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Alteration in morphological features of *Puntius javanicus* liver exposed to copper sulfate

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Abstract

The environmental toxicants such as copper are known to affect vital organ especially liver. This study examined the effects of copper sulfate ($CuSO_4$) on the liver morphological structure of *P. javanicus*. The untreated control, 0.1 and 0.3 mg/L $CuSO_4$ treated groups displayed normal polygonal structure of the hepatocyte. However, at the concentrations of 0.5, 1.0 and 5.0 mg/L $CuSO_4$, the hepatostructure was significantly affected, as shown by the increasing number of dilation and congestion of sinusoids, vacuolation, macrophage activities and peliosis. The damage level and HSI value were increased while the number of hepatic nuclei per mm^2 was decreased with the increasing of copper concentration. In conclusion, this study shows that the degree of liver damage in *P.javanicus* is dependent to the dose exposure.

Introduction

Copper (Cu) is widely used to manufacture products such as pesticides, fertilizers, piping, electrical and electronic components However, the uncontrolled release of Cu-containing compounds into the water bodies may negatively affect aquatic organisms. Prolonged and continuous exposure may caused the elevation of internal Cu concentration that lead to impairment of biological activities such as enzyme inhibition, ion homeostasis imbalance and retardation of cell development (Peña *et al.*, 1999; Gaetke and Chow, 2003; Saluja and Kumar, 2005; Langley and Dameron, 2013; Sabullah *et al*., 2015a). In addition, elevated level of Cu exposure was shown to decrease hemoglobin and heamatocrit levels for organism such as pigs and sheep (Kline *et al.*, 1971) . In trace amounts, Cu is essential for the regulation of body metabolism such as for the stabilization of human ceruloplasmin, thermal, redox reaction and respiration (Miranda *et al*., 2000; Tapiero *et al*., 2003; Sedlák *et al*., 2008). However, it can be toxic at higher concentrations that may lead to the inhibition of several biochemical reactions and alteration of liver morphology (Ali *et al*., 2003; Sarnowski and Witeska, 2008; Ajani and Akpoilih, 2010; Tilton *et al*., 2011; Lauer *et al*., 2012).

The morphological alterations in the liver of Cu-exposed fish varies throughout the available literature (Stephensen *et al*., 2000; Varanka *et al*., 2001; Figueiredo-Fernandes *et al*., 2007; Liu *et al.*, 2010). Monitoring these morphological changes is an alternative way that is considered to be effective and sensitive in evaluation of the effects of Cu exposure since the variability of cellular responses are related to the concentration and exposure duration (Campagna *et al.*, 2008). Liver has multiple functions such as its involvement in the metabolism of glycogen, proteins and vitamins; as a primary target for accumulation of xenobiotic compounds prior to detoxification and neutralization of toxins to nontoxic form or eliminated through macrophage or degraded by biochemical reaction of the enzyme (Cao *et al.*, 2013; Shin *et al*., 2013). Cu toxicity leads to the increasing level

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Table 1. Hepatossomatic index (HSI) and number of hepatocyte nucleus per mm^2 of hepatic tissue (Hepat. nucl/mm2) measured in *P. javanicus* exposed to different $CuSO_4$ concentrations for 96 hours

$CuSO_4$ concentration (mg/L)	HSI	Hepat. nucl./mm^2
Control	1.12 ± 0.01^{a}	2566.80 ± 27.55^{a}
0.1	1.11 ± 0.02^{a}	2555.76 ± 76.88^{a}
0.3	1.34 ± 0.12^{ab}	$2244.45\pm99.94^{b*}$
0.5	$1.43\pm0.11^{bc*}$	$2070.67\pm57.94^{bc*}$
1.0	$1.61\pm0.11^{bc**}$	$1815.03\pm58.90^{c**}$
5.0	$1.66\pm0.18^{c**}$	$1559.68\pm5.66^{c**}$

Similar superscript letter with control indicates no significant difference ($p>0.05$)
*: Slightly significantly different between treatment and control ($p<0.05$)
**: Strongly significantly different between treatment and control ($p<0.01$)

of reactive oxygen species (ROS; H_2O_2 and •OH), lipid peroxidation, enzyme inhibition (cholinesterase and glutathione S-transferase) and induces apoptotic factors (BAX and caspase) associated with DNA damage and development of apoptosis and necrotic cell (Santra *et al*., 2000; Manzl *et al*., 2004; Faix *et al*., 2005; Valko *et al*., 2006; Yu *et al*., 2008; Letelier *et al*., 2010; Tilton *et al*., 2011, Sabullah *et al*., 2014, Sabullah *et al*., 2015b). Therefore, morphological changes are proven useful not only as biomarker of the presence of toxicant, but also in the evaluation of fish health status. Hence, in this study, *Puntius javanicus* was selected as a test model for the evaluation of Cu exposure effect(s) on its liver morphology. This freshwater fish species was selected due to its potential commercial value, health benefits and regular consumption in the diet of local Orang Asli (native tribe) and villager community.

Materials and Methods

Specimen and sample preparation

Live *P. javanicus* samples were purchased from the Aquaculture Development Center, Bukit Tinggi, Pahang, Malaysia. Fish specimens weighing between 400-500 g were randomly separated into six groups of nine fish each. Acclimatization was carried out in 80 L of chlorine-free water with full aeration for 15 days. Water pH, temperature and a 12 hours (light: dark) photoperiod were replicated in the laboratory to mimic *P. javanicus* natural habitat conditions. At the end of the acclimatization period, each group of fish was separately exposed to different concentration of copper sulfate (Acros Organic,UK); $CuSO_4$ (0.1, 0.3, 0.5, 1.0 and 5.0 mg/L) for 96 hours except for the first group which was assigned as an experimental control. The fish was then humanely killed and the liver was dissected out and weighed to measure the hepatossomatic index (HSI). This was immediately followed by sectioning of the liver into approximately 1mm3 slices. Primary fixation was performed by immersing the sample in 4% of glutaraldehyde (Fisher BioReagents, UK) for 24 hours at 4°C. Samples were then washed three times with 10 minutes immersion in 0.1M sodium cacodylate buffer (Sigma-Aldrich, USA). One percent of osmium tetroxide (Sigma-Aldrich, USA) was used for two hours of post fixation of the samples then washed another three times with 0.1M sodium cacodylate for 10 minutes. The samples were dehydrated in ascending series of acetone (JT-Baker, USA) and embedded in resin, encapsulated then polymerized in the oven for 48 hours at 60°C. The thick section was cut to 1µm using an ultramicrotome then stained with toluidine blue (TB) (Merck, Germany). The section was dried on the hotplate then washed to remove the stain. The sections were examined under a light microscope (Leica DMRA II) and the selected areas were photographed.The amount of hepatocytes nucleus per mm2 of hepatic tissue was obtained according to Figueiredo-Fernandes *et al.*, (2007) without calculating the cells present in sinusoids.

Statistical analysis

The data reported in the Table 1 were averages of triplicate observations. The data were subjected to One-Way Analysis of Variance (ANOVA) and Tukey post-hoc analyses were carried out to further determine their differences. All of statistical analyses in this study were performed using GraphPad Prism version 5.0 (GraphPad Software, California, USA *[http://www.graphpad.com/]*). A significant

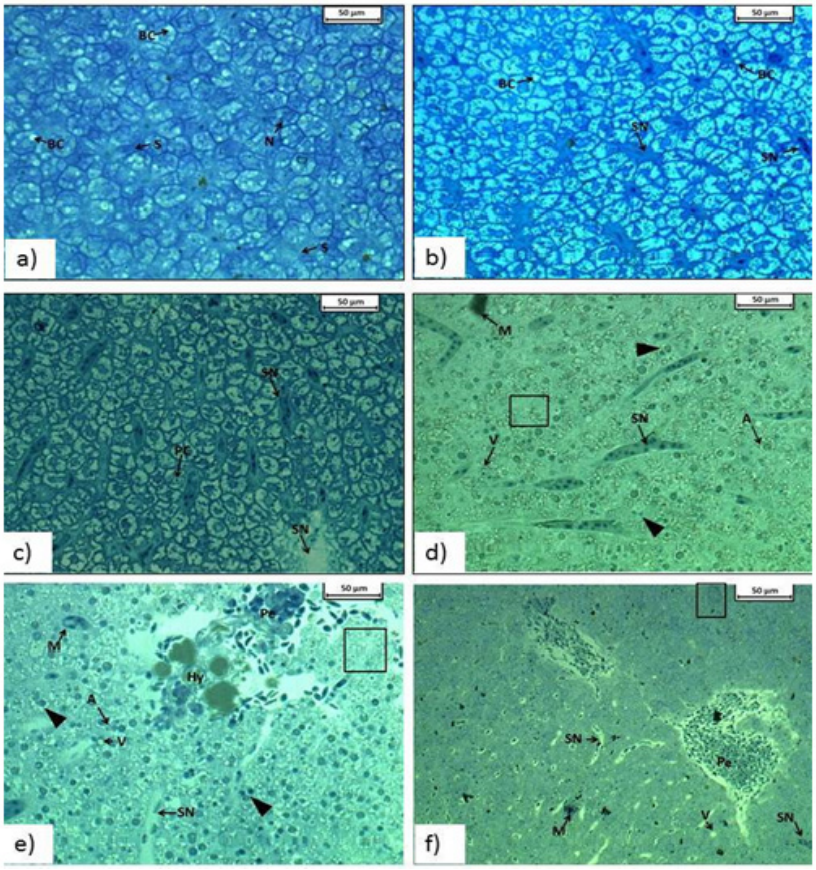

Figure 1. Light micrograph of section images of *P. javanicus* liver which are the representative for control and different concentrations of $CuSO_4$ treatment. a) Control. Observed the normal polygonal structure of hepatocyte: 20x, b) 0.1 mg/L. Increase in sinusoidal spaces: 20x, c) 0.3mg/L. Increasing number of congested sinusoids. Note the presence of pyknotic cell and abnormal sinusoid: 20x, d) 0.5mg/L. The present of vacuolation, necrotic area and macrophage activity: 20x, e) The accumulation of red blood cells. Note the hyalinization of hepatocyte: 20x, f) The increasing accumulation of red blood cells: 10x. N, Nucleus; S, Normal sinusoid; SN, Abnormal sinusoid; BC, Bile canaliculi; PC, Pyknotic cell; A, Apoptotic cell; M, Macrophage; Box area, Necrotic area; V, Vacuolation; Arrowhead, binucleaition; Hy, Hyalinization; Pe, Peliosis. TB stained

difference was considered at the level of $p<0.05$.

Results

At the end of 96 hours of Cu exposure, all of the fish were still alive, though a decrease in swimming performance at 1 and 5 mg/L of $CuSO_4$ concentration was observed. *P. javanicus* liver morphology from control and treated fish groups are shown in Figure 1. In the control (Figure 1a), 0.1 and 0.3 mg/L groups, the hepatocyte displayed a normal structure without any cellular impairment, with presenting a homogenous cytoplasm and identical spherical nucleus and several bile canaliculi was also observed. Compared to the control, 0.1 mg/L treatment shows the presence of dilated longitudinal and cross section of sinusoids (Figure 1b) while 0.3 mg/L treatment displayed the increasing number of pyknotic cell along with congested sinusoids by red blood cell (Figure 1c). At the exposure concentration of 0.5 mg/L, the abnormal hepatocyte was visualized with an increasing vacuolation of cytoplasm, congestion of sinusoids, necrosis, apoptotic cell and macrophage activity (Figure 1d). Hepatocytehyalinization and binucleation was visualized at treatment concentration of 1 mg/L (Figure 1e). 1 mg/L and 5 mg/L treatment show peliosis between the hepatocyte and the increasing number of macrophage activities (Figure 1e and 1f). This study shows the beginning of programmed cell death at 0.5 mg/L treatment and increasing number of hepatocellular abnormalities associated with increasing concentration of $CuSO_4$ exposure. The number of hepatocytes nucleus per mm^2 of *P. javanicus* hepatic tissues were decreased

while HSI values were increased with the increasing of $CuSO_4$ concentration (Table 1).

Discussion

Similar fish liver morphological alterations due to Cu exposure were observed by previous authors (Rojik *et al*., 1983; Liu *et al.*, 2010; Ajani and Akpoilih, 2010) such as the formation of pyknosis cell, necrosis, inflammation and loss of shape, size and number of nuclei of hepatocyte (Deore and Wagh, 2012). Usually, at the beginning of the toxicity duration effect especially at the lower concentrations of toxicant, the affected hepatocyte will undergo vacuolation as reported by Ajani and Akpoilih (2010), Kaoud *et al*. (2012), Rakhi *et al*., (2013) and Younis *et al.* (2013). Hepatocyte vacuolation occur due to the metabolic disorder caused by imbalanced synthesis and release of the substances from the parenchyma cells, disturbance of protein synthesis, energy reduction, disaggregation of microtubules and alteration in substrate usage which disrupt vesicle movement and result indeposition of secretion products (Gingerich, 1982; Hinton and Laurén, 1990; Ribeiro *et al.*, 2005). The lipid-filled vacuoles may contain substances such as glycogen and degenerative organelles especially mitochondria (Narayana and Al-Bader, 2011). Hepatocyte vacuolation was commonly observed in other fish studies and is an early morphological biomarker of toxicant exposure, especially exposure to metals (Fernandez *et al*., 2012; Mela *et al*., 2013). A review by Henics and Wheatley (1999) mentioned that vacuolation is reversible at low toxicant concentrations, though it requires time to return to their normal state but it tends to become irreversible at the high toxicant concentrations which may lead to cell death. In this study, vacuolation along with the sinusoids congestion, hyalinization, macrophage activity, apoptosis and necrosis are observed with the increasing concentration of $CuSO_4$ treatments.

Hyalinization also called hyaline droplet, is a side effect of vacuolization which is more towards the inhibition of protein synthesis in parenchyma cell (Van Dyk *et al*., 2007). Additionally, protein absorption forms are normally connected with metal toxicity. Macrophage aggregation in hepatocyte due to heavy metals exposure such as cadmium (Younis *et al*., 2013), gold nanoparticle (Sadauskas *et al*., 2007; Abdelhalim and Jarrar, 2012) and Cu (Al-Bairutya *et al*., 2013) has been reported. Kupffer cells are related to the monocyte-macrophage system which play an important role in hepatic immune response, "filtration" or specifically in removal of dead cell and cellular debris (Naito *et al*., 2004; Sadauskas *et al.*, 2007). There is a presence of large blood cysts (so called peliosis) whereby its morphological pattern has been divided into two (Yanoff and Rawson, 1964): (1) phlebectatic type, in which large blood cysts are lined by endothelial cells, and (2) parenchymal type, in which the blood-filled spaces are not lined by endothelial cells associated with hemorrhagic parenchymal necrosis. Figure 1e and 1f shows the phlebectatic type peliosis as a result of excessive dilatation of single sinusoids or central vein.

Previous studies have mentioned the increasing number of hepatossomatic index (HSI) in the liver of species such as *Myoxocephalus scorpius* (Stephensen *et al*., 2000) and Oreochromis niloticus (Figueiredo-Fernandes *et al*., 2007) exposed to contaminants. Paris-Palacios *et al.* (2000) reported that the exposure of $CuSO_4$ affected the nucleus size of Brachydanio rerio hepatocyte. This study also shows that the decreasing number of Hepat. Nucl. / mm^2 is related to the increasing activity of deleterious effect through apoptosis and necrosis. However, the changes observed depend on the exposure to different kinds of heavy metals, such as chromium (Mishra and Mohanty, 2009), cadmium (Hirano *et al*., 1991; Thophon *et al*., 2004), zinc (Abdel-Warith *et al.*, 2011), lead (Franchini *et al*., 1991) and arsenic (Straub *et al*., 2007) and other pollutant such as pesticides (Dezfuli *et al.*, 2006; Abd-Algadir *et al*., 2011), drugs (Moutou *et al*., 1997), surfactant (Kumar *et al*., 2007) and nitrosamine (Braunbeck *et al.*, 1992). This proves that Cu is not only an inducer of hepatocyte abnormalities but the degree of alteration also depends on its interaction with other accumulated toxic metals. Campagna *et al*. (2008) reported that the elevated level of Cu may caused irreversible effects on hepatocyte morphology. This deleterious effect of Cu toxicity may subsequently lead to inhibition of the interconversion of foodstuff and storage, impairment of absorption and excretion of waste product and complete imbalance of homeostatic control and cell rejuvenation.

In conclusion, we have observe the morphological alterations of *P. javanicus* hepatocyte by Cu were dependent on the dose of exposure. *P. javanicus* liver model is proven as a reliable alternative indicator to evaluate any deleterious effects in the course of Cu toxicity. Future studies are needed to evaluate the exposure effect of Cu together with other heavy metals, pesticides and drugs on the *P.javanicus* liver model.

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References

Abd-Algadir, M. I., Elkhier, M. K. S. and Idris, O. F. 2011. Changes of fish liver *(Tilapia nilotica)* made by herbicide *(Pendimethalin)*. Journal of Applied Biosciences 43: 2942-2946.

Abdel-Warith, A. A., Younis, E. M., Al-Asgah, N. A. and Wahbi, O. M. 2011. Effect of zinc toxicity on liver histology of Nile tilapia, *Oreochromis niloticus.* Scientific Research and Essays 6: 3760-3769.

Ajani, E. K. and Akpoilih, B. U. 2010. Effect of chronic dietary copper exposure on haematology and histology of common carp (*Cyprinus carpio* L.). Journal of Applied Sciences and Environmental Management 14: 39-45.

Al-Bairuty, G. A., Shaw, B. J., Handy, R. D. and Henry, T. B. 2013. Histopathological effects of waterborne copper nanoparticles and copper sulphate on the organs of rainbow trou*t (Oncorhynchus mykiss)*. Aquatic Toxicology 126: 104-115.

Ali, A., Al-Ogaily, S. M., Al-Asgah, N. A. and Gropp, J. 2003. Effect of sublethal concentrations of copper on the growth performance of *Oreochromis niloticus.* Journal of Applied Ichthyology 19: 183-188.

Braunbeck, T. A., Teh, S. J., Lester, S. M. and Hinton, D. E. 1992. Ultrastructural alterations in liver of Medaka *(Oryzias latipes)* exposed to diethylnitrosamine. Toxicologic Pathology 20: 179-196.

Campagna, A. F., Fracácio, R., Rodrigues, B. K., Eler, M. N., Fenerich-Verani, N. and Espíndola, E. L. G. 2008. Effects of the copper in the survival, growth and gill morphology of *Danio rerio* (Cypriniformes, Cyprinidae). Acta Limnologica Brasiliensia 20: 253-259.

Cao, J., Chen, J., Wang, J., Jia, R., Xue, W., Luo, Y. and Gan, X. 2013. Effects of fluoride on liver apoptosis and Bcl-2, Bax protein expression in freshwater teleost, *Cyprinus carpio*. Chemosphere 91: 1203-1212.

Deore, S. V. and Wagh, S. B. 2012. Heavy metal induced histopathological alterations in liver of Channa gachua (Ham). Journal of Experimental Biology 3: 35-38.

Dezfuli, B. S., Simoni, E. Giari, L. and Manera, M. 2006. Effects of experimental terbuthylazine exposure on the cells of *Dicentrarchus labrax* (L.). Chemosphere 64: 1684-1694.

Faix, S., Faixova, Z., Boldizarova, K. and Javorsky, P. 2005. The effect of long-term high heavy metal intake on lipid peroxidation of gastrointestinal tissue in sheep. Veterinary Medicine – Czech 50: 401-405.

Fernandez, W. S., Dias, J. F., Ribeiro, C. A. O. and Azevedo, J. S. 2011. Liver damages and nuclear abnormalities in erythrocytes of *Atherinella brasiliensis* (actynopterigii, atherinopsidade) from two beaches in southeast of brazil. Brazilian Journal of Oceanography 59: 163-169.

Figueiredo-Fernandes, A., Ferreira-Cardoso, J. V., Garcia-Santos, S., Monteiro, S. M., Carrola, J., Matos, P. and Fontaínhas-Fernandes, A. 2007. Histopathological changes in liver and gill epithelium of Nile tilapia, *Oreochromis niloticus*, exposed to waterborne copper. Pesquisa Veterinária Brasileira 27(3): 103-109.

Franchini, A., Barbanti, E. and Fantin, A. M. B. 1991. Effects of lead on hepatocyte ultrastructure in *Carassius carassius* (l.) var. *auratus*. Tissue and Cell 23: 893-901.

Gaetke, L. M. and Chow, C. K. 2003. Copper toxicity, oxidative stress, and antioxidant nutrients. Toxicology 189: 147-163.

Gingerich, W. H. 1982. Hepatic toxicology of fishes. In:Aquatic toxicology (ed. L.J. Weber), Raven Press, New York.

Kumar, M., Trivedi, S. P., Misra, A. and Sharma, S. 2007. Histopathological changes in testis of the freshwater fish, *Heteropneustes fossilis* (Bloch) exposed to linear alkyl benzene sulphonate (LAS). Journal of Environmental Biology 28: 679-684.

Henics, T., and Wheatley, D. N. 1999. Cytoplasmic vacuolation, adaptation and cell death: A view on new perspectives and features. Biology of the Cell 91: 485-498.

Hinton, D. E. and Laurén, D. J. 1990. Integrative histopathological effects of environmental stressors on fishes. American Fisheries Society Symposium 8: 51–66.

Hirano, T., Ueda, H., Kawahara, A. and Fujimoto, S. 1991. Cadmium toxicity on cultured neonatal rat hepatocytes: biochemical and ultrastructural analyses. Histology and Histopathology 6: 127-133.

van Dyk, J. C., Pieterse, G. M. and van Vuren, J. H. J. 2007. Histological changes in the liver of *Oreochromis mossambicus* (Cichlidae) after exposure to cadmium and zinc. Ecotoxicology and Environmental Safety 66: 432-440.

Kaoud, H. A., Mahran, K. M. A., Rezk, A. and Khalf, M. A. 2012. Bioremediation the toxic effect of mercury on liver histopathology, some hematological parameters and enzymatic activity in Nile tilapia, *Oreochromis niloticus*. Researcher 4(1): 60-70.

Kline, R. D., Hays, V. E. and Cromwell, G. L. 1971. Effects of copper, molybdenum and sulfate on performance, hematology and copper stores of pigs and lambs. Journal of Animal Science 33: 771-779.

Langley, A. and Dameron, C. T. 2013. Copper and anesthesia: Clinical relevance and management of copper related disorders. Anesthesiology Research and Practice 2013: 750901.

Lauer, M. M., de Oliveira, C. B., Yano, N. L. and Bianchini, A. 2012. Copper effects on key metabolic enzymes and mitochondrial membrane potential in gills of the estuarine crab Neohelice granulata at different salinities. Comparative Biochemistry and Physiology,

Part C 156(3-4): 140-147.

Letelier, M. E., Sánchez-Jofré, S., Peredo-Silva, L., Cortés-Troncoso, J. and Aracena-Parks, P. 2010. Mechanisms underlying iron and copper ions toxicity in biological systems: Pro-oxidant activity and protein-binding effects. Chemico-Biological Interactions 188: 220-227.

Liu, X. J., Luoa, Z., Xiong, B. X., Liu, X., Zhao, Y. H., Hu, G. F. and Lv, G. J. 2010. Effect of waterborne copper exposure on growth, hepatic enzymatic activities and histology in Synechogobius hasta. Ecotoxicology and Environmental Safety 73: 1286–1291

Manzl, C., Enrich, J., Ebner, H., Dallinger, R. and Krumschnabel, G. 2004. Copper-induced formation of reactive oxygen species causes cell death and disruption of calcium homeostasis in trout hepatocytes. Toxicology 196: 57-64.

Mela, M., Guiloski, I. C., Doria, H. B., Rabitto, I. S., da Silva, C. A., Maraschi, A. C., Prodocimo, V., Freire, C. A., Randi, M. A., Ribeiro, C. A. and de Assis, H. C. 2013. Risks of waterborne copper exposure to a cultivated freshwater Neotropical catfish *(Rhamdia quelen)*. Ecotoxicology and Environmental Safety 88: 108–116.

Miranda, S., Opazo, C., Larrondo, L. F., Munoz, F. J., Ruiz, F., Leighton, F. and Inestrosa, N. C. 2000. The role of oxidative stress in the toxicity induced by amyloid beta-peptide in Alzheimer's disease. Progress in Neurobiology 62: 633–648.

Mishra, A. K. and Mohanty, B. 2009. Chronic exposure to sublethal hexavalent chromium affects organ histopathology and serum cortisol profile of a teleost, *Channa punctatus* (Bloch). Science of the Total Environment 407: 5031–5038.

Moutou, K. A., Braunbeck, T. and Houlihan, D. F. 1997. Quantitative analysis of alterations in liver ultrastructure of rainbow trout *Oncorhynchus mykiss* after administration of the aquaculture antibacterials oxolinic acid and flumequine. Diseases of Aquatic Organisms 29: 21-34.

Naito, M., Hasegawa, G. Ebe, Y. and Yamamoto, T. 2004. Differentiation and function of kupffer cells. Medical Electron Microscopy 37: 16-28.

Narayana, K. and Al-Bader, M. 2011. Ultrastructural and DNA damaging effects of lead nitrate in the liver. Experimental and Toxicologic Pathology 63: 43-51.

Paris-Palacios, S., Biagianti-Risbourg, S. and Vernet, G. 2000. Biochemical and (ultra)structural hepatic perturbations of *Brachydanio rerio* (Teleostei, Cyprinidae) exposed to two sublethal concentrations of copper sulfate. Aquatic Toxicology 50: 109-124.

Peña, M. M. O., Lee, J. and Thiele, D. J. 1999. A delicate balance: homeostatic control of copper uptake and distribution. Journal of Nutrition 129: 1251-1260.

Ribeiro, C. A. O., Vollaire, Y., Sanchez-Chardi, A. and Roche, H. 2005. Bioaccumulation and the effects of organochlorine pesticides, PAH and heavy metals in the Eel *(Anguilla anguilla)* at the Camargue Nature Reserve, France. Aquatic Toxicology 74(1): 53-69.

Rojik, I., Nemcsók, J. and Boross, L. 1983. Morphological and biochemical studies on liver, kidney and gill of fishes affected by pesticides. Acta Biologica Hungarica 34(1): 81-92.

Sabullah, M.K., Ahmad, S.A., Shukor, M.Y., Shamaan, N.A., Khalid, A., Gansau, A.J., Dahalan, F.A., and Sulaiman, M.R. 2015. Acetylcholinesterase from *Puntius javanicus* for the detection of carbamates and organophosphates. Journal of Chemical and Pharmaceutical Sciences 8(2): 348-353.

Sabullah, M.K., Sulaiman, M.R., Shukor, M.Y., Syed, M.A., Shamaan, N.A., Khalid, A. and Ahmad, S.A. 2014. The Assessment of cholinesterase from the liver of *Puntius javanicus* as detection of metal ions. The Scientific World Journal 2014: ID 571094.

Sabullah, M.K., Sulaiman, M.R., Shukor, M.S., Yusof, M.T., Wan Johari, W.L., Shukor, M.Y., and Syahir, A. 2015. Heavy metals biomonitoring via inhibitive assay of acetylcholinesterase from *Periophthalmodon schlosseri*. Rendiconti Lincei 22(2): 151-158.

Sadauskas, E., Wallin, H., Stoltenberg, M., Vogel, U., Doering, P., Larsen, A. and Danscher, G. 2007. Kupffer cells are central in the removal of nanoparticles from the organism. Particle and Fibre Toxicology 4:10.

Saluja, U. and Kumar, S. 2005. Inhibitory chronic effect of copper sulphate on acetylcholinesterase activity and enzyme kinetics with its subsequent reactivation in the stomach of Rattus norvegicus. Asian Journal of Experimental Sciences 19: 65-71.

Santra, A., Maiti, A., Das, S., Lahiri, S., Charkaborty, S. K. and Mazumder, D. N. 2000. Hepatic damage caused by chronic arsenic toxicity in experimental animals. Journal of Clinical Toxicology 38: 395-405.

Sarnowski, P. and Witeska, M. 2008. The effects of copper and cadmium in single exposure or co-exposure on growth of common carp (*Cyprinus carpio* l.) Larvae. Polish Journal of Environmental Studies 17: 791-796.

Sedlák, E., Žoldák, G. and Wittung-Stafshede, P. 2008. Role of copper in thermal stability of human ceruloplasmin. Biophysical Journal 94: 1384-1391.

Rakhi, S. F., Reza, A. H. M. M., Hossen, M. S. and Hossain, Z. 2013. Alterations in histopathological features and brain acetylcholinesterase activity in stinging catfish *Heteropneustes fossilis* exposed to polluted river water. International Aquatic Research 5:7.

Shin, S. M., Yang, J. H. and Ki, S. H. 2013. Role of the Nrf2-ARE pathway in liver diseases. Oxidative Medicine and Cellular Longevity 2013:763257.

Stephensen, E., Svavarsson, J., Sturve, J., Ericson, G., Adolfson-Erici, M. and Förlin, L. 2000. Biochemical indicators of pollution exposure in shorthorn sculpin *(Myoxocephalus scorpius)*, caught in four harbours on the south-west coast of Iceland. Aquatic Toxicology 48: 431-442.

Straub, A. C., Stolz, D. B., Ross, M. A., Hernández-Zavala, A., Soucy, N. V., Klei, L. R. and Barchowsky, A. 2007. Arsenic stimulates sinusoidal endothelial cell capillarization and vessel remodeling in mouse liver. Hepatology 45: 205-212.

Tapiero, H., Townsend, D. M. and Tew, K. D. 2003. Trace elements in human physiology and pathology. Copper.

Biomedicine and Pharmacotherapy 57: 386–398.
Thophon, S., Pokethitiyook, P., Chalermwat, K., Upatham, E. S. and Sahaphong, S. 2004. Ultrastructural alterations in the liver and kidney of white sea bass, *Lates calcarifer*, in acute and subchronic cadmium exposure. Environmental Toxicology 19: 11-19
Tilton, F. A., Bammler, T. K. and Gallagher, E. P. 2011. Swimming impairment and acetylcholinesterase inhibition in zebrafish exposed to copper or chlorpyrifos separately, or as mixtures. Comparative Biochemistry and Physiology, Part C 153(1): 9-16.
Valko, M., Rhodes, C. J., Moncol, J., Izakovic, M. and Mazur, M. 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chemico-Biological Interactions 160: 1–40.
Varanka, Z., Rojik, I., Varanka, I., Nemcśok, J. and Ábraĥam, M. 2001. Biochemical and morphological changes in carp (*Cyprinus carpio* L.) liver following exposure to copper sulfate and tannic acid. Comparative Biochemistry and Physiology, Part C 128: 467-478.
Younis, E. M., Abdel-Warith, A. A., Al-Asgah, N. A., Ebaid, H. and Mubarak, M. 2013. Histological changes in the liver and intestine of Nile Tilapia, *Oreochromis niloticus,* exposed to sublethal concentrations of cadmium. Pakistan Journal of Zoology 45(3): 833-841.
Yanoff, M. and Rawson, A. J. 1964. Peliosis hepatis: an anatomic study with demonstration of two varieties. Archives of Pathology and Laboratory Medicine Online 77: 159-165.
Yu, Z. L., Zhang, J. G., Wang, X. C. and Chen, J. 2008. Excessive copper induces the production of reactive oxygen species, which is mediated by phospholipase D, nicotinamide adenine dinucleotide phosphate oxidase and antioxidant systems. Journal of Integrative Plant Biology 50: 157-167.