EnvironmentAsia
The international journal published by the Thai Society of Higher Education Institutes on Environment

Available online at www.tshe.org/ea/index.html
EnvironmentAsia 9(2) (2016) 72-79
DOI 10.14456/ea.2016.10

Histological Alterations in Asian seabass (*Lates calcarifer*) during Exposed to Non-Essential and Essential Elements

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Abstract

The aim of this study was to investigate the histological alterations and melanomacrophage and pigmented macrophage counting in Asian seabass (*Lates calcarifer*) after exposed to non-essential ($HgCl_2$) and essential ($NiCl_2$, $ZnSO_4$ and $CuSO_4$) elements by duration time (24, 48 and 72 h) and concentration (2, 4 and 6 mg/kg) of exposure. We found that the type of element and concentration of exposure causes the alterations of gill, liver, and kidney. Observable damages to gills include epithelial lifting, hyperplasia, oedema, and partial fusion of lamellae. In the liver tissue, enlargement of sinusoid and vacuolation were observed. As for kidney tissue, there was occurrence of degeneration of tubular, vacuolation, melanomacrophage aggregate, Bowman' space increase, and dilation of renal tubular. The highest of alteration exhibited in kidney. Melanomacrophage and pigmented macrophage counting showed the highest values of 173±3 at 6 mg/kg. $HgCl_2$ concentration for 72 h which was 2.1 time higher than control and vehicle control group, in contrast, the lowest was observed in $ZnSO_4$ at 2 mg/kg for 24 h. Both in non-essential and essential treated group melanomacrophage and pigmented macrophage counting were significant different as compared to control and vehicle control group with 95% confidence ($p < 0.05$). In addition, the comparisons of severity of tissues alteration showed that the $HgCl_2$ caused the highest alteration while the $ZnSO_4$ caused minimal alteration ($HgCl_2$> $NiCl_2$> $CuSO_4$> $ZnSO_4$). Kidneys exhibited the highest level of tissue alteration, while gills showed the least alteration.

Keywords: histology; non-essential and essential element; Asian seabass; *Lates calcarifer*; melanomacrophage

1. Introduction

An increasing in industrial development adversely causes heavy metal contamination in the environment especially in the aquaria. These heavy metal elements can be divided into 2 groups; non-essential and essential elements (Wood *et al*., 2011; Wood *et al*., 2012). Though essential element; such as Zn and Cu, benefits to the organisms (Wood *et al*., 2012) but in the excessive amount it can cause harmful effect (Adhikari *et al*., 2009). This adverse effect can be increased when passing through food chain and eventually reach human being. Thus, heavy metal contamination monitoring in aquatic organism, especially in fish, should be established as warning program.

Histology can detect sign of disease not easily recognized on gross examination and can therefore be of interest in fish health supervision. Nearly 60% of all vertebrate are fish species and are economically of major importance. Fish health in fisheries is therefore an important concern and it is not always possible to diagnose fish disease purely on the basis of behavior or physical changes. Further evaluations and test are often crucial to arrive at a definite analysis (Genten *et al*., 2009).

The study of histological alteration would be useful for health monitoring and management of fish population. Moreover, this phenomenon provides a better understanding of the polluted-related health effect in fish. Several tissues can be damage during sublethal of non-essential and essential element. Such as Mela *et al*. (2007) reported that methylmercury had effect on liver and kidney in neotropical fish (*H. malabaricus*). Furthermore, Cerqueira and Fernandes (2002) investigated the gill of tropical fish (*P. scrofa*) after

exposure to $CuSO_4$, the changes included epithelial lifting, cell swelling, pavement, chloride and mucous cell proliferation, and blood vessel anomalies.

In this work, we investigated the histological alterations in gill, liver, and kidney of Asian seabass after exposure to non-essential ($HgCl_2$) and essential ($NiCl_2$, $ZnSO_4$ and $CuSO_4$) elements by difference duration times and concentrations of exposure. Moreover, we applied the melanomacrophage and pigmented macrophage counting in kidney to assess the metal exposure in Asian seabass.

2. Materials and Methods

2.1 Animals husbandry

Healthy specimens of juvenile Asian seabass (*Lates calcarifer*) were collected from farm in Chachoengsao Province, Thailand. Fish were maintained in 2000-L tanks, aerated freshwater at 27 ºC and acclimated for 5 days and no feeding during the studies period.

2.2 Non-essential and essential element dosing

Non-essential ($HgCl_2$) and essential element ($NiCl_2$, $ZnSO_4$ and $CuSO_4$) were added to normal saline buffer solution. After acclimation, juvenile Asian seabass ($n=3$) with an average body weight of 100±8.5 g and length of 15±2 cm were injected intra-peritoneally with non-essential or essential element to a final concentration of 2, 4, and 6 mg/kg BW. Three fishes were used as a control group (non-treated) and a vehicle control group, which were injected the same volume of normal saline buffer solution without non-essential and essential elements. Fished were sacrificed after 24, 48 and 72 h of exposure. Then, they were placed in 10% phosphate buffer formalin for fixation.

2.3 Histological studies

Tissue samples from healthy Asian seabass (i.e. gill, liver, and kidney) of control, vehicle control, non-essential and essential element of treated fish were cut into small pieces and fixed in 10% phosphate buffered formalin solution for 24 hours. After dehydration in graded series concentration of ethanol (50%, 70%, 80%, 90%, and absolute ethanol), samples were cleared with xylene, infiltrated, and embedded in paraffin wax. Histological sections (6 μm thickness) were cut and stained with hematoxylin and eosin. The change in tissues were observed under the light microscope (Primo Star, ZEISS) and photographed by digital camera (Nikon coolpix S 5100).

2.4 Histological alterations scoring

Three fish from control, vehicle control, and treated group were analyzed for histology. Approximately, 10 serial sections were constructed from each fish for analysis. The slides were blinded to observer and histological alteration scoring was carried out on all serial sections. Histology was determined following by observing the severity of changes of the treated group as compared with control and vehicle control group. Base on completion of the scoring, the slides was revealed. Score was based on a combination of the level of severity and the number of slides out of the total in which the histological changes were observed with (0) unchanged or changing less than 10%, (1) mild occurrence with changing 10-30%, (2) moderate occurrence with changing 31-70%, and (3) severe occurrence with changing 71-100%.

2.5 Melanomacrophage center and pigmented macrophage analysis

In kidney, the melanomacrophage center (MMC) and pigmented macrophage sections of 3 fishes from all treatments were observed under light microscope (40x) followed the method proposed by Agius and Roberts (2003). Melanomacrophage center and pigmented macrophage in each fish was recorded in sections using an eyepiece graticule coupled to the light microscope. The average count was determined and results were presented as average count per mm^2.

2.6 Statistic analysis

The results presented as mean±S.E. values. Two-way analysis of variance (ANOVA) was used to test the significance of melanomacrophage and pigmented macrophage in each group of the element and concentration of exposure. The adopted significance level was $p<0.05$. The statistical analysis were performed using SPSS 10.0 software.

3. Results

3.1 Histological alterations

3.1.1 Gill

The gill of fish in control and vehicle control groups exhibited normal conditions, such as gill filament and gill lamellae, as shown in Fig. 1 (A-B). In the fish exposed to at least 2 mg/kg of $HgCl_2$ (non-essential element), it showed histological changes. These changes increased with an increasing in concentration of exposure. The histological changes

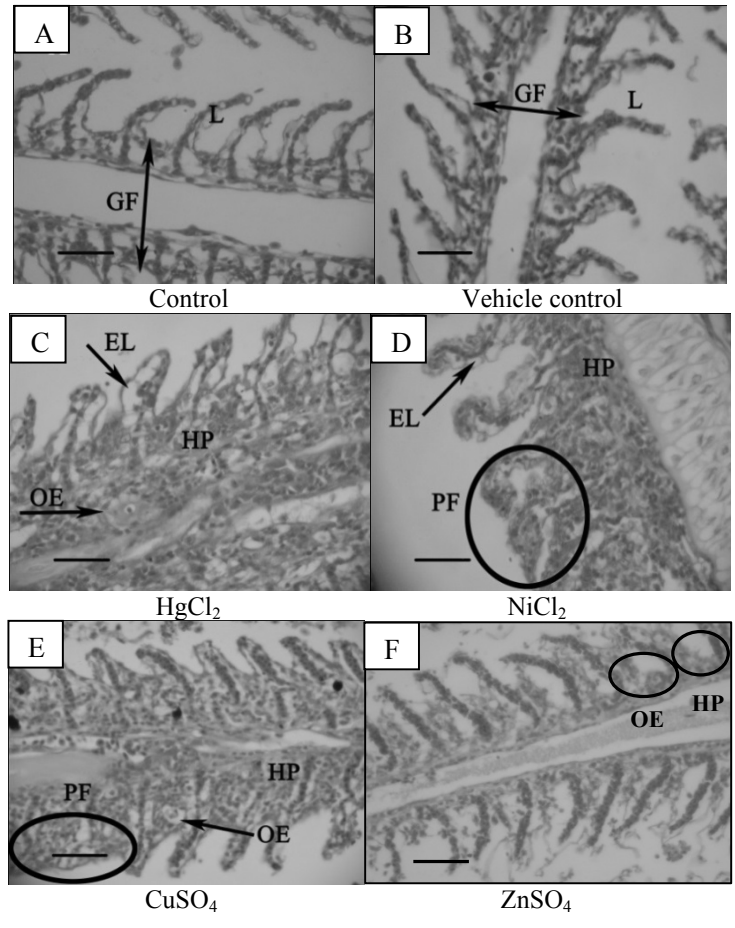

Figure 1. Histological alteration in gill tissue of Asian seabass in control (A), vehicle control (B) and treatment of non-essential element ($HgCl_2$) (C) and essential element comprising $NiCl_2$ (D) $CuSO_4$ (E) and $ZnSO_4$ (F) where EL: Epithelial lifting, HP: Hyperplasia, OE: Oedema, PF: Partial fusion of lamella, and scale bars 25 µm.

included epithelial lifting, hyperplasia oedema, and partial fusion of lamellae (Fig. 2(C)). In the fish exposed to essential element ($NiCl_2$, $CuSO_4$ and $ZnSO_4$) at the concentration of 2 mg/kg, histological changes was not found as compared to control and vehicle control groups. Nevertheless, histological changes were observable as the concentration increase, where the highest change occurred at 6 mg/kg after 72 h of exposure (Table 1). Histological changes, such as epithelial lifting, hyperplasia, oedema, and epithelial lifting, of the group treated with high concentration of essential elements are shown in Fig. 1(D-F).

3.1.2 Liver

Liver cells in control and vehicle control groups expressed normal condition; round shape nucleus locates in the middle, obvious cell membrane, and normal size sinusoid (Fig. 2(A-B)). In the group exposed to non-essential ($HgCl_2$) at 2 mg/kg for 24 h, liver cell showed histological change as compared with the group exposed to essential ($CuSO_4$ and $ZnSO_4$), which expressed measurable change at 48 h after exposed to the concentration of 4 mg/kg. Histological change found was enlargement of sinusoid and vacuolation of hepatocytes. The severe increased with an increasing of concentration of exposure and the highest was found at the concentration of 6 mg/kg after 72 h of exposure (Fig. 2(C-F) and Table 1)

3.1.3 Kidney

In the control and vehicle control group, kidney tissues expressed the normal condition as shown in Fig. 3(A-B). Samples that exposed to non-essential ($HgCl_2$) and essential ($NiCl_2$, $CuSO_4$ and $ZnSO_4$) elements showed the same histological change; degeneration of tubular, vacuolation, melanomacrophage center aggregate, Bowman's space increase, dilation of tubular, and necrosis. The highest changes occurred at the concentration of 6 mg/kg after 72 h of exposure (Fig. 3(C-F)). However, $HgCl_2$, which is a non-essential element, showed the most harmful effect since the alterations were observable at the concentration of only 2 mg/kg and after only 24 h of exposure, which was faster than that of the essential element, which caused the measurable harms at the concentration of 6 mg/kg (Table 1).

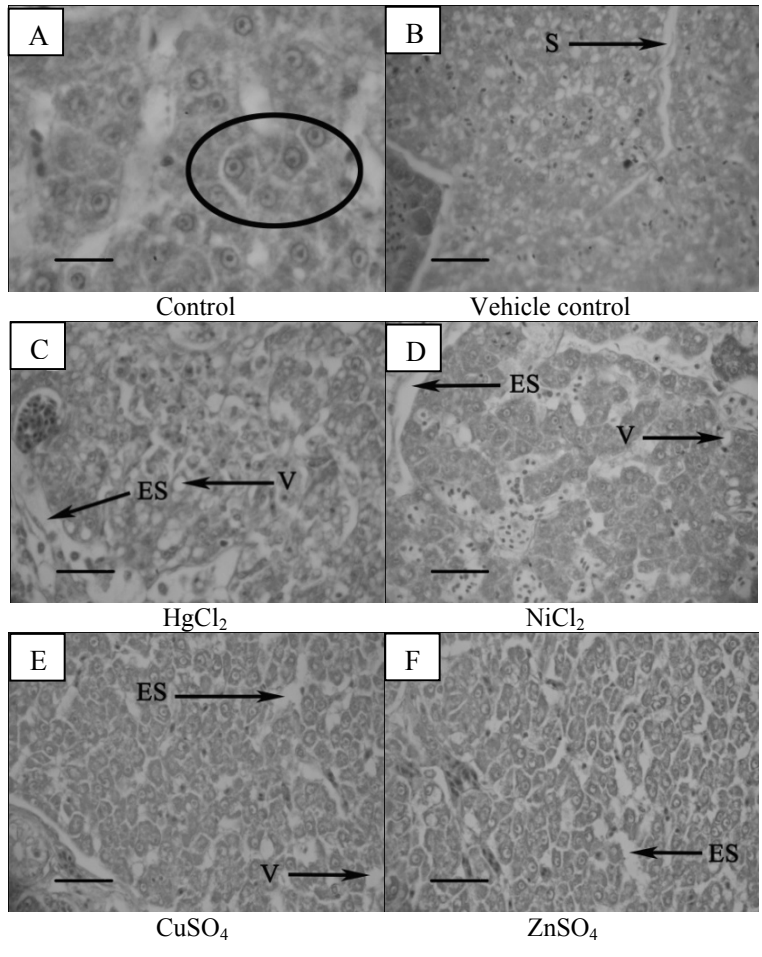

Figure 2. Histological alteration in liver tissues of Asian seabass in control (A), vehicle control (B) and treatment of non-essential element ($HgCl_2$) (C) and essential elements comprising $NiCl_2$ (D), $CuSO_4$ (E) and $ZnSO_4$ (F), where ES: Enlargement of sinusoid, V: Vacuolation, and scale bars 25 μm.

3.2 Melanomacrophage center and pigmented macrophage observation

Melanomacrophage center and pigmented macrophage counting were assess in kidney of Asian seabass exposed to non-essential ($HgCl_2$) and essential element ($NiCl_2$, $CuSO_4$ and $ZnSO_4$) at concentration of 2, 4 and 6 mg/kg for 24, 48 and 72 h. The results show that the element and concentration of exposure had effect on melanomacrophage and pigmented macrophage counting (Fig.4(A-C)). The highest of melanomacrophage center and pigmented macrophage counting were 173±3 at 6 mg/kg $HgCl_2$ (non-essential element) concentration after 72 h, which was 2.1 times higher than control and vehicle control group. In contrast, the lowest of melanomacrophage center and pigmented macrophage counting were observed after exposed to $ZnSO_4$ (essential element) at 2 mg/kg for 24 h (133±2). Moreover, in both cases of non-essential and essential element treatments, melanomacrophage center and pigmented macrophage counting were significantly different as compared with the control and vehicle control groups with 95% confidence ($p < 0.05$).

4. Discussion

4.1 Histological alterations

As mentioned above, both non-essential and essential element can cause the measurable adverse effect in the target tissue and the different effect of two element types of was not found. However, the results showed that histological change was increased with an increasing in heavy metal concentration and our finding is in agreement with Gupta and Srivastava (2006) which found that the histological alteration depend upon dose and exposure duration.

Gills are the main site of gas exchange process, ionic and osmotic regulation, and acid-base equilibrium. Histological changes in gill of Asian seabass, both in non-essential and essential element treated groups were similar. This finding is in agreement with that reported by Cerqueira and Fernandes (2002) that studied the adverse effect of Cu in gill in the tropical fish (*P. scrofa*). Furthermore, the results corresponds to the study by Thophon *et al*. (2003), which found that the damages of gill in Seabass (*L. calcarifer*) after exposed to $CdCl_2$ comprising edema of epithelial

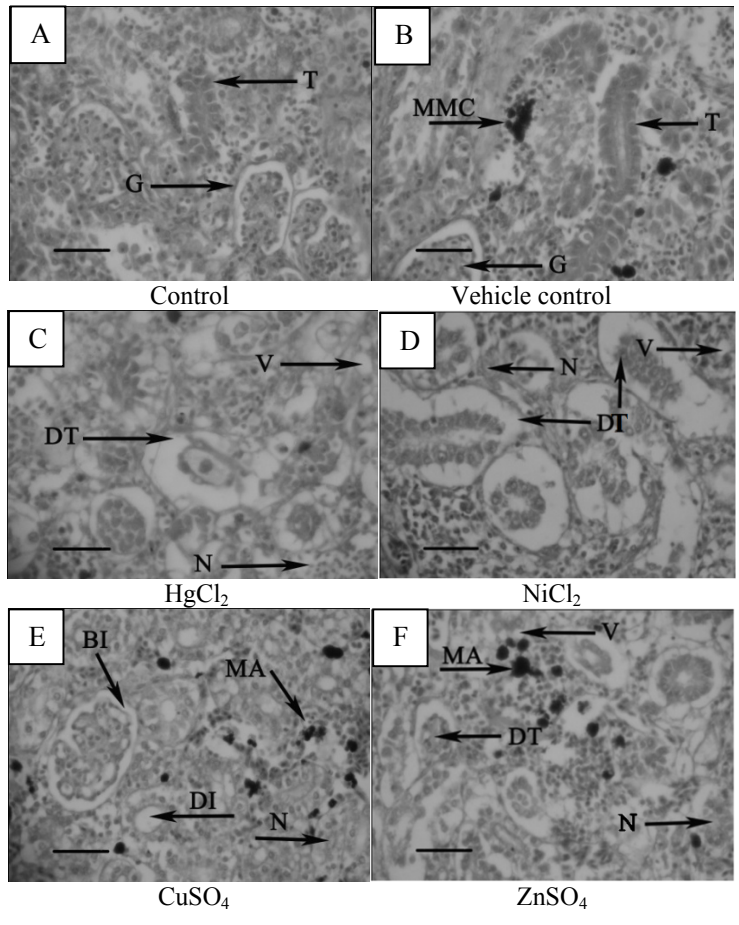

Figure 3. Histological alteration in kidney tissue of Asian seabass in the control (A), vehicle control (B) and treated with non-essential element comprising $HgCl_2$ (C) and essential elements containing $NiCl_2$ (D), $CuSO_4$ (E) and $ZnSO_4$ (F), where N: Necrosis, DT: Degeneration of tubular, V: Vacuolation, MA: Melanomacrophage center aggregate, BI: Bowman's space increase, DI: Dilation of tubular and scale bars 25 μm.

Table 1. Summary of histological alterations in control, vehicle control and non-essential and essential element treatment groups of Asian seabass.

Organ	Dose (mg/kg) and time (h) of exposure	Histological alterations in treatment											
		$HgCl_2$			$NiCl_2$			$ZnSO_4$			$CuSO_4$		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Gill	Control	-	-	-	-	-	-	-	-	-	-	-	-
	Vehicle control	-	-	-	-	-	-	-	-	-	-	-	-
	2 mg	+	+	++	-	-	-	-	-	-	-	-	-
	4 mg	+	++	+++	-	-	-	-	-	-	-	-	-
	6 mg	++	+++	++++	+	++	+++	+	++	+++	+	++	+++
Liver	Control	-	-	-	-	-	-	-	-	-	-	-	-
	Vehicle control	-	-	-	-	-	-	-	-	-	-	-	-
	2 mg	+	+	++	-	-	-	-	-	-	-	-	-
	4 mg	+	++	++	+	++	++	-	-	-	-	-	-
	6 mg	+	++	+++	+	++	+++	-	+	+	-	+	++
Kidney	Control	-	-	-	-	-	-	-	-	-	-	-	-
	Vehicle control	-	-	-	-	-	-	-	-	-	-	-	-
	2 mg	+	+	++	-	-	+	-	-	-	-	-	-
	4 mg	+	++	+++	+	+	++	-	-	+	-	+	++
	6 mg	++	+++	++++	+	++	+++	+	++	++	+	++	+++

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

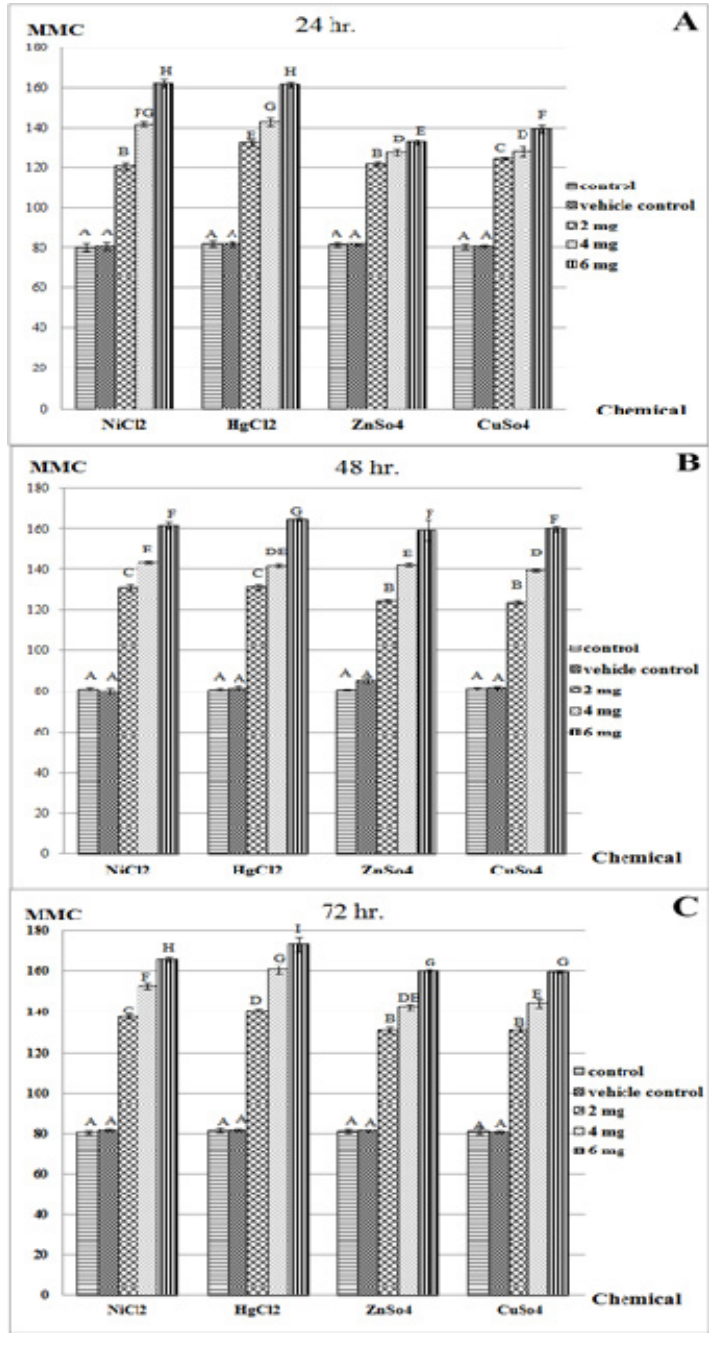

Figure 4. Summary of melanomacrophage center and pigmented macrophage counting in kidney of Asian seabass exposed to non-essential ($HgCl_2$) and essential ($NiCl_2$, $ZnSO_4$ and $CuSO_4$) elements at concentration of 2, 4 and 6 mg/kg for 24, 48 and 72 h. The different letters shown on the graph represent the various studied groups and different statistically significant levels. The values shown is mean±S.E. at 95% ($p < 0.05$) statistically significant level.

cells, hyperplasia of epithelial, and chloride cells. In addition, Pantung *et al*. (2008) studied the alterations in Hybrid Walking Catfish (*Clarias macrocephalus* x *Clarias gariepinus*) after exposed to cadmium in both acute and subacute. They found gill alterations consisting of an increase in chloride cells, breakdown of the pillar cells and edema of the epithelial cells

Fish liver serves functions similar to those in mammals, containing assimilation of nutrient, production of bile detoxification, and maintenance of the body metabolic homeostasis (Genten *et al*., 2009). We observed alterations of liver tissues of Asian seabass, such as enlargement of sinusoid and vacuolation of hepatocytes. The finding corresponded with Thophon *et al*. (2003) that studied the effects of $CdCl_2$ in liver of Seabass (*L. calcarifer*) and Hybrid Walking Catfish (*Clarias macrocephalus* x *Clarias gariepinus*) after exposed to cadmium in both acute and subacute which found blood conjestion in sinusoids and swelling of hepatocytes in the liver. Moreover,

the results is also similar to the results of Mohamed (2009), which found that the liver in fish from contaminated area (Lake Qarun, Egypt) exhibited vacuole degeneration in hepatocytes and dilation and congestion of blood sinusoids.

The kidney of teleost fish is a mixed organ, consisting of hematopoietic, phagocytic, endocrine, and excretory elements. The kidney varies greatly between different species of fish, both grossly and histologically (Genten *et al*., 2009). In this study, we found that the change of kidney tissue were highest as compared to gill tissues. The changes comprise degeneration of tubular, vacuolation, melanomacrophage center aggregate, Bowman's space increase, dilation of tubular, and necrosis. This is corresponds to Gupta and Srivastava (2006) that the kidney of *C. punctatus* exposed to sublethal of zinc exhibited enlargement of renal tubules, loss of cellular integrity of tubular cell, oedema, dilation of renal tubule pyknotic nuclei vacuolation and necrosis. Moreover, Thophon *et al*. (2003) who found the changes of Seabass (*L. calcarifer*) kidney tissues after exposed to $CdCl_2$ and reported observation of hydropic swelling of tubular cell, vacuolation, tubular degeneration, and necrosis cell. In additions, Pantung *et al*. (2008) found that Hybrid Walking Catfish (*Clarias macrocephalus* x *Clarias gariepinus*) after exposed to cadmium in both acute and subacute developed vacuolation and necrosis of proximal tubular cells in their kidney.

4.2 Melanomacrophage center and pigmented macrophage observation

Melanomacrophage center is distinctive grouping of pigment-containing cells. Normally, in fish, they are located in the stroma of the haemopoietic tissue of the spleen and kidney. Functions of melanomacrophage center are many. For instance, in fish, malanomacrophage center plays an importance role in the response to foreign materials, including infection agents. Melanomacrophage center increases in size or frequency in the condition of environmental stress. As a result, it could be a reliable biomarker for evaluation of environment pollution (Agius and Roberts, 2003) The similar results of the high incidence of melanomacrophage and the pigmented macrophage presented in this study was also reported by Rabitto *et al*. (2005) who studied on *H. malabaricus* exposed to Pb (II) and TBT (Tributyltin) via oral administration. In addition, in this study, we verified that the melanomacrophage and the pigmented macrophage counts also depended upon the element and concentration of exposure.

Some increase in melanomacrophage was previously observed in the kidney exposed to 10 mg/kg of mercuric chloride for 42 days (Handy and Penrice, 1993). Furthermore, Mela *et al*. (2007) reported that *H. malabicus* after exposured to dietary methylmercury showed increasing number of melanomacrophage center both in kidney and liver, however, melanomacrophage center in kidney and liver are distinct functions. (Manera *et al*., 2001) suggested the prevalence of melanomacrophage in liver and head kidney is a great biomarker to evaluate the pollution and degradation of the environment.

5. Conclusion

In conclusion, we investigated histological alterations in Asian seabass correlated with exposure to various types of chemical elements, both essential (i.e. $NiCl_2$, $ZnSO_4$, and $CuSO_4$) and non-essential (i.e. $HgCl_2$) elements, at different exposure durations (i.e. 24, 48, and 72 h) and different exposure levels (i.e. 2, 4, and 6 mg/kg). The result showed that the non-essential element caused tissues alterations even at small dose and short time of exposure as compared with the essential elements. Among essential elements, $NiCl_2$ cause the most severe effect, where the tissues alterations were observable starting from 2 mg/kg dose and 72 h exposure time, and $ZnSO_4$ caused the minimal alteration, where the tissues alterations were observable starting from 6 mg/kg dose and 72 h exposure time. Among different types of observed tissues, kidney was shown to be the most venerable, exhibiting the highest level of alterations in all elements. Furthermore, the results from melanomacrophage center and pigmented macrophage counting in kidney tissues showed an agreement with that from histology, where the highest count was found in the case of $HgCl_2$ treatment and the lowest count in the $ZnSO_4$ treatment. Nevertheless, both cases of essential and non-essential treatment showed significant difference count compared with that of the control and vehicle control groups.

Acknowledgements

This work was supported by Faculty of Science, Burapha University, Thailand.

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Received 7 April 2016
Accepted 13 May 2016

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