates calcarifer*) after exposure at 24, 48, 72 and 96 h , and the concentrations of 0, 2.5, 5, 7.5 and 10 mg L^{-1} by measuring behavior change, assess mortality rate and gill tissue alterations of fish were done. The results showed that the exposed glyphosate fish swam erratically with jerky movement. Their movement was loss of balance and faster than that of in the non-exposed. Their body color and gill were fade out. Moreover, edema of gill and abdominal were observed. Hemorrhage was found in various parts of the body. The mortality percentage increased with an increasing in exposure time. LC_{50} of glyphosate in Asian sea bass after 24, 48, 72 and 96 h of exposure were 5.57, 3.55, 2.5 and 0.76 mg L^{-1}, respectively. The alteration found could be distinguished as 3 stages: (1) edema, fusion of lamellae irregular thickening of primary lamellae epithelium and epithelial lifting, (2) blood congestion and (3) lamellar aneurysm and necrosis of lamellae. This responsiveness of Asian sea bass can be used as an early warning signal monitoring glyphosate pollution in the water.

Keywords: Asian sea bass, Behavior, Glyphosate, Histology, Herbicide

Introduction

As generally known, the excessive application of pesticide and herbicide results in a serious problem to environments. Although almost agrochemicals especially pesticide and herbicides are used in farmland or agricultural area; however, became of their fates they can find their way reaching to the aquatic environment is inevitably by drift, runoff, drainage and leaching. After reaches the waters, they cause a serious environmental contamination especially in the shallow and stagnant waters. Organophosphate herbicide is classified as the most dangerous for the aquatic environment (Guilherme *et al*., 2012). Of various commercial formulation, glyphosate has been widely detected in water bodies and affect to both target and non-target organisms especially on fishes (Cavas & Könen, 2007).

Asian sea bass is very economic important. It has been found that the fish grow quite fast and being more resistant to environmental conditions such as temperature and salinity in a wide range. It can live in freshwater, brackish water and seawater (Turchini *et al*., 2009). In present, Thailand is the top leader exporter for Asian sea bass and followed by Taiwan, Indonesia, and Malaysia, respectively (FIGIS, 2006). For the reason mentioned above, the excessive application of glyphosate inevitable causes the contamination in waters and eventually in fishes. Finally, it will find a way to human being and cause the adverse effect. Thus, its toxicity and effect of glyphosate in Asian sea bass must be studied for setting management plan.

Glyphosate is a broad-spectrum herbicide generally applied for controlling a great variety of annual, biennial and perennial grasses, sedges, broad leaved weeds and woody shrubs. Moreover, it is also used for

eliminating aquatic weed in fish ponds, lakes and canals having slow water flows (Tsui & Chu, 2008). Because it is widely used in agricultural areas, WHO (1994) stated that glyphosate is perhaps the most important herbicide that has been ever developed. It is classified as a moderate to very slight toxicant to aquatic animals because of its high water solubility varying from 10,000 to 15,700 mg/L at 25°C (USEPA, 1993; Nwani *et al*., 2013).

In Thailand, glyphosate is a herbicide imported in very high volume although it has been banned in many countries (Praneetvatakul *et al*., 2013). It has many trade names and is still used worldwide. Its effect mechanism is to inhibit the synthesized aromatic amino acids i.e. phenylalanine, tyrosine and tryptophan which are very important in the synthesis of protein and other essential agents for plant growth. It competitively binds with phosphoenolpyruvate (PEP) on the active site of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), thus it is called a competitive inhibitor. PEP is a precursor for aromatic amino acid synthesis, when PEP cannot bind with EPSPS; caused a halt of amino acid synthesis (Tu *et al*., 2001). Moreover, it can inhibit the synthesis of other enzymes such as chlorismatemutase and prephrenatehydratas which also play an important role in the same amino acid synthesis. The disturbed synthesis process is shikimic acid pathway which is found in multi-cellular plants and microorganisms but not found in the animals (Gilchrist & Kosuge, 1980). In addition, shikimate biosynthetic pathway is not only important for aromatic amino acids synthesis but also for the synthesis of auxin, phytoalexins, folic acid, lignin, plastoquinones and secondary products. Because of glyphosate affect only on shikimate pathway, it is believed that it has very low effect on other organisms (WHO, 1996).

The study of histological alteration can be used to assess fish health in contaminated area and moreover it can be applied to establish a causal relation between toxicant exposure and the various biological responses (Schwaiger *et al*., 1997). The dispersion of diseases or pathogens in aquatic organisms especially in fish can be used as indicators of environmental stress (Matthiessen *et al*., 1993). Histological alterations occurred in each organ indicate the impact of endogenous and exogenous on the organism expressing in lesser levels of biological organization (Stebbing, 1985). Recently, both physiological and histological biomarkers are applied extensively in documenting and quantifying exposure and effects of toxicants. For exposure monitoring, the important advantage of biomarker is expressing only pollutants that are biologically available. In the case of effects, biomarkers can indicate the effects of multiple stressors and demonstrate mechanisms of action (Adams, 1990; Flores-Lopes & Thomaz, 2011). There are many researchers such as Ayoola (2008), Jiraungkoorskul *et al.* (2002) and Flores-Lopes and Thomaz, 2011 reported that histological alteration in gill tissue could be used as bio-indicator for toxicant exposure in Tilapia (*Oreochromis niloticus*) and African catfish (*Clarias gariepinus*). The alterations observed were cellular infiltration, filament cell proliferation, lamellar cell hyperplasia, lamellar fusion epithelial lifting and aneurysm. In this study, we investigated toxicity level and the effect of glyphosate on behavior, mortality rate and gill

tissues alterations in Asian sea bass which found in freshwater, brackish water, and seawater. It has been generally known that there is little information on toxicity to brackish water fish. Therefore, we selected the native fish in coastal areas for candidate in the toxicity testing in this study.

Methods

Animals and glyphosate treatments

Glyphosate (41%) was purchased from Sigma Chemical (Thailand). The fish samples with an average body weight of 110 ± 6 g and length of 16 ± 1.2 cm (n=100) were randomly separated into 5 aquaria. They were kept in 250 L of aquaria with 12:12 h photoperiod. The aquaria was aerated with external filtration and a layer of gravel in the bottom. Fish was daily fed with pelleted commercial food for 2 times. They were acclimated to captivity conditions for 2 weeks before taking the tissue samples. Then, it was carefully netted and handled to minimize stress. The concentrations of glyphosate used in this study were 2.5, 5.0, 7.5 and 10.0 mg L^{-1} which prepared by dissolving glyphosate in distilled water. The exposure times applied in each experiment were 24, 48, 72 and 96 h. The fish behavior such as swimming characteristic, operculum opening and body balance were monitored and recorded.

Toxicity testing

LC_{50} of glyphosate which means that the concentration making 50% of accumulative mortality in given exposure time in Asian Sea bass was identified. The gill is very important organ; playing its role in respiratory and gas exchange, was used to evaluate the effect of glyphosate because it is a target organ. The mortality percentage was calculated by using the following equation.

$$\text{Mortality of fish (\%)} = \frac{\text{No. of fish died aquaria}}{\text{Total No. of fish stocked aquaria}} \times 100 \qquad (1)$$

Throughout the experimental period, the water temperature, pH, dissolved oxygen values, turbidity salinity and conductivity were kept at 28.1±0.4ºC, 6.84± 0.02, 3.6±0.20 mg L^{-1}, 21.5 ±3.2 ppm, 18 ppt and 115.1±1.02 µmhos/cm, respectively.

Histological alterations

The method used to study histological alteration was described in Genten et al. (2009). Firstly, gill tissue was fixed by using 10% buffer formalin for at least 24 h. Then, the tissue was sectioned to the thickness less than 0.5 cm. Next, it was filled in cassette block and soaked in decalcification solution in order to 6 h for softening. It was washed by running tap water and then soaked in 5% Na_2SO_4 for 4-6 h for acid-base conditioning. After that, it was inserted in paraffin block and sectioned to be thickness of 5 µm using microtome. The sectioned tissue was placed on glass slide and warmed to fix tissue onto the slide at least 12

h. Next, it was stained by Mayer's hematoxylin and Eosin and then made permanent slide using permount solution. Finally, its histological alteration was studied under high magnified microscope and photographed.

The histological alteration was scored as 0 to 3; where, 0 = no alteration, 1 = slight alteration, 2 = moderate alteration and 3 = severe alteration (Hose *et al*., 1996). For slight alteration, the changes do not permanently damage gill tissues. The change is limited into small parts of the gills or some filaments such as the epithelium of the primary lamella. For moderate alteration, the changes do more severe and lead to effects in tissues associated with the functioning of the organ such as reparable lesions. The most areas of gills are affected in situations of chronic pollution and lead to severe alterations. However, it occurs on only the surface of the gills such as epithelial lifting of secondary lamella. The last stage severe alteration, the recovery of the gill structure is not possible although water quality is improved such as aneurysms. This score was used to determine the severity of alterations.

Results and Discussion

Glyphosate is the herbicide wildly used. It is the top imported agro-chemicals in Thailand. As the producer claim that it can be absorbed on soil particles and the probability of leaching to the environment is very less. Many studies revealed that it can be occurred especially in the case of spraying. It will contaminate to the freshwater and finally the sea water. The contamination level is depend on the quantity of glyphosate applied and rain water which can leach the adsorbed glyphosate from the soil (Peruzzo *et al*., 2008). In this study, after the fish exposed to glyphosate it swam unusually and uncontrollably showing spinning movement with loss of balance. Their body color faded out and gill was swollen. The hemorrhage of air and restlessness which opposite to non-exposed fish, Figure 1 shows percentage cumulative mortality rates in the fish after exposed to glyphosate in the concentrations of 2.5, 5, 7.5 and 10 mg L^{-1}. It was found 100% mortality in glyphosate exposure in the concentrations of 7.5 and 10 mg L^{-1} for 24 h. However, in the fish exposed to glyphosate in the concentration of 2.5 mg L^{-1}, the mortality rate increased with an increasing in exposure time. The mortality rates after 24, 48, 72 and 96 h of exposure were 10%, 20%, 60% and 90%, respectively. For the fish exposed to glyphosate in the concentration of 5. 0 mg L^{-1}, the mortality rates at 48 h, 72 h and 96 h were 60%, 80% and 100%, respectively.

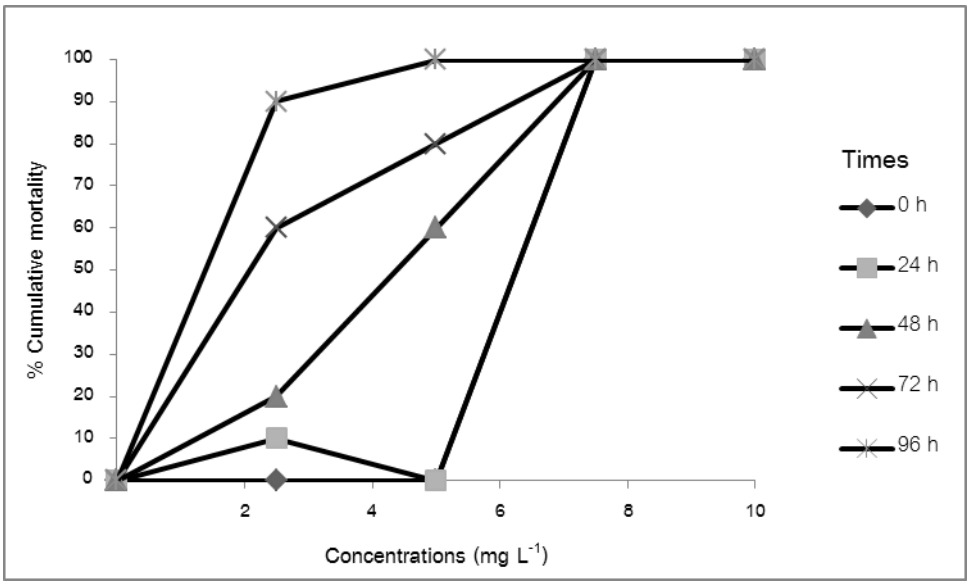

Figure 1 Percentage of cumulative mortality rates of fish with and without exposure to glyphosate in the concentration of 2.5, 5.0, 7.5 and 10.0 mg L^{-1} for 24, 48, 72 and 96 h

For LC_{50}, figure 2 shows the relations of probits value and logarithm function of glyphosate concentration which being converted from accumulative percentage and glyphosate concentration. LC_{50} can be calculated from the relations showing in each graph. For the example, in the case of y = 5 (50%) which getting x = log mg L^{-1} applying in the equation as y = 9.1153x-1.7976, y = 7.2427x+1.0163, y= 5.2764x+2.8971 and y =3.0541x+5.3683 at 24, 48, 72 and 96 h of exposure time, respectively.

The LC_{50} in each exposure time (24, 48, 72 and 96 h) was 5.57, 3.55, 2.5 and 0.76 mg L^{-1}, respectively which shown in table 1. In the case of quite low relation of LC_{50} at 24 h might be explained by the very low mortality rate occurred (< 50%). Thus, when applied it to calculate in the linear equation, thus the achieved value is low. This result was different from the study performed in cachama blanca fish (*Piaractus brachypomus*) which having LC_{50} value of 97.47 mg L^{-1} for the Roundup (Ramírez-Duarte *et al.,* 2008). Moreover, it was not in agreement with the LC_{50} value of glyphosate in young Nile tilapia (*Oreochromis niloticus*) which having 17.5. 17.1 16.9 and 16.8 l ppm. For matured fish, it was 46.9, 44.4, 44.0 and 36.8 ppm at 24, 48 72 and 96 h after exposure (Jiraungkoorskul *et al*., 2002). Based on this finding, it can be concluded that the difference of species and size influenced on LC_{50} determination.

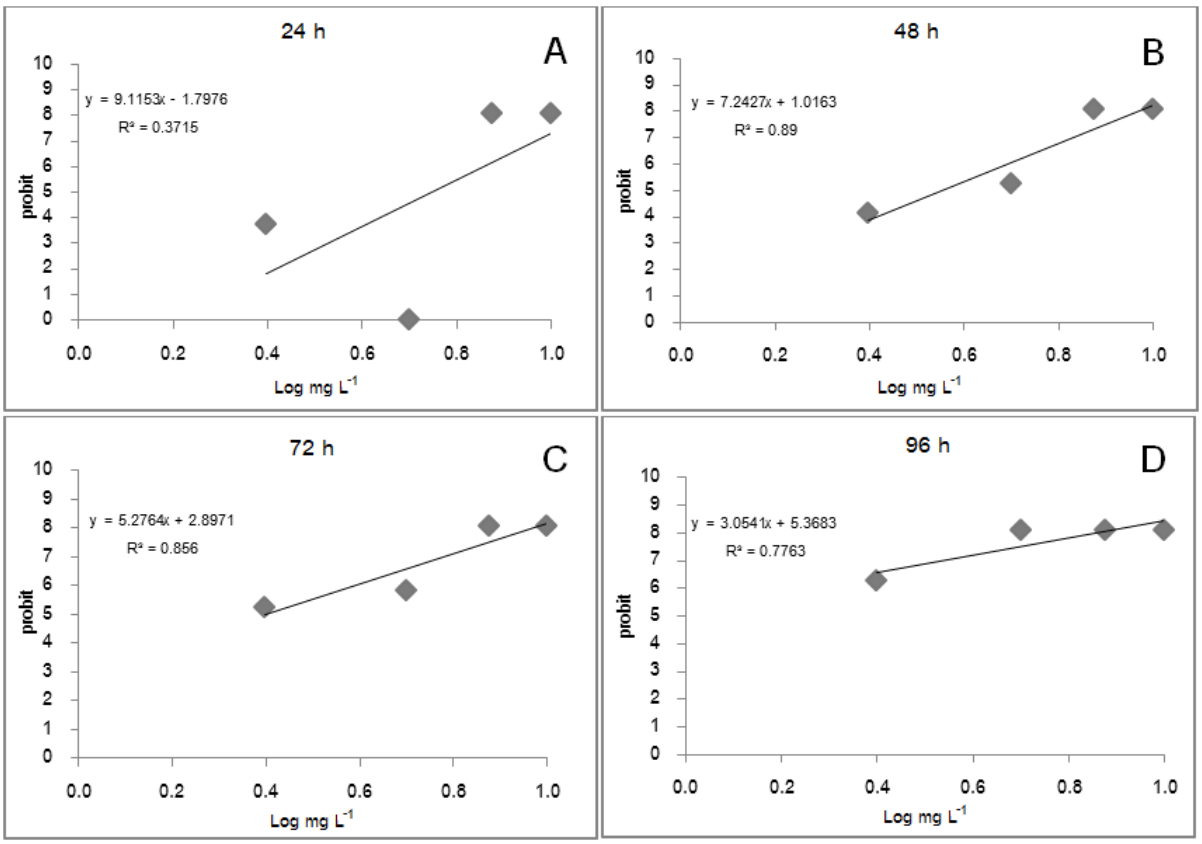

Figure 2 The determination of LC_{50} in each exposure time 24 h (A), 48 h (B), 72 h (C) and 96 h (D)

Table 1 LC_{50} of glyphosate in Asian sea bass at different exposure time

Time of Exposure	Concentrations (log mg L^{-1})	LC_{50} (mg L^{-1})
24 h	0.74573519	5.57
48 h	0.55002969	3.55
72 h	0.39854825	2.5
96 h	-0.120592	0.76

Histological alterations in gill tissue of the fish after exposed to glyphosate in the concentration of 0, 2.5, 5, 7.5 and 10 mg L^{-1} for 24, 48, 72 and 96 h were studied. It was found that the alteration was increased with an increasing in exposure time and concentration. The alteration could be separated into 3 periods as shown in Table 2 and Figure 3.

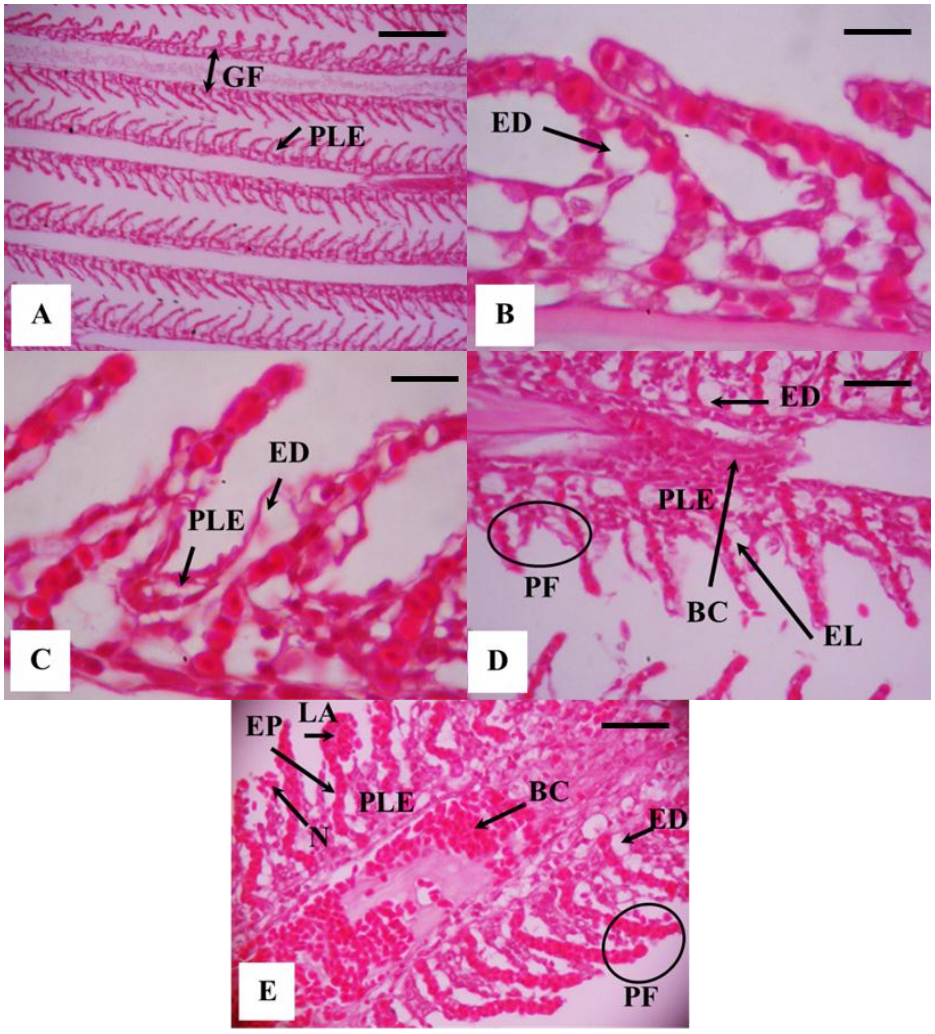

Figure 3 Transverse section of fish in non-exposed and exposed to glyphosate.

A. Non-exposed fish showing normal appearance of gill filament and secondary lamellae where GF: Gill filament, PLE: Primary lamellae epithelium, scale bar replacement 100 µm (x10)

B. Fish exposed to glyphosate in the concentration of 2.5 mg L^{-1} for 72 h, where ED: Edema, scale bar replacement 10 µm (x100)

C. Fish exposed to glyphosate in the concentration of 5.0 mg L^{-1} for 48 h, where ED: Edema, PLE: primary lamellar epithelium, scale bar replacement 10 µm (x100)

D. Fish exposed to glyphosate in the concentration of 5.0 mg L^{-1} for 72 h, where ED: Edema, PLE: primary lamellar epithelium, PF: Partial fusion of lamellae, BC: Blood congestion, EL: Epithelial lifting, scale bar replacement 25 µm (x40)

E. Fish exposed to glyphosate in the concentration of 5.0 mg L^{-1} for 96 h, where ED: Edema, PLE: primary lamellar epithelium, PF: Partial fusion of lamellae, BC: Blood congestion, EL: Epithelial lifting ,LA: Lamellar aneurysm, scale bar replacement 25 µm (x40)

Table 2 Histological alterations in the gills of non-exposed and exposed fish with glyphosate in the concentrations of 2.5, 5.0, 7.5 and 10.0 mg L^{-1} for 24, 48, 72 and 96 h

Histological alterations stage	Exposure time and concentrations															
	24 h				48 h				72 h				96 h			
	2.5 mg/L	5 mg/L	7.5 mg/L	10 mg/L	2.5 mg/L	5 mg/L	7.5 mg/L	10 mg/L	2.5 mg/L	5 mg/L	7.5 mg/L	10 mg/L	2.5 mg/L	5 mg/L	7.5 mg/L	10 mg/L
Stage I	0	0	+	++	0	+	++	+++	++	+++	N/A	N/A	+++	+++	N/A	N/A
Stage II	0	0	0	0	0	+	++	++	0	+	N/A	N/A	+	+++	N/A	N/A
Stage III	0	0	0	0	0	0	0	0	0	++	N/A	N/A	0	+++	N/A	N/A

Remark 1: N/A cannot detected, where 0 = no alteration, 1 = slight alteration, 2 = moderate alteration and 3 = severe alteration

Remark 2: Stage 1 comprising; edema, fusion of lamellae irregular thickening of primary lamellae epithelium and epithelial lifting

Stage 2 comprising; blood congestion

Stage 3 comprising; lamellar aneurysm and necrosis of lamellae

In the first period, the alteration could be observed in the fish exposed glyphosate in the concentration of 2.5 mg L^{-1} for 72 h. The histological alterations found in gill was edema, fusion of lamellae irregular thickening of primary lamellae epithelium and epithelial lifting. In the second period, it was found blood congestion in the fish exposed to glyphosate for 72 h. And the third period, the most serious alteration was lamellar aneurysm and necrosis of lamellae was observed in fish exposed to glyphosate in the concentration of 5.0 mg L^{-1} for 96 h. The fish exposed to glyphosate in the concentration of 7.5 and 10.0 mg L^{-1} for 24 h showed 100% mortality and the symptoms observed were the same as previously with more serious. The studied alteration which was separated into three states followed the report of Flores-Lopes and Thomaz (2011). They demonstrated that the alteration of gill tissue could be used as bio-indicator to monitor environmental condition. Histopathological alteration in gills were used to determine semi-quantitatively regarding to the degree of tissue alteration (Histopathologic alterations index - HAI) based on the severity of the lesions. Jiraungkoorskul *et al*. (2002) reported that tilapia (*O. niloticus*) which exposed to glyphosate at the concentrations of 50 to 97.47mg L^{-1} showed filament cell proliferation, lamellar cell hyperplasia, lamellar fusion, epithelial lifting and aneurysm in the gill. In catfish (*C. gariepinus*) after exposed to glyphosate, it was observed edema, epithelial lifting and fusion of secondary lamellae in their gill (Ayanda & Egbamuno, 2012). Moreover, it was found that LC_{50} at 48 h of glyphosate in *Piaractus mesopotamicus* was 3.74±0.2 mg L^{-1} and expressing important alteration in hyperplasia, pillar cell system enlargement and edema (Shiogiri *et al*., 2012). This alteration was also found in this study. The gills of fish are sensitive organ easily affected by many toxicants even though in low concentrations (Karlsson, 1983). Fish gill plays the important role in various functions; respiration, osmoregulation and excretion, and has a large surface area contacting with the

external environment. Hence, it is sensitive to chemical and physical changes in the aquatic environment (Mallatt, 1985; Mazon *et al*., 1999; Cerqueira & Fernandes, 2002). Gill is the route of chemicals in the aquatic environment to entry into fish body via the gill lamellar sieve by the branchial pump, diffusion of the chemical across the water flow channel and the gill epithelium and further into the blood (McKim & Eriekson, 1991). Therefore, the alteration of gill tissue of exposed fish is of paramount importance to study.

Conclusion

In conclusion, the result indicated that glyphosate had the effect on Asian sea bass in both physiological and behavior alteration, mortality rate and gill tissue alteration. The histological alteration was seen obviously and indicated that glyphosate in the low concentration can cause the adverse effect. Moreover, the alteration in gill tissue was also depended on exposure time. Thus, these responsiveness of Asian sea bass can be used as an early warning signal monitoring glyphosate pollution in the water.

References

Adams, D.M., Shugart, L.R., Southworth, G.R., & Hinton, D.E. (1990). Application of bioindicators in assessing the health of fish populations experiencing contaminant stress. In McCarthy JF & Shugart, L.R. (Eds.), *Biomarkers of environmental contamination.* (pp. 333-353).Boca Raton, USA: Lewis Publishers.

Ayanda, O.I., & Egbamuno, E.A. (2012). Histology examination of the liver and gills of *Clarias gariepinus* treated with glyphosate. *Environmental Research Journal 6*(3), 228-234.

Ayoola, S.O. (2008). Toxicity of glyphosate herbicide on Tilapia (*Oreochromis niloticus*) juvenile. *African Journal of Agricultural research, 3*(12), 825-834.

Cavas, T., & Könen, S. (2007). Detection of cytogenetic and DNA damage in the peripheral goldfish (*Carassius auratus*) exposed to a glyphosate formulation using the micronucleus test and comet assay. *Mutagenesis, 22*, 263-268.

Cerqueira, C.C.C., & Fernandes, M.N. (2002). Gill tissue recovery after copper exposure and blood parameter responses in the tropical fish, *Prochilodus scrofa*. *Ecotoxicology and Environment Safety*, *52*, 83-91.

Flores-Lopes, F., & Thomaz, A.T. (2011). Histopathologic alterations observed in fish gills as a tool in environmental monitoring. *Brazilian Journal of Biology*, *71*(1), 179-188.

FIGIS. (2006). *Global aquaculture production 1950-2004*. Rome, Italy: FAO

Gilchrist, D., & Kosuge, T. (1980). Aromatic amino acid biosynthesis and its regulation (pp. 507-531). In Miflin BN (Eds.), New York: The Biochemistry of Plants Academic Press.

Guilherme, S., Gaivãob, I., Santosa, M.A., & Pachecoa, M. (2012). DNA damage in fish (*Anguilla anguilla*) exposed to a glyphosate-based herbicide-Elucidation of organ-specificity and the role of oxidative stress. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. *743*, 1-9.

Hose, J.E., McGurk, M.D., Marty, G.D., Hinton, D.E.B., & Baker, T.T. (1996). Sublethal effects of the Exxon Valdez oil spill on herring embryos and larvae: morphological, cytogenetic, and histopathological assessments, 1989-1991. *Canadian Journal of Fish and Aquatic Science, 53*, 2355-2365.

Jiraungkoorskul, W., Upathum, E.S., Kruatrachue, M., Sahaphong, S., Vichasri-Grams, S., and Pokethitiyook, P. (2002). Histopathological effects of Roundup, a glyphosate herbicide, on Nile tilapia (*Oreochromis niloticus*). *ScienceAsia*. *28*, 121-127.

Karlsson, L. (1993). Gill morphology in the zebrafish, *Brachydanio rerio* (Hamilton-Buchana). *Journal of fish biology*, *23*, 511-524.

Mallatt, J. (1985). Fish gill structure changes induced by toxicants and other irritants: a statistical review. *Canadian Journal of Fisheries and Aquatic Sciences*, *42*, 630-648.

Matthiessen, P., Thain, J.E., Law, R.J., & Fileman, T.W. (1993). Attempts to assess the environmental hazard posed by complex mixtures of organic chemicals in UK estuaries. *Marine pollution bulletin*. *26*, 90-95.

Mazon, A.F., Cerqueira, C.C.C., Monteiro, E.A.S., & Fernandes, M.N. (1999). Acute copper exposure in freshwater fish: Morphological and physiological effect. In A.L. Val, VMF. Almeida-Val. (Eds.), *Biology of tropical fishes*. (pp.263-275). Manaus: INPA.

McKim, J.M., & Erickson, R.J. (1991). Environmental impacts on the physiological mechanisms controlling xenobiotic transferacross fish gills. *Physiological zoology*, *64*,39-67.

Nwani, C.D., Ibiam, U.A., Ibiam, O.U., Nworie, O., Onyishi, G., & Atama, C. (2013). Investigation on Acute toxicity and behavioral changes in *Tilapia zillii* due to glyphosate- based herbicide, forceup. *The journal of Animal and Plant Sciences*. *23*(3), 888-892.

Praneetvatakul, S., Schreinemachers, P., Pananurak, P., & Tipraqsa, P. 2013. Pesticides, extermal coasts and policy options for Thai agriculture. *Environmental Science and Policy, 27*, 103-113.

Peruzzo,P. J., Porta, A. A. and Ronco, A E. (2008). Levels of glyphosate in surface waters, sediments and soils associatedwith direct sowing soybean cultivation in north pampasic region of Argentina. *Environmental Pollution*, *156* (1). 61- 66

Ramírez-Duarte, W.F., Rondón-Barragán, I. S. & Eslava-Mocha, P.R.(2008). Acute toxicity and histopathological alterations of Roundup® herbicide on cachama blanca (*Piaractus brachypomus*). *Pesquisa Veterinary Brasileir*. *28* (11).547-554

Schwaiger, J., Wanke, R., Adam, S., Pawert, M., Honnen, W., & Triebskorn, R. (1997). The use of histopathological indicators to evaluate contaminant-related stress in fish. *Journal of Aquatic Ecosystem stress and Recovery*, *6*, 75-86.

Shiogiri, N.S., Paulino, M.G., Carraschi, S.P., Baraldi, F.G., Cruz, C., & Fernandes, M.N. (2012). Acute exposure of a glyphosate-based herbicide affects the gills and liver of the Neotropical fish, *Piaractus mesopotamicus*. *Environmental Toxicology and Pharmacology*, *34*, 388-396.

Stebbing, A.R.D. (1985). *A possible synthesis*. In B.L., Bayne. (Eds.). The effects of stress and pollution on marine animals, New York: Praeger.

Tsui, M.T., & Chu, L.M. (2008). Environmental fate and non-target impact of glyphosate-based herbicides (Roundup) in a subtropical wetland. *Chemosphere*, *71*, 439-446.

Tu, M., Hurd, C., & Randall, J.M. (2001). *Weed control method handbook: Tools and Techniques for use in natural Areas*. California: The nature Conservancy.

Turchini GM, Torstensen BE, Ng WK. (2009). Fish oil replacement in finfish nutrition. *Reviews of Aquaculture*, *1*, 10-57.

US Environmental protection Agency (USEPA).(1993). *Registration Eligibility Decision (RED): Glyphosate.* US Environmental Protection Agency, Office of prevention, Pesticides and Toxic Substances, Washington, DC.

World Health Organization (WHO). (1994). *Glyphosate*. Environmental Health Criteria 159. WHO, Geneva, Switzerland.

World Health Organization (WHO). (1996). *Data sheets on pesticides: glyphosate. International Programme on Chemical safety*, Food and Agriculture Organization, Geneva, Switzerland. Differential effects of glyphosate and roundup on human placental cells and aromatase.