Journal of Environmental Science and Management 24-1: 25-35 (June 2021) ISSN 0119-1144

Melanomacrophage Centers in Nile Tilapia (*Oreochromis niloticus* L.) as Biomarker for Carbamate Exposure

Jefferson C. Marteja[1]
Ris Menoel R. Modina[1*]

ABSTRACT

Melanomacrophage centers are aggregates of pigment-containing cells found in the animal's hematopoietic tissues. Changes in its characteristics have been used to assess the influence of pesticide exposure, and as tools for potential monitoring for fish and environmental health. This study aimed to evaluate the pesticide-induced hepatic and splenic melanomacrophage center responses in Nile tilapia (Oreochromis niloticus L.) following exposure to fenobucarb in varying lengths of exposure. Five test groups were exposed to constant dose of fenobucarb at 0.08 mg L^{-1} at different periods (0, 7, 14, 21, and 28 days). Fenobucarb only induced significant changes in the splenic melanomacrophage centers. Splenic melanomacrophage centers significantly increased in number in response to the increasing lengths of exposure. Increasing trend of size and cover was also observed, however, significant difference was only detected at 28 days exposure period. Significant difference in hemosiderin and lipofuscin pigments was also detected at 28 days exposure which suggests tissue destruction after prolonged exposure. This study confirms the potential of melanomacrophage centers as a sensitive biomarker for fenobucarb exposure indicated by the changes in its characteristics, particularly in the spleen.

Keywords: *pesticide, fenobucarb, spleen, melanomacrophage center, biomarker*

[1] Department of Biological Sciences, College of Arts and Sciences, Visayas State University, Visca, Baybay City, 6521-A Leyte, Philippines

[*] corresponding author: rismenoel.modina@vsu.edu.ph

INTRODUCTION

Pesticides have been of great help in agriculture by decreasing crop losses due to pests. However, problems due to its widespread use have been evident worldwide. One of these is the off-site migration of the pesticides in different bodies of water, such as irrigation canals, lakes, rivers, and oceans (*Claver et al. 2006; Leong et al. 2007; Gao et al. 2008; Añasco et al. 2010; Varca 2012; Lu 2010; Toan et al. 2013; Allinson et al. 2016; Navarrete et al. 2018; Bonmatin et al. 2021*). These pesticides may harm non-target plants and animals, as well as human beings (*Ohkawa et al. 2007*). Pesticides also has the capacity to bioaccumulate in exposed organisms such as frogs (*Katagi and Ose 2014*), aquatic insect larvae (*Katagi and Tanaka 2016*), and fish (*Cullen and Connell 1992; Streit 1998; Satyanarayan et al. 2007; Rossi et al. 2020*).

One of the most widely used insecticides worldwide is fenobucarb (*Kim et al. 2013*). In the Philippines, surveys revealed that farmers in Laguna used fenobucarb (*Fabro and Varca 2012*). It is a type of carbamate insecticide that is a potent inhibitor of acetylcholinesterase (AChE) which results in sympathetic and parasympathetic overstimulation, respiratory failure, and skeletal muscle paralysis (*Murray et al. 2015*). *Modina* (*2017*) studied the effects of fenobucarb in the AChE activity of Nile tilapia and found out that brain and muscle AChE activities were significantly inhibited despite the minimal concentration used. Fenobucarb has been recorded to have the capacity to move offsite from the point of application. *Lamers et al.* (*2011*) detected a maximum of 0.17 µg L^{-1} of Fenobucarb in nearby streams and rivers after it was sprayed in rice paddies. Fenobucarb residues were even detected in the coastal waters of southern Japan that receives effluents from a wastewater reservoir that temporarily stores surface runoffs of rice paddy fields with a maximum concentration of 0.27 µg L^{-1} (*Añasco et al. 2010*). Aside from this, fenobucarb has the capacity to bioaccumulate in fishes (*Pucher et al. 2014*).

There is a need to routinely monitor the concentration of off-site migration of pesticides in different bodies of water. However, pesticide concentration monitoring is cost intensive and requires skilled laboratory staff and sophisticated equipment. One alternative in environmental monitoring is the biological approach. Contaminants in low concentrations in aquatic ecosystems could interact

with animals and cause measurable physiological and histological responses. These responses are referred to as biomarkers (*Sparling 2016*). Melanomacrophage Centers (MMCs) or macrophage aggregates is one of many potential biomonitoring tools in determining the effects of minute concentrations of pesticide contaminants (*Ugaddan and Ocampo 2009; Tellez-Bañuelos et al. 2009; Manrique et al. 2014; Beso et al. 2016; Tjahjaningsih 2017; Manrique et al. 2019*). These are pigment-containing cells found in the fish's hematopoietic tissue of the spleen, kidney, and sometimes in the liver of teleost fishes. MMCs are normally packed to form large aggregates and are enlarged after phagocytosis of heterogeneous materials, such as melanin pigments, cell debris, lipofucsin residues and hemosiderin granules. Number, size, and pigment content of MMCs are known to change in relation to fish health and environmental degradation (*Wolke 1992*).

Among the fishes found in freshwater ecosystems, Nile tilapia (*Oreochromis niloticus* L.) is one of the most commonly studied bioindicator. It thrives well in any freshwater environment making it vulnerable to pesticide exposure as it searches for food at the bottom of their aquatic habitats such as rice paddies, ponds, and lakes (*Sagun and Ocampo 2006*). *O. niloticus* are widely used in experimental work for its availability and good adaptation under captive conditions (*Roxas-Aragon et al. 2003*). *O. niloticus* have been used to investigate pollutants in estuarine environments (*Bacolod et al. 2017*), freshwater contaminants, insecticides (*Toledo-Ibarra et al. 2016; Zahran et al. 2018*), herbicides (*de Almeida et al. 2018; Felício et al. 2018*), cyanotoxin (*Guzmán-Guillén et al. 2015*), and pharmaceutical drugs (*Ajima et al. 2017*).

Studies which used MMC responses of tilapia following exposure to different stressors and contaminants have been recorded (*Ugaddan and Ocampo 2009; Tellez-Bañuelos, et al. 2009; Kaewamatawong et al. 2013; Manrique et al. 2014; Lituañas and Ocampo 2015; Beso et al. 2016; Manrique et al. 2019*). Environmental Protection Agency's Environmental Monitoring and Assessment Program–Estuaries (EMAP-E) used splenic MMCs as an indicator of fish exposure to degraded environments in different estuaries (*Fournie et al. 2001*). *Guevarra et al.* (*2020*) used MMCs as one of the biomarkers to assess the health condition of cage aquaculture areas in the Seven Lakes of San Pablo, Philippines.

This study evaluated the response of hepatic and splenic MMCs of Nile tilapia in terms of count, size, percent cover, and pigment composition. The Nile tilapia were subjected hepatic and splenic MMCs of Nile tilapia subjected to fenobucarb with varying lengths of exposure. The responses of MMCs recorded in this study could give insights to the potential of Nile tilapia as a bioindicator and MMC as biomarker of carbamate contamination in different aquatic environment.

MATERIALS AND METHODS

Procurement and Selection of Tilapia Samples

A total of 50 fry of Nile Tilapia (*Oreochromis niloticus* L.), with body lengths ranging from 3 to 4 cm and average weight of 7 g, were used in this study. The test animals were obtained from the Demonstration Farm of the Bureau of Fisheries and Aquatic Resources (BFAR) and were brought to the laboratory inside plastic bags half-filled with water and adequately provided with oxygen. The fishes were then transferred to a 40-L (25 × 50 × 30 cm^3) aquarium filled with 35 L of aged tap water. The tanks were fully aerated using a compressor and were allowed to acclimate for two weeks. They were given fish commercial starter crumble pellets regularly. Water in the aquaria was changed once a day.

Description of Test Chemical

Fenobucarb or BPMC (2-sec-butylphenyl methylcarbamate) Hopcin®, a carbamate insecticide, was used in this study. Hopcin 50 EC (Wuhan Chemwish Technology Company) is a formulation with 500 g BPMC kg^{-1}. This was chosen among several insecticides because it is commonly used in rice fields and farms (*Bajet et al. 2012; Varca 2012*).

Time-course pattern experiment

All fish were randomly distributed into five 25 x 50 x 30 cm^3 tanks (labeled A to E) and were filled with 40 L aged tap water. The test concentration of fenobucarb used was 0.08 mg L^{-1}, which was reported by *Calumpang et al.* (*1997*) as the detectable concentration of fenobucarb residues in irrigation areas. At the start of the experiment, all the fish except those in tank A, were exposed to the treatment dose. Fishes in tank A was assigned as Group A and served as the control wherein no treatment was applied. Four groups of fish subjected at varying lengths of exposure were assigned as follows: Group B: 7 days exposure; Group C: 14 days exposure; Group D: 21 days exposure; and Group E: 27 days exposure. Fresh doses of the same concentration of the test chemical was

introduced every day right after the water in the tanks were replaced. The general management and feeding of the fish were the same as already described in the first section. The fish were sacrificed at the end of each exposure period. Rapid cooling using iced water (about 4°C) was applied as anesthetic before the fishes were euthanized through decapitation (*Jenkins et al. 2014*). Liver and spleen samples were obtained for MMCs evaluation.

Histological Processing and Analysis

The excised liver and spleen were preserved in 10% buffered formalin solution prior to the preparation of the paraffin cross sections. The preserved organs were dehydrated by placing in a series of ethyl alcohol concentration (50-100%). After dehydration, tissue samples were cleared in chloroform and were immersed in melted soft paraffin wax with chloroform (1:1 ratio). This step was repeated for another three hours. The tissue samples were then transferred in absolute melted soft paraffin (50- 55°C) and kept in the oven. After three hours in melted paraffin, samples were transferred in melted hard paraffin (50- 55°C) and were kept inside the oven. This was repeated once and was kept in the oven for another three hours. Using small paper boats, tissue sections were embedded using the hard paraffin. Sample tissues were sectioned using rotary microtome set at 5.0 µm with a correction factor of 2. Sections were fastened on glass slides and were stained using Perls' technique (*Wan 2011*). The sectioned tissues were deparaffinized and were hydrated in distilled water. Tissue samples were dehydrated (95% to 100% alcohol) and were cleared in xylene and then mounted using Eukitt® (quick-hardening mounting medium).

The counting and measurement of MMCs followed the procedure used by *Sagun and Ocampo* (*2006*). Three focal fields of views per tissue section were examined per slide for MMC counts and cover. In each selected field, MMCs were counted visually, and the number of MMCs per mm^2 under 100× magnification were noted. Cover is the ratio of the area occupied by the MMC over the total area of the sectioned tissues expressed as percent. Three MMCs were randomly selected per slide for the examination of MMC sizes, and pigment composition which was expressed as percentage. To determine the percent composition of pigments in MMCs, the measured size of each pigment was divided by the total MMC size multiplied by 100. The tissues were examined and photographed under a light microscope. Measurements were done using ImageJ analysis program (Sun Microsystems, Inc.) calibrated under 200× magnification. The mean size (μm^2) and count (number of MMCs per mm^2) were noted from each replicate.

Statistical Analysis

Statistical analysis was carried out using R Statistical Software. Kruskall-Wallis H Test Analysis of Variance was used to analyze the significant difference among treatment groups for the different parameters: size (μm^2); cover (percent); and count (number of MMCs mm^{-2}), and pigment composition (percent), of the MMCs of the liver and spleen tissues. To test which groups in the different parameters are distinct from each other, Dunn's post hoc test was carried.

RESULTS AND DISCUSSION

No MMCs in the liver of Group A were observed (**Figure 1**). *Manera et al.* (*2000*) reported that hepatic MMCs were relatively scarce and may be attributed to their slow formation compared to renal and splenic MMCs. The same trend was also observed by *Sagun and Ocampo* (*2006*) in their study on MMC formation in Nile tilapia following exposure to imidacloprid. *Vitualla* (*2014*) also stated that splenic MMCs appear to be more obvious than hepatic MMCs.

In contrast, splenic MMCs of Group A could be easily observed and located due to the pigment aggregates (**Figure 2**). The measured mean MMC count in spleen tissues was 5.98 MMCs mm^{-2}. The mean splenic MMC size was 40.38 μm^2. The measured mean splenic MMC cover observed was 2.69%. Pigment composition of splenic MMCs of Group A were generally composed of hemosiderin (70.89%) and lipofucsin (29.11%). Hemosiderin is composed of protein and ferric iron and is derived from the catabolism of hemoglobin from effete erythrocytes. It occurs during recycling of components for erythropoiesis (*Kranz 1989*). Spleen is a hematopoietic organ in fishes; thus, the presence of hemosiderin indicates the role of MMC in erythropoiesis and the storage of erythrolysis-by-products by macrophages (*Wolke 1992; Tayel et al. 2013*). On the other hand, lipofucsin, or ceroid, results from the oxidative polymerization of polyunsaturated fatty acids of effete subcellular membranes and may also accumulate in relation to age and tissue destruction (*Agius 1981*).

It was established in this study that fenobucarb induced the formation of MMCs in all of the treated groups (i.e., Groups B to E). However, MMCs were only observed in the spleen (**Figure 3 and 4**). No MMC were observed in the liver in all treatment groups. Excessive hemosiderosis in the spleen results to the spleen

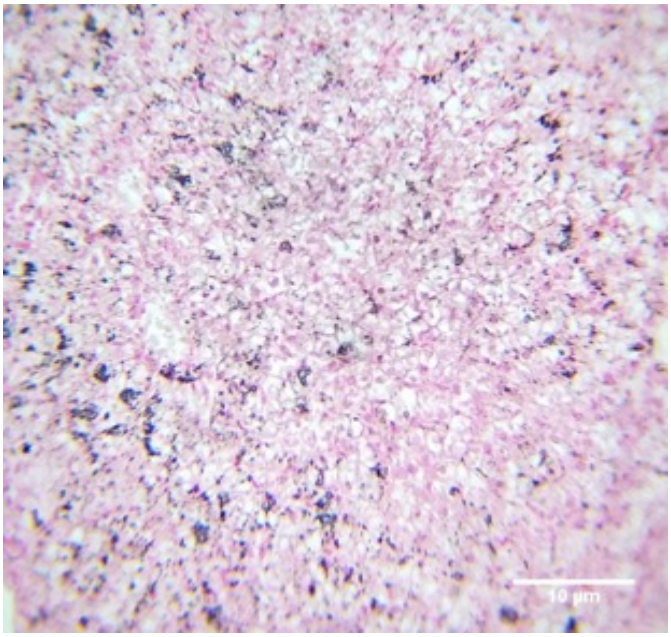

Figure 1. Hepatic tissues of untreated *Oreochromis niloticus* (Blue arrow= hemosiderin, yellow arrow= lipofuscin). 400X. Perls' stain technique.

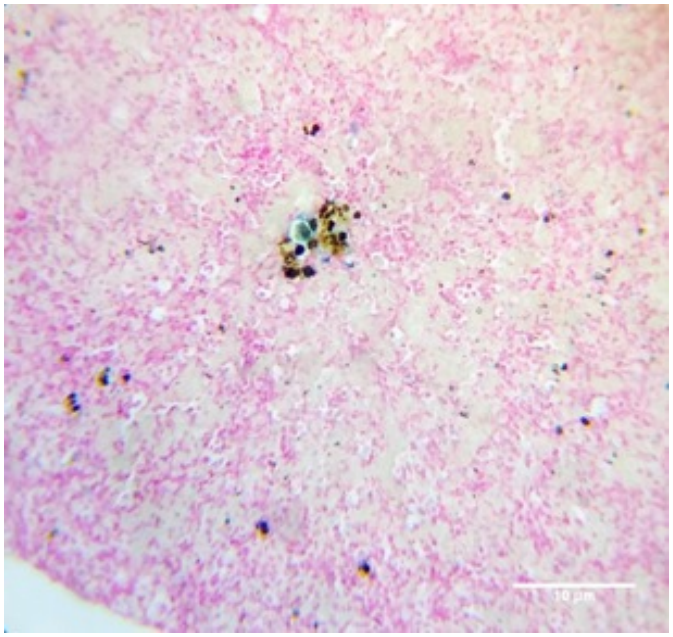

Figure 2. Splenic tissues of untreated *Oreochromis niloticus* (Blue arrow= hemosiderin, yellow arrow= lipofuscin). 400X. Perls' stain technique.

reaching its maximum capacity to contain hemosiderin and can result to spill-over effect wherein pigments "spills" into the liver (*Ocampo 2007*). Absence of these pigments suggests that the concentration used in this study was not enough to cause excessive hemosiderosis and did not induce the formation of MMC in the liver.

Fish exposed to increasing lengths of exposure to fenobucarb have resulted to increased proliferation in splenic MMCs compared to the control fish (**Table 1; Figure 4 and 5**).

Analysis showed that there were significant differences on the mean splenic MMC count among the treatment groups (KW = 13.5, $p<0.10$). Group B (exposed for seven days) has no significant difference from Group A (control group) (**Figure 5**). The MMC counts of Groups C, D, and E differed significantly with that of Group A. Although no significant difference was detected in Groups C to E, an increasing trend of MMC count was observed with respect to the increasing lengths of exposure.

In terms of mean splenic MMC size (μm^2), the control group has the smallest mean size (**Table 1 and Figure 6**). It was established that MMCs become larger after exposure to infectious agents and toxicants (*Blazer et al. 1987*). Analysis showed that there were significant differences on the mean splenic MMC size among the treatment groups (KW= 12.17, $p<0.10$). MMC size of Group A differ significantly compared to the treated groups (B-E). However, no definite pattern of MMC size with respect to increasing length of exposure was observed (**Figure 6**). Results suggest that MMC size is not effective in discerning the effects of continued exposure to minimal concentration of fenobucarb and only had significant effect at the longest exposure period.

Analysis showed significant differences on the mean splenic MMC cover among the treatment groups

Table 1. Size, count, and cover of splenic MMCs in *Oreochromis niloticus* exposed to fenobucarb at varying length of exposure.

Exposure Period	Size (μm^2)			Count (no./mm^2)			Cover (%)		
Group A (Control)	40.383^{d}	±	5.733	5.980^{b}	±	0.260	2.687^{b}	±	0.454
Group B (7 days)	46.823^{c}	±	2.010	7.760^{b}	±	0.248	6.130^{b}	±	1.367
Group C (14 days)	61.867^{b}	±	5.607	13.073^{a}	±	1.168	7.770^{b}	±	0.821
Group D (21 days)	59.713^{c}	±	2.283	16.490^{a}	±	0.162	11.207^{b}	±	0.678
Group E (28 days)	118.543^{a}	±	18.684	19.327^{a}	±	0.370	15.830^{a}	±	0.819

(KW = 13.033, p<0.10). However, only Group E (28 days exposure) had a significant difference with the Control group (**Figure 7**). This result is similar with the MMC size wherein it took 28 days for the insecticide to have significant influence on the MMC cover.

One of the main functions of MMCs is to localize the products of pathological tissue destruction which can be attributed to acetylcholinesterase (AChE) inhibition. AChE inhibition is associated with increased cellular damage due to oxidative damage (*Yang et al. 1996; Yang and Dettbarn 1996; Kazi and Oommen 2012*). *Modina (2017)* studied the effect of the same concentration of fenobucarb on AChE activity in Nile tilapia. The study observed significant AChE inhibition of treated fish. Since the main mechanism of action of fenobucarb is AChE inhibition, and MMCs are active in the accumulation of the products of cell destruction (*Blazer and Dethloff 2000*), it can be deduced that fishes in this study experienced cellular and tissue damage. This ultimately caused the increase in the severity and presence of MMC which is evident in the significant difference of MMC number, size, and cover of control group with that of the treated groups. Increasing pattern of all the MMC parameters

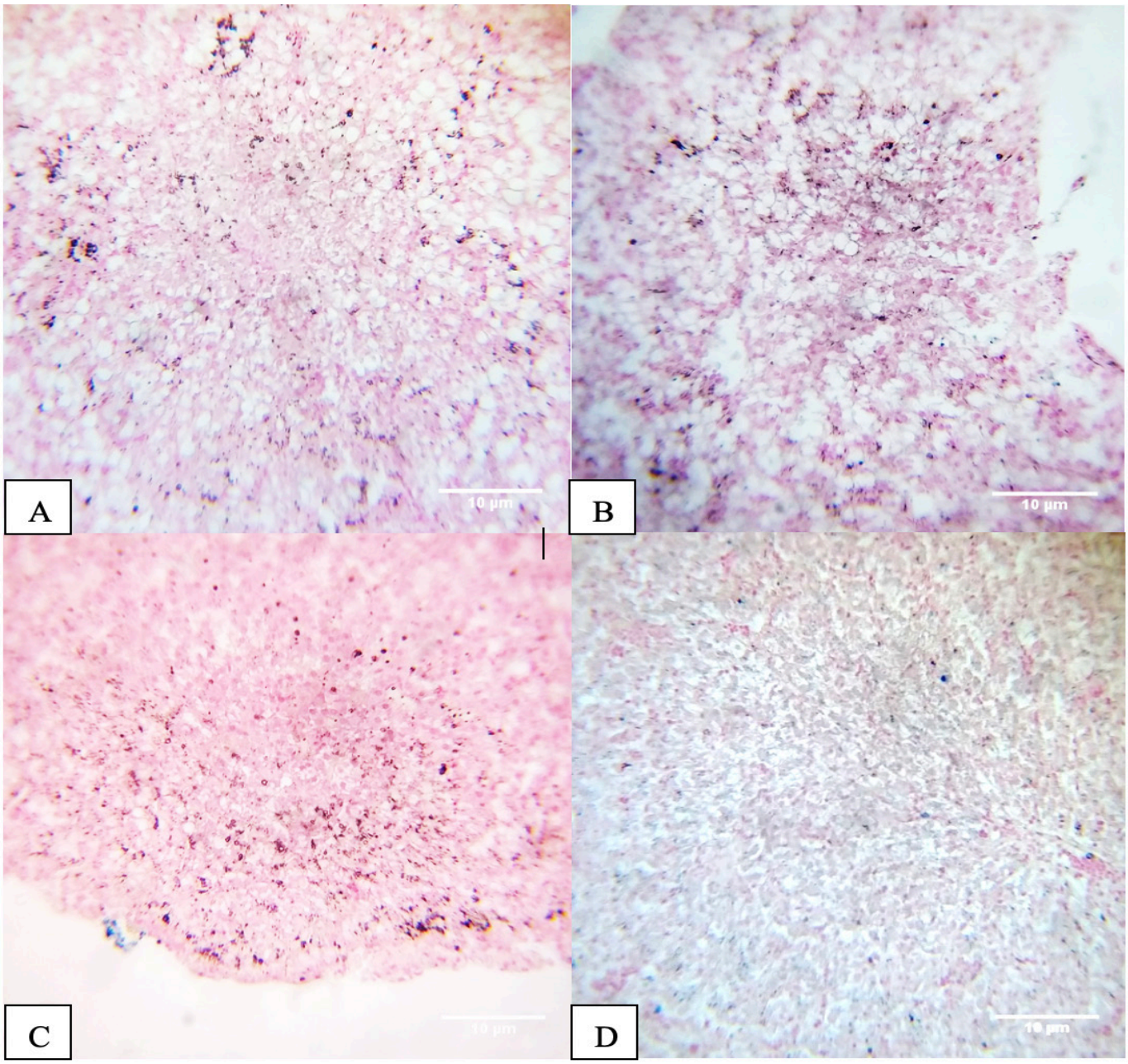

Figure 3. Hepatic tissues of *Oreochromis niloticus* exposed to nonlethal dose of fenobucarb in different lengths of exposure (A= 7 days; B= 14 days; C= 21 days; and D= 28 days) 400X. Perls' stain technique.

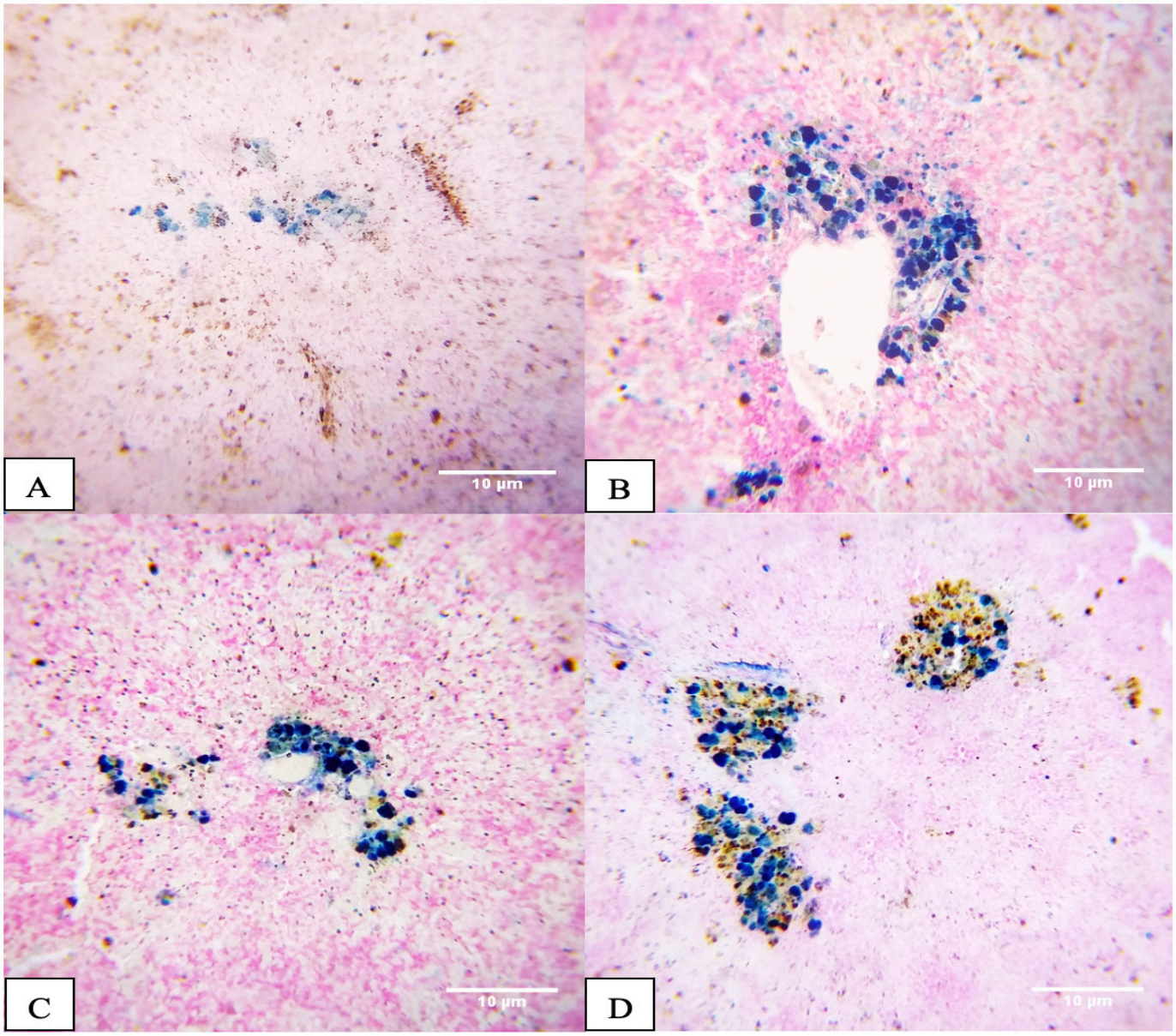

Figure 4. Splenic tissues of *Oreochromis niloticus* exposed to nonlethal dose of fenobucarb in different lengths of exposure (A= 7 days; B= 14 days; C= 21 days; and D= 28 days) 400X. Perls' stain technique.

with increasing lengths of exposure, especially MMC count, suggests that fishes experienced continued effects of fenobucarb. This is supported by the results of *Ugaddan and Ocampo* (*2009*) which observed an increasing pattern of MMC count with increasing length of exposure to glyphosate, another AChE inhibitor, in Nile tilapia.

The pigment composition of MMCs in relation to increasing length of exposure to fenobucarb showed an initial increase in hemosiderin in Group B (7-days-exposed fish) and decreased afterwards. Initial increase in hemosiderin appear to be the first response of the organism to the exposure to fenobucarb (**Table 2 and Figure 8**).

Increase in the content of hemosiderin in the spleen suggests increased degradation of erythrocytes and the release of iron, which could be the consequence of damage of these cells caused by exposure to toxins (*Pronina et al. 2014*). A number of studies already reported the hemolytic effects of pesticides exposure. *Gaafar et al.* (*2010*) reported the hemolytic effects of edifenphos on Nile tilapia (*O. niloticus*). *Al-Rudainy* and *Kadhim* (*2012*) reported increased rate of erythrocyte destruction in hematopoietic organs (i.e., spleen) on common carp (*Cyprinus carpio*) following exposure to endosulfan. However, statistical analysis did not detect significant differences of the hemosiderin of Groups A-D. Only the group exposed the longest differed significantly with the rest of the treatment groups.

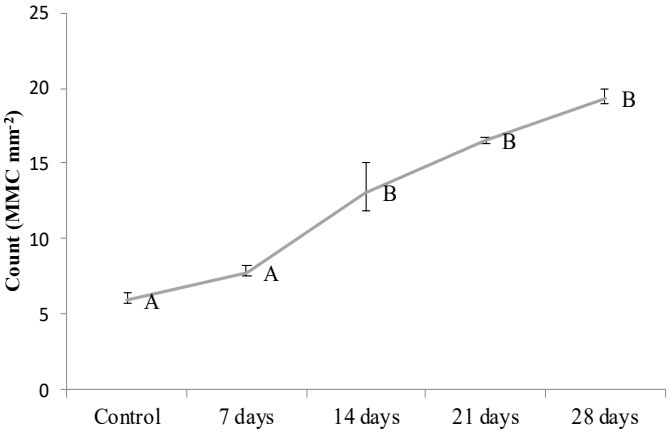

Figure 5. Mean±SE splenic melanomacrophage centers count (MMC mm^{-2}) in *Oreochromis niloticus* exposed to nonlethal dose of fenobucarb at increasing length of exposure N=10 fish per group. (KW = 13.5, p <0.10).

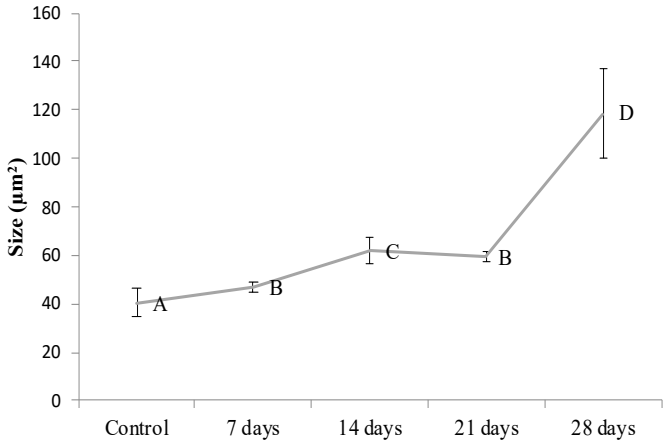

Figure 6. Mean±SE splenic melanomacrophage centers (MMCs) size (μm^2) in *Oreochromis niloticus* exposed to nonlethal dose of fenobucarb at increasing length of exposure. N=10 fish per group. (KW = 12.17, p <0.10).

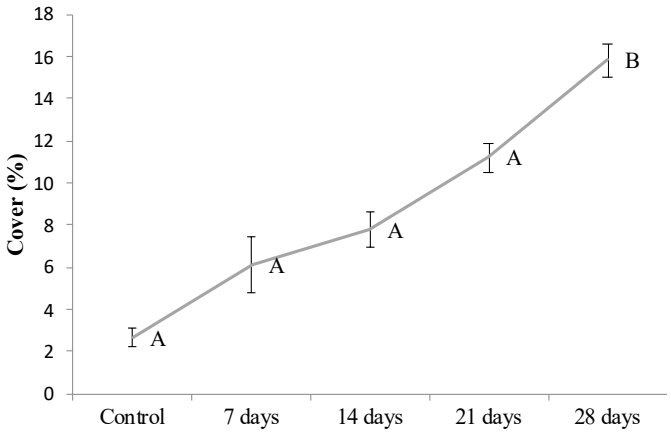

Figure 7. Mean±SE splenic melanomacrophage centers (MMCs) percent cover in *Oreochromis niloticus* exposed to nonlethal dose of fenobucarb at increasing length of exposure. N=10 fish per group. (KW = 13.03, p <0.10).

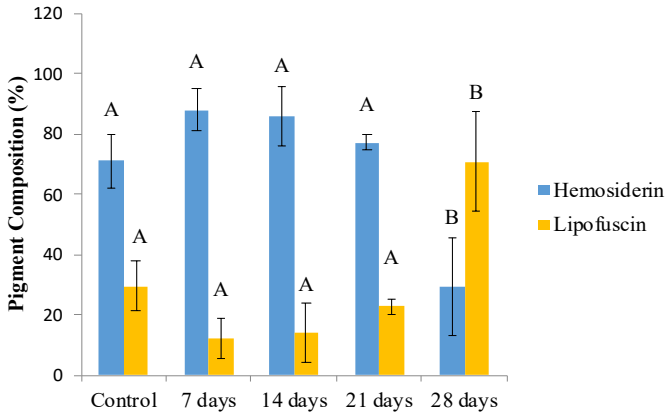

Figure 8. Pigment composition±SE of splenic MMCs in *Oreochromis niloticus* exposed to nonlethal dose of fenobucarb at increasing length of exposure. N=10 fish per group. (Hemosiderin KW=8.17, p <0.10; Lipofuscin KW=8.66, p <0.10).

Table 2. Pigment composition of splenic MMCs in *Oreochromis niloticus* exposed to fenobucarb at varying length of exposure.

Exposure	Haemosiderin (%)			Lipofucsin (%)		
Group A (Control)	70.890^{ab}	±	8.750	29.403^{ab}	±	8.296
Group B (7 days)	87.963^{a}	±	6.861	12.043^{b}	±	6.864
Group C (14 days)	85.910^{a}	±	9.718	14.090^{b}	±	9.718
Group D (21 days)	77.110^{a}	±	2.544	22.887^{b}	±	2.545
Group E (28 days)	29.287^{b}	±	16.477	70.717^{a}	±	16.479

Lipofuscin content of the MMC differed significantly (KW = 8.66, p<0.10). Lipofucsin results from the oxidation of unsaturated lipids and accumulates with age and tissue destruction caused by toxicants (*Agius 1981*). Data showed that increasing length of exposure to fenobucarb induce higher composition of lipofucsin in MMCs. Lipofucsin composition increases with increasing lengths of exposure due to higher oxidation of unsaturated lipids and effete subcellular membranes rather than hemolysis (*Wolke et al. 1985*). According to *Pronina et al.* (*2014*), increase in lipofuscin content suggests structural and functional abnormalities in the organs of fish. However, only group E had significant difference in terms of lipofuscin content. This suggests

that prolonged exposure to the toxicant have an additive effect on tissue destruction. This also means that the concentration used in this study was minimal to have caused destruction at a shorter exposure period.

The results of this study showed the potential of MMCs to be used as biomarker of fenobucarb exposure in aquatic environments. Even at minimal concentration, significant difference was detected between control and treated groups, especially with the longest exposure period. According to *Fournie et al.* (*2001*), MMCs respond relatively rapidly to environmental stressors including pesticide exposure. Changes in MMCs in fishes can be considered as early warning system which could serve to notify regulatory agencies of environmental problems (e.g., pesticide contamination) before opting for cost intensive chemical analysis of water and sediments.

CONCLUSION AND RECOMMENDATIONS

Exposure to minimal concentration of fenobucarb induced changes in splenic MMC parameters (i.e., count, size, percent cover, and pigment composition) in relation to varying lengths of exposure. All the MMC parameters under study detected significant differences between control and treated groups. Increasing trend of MMC count, size, and percent cover, in relation to increasing lengths of exposure was evident in this study. Significant increase was detected at the longest length of exposure of 28 days. Significant effect of fenobucarb exposure on pigment compositions of MMCs was only detected at the longest exposure period. This suggests that the minimal concentration used in this study took longer to have significant influence on the pigment composition.

Although all of the splenic morphometric parameters were sensitive enough to detect the influence of fenobucarb, results of the study show that MMC count and size are the most sensitive and ideal parameter which detected pattern of the effect of fenobucarb through time.

The long-term harmful effects of toxicants are of ultimate importance. Longer exposure may intensify the toxicity of insecticides. Even at low concentrations, contamination of pesticides in aquatic environments at longer periods could be harmful to fish and other non-target organisms. Even at non-lethal concentration of fenobucarb, the proliferation of splenic MMCs is induced as the length of exposure increases. Such effects can occur in real field situations such as in irrigation canals, ponds, rivers, lakes, estuaries, and even in coastal waters.

Since concentration of contaminants in aquatic environments vary with respect to frequency and amount of pesticide application, distance from point source, and different seasons, it is recommended to study the effects of increasing sublethal concentration of the test chemical in relation to increasing duration of exposure. Since more than one insecticide are normally used in field settings, other test chemicals can be used to assess the applicability of MMCs as a biomarker for other sources of stress to the animal, such as other pesticides, heavy metals, and eutrophication.

REFERENCES

Ajima, M., Pandey, P., Kumar, K., and Poojary, N. 2017. "Neurotoxic effects, molecular responses and oxidative stress biomarkers in Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758) exposed to verapamil". *Comparative Biochemistry and physiology. Toxicology & Pharmacology: CBP*. 196, 44-52. https://doi.org/10.1016/j.cbpc.2017.03.009

Agius, C. 1981. "Preliminary studies on the ontogeny of the melano-macrophages of teleost haemopoietic tissue and age-related changes". *Developmental and Comparative Immunology* 5:597-606.

Agius, C. and Roberts, R.J. 2003. "Melano-macrophage centres and their role in fish pathology". *Journal of Fish Diseases* 26: 499-509. https://doi.org/10.1046/j.1365-2761.2003.00485.x

Al-Rudainy, A.H. and Kadhim, M.H. 2012. "Hematological and Neurotoxic Effects of Endosulfan Pesticide on Common Carp *Cyprinus carpi*". *The Iraqi Journal of Veterinary Medicine* (1): 58 – 67. https://www.iasj.net/iasj/download/97929d43bc7d384136

Allinson, G., Allinson M., Bui A., Zhang P., Croatto G., Wightwick A., Rose G., Walters R. 2016. "Pesticide and trace metals in surface waters and sediments of rivers entering the Corner Inlet Marine National Park, Victoria, Australia". *Environmental Science and Pollution Research International* ;23(6):5881-91. doi: 10.1007/s11356-015-5795-6. Epub 2015 Nov 23. PMID: 26593725.

Añasco, N.C., Koyama J. and Uno S. 2010. "Pesticide residues in coastal waters affected by rice paddy effluents temporarily stored in a wastewater reservoir in southern Japan". *Archives of Environmental Contamination and Toxicology* 58(2):352-60. doi: 10.1007/s00244-009-9364-1. Epub 2009 Jul 17. PMID: 19609592.

Bacolod, E., Uno, S., Villamor, S., and Koyama, J. 2017. "Oxidative stress and genotoxicity biomarker responses in tilapia (*Oreochromis niloticus*) exposed to environmental concentration of

1-nitropyrene". *Marine Pollution Bulletin*. 124, 786-791.https://doi.org/10.1016/j.marpolbul.2017.01.077

Bajet, C.M., Kumar, A., Calingacion, M.N. and Narvacan, T.C. 2012. "Toxicological assessment of pesticides used in the Pagsanjan-Lumban catchment to selected non-target aquatic organisms in Laguna Lake, Philippines". *Agricultural Water Management* 106: 42-49. https://doi.org/10.1016/j.agwat.2012.01.009.

Beso, G.A., Candelaria, V.Y., dela Cruz, J.F., Tolentino, M.S., Tameta, A D.C. and Espinosa, A. 2016. "Effects of unleaded petroleum on the macrophage aggregates (MA) formation in red king tilapia (*Oreochromis* sp.) Fingerlings" *bioRxiv* 044537; doi: http://dx.doi.org/10.1101/044537

Blazer, V.S., Facey D.E., Fournie J.W., Courtney L.A., and Summers, J.K. 1994. Macrophage aggregates as indicators of environmental stress. Modulators of Fish Immune Responses: Volume 1, Models for Environmental Toxicology, Biomarkers, Immunostimulators. pp. 169-186.

Bonmatin, J.M., Mitchell, E.A.D., Glauser, G., Lumawig-Heitzman, E., Claveria, F., van Lexmond, M.B., Taira, K., Sánchez-Bayo, F. 2021. Residues of neonicotinoids in soil, water and people's hair: A case study from three agricultural regions of the Philippines. *Science of The Total Environment* (757) Article 143822. https://doi.org/10.1016/j.scitotenv.2020.143822.

Calumpang, S.M.F., Medina, M.J.B., Tejada, A.W. and Medina, J.R. 1997. "Toxicity of chlorpyrifos, fenubucarb, monocrotophos, and methyl parathion to fish and frog after a stimulated overflow of paddy water." *Bulletin of Environmental Contamination and Toxicology*. 58(6): 909-14.

Cullen, M.C. and Connell, D.W.. 1992. "Bioaccumulation of chlorohydrocarbon pesticides by fish in the natural environment". *Chemosphere* 25, Article 1579e1587 1579e1587. https://doi.org/10.1016/0045-6535(92)90306-C

Claver, A., Ormad, P., Rodríguez, L. and Ovelleiro, J.L. 2006. "Study of the presence of pesticides in surface waters in the Ebro river basin (Spain)". *Chemosphere* ;64(9):1437-43. doi: 10.1016/j.chemosphere.2006.02.034. Epub 2006 Mar 29. PMID: 16574191.

De Almeida, M., Pereira, T., Batlouni, S., Boscolo, C., and De Almeida, E. 2018. "Estrogenic and anti-androgenic effects of the herbicide tebuthiuron in male Nile Tilapia (*Oreochromis niloticus*)". *Aquatic Toxicology* 194, 86-93. https://doi.org/10.1016/j.aquatox.2017.11.006

De Silva, C.D. and Ranasinghe, J. 1989. "Toxicity of four commonly used agrochemicals on *Oreochromis niloticus* (L.) Fry". *Asian Fisheries Science*, 2:135-145. Fabro, L. and Varca, L. 2012. 'Pesticide residues in surface waters of Pagsanjan-Lumban catchment of Laguna de Bay, Philippines". *Fuel and Energy Abstracts*. 106. 10.1016/j.agwat.2011.08.006.

Felício, A., Freitas, J., Scarin, J., Ondei, L., Teresa, F., Schelenk, D., and De Almeida, E. 2018. "Isolated and mixed effects of diuron and its metabolites on biotransformation enzymes and oxidative stress response of Nile tilapia (*Oreochromis niloticus*)". *Ecotoxicology and Environmental Safety.* 149, 248-256. https://doi.org/10.1016/j.ecoenv.2017.12.009

Fournie, J., Summers, J., Courtney, L.A., Engle, V.D. and Blazer, V.S. 2001. "Utility of Splenic Macrophage Aggregates as an Indicator of Fish Exposure to Degraded Environments". *Journal of Aquatic Animal Health*. 13. https://doi.org/10.1577/1548-8667(2001)013%3C0105:UOSMAA%3E2.0.CO;2.

Gaafar, A.Y., El-Manakhly, E.M., Soliman, M.K., Soufy, H., Zaki, M.S., Mohamed, S.G. and Hassan, S.M. 2010. "Some pathological, biochemical and hematological investigations on Nile tilapia (*Oreochromis niloticus*) following chronic exposure to edifenphos pesticide". *Journal of American Science*. 6 (10) 542-551.

Gao J, Liu L, Liu X, Lu J, Zhou H, Huang S, Wang Z, Spear PA. 2008. "Occurrence and distribution of organochlorine pesticides - lindane, p,p'-DDT, and heptachlor epoxide - in surface water of China". *Environ Int*.;34(8):1097-103. doi: 10.1016/j.envint.2008.03.011. PMID: 18508123.

Guevarra, R.D., Paraso, M.G. and Lola, M.S. 2020. "Biomarker Evaluation in Nile Tilapia (*Oreochromis niloticus*) to Assess the Health Status of Aquaculture Areas in the Seven Lakes of San Pablo". *Philippine Journal of Science*. 149. 833-840.

Guzmán-Guillén, R., Ortega, A, Martín-Caméan, A, and Cameán, A. 2015. "Beneficial effects of vitamin E supplementation against the oxidative stress on Cylindrospermopsin-exposed tilapia (*Oreochromis niloticus*)". *Toxicon*. 104, 34-42. https://doi.org/10.1016/j.toxicon.2015.07.336.

Jenkins, J.A., Bart Jr., H.L., Bowker, J.D., Bowser, P.R., MacMillan, J.R., Nickum, J.G., Rachlin, J.W., Rose, J.D., Sorensen, P.W., Warkentine, B.E. and Whitledge, G.W. 2014. "Guidelines for Use of Fishes in Research—Revised and Expanded" *Fisheries*, 39:9, 415-416, https://doi.org/10.1080/03632415.2014.924408

Kaewamatawong, T., Rattanapinyopituk, K., Ponpornpisit, A., Pirarat, N., Ruangwises, S. and Rungsipipat, A. 2013. "Short-term exposure of Nile Tilapia (*Oreochromis niloticus*) to mercury: histopathological changes, mercury bioaccumulation, and protective

role of metallothioneins in different exposure routes". *Toxicologic Pathology* 41(3):470-9. https://doi.org/10.1177%2F0192623312457269.

Katagi, T. and Ose, K. 2014. "Bioconcentration and metabolism of pesticides and industrial chemicals in the frog". *Journal of Pesticide Science*. 39(2): 55-68 https://doi.org/10.1584/jpestics.D13-047

Katagi, T., and Tanaka, H. 2016. "Metabolism, bioaccumulation, and toxicity of pesticides in aquatic insect larvae". *Journal of Pesticide Science*, 41(2), 25–37.https://doi.org/10.1584/jpestics.D15-064

Kazi, A.I., and Oommen, A. 2012. "Monocrotophos induced oxidative damage associates with severe acetylcholinesterase inhibition in rat brain". *Neurotoxicology*, 33(2): 156-61

Kim, I., Kim, DU., Kim, NH. , Ka, J.O. 2014. "Isolation and characterization of fenobucarb-degrading bacteria from rice paddy soils". *Biodegradation* 25: 383–394. https://doi.org/10.1007/s10532-013-9667-9

Kranz, H. 1989. "Changes in splenic melano-macrophage centres of dab, *Limanda limanda* during and after infection with ulcer disease". *Diseases of Aquatic Organisms*.6:167-173.

Lamers, M., Anyusheva M., La N., Nguyen V.V. and Streck T. 2011. "Pesticide pollution in surface- and groundwater by paddy rice cultivation:a case study from Northern Vietnam (short communication) CLEAN'. *Soil Air Water* 39: 356−361

Leong, K. H., Benjamin Tan, L. L., and Mustafa, A. M. 2007. "Contamination levels of selected organochlorine and organophosphate pesticides in the Selangor River, Malaysia between 2002 and 2003". *Chemosphere*, 66(6): 1153-1159.

Lu, J. L. 2010. "Analysis of Trends of the Types of Pesticide Used, Residues and Related Factors among Farmers in the Largest Vegetable Producing Area in the Philippines". *Journal of Rural Medicine* 5(2): 184–189. https://doi.org/10.2185/jrm.5.184

Manera, M., Serra, R., Isani, G., and Carpeñe, E. 2000. "Macrophage aggregates in gilthead sea bream fed copper, iron, and zinc enriched diets". *Journal of Fish Biology* 57(2):457-465.

Manrique, W.G., Claudiano, G., Petrillo, T.R., Castro, M.P., Figueiredo, M.A.P., Belo, M.A., Moraes, J.R. and Moraes F.R. 2014. "Response of splenic melanomacrophage centers of *Oreochromis niloticus* (Linnaeus, 1758) to inflammatory stimuli by BCG and foreign bodies". *Journal of Applied Ichthyology*. 30: 1001-6. https://doi.org/10.1111/jai.12445.

Manrique, W.G., Figueiredo, M.A., Charlie-Silva, I., Belo, M.A. and Dib, C.C. 2019. "Spleen melanomacrophage centers response of Nile tilapia during *Aeromanas hydrophila* and *Mycobacterium marinum* infections". *Fish Shellfish Immunology.*;95:514-518. https://doi.org/10.1016/j.fsi.2019.10.071. Epub 2019 Nov 1. PMID: 31682998.

Modina, R.M.R. 2017. "Acetylcholinesterase activity in Nile tilapia (*Oreochromis niloticus* L.) following exposure to carbamate insecticide". *Annals of Tropical Research.* 39(2): 78-87

Murray, J., Nadel, J., Mason, R., Slutsky, A., and Gotway, M. 2015. "Murray and Nadel's textbook of respiratory medicine, 6th edition". *Annals of the American Thoracic Society*. 12(8): 1257-8.

Navarrete, I.A., Tee, K.A.M., Unson, J.R.S., and Hallare, A.V. 2018. "Organochlorine pesticide residues in surface water and groundwater along Pampanga River, Philippines". *Environmental Monitoring and Assessment* 190(5):289. https://doi.org/10.1007/s10661-018-6680-9.

Ocampo, P.P. 2007. "Piscine melanomacrophage centers (MMCs) as potential indicator of pesticide exposure". *Philippine Entomologist.* 21(1): 100-114.

Ogundiran, M.A. and Fawole, O.O. 2018. "Toxic effects of water pollution on two bio- indicators of aquatic resources of Asa River, Nigeria". *Journal of Fisheriessciences.com*. 12(2): 020-027. https://doi.org/10.21767/1307-234X.1000148

Ohkawa, H. Miyagawa, H. and Lee, Philip. .2007. Pesticide Chemistry: Crop Protection, Public Health, Environmental Safety. 10.1002/9783527611249.

Pronina, S.V., Batueva, M.D.-D., and Pronin, N.M. 2014. "Characteristics of Melanomacrophage Centers in the Liver and Spleen of the Roach *Rutilus rutilus* (Cypriniformes: Cyprinidae) in Lake Kotokel during the Haff Disease Outbreak". *Journal of Ichthyology*. 54 (1): 104-110.

Pucher, J., Gut T., Mayrhofer, R., El-Matbouli, M., Viet, P.H., Ngoc, N.T., Lamers, M., Streck, T. and Focken, U. 2014. "Pesticide-contaminated feeds in integrated grass carp aquaculture: toxicology and bioaccumulation". *Diseases of Aquatic Organisms* 108(2):137-47. https://doi.org/10.3354/dao02710.

Rossi, A.S, N. Fanton, M.P. Michlig, M.R. Repetti, J. Cazenave. 2020. "Fish inhabiting rice fields: Bioaccumulation, oxidative stress and neurotoxic effects after pesticides application". *Ecological Indicators* 113. https://doi.

org/10.1016/j.ecolind.2020.106186.

Roxas-Aragon, M.G., Ocampo, P.P., Alviola III, P.L. and Lontoc, B.M. 2003. "Cytochrome P450 induction, hematological changes and melanomacrophage accumulation in Nile tilapia (*Oreochromis niloticus* L.) after exposure to chlorpyrifos". *Philippine Entomologist* 17(1): 66-76.

Sagun, V.G. and Ocampo, P.P. 2006. "Proliferation of melanomacrophage centers (MMCs) in Nile tilapia (*Oreochromis niloticus* L.) as induced by exposure to imidacloprid insecticide". *Philipp. Ent*. 20(2): 137-149.

Satyanarayan, S., P. L. Muthal, K. P. Krishnamoorthy and S. N. Kaul. 1999. "Bioaccumulation of organochlorine pesticides in different fish tissues, International" *Journal of Environmental Studies*, 56(2): 201-13 201-213, https://doi.org/10.1080/00207239908711200

Sparling, D. 2016. Bioindicators of Contaminant Exposure. In: Sparling, D.W. (Ed.) Ecotoxicology Essentials. Academic Press. pp 45-66. http://doi.org/10.1016/B978-0-12-801947-4.00003-2

Streit, B. 1998. Bioaccumulation of contaminants in fish. In: Braunbeck T., Hinton D.E., Streit B. (Eds) Fish Ecotoxicology. EXS, vol 86. Birkhäuser, Basel. https://doi.org/10.1007/978-3-0348-8853-0_12

Tayel, S. I., Ibrahim, S. A. and Mahmoud S. A. 2013. "Histopathological and muscle composition studies on *Tilapia zillii* in relation to water quality of Lake Qarun, Egypt." *Journal of Applied Sciences Research* Vol.9 9(6): 3857-72

Téllez-Bañuelos, M.C., Santerre, A., Casas-Solís, J., Bravo-Cuellar, A., and Zaitseva, G. 2009. "Oxidative stress in macrophages from spleen of Nile tilapia (*Oreochromis niloticus*) exposed to sublethal concentration of endosulfan".*FishandShellfishImmunology*,27(2),105-11.

Tjahjaningsih, W., Tri Pursetyo K. and Sulmartiwi L. 2017. Melanomacrophage centers in kidney, spleen and liver: A toxic response in carp fish (*Cyprinus carpio*) exposed to mercury chloride. AIP Conference Proceedings. 1813. 020012. 10.1063/1.4975950.

Toan, P., Sebesvari, Z., Bläsing, M., Rosendahl, I. and Renaud, F.G., 2013. "Pesticide management and their residues in sediments and surface and drinking water in the Mekong Delta, Vietnam". *Science of the Total Environment* 452-453, 28–39. https://doi.org/10.1016/j.scitotenv.2013.02.026

Toledo-Ibarra, G. Díaz Resendiz, K. Ventura-Ramón, G. González-Jaime, F. Vega-López, A Becerril-Villanueva, E. Pavón, L. and Girón-Pérez, M. 2016. "Oxidative damage in gills and liver in Nile tilapia (*Oreochromis niloticus*) exposed to diazinon". *Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology* 200, 3-8. http://doi.org/10.1016/j.cbpa.2016.05.007

Ugaddan, G. and Ocampo, P. 2009. "Brain acetylcholinesterase (AChE) activity and liver melanomacrophage centers (MMCs) formation in Nile tilapia (*Oreochromis niloticus* L.) following exposure to glyphosate herbicide". *Asia Life Sciences*. 18. 73-85.

Varca, L.M. 2012. 'Pesticide residues in surface waters of Pagsan-Lumban catchment of Laguna de Bay, Philippines". *Agricultural Water Management* 106: 35-41.

Vitualla, J.L. 2014. Morphometrics of the hepatic and splenic melanomacrophage centers (MMCs) of Nile tilapia (*Oreochromis niloticus,* Linnaeus) after exposure to induced stress. Unpublished MSc thesis, University of the Philippines, Los Banos, Philippines.

Wan, M. 2011. Perls' technique for the demonstration of haemosiderin – methods and tips. Blog Skin Pathol. 4 p.

Wolke, R.E. 1992. "Piscine macrophage aggregates: A review." *Annual Review of Fish Diseases*. 2:91-108.

Wolke, R.E., Murchelano, R.A., and George, C.J. 1985. "Preliminary evaluation of the use of macrophage aggregates (MA) as fish health monitors". *Bulletin of Environmental Contamination and Toxicology.* 35:222-227.

Yang, C.C., Alvarez, R.B., Engel W.K. and Askanas, V. 1996. "Increase of nitric oxide synthases and nitrotyrosine in inclusion-body myositis". *Neuroreport* 8: 153–8.

Yang, Z.P. and Dettbarn, W.D. 1996. "Diisopropylphosphorofluoridate-induced cholinergic hyperactivity and lipid peroxidation". *Toxicology and Applied Pharmacology* 138: 48–53.

Zhang, M., Zeiss, M.R. and Shu Geng S. 2015. "Agricultural pesticide use and food safety: California's model". *Journal of Integrative Agriculture* 14 (11):2340–2357.

Zahran, E., Risha, E., Awadin, W. and Palic, D. 2018. "Acute exposure to chlorpyrifos induces reversible changes in health parameters of Nile tilapia" (*Oreochromis niloticus*). *Aquatic Toxicology* 197: 47-59. http://doi.org/10.1016/j.aquatox.2018.02.001