UNIVERSITI PUTRA MALAYSIA

HISTOPATHOLOGICAL EVALUATION OF THE GIANT MUDSKIPPERS (*PERIOPHTHALMODON SCHLOSSERI*) LIVER AS A POTENTIAL POLLUTION BIOINDICATOR IN MORIB, SELANGOR

AMMAR BIN NORAZMAN

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AMMAR BIN NORAZMAN

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CERTIFICATION

It is hereby certified that we have read this project entitled "Histopathological Evaluation of the Giant Mudskippers (*Periophthalmodon schlosseri*) Liver as a Potential Pollution Bioindicator in Morib, Selangor", by Ammar bin Norazman and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 - Final Year Project.

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ASSOCIATE PROFESSOR DR INTAN SHAMEHA ABDUL RAZAK
DVM (UPM), PhD (UPM)
Lecturer,
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Supervisor)

____

DR MOHD FUAD BIN MATORI
DVM (UPM)
Veterinary Officer
Aquatic Animal Health Unit
Universiti Putra Malaysia
(Co-Supervisor)

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LIST OF ABBREVIATIONS

%	Percent
<	Less than
>	More than
MMC	Melanomacrophage centre
GC	Germinal centre
MM	Melanomacrophage
AUP	Animal Utilisation Protocol
IACUC	Institutional Animal Care and Use Committee
H&E	Haematoxylin & Eosin
DPX	Distyrene Plasticizer Xylene
SPSS	Statistical Package for the Social Sciences
EDX	Energy Dispersive X-ray
FESEM	Field Emission Scanning Electron Microscopy
g	Gram
ICI	Inflammatory Cells Infiltration
PMN	Polymorphonuclear
AAS	Atomic Absorption Spectroscopy

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 - Projek Tahun Akhir.

PENILAIAN KEBOLEHAN HISTOPATOLOGI HATI IKAN BELACAK (*Periophthalmodon schlosseri*) SEBAGAI PENUNJUK BIOLOGI PENCEMARAN DI MORIB, SELANGOR.

Oleh

AMMAR NORAZMAN

2022

Penyelia: Prof. Madya Dr. Intan Shameha Abdul Razak

Penyelia bersama: Dr. Mohd Fuad Matori

Ikan tembakul *(Periophthalmodon schlosseri)* menghuni setiap lapisan zon pasang surut laut di seluruh semenanjung Malaysia. Ikan tembakul sensitif kepada keadaan persekitaran dan tabiatnya yang suka menenggelamkan diri di dalam lumpur menjadikannya calon yang sesuai sebagai petunjuk biologi pencemaran alam sekitar. Tujuan kajian ini adalah untuk menentukan sama ada hati ikan tembakul boleh digunakan sebagai petunjuk biologi pencemaran alam sekitar di kawasan ini. Kumpulan umur berbeza juga dibandingkan untuk mengaitkannya dengan tempoh pendedahan kepada pencemaran. 5 ikan tembakul (2 dewasa, 3 juvenil) ditangkap di Kuala Kelanang, Morib, Selangor menggunakan joran. Ikan ditimbang, dimatikan dan dibedah siasat. Hati ikan ditimbang dan ciri fizikal dicatat. Hati tersebut diawet di dalam formalin, diproses dan diwarnai menggunakan pewarna H&E. Perubahan histopatologi dinilai dan kumpulan melanomakrofaj (MMC) dan penyusupan sel radang (ICI) dikelaskan. Penilaian kualitatif kewujudan unsur di dalam sampel lumpur dan hati dilakukan menggunakan *energy dispersive x-ray* (EDX). Bedah siasat menunjukkan bahawa hati ikan belacak berwarna merah jambu, mempunyai 2 lobus, dan lembut. Penilaian histopatologi menunjukkan terdapat perbezaan ketara ($p<0.005$) skor MMC antara kumpulan ikan dewasa dan juvenil, dan tiada perbezaan ketara ($p>0.005$) skor ICI. Analisis EDX mendapati tiada unsur wujud di dalam sampel hati melainkan hanya terdapat Aluminium di dalam lumpur, menunjukkan bahawa kawasan kajian kurang tercemar. Oleh itu, disarankan bahawa kajian dilakukan di kawasan yang terbukti tercemar sebagai perbandingan.

Kata kunci: *perubahan histopatologi, hati, petunjuk biologi, pencemaran alam, ikan tembakul (Periophthalmodon schlosseri)*

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the course VPD 4901- Project.

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999 – Project.

By

AMMAR NORAZMAN

2022

Supervisor: Assoc. Prof. Dr. Intan Shameha

Co-supervisor: Dr. Mohd Fuad Matori

Giant mudskipper (*Periophthalmodon schlosseri*) inhabits all intertidal zone levels across peninsular Malaysia. Mudskippers are sensitive to the ambient environment and their mud burrowing lifestyle makes them a good candidate for pollution bioindicator. The purpose of this study is to determine whether the liver of giant mudskippers can be used as an environmental pollution bioindicator in this area. Age groups were compared to relate to the duration of exposure. Five mudskippers (3 juveniles, 2 adults) were caught at Kuala Kelanang, Morib, Selangor using fishing rods. The fish were weighed, euthanized and necropsied. The livers were weighed and gross features were recorded. The livers were fixed in formalin, processed, and stained with H&E staining. The histopathological changes of the liver were evaluated and melanomacrophage centres (MMC) and inflammatory cells infiltration (ICI) were graded. Qualitative analysis of the elements present in the soil and liver samples was done using energy dispersive x-ray (EDX). Necropsy revealed that the liver was pink, bilobed and soft. Histopathological evaluation showed significant difference ($p<0.005$) in MMC scores between the juvenile and adult group, while there was no significant difference ($p>0.005$) in ICI. EDX analysis showed absence of elements in the liver except for Aluminium in the soil. In conclusion, we could not conclude the livers as good bioindicators in this study, since no element traces found in the liver and soil, indicating the sampling area was less polluted. Thus, it is suggested to choose the sampling area known to be polluted for comparison.

Keywords: *histopathological change, liver, bio-indicator, environmental pollution, giant mudskipper (Periophthalmodon schlosseri)*

1. 0 INTRODUCTION

Periophthalmodon schlosseri, known as the giant mudskipper, is an obligatory air-breathing amphibious fish that roams freely through the tropical mangrove mudflats in Southeast Asia's intertidal zone (Randall, 2004). It is a carnivore with a mud burrow high up in the intertidal zone that is filled with water. It is one of the biggest mudskippers and is relatively common along Peninsular Malaysia's western coast (Mazlan, 2008).

Like any other organism, the toxins that are absorbed into its body will cause physiological, morphological, or genetic changes that may disrupt normal processes of the mudskippers. The structure of the tissues can also be defective due to the accumulation of Lead, Cadmium, and Copper. This is further backed by the fact that mudskippers could accumulate and increase the levels of Chromium, Nickel, Lead, Silver, and Cadmium in their tissue without physical stress and seasonal influences (Moslen & Miebeka, 2016). According to Renieri *et al.* (2014), Cadmium, Lead and Mercury were found in higher levels inside the liver and muscle, compared to gonads, depending on the age and season.

Aquatic animals have been used as environmental bioindicators in various places for a long time. In Malaysia, besides mudskippers, some other species used include cockles (Sany *et al,* 2014), oysters, Carassius spp. (Shazili *et. al,* 2006), and many more. This is because a lot of waste material from human activities is released into waterways, which will then flow into the ocean. These heavy metals are then absorbed by marine animals, some of which are consumed by humans.

Mud, the main substrate of mudflats and intertidal zones, is rich in nutrients and minerals, including heavy metal pollutants. The mudskippers, who spend their whole life burrowing or skipping on the mud, may have direct contact with the heavy metals. They are very tolerant to organic and inorganic pollutants. So, they potentially accumulate a high level of heavy metals disposed of from anthropogenic activities. So, this fish may be a good species to be used as a marine bioindicator for environmental pollution (Sangur *et al.*, 2021).

Thus, the objectives of the study are:

1. To identify the gross and histopathological changes in the liver of giant mudskippers (*Periophthalmodon schlosseri*) captured from Kampung Kelanang estuary.

2. To determine whether the morphological changes of the liver of giant mudskippers (*Periophthalmodon schlosseri*) have the potential to be used as a bioindicator for environmental pollution.

3. To compare the gross and histopathological changes between juvenile and adult giant mudskippers.

2.0 LITERATURE REVIEW

2.1 Mudskippers

Oxudercine gobies, also known as mudskippers, are amphibious gobies that usually inhabit mudflats or intertidal zones and mangrove swamps throughout tropical and subtropical regions, including peninsular Malaysia. In Malaysia, 19 species from 12 genera can be found. The giant mudskipper (*Periophthalmodon schlosseri*) is the largest species and can be found at all levels and stages of the intertidal zones (Ng*et al.*, 2018). *P. schlosseri* can be identified by their huge size and, when agitated or frightened, exhibit a bold, black longitudinal stripe that runs from the eye to the tail (Mazlan, 2008).

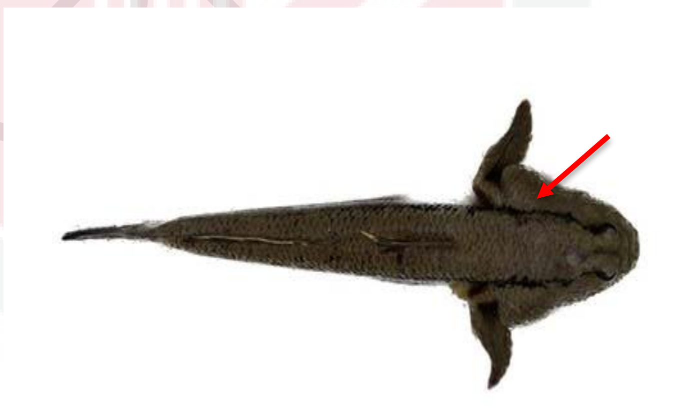

Figure 1: Giant mudskipper. Notice the lateral black line running from the eye to the tail (pointed by the red arrow).

The mudskippers can be found in mangrove swamps and tidal flats that formed during low tide in creeks, estuaries, and coastal seas. They spend a lot of time on land and have a variety of physiological, morphological, and behavioural adaptations for an amphibious lifestyle (Zulkifli *et al.*, 2012). Among the modifications are aerial vision and olfaction, higher ammonia tolerance, aerial respiration, increased immune response towards terrestrial pathogens, as well as utilising its pectoral fins to move on land (You *et al*., 2018). They also have a modified pair of ventral fins that forms a sucking disc, which allows them to attach to trees.

Mudskippers are economically significant in some countries because people consume them, for example in China, Taiwan and India, and create an alternative fishery in Mumbai during the monsoon season (Mutsaddi, 1973). In local tradition, this amphibious fish, also known as *ikan tembakul,* is famous for its appearance in the folklore of *Batu Belah Batu Bertangkup*. Although it is not usually consumed as food, it is believed to have aphrodisiac properties and is very commonly used to produce massage oil.

2.2 Environmental Pollution

There are three forms in which heavy metal concentration in aquatic environments can be measured, which are water, sediment and living organisms. In water, it will have the lowest concentration, followed by soil. The heavy metals will then accumulate in living organisms (Ebrahimpour & Mushrifah, 2008).

Heavy metal contamination of water is one of the main types of pollution that might impact the living organisms inside the aquatic ecosystem. Some heavy metals are released into the water from sediments. Sediments are a combination of a few mineral species and serve as an important sink in aquatic systems, including heavy metals (Yunus *et al*., 2020). Normal aquatic fishes are mostly affected by the heavy metals in the water (Ebrahimpour & Mushrifah, 2008). However, mudskippers are affected by heavy metals from both the water and the sediment due to their amphibious behaviour.

The west coast of peninsular Malaysia, where the research was done, is an area where multiple studies on heavy metal pollution have been performed. It is an area with a dense human population and intense agricultural and economic activities (Abdullah *et al.*, 1999). The Straits of Malacca, where the west coast is located, is one of the most important international shipping routes connecting Europe, the Middle East, and East Asian countries. This and other maritime operations could also be a factor in Malaysia's marine environment becoming contaminated (Ikram *et al.*, 2010).

Until recently, there were few reports on Malaysia's aquatic environment's trace element status, particularly in connection to intertidal sediments (Ikram *et al*., 2010). However, ongoing long-term monitoring is required to evaluate the hazardous heavy metals in this river because they may eventually have an impact on the fish, which may then be consumed by humans (Yunus *et al*., 2020)

2.3 Liver of Fish

The liver is both the largest gland tissue and the largest internal organ in the body of a fish. It is the organ where nutrients are taken in during digestion and are stored for later use by other body components. The metabolism serves several purposes, for example, protein synthesis, metabolite storage, bile secretion, detoxification, and inactivation. Although the liver is thought to be the most crucial organ for detoxification, several studies indicate that it is the main organ for some heavy metals accumulation (Alijani *et al.*, 2017).

In most fishes, including mudskippers, there are no hepatic lobules present. Hepatocytes made up most of the liver, which also contained islands of connective tissue that encased the bile duct and artery circulation. The arrangement of hepatocytes ranges from cord-like form, as in Girella *spp*., tubular form, as in lionfish, solid form, as in stonefish, and fat-filled such as in Gobioides (eg., mudskippers) and Tetraodontiformes. In all species of Gobioidei, including mudskippers, the hepatocytes are rounded and contain numerous fat droplets, since the liver acts as a fat storage organ (Akiyoshi & Inoue, 2004).

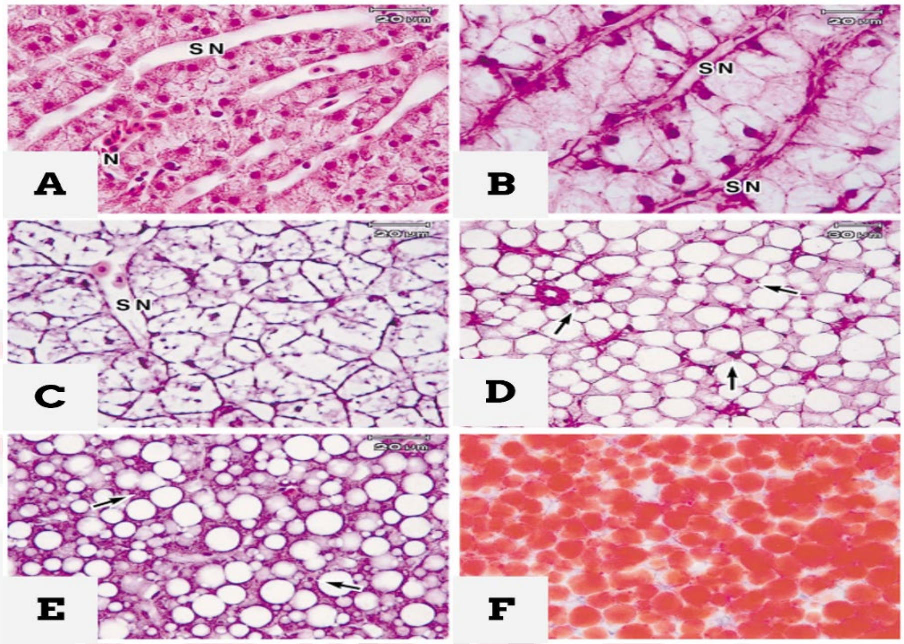

Figure 2: Different arrangements of hepatocyte in different species of fishes. (A) Cord-like arrangement as in Girella *spp*. (B) Tubular form as in Lion fish. (C) Solid form as in Stonefish. (D) Fat-filled as in Gobioides (*e.g.* Mudskipper). (E) Fat-filed as in Tetraodontiformes (e.g. puffer fish). (F) fat-filled hepatocyte that is stained with oil red-O stain. Obtained from Akiyoshi & Inoue, 2004.

2.4 Melanomacrophage centres (MMC)

Melanomacrophage centres (MMC) are collections of highly pigmented phagocytes that can occasionally be seen in the liver, spleen, and head of kidney of various vertebrate species. According to preliminary histological studies, MMCs and mammalian germinal centres (GC) in the spleen share structural similarities, which supports the idea that the MMC is involved in the humoral adaptive immune response. MMC is a potentially vital tool in assessing disease conditions in wild fishes since it is an easy, affordable, and widely applicable method to evaluate adaptive immunity in poikilotherms (Steinel & Bolnick, 2017).

MMCs are composed of aggregates of macrophages that contain melanin, hemosiderin, and lipofuscin as well as other pigments (Riberio *et al.*, 2011). Agius (1985) mentioned that MMCs are a crucial component of the fish reticuloendothelial system. It is believed that the MMC participates in both immune defences and typical, physiological functions that are not immune-related (Steinel & Bolnick, 2017).

The main job of melanomacrophage centres (MMCs), like other macrophages, is phagocytosis. It also phagocytoses degenerated red blood cells, and the product of haemoglobin breakdown gives the MM its colour. It is termed "metabolic dumps" referring to the existence and long-term storage of unmetabolized, effete compounds in MMCs (Agius, 2009). MMCs also accumulate exogenous elements from natural or experimental sources. This underlines the significance of MMCs in the removal of waste and the long-term storage of highly toxic and/or indigestible compounds (Steinel & Bolnick, 2017).

MMCs are also involved in innate and adaptive immune responses. As mentioned before, MMC resembles the germinal centres of mammals. The close relationship between MMCs and the ellipsoids, or specialised capillaries in the spleen, shows that they may scavenge blood-borne pathogens. Like mammalian GC, the number and/or size of MMCs increase after immunisation/infection

(Steinel & Bolnick, 2017). MMC may also occur in developing chronic lesions (Agius & Roberts, 2003). However, in normal fish, the MMC numbers may increase physiologically with age (Agius & Roberts, 2003).

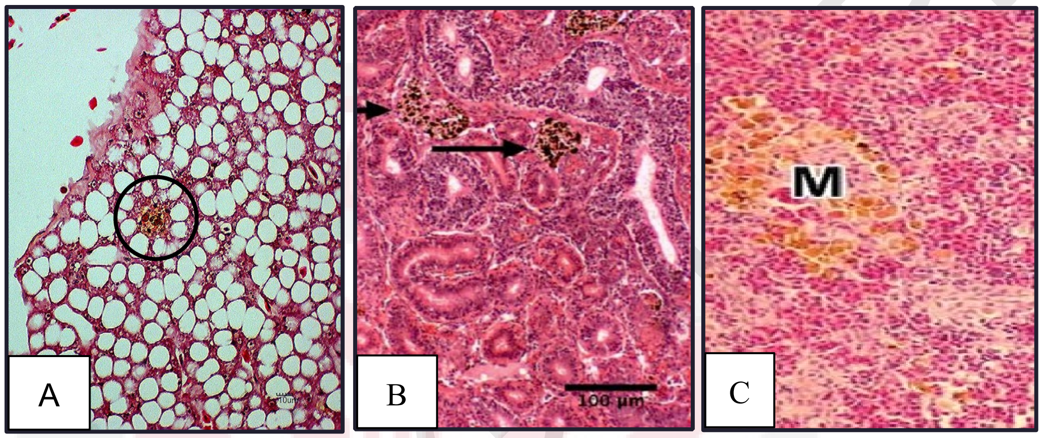

Figure 3: Micrograph showing MMC in different organs. (A) MMC in the liver of a giant mudskipper, *Periophthalmodom schlosseri* (black circle). (B) MMC in the spleen of a bluefin tuna (black arrows) (Evans & Nowak, 2016). (C) MMC in the spleen of a tilapia (M) (Kaewamatawong, 2012).

3.0 MATERIALS AND METHODS

3.1 Experimental Fish

This study was approved by the Institutional Animal Care and Use Committee (IACUC, UPM) with the AUP number UPM/IACUC-U007/2022. A total of 3 juvenile (<3 inches in length) and 2 adults (>6 inches in length) giant mudskippers were used in this study. All the giant mudskippers were caught using a rod and line at Jeti Kampung Kelanang, Morib.

3.2 Experimental Design

The fish was anaesthetised by submerging it inside an ice bucket and then euthanized by cervical dislocation without transection. Then the abdominal cavity was then opened using a scalpel blade, and the in situ of the viscera is observed. The liver, spleen and kidney were removed, and the liver was weighed using an electronic scale, and the gross features are noted if there were any abnormalities. All the livers were transacted into 2 parts to be fixed in two types of fixative, 20% formalin and Bouin's solution. All the spleen and kidney are fixed using 20% buffered formalin, before being transferred to 70% ethanol for preservation. Finally, all the samples were processed for histological viewing using the routine histological technique (H&E staining).

3.3 Histology Slides Preparation

Before euthanizing the fish, the 10% buffered formalin solution, 20% buffered formalin solution, Bouin's solution and 70% ethanol were prepared. The 20% buffered formalin and Bouin's solution were kept at 4^{o}C.

Immediately after euthanasia, the livers were removed from each fish and transacted into 2 parts. The first part was inserted into a 20% buffered formalin solution, while the other part was inserted into Bouin's solution. For the first 4 hours of fixation, the temperature of both fixatives was maintained at 4^{o}C to reduce the lysosomal activities inside the tissue, which would lead to autolysis. After 4 hours, the samples inside the 20% buffered formalin were transferred to 10% buffered formalin. The samples inside Bouin's solution were kept inside the same solution. The samples were then fixed for another 20 hours at room temperature. After 20 hours the samples inside the 10% buffered formalin were transferred to 70% ethanol for preservation, while the samples inside Bouin's solution were washed 3 times, 30 minutes each time using 70% ethanol, before finally keeping them inside 70% ethanol for preservation. For the spleen and kidney, they underwent the same process as the livers that were first fixed with 20% buffered formalin.

All the samples were dried and inserted into the tissue processor. Next, the samples were embedded with paraffin wax. Then, after cooling and trimming, the samples were sectioned using a microtome at 4µm each slice. It was then left to dry overnight, before being stained using Haematoxylin and Eosin (H&E) stain. After that, the glass slides were mounted using Distyrene Plasticizer Xylene (DPX). After the DPX has dried, the slides are ready to be observed.

3.4 Histological and Morphological Observation

All the slides were viewed under the light microscope equipped with a digital camera (Image Analyzer (Olympus® BX51) and the pathological changes were observed and recorded. For grading, 10 random 400x magnification fields were observed for each sample, and the grading was done based on the size of the area with changes. The grading was done based on the modified scoring system as summarised in table 1 below. To grade the lesion, the mean from all 10 fields was taken, recorded, and analysed (Gibson-Corley *et al.*, 2013).

Scoring Table			
Score	**Description**	**Percentage**	**Lesions**
0	No tissue damage	0	No lesion
1	Minimal tissue damage	1-24	1-3 Spots
2	≥25% tissue damage	25-49	Multifocal
3	≥50% tissue damage	50-74	Diffuse 50% or more
4	≥75% tissue damage	75-100	Diffuse 75% or more

Table 1: Histopathological Lesion Scoring system. Modified from Gibson-Corley *et al*. (2013).

3.5 Statistical Analysis

Statistical analysis between groups was examined by using SPSS (version 26) with Mann-Whitney U test.

3.6 Elemental Analysis

For elemental analysis, an energy dispersive x-ray (EDX) was performed on the soil sample and the liver sample.

For elemental analysis of the mud, the sample must first be dried in the oven at 60^{o} C overnight. The soil was then crushed into powder, and the powder was mounted onto aluminium stubs using carbon tape. The samples were coated with gold using a sputter coater and were viewed under Scanning Electron Microscope - JEOL 7600F FESEM

For elemental analysis of the liver sample, the sample was first trimmed to 1cm^{3}. The sample was then fixed in 4% glutaraldehyde at 4^{o} C for 24 hours. The sample was then washed in 0.1M sodium cacodylate buffer three times, approximately 10 minutes each. Next, it was post-fixed in 1% aqueous Osmium tetroxide for approximately 2 hours at approximately 4^{o}C. The sample was washed 3 times in 0.1M sodium cacodylate buffer for approximately 10 minutes each. Then, it was dehydrated in a graded series of acetone (35%, 50%, 75%, 95%) for 10 minutes each, and 100% acetone 3 times, 15 minutes each time. The sample was then transferred into a pre-labelled specimen basket. The basket was placed in a Critical Point Dryer (BALTEC). The sample was mounted onto the stub using carbon double-sided tape. Finally, the sample was viewed under Scanning Electron Microscope-JEOL 7600F FESEM

4.0 RESULTS

4.1 Relative Weight

The weight and body length of the fish and the weight of the liver were taken during the necropsy as showed in Table 2 below.

Group	Body Weight (g)	Liver weight (g)	Relative Liver to Body Weight (%)
Adult	55.80	1.03	1.85%
Juvenile	41.40	0.67	1.61%

Table 2: Relative weight of the liver to the body

4.2 Gross Observation

The in situ of liver and other visceral organs were observed for each fish during the necropsy. All the livers observed were normal, which were pinkish and bilobed with a soft consistency.

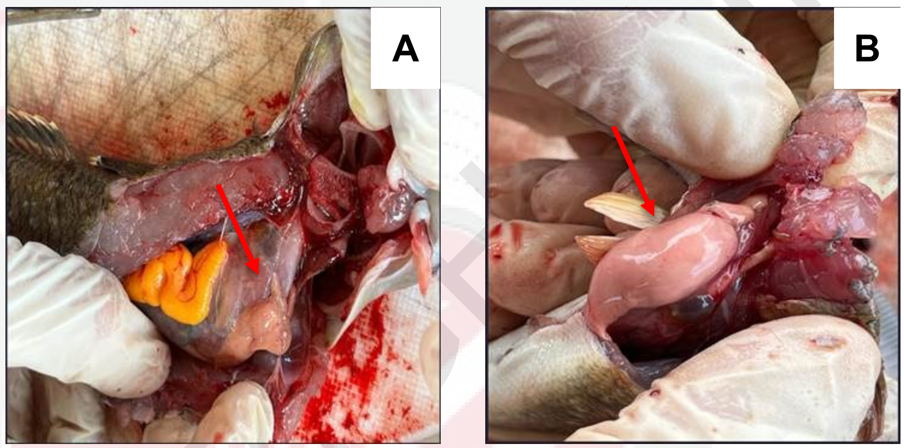

Figure 4: *In-situ* photographs of the liver of giant mudskippers caught in Kuala Kelanang. (A) The liver of an adult giant mudskipper. (B) The liver of a juvenile giant mudskipper. The livers were soft and pinkish.

4.3 Histological Observation

All the hepatocytes are round-shaped with lipid droplets inside the cells, and the nucleus pushed to the sides. There was no portal triad inside the liver, and the hepatocytes are not arranged in lobules. It was also observed that the sinusoids are not parallelly arranged. There were also no pancreatic tissues in the mudskipper liver because it is not a hepatopancreatic liver. There was no difference in terms of general appearance between the adult and juvenile group.

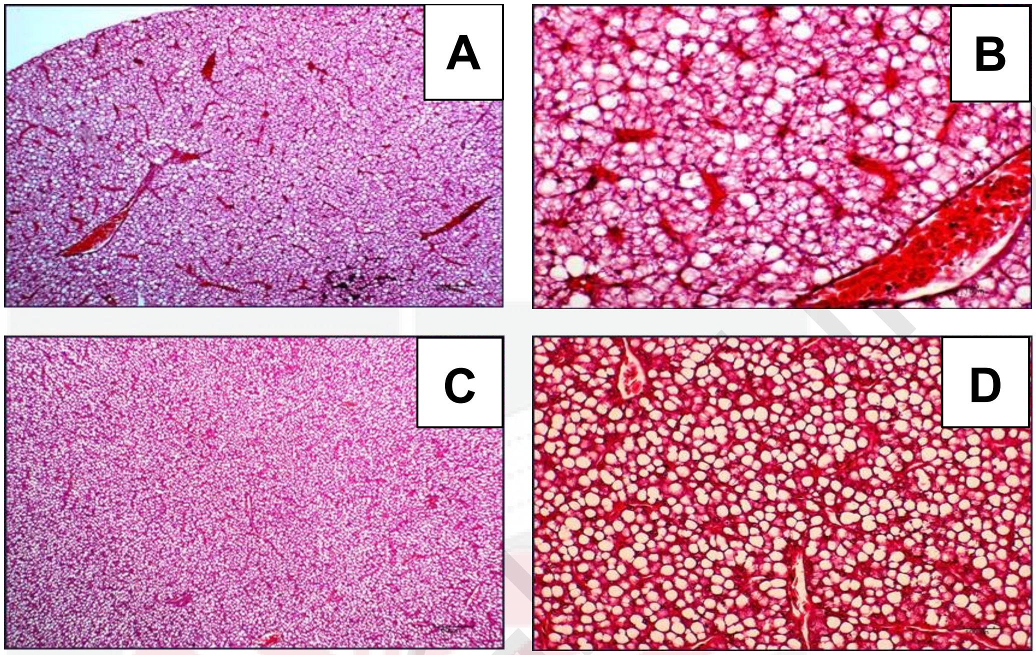

Figure 5: Photomicrographs of the general view of giant mudskippers liver tissues. (A) The sinusoids and blood vessels arrangements. (100x magnification) (B) Higher magnification of the region as in image A (400x magnification). Note that the sinusoids are not parallelly arranged. (C) The hepatocytes arrangements. (100x magnification). (D) Higher magnification of the region as in image C (400x magnification). H&E staining.

There were several abnormalities observed in most of the livers, such as presence of melanomacrophage centres (MMC), inflammatory cells infiltration (ICI) and polymorphonuclear (PMN) cells. PMN is a mixture of necrotised cells and inflammatory cells that respond to clear the dead cells. Scoring was done for the presence of MMC and ICI. PMN was graded together with ICI and the mean was taken. The total scoring for both MMC and ICI were also recorded, and the example of their presence is shown in Figure 6.

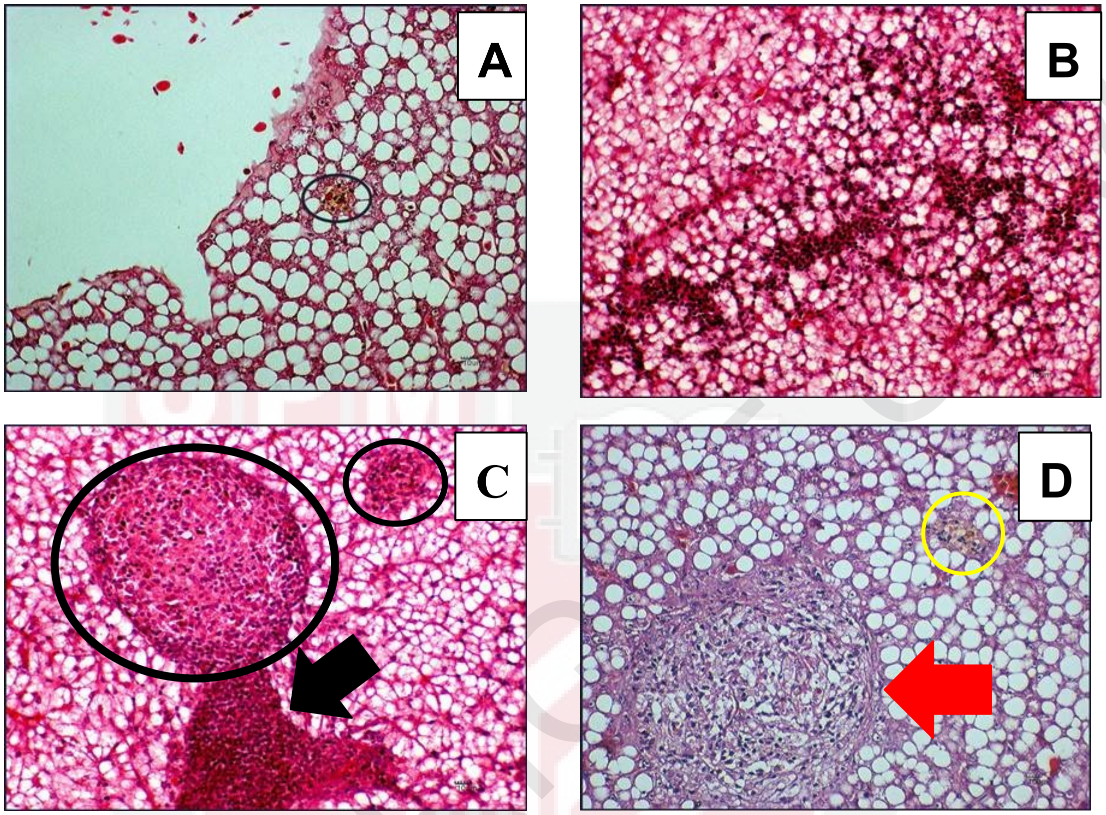

Figure 6: Photomicrographs of the liver of giant mudskipper from different fish stained with H&E. (A) Presence of melanomacrophage centre (black circle). (B) Presence of inflammatory cells infiltration (basophilic stained cells). (C) Polymorphonuclear cells (PMN) (black circles), a mixture of necrotised hepatocytes with inflammatory cells. The black arrow shows a severely congested blood vessel that is also filled with inflammatory cells. (D) Presence of an MMC (yellow circle) that was closely associated with a PMN (red arrow).

The mean score for MMC in adult fish was 1.6, while for juvenile fish it was 0.9, and the score for ICI in adult fish is 1.4, while for juvenile fish it was 1.0. The adult fish has higher mean score of MMC compared to the juvenile fish. For ICI, the juvenile fish has slightly higher mean score than juvenile fish. The means are summarised in the Figure 7 below.

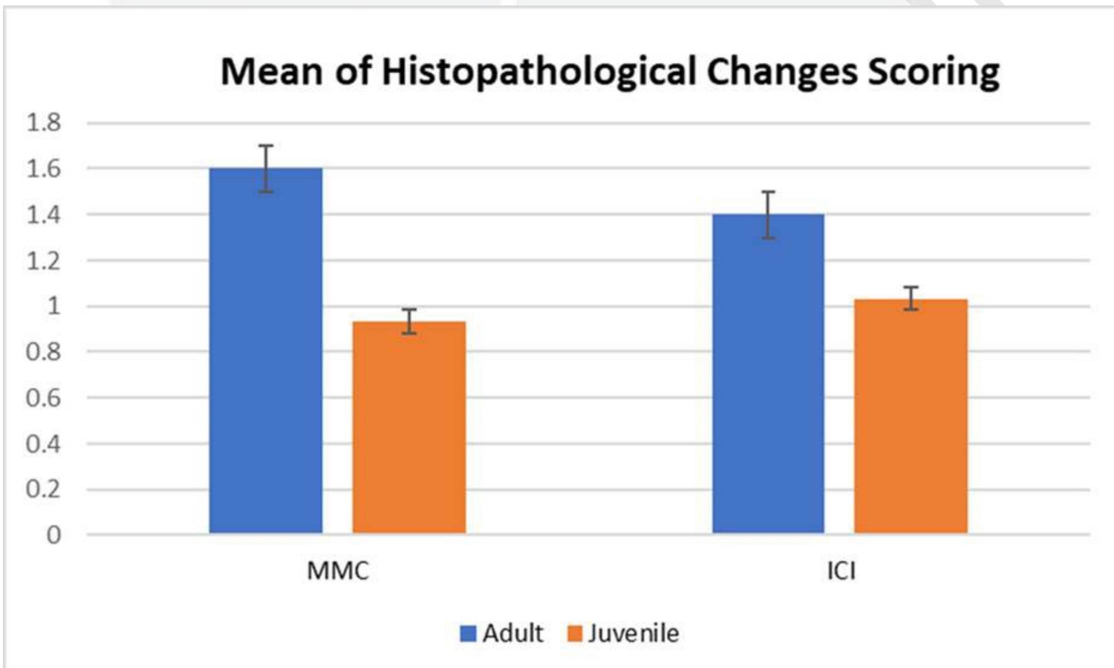

Figure 7: The mean score for the presence of melanomacrophage centres and inflammatory cells infiltration.

Overall, the total score for both MMC and ICI were quite low. For MMC, score 1 was the most scored field at 40% followed by score 2 at 30%, score 0 at 18%, and score 3 at 4%. None of the fields were scored with score 4. This is also similar in ICI, where score 1 was also the highest at 40%, score 2 at 30%, score 0 at 28%, and score 3 at 6%, while none of the fields were scored 4. The totals scorings were summarised in Figure 8 below.

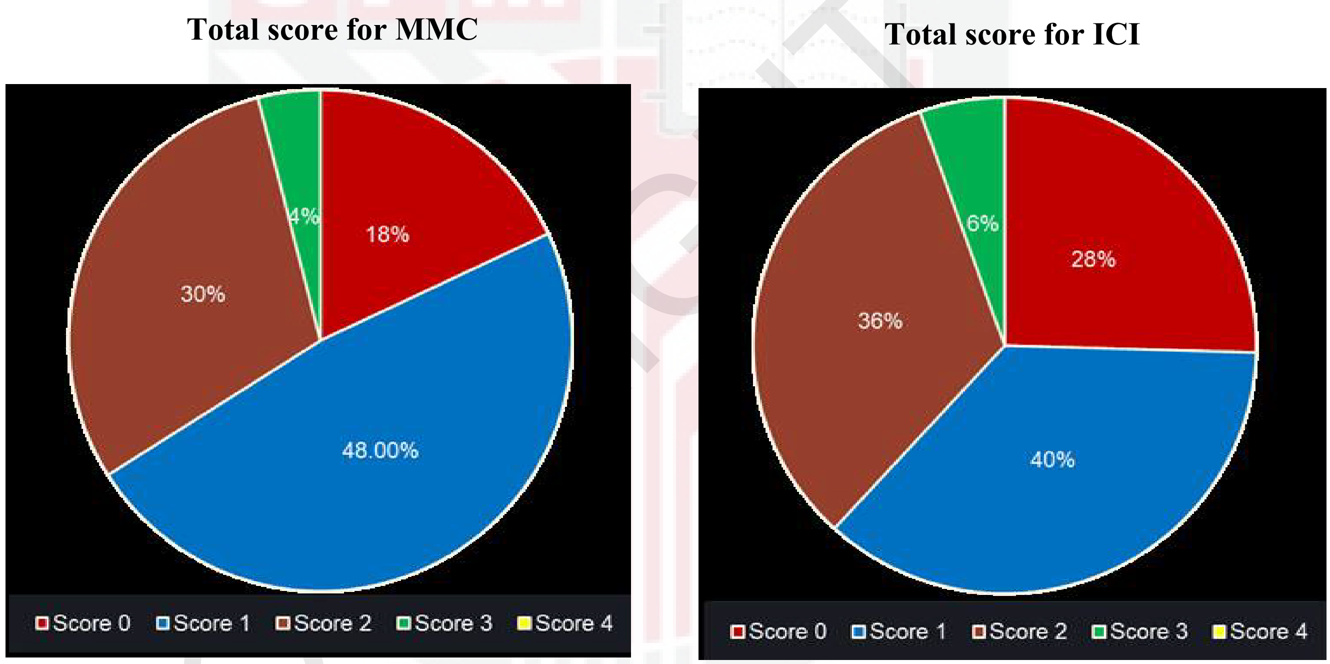

Figure 8: The total scoring for melanomacrophage centre and inflammatory cells infiltration.

4.4 Elemental Analysis

From elemental analysis, it was discovered that there were no traces of any heavy metals found in the liver tissues of the adult and juvenile giant mudskippers. In the mud sample, traces of Sodium, Silica and Aluminium were found. However, sodium and silica are normal compositions of soil.

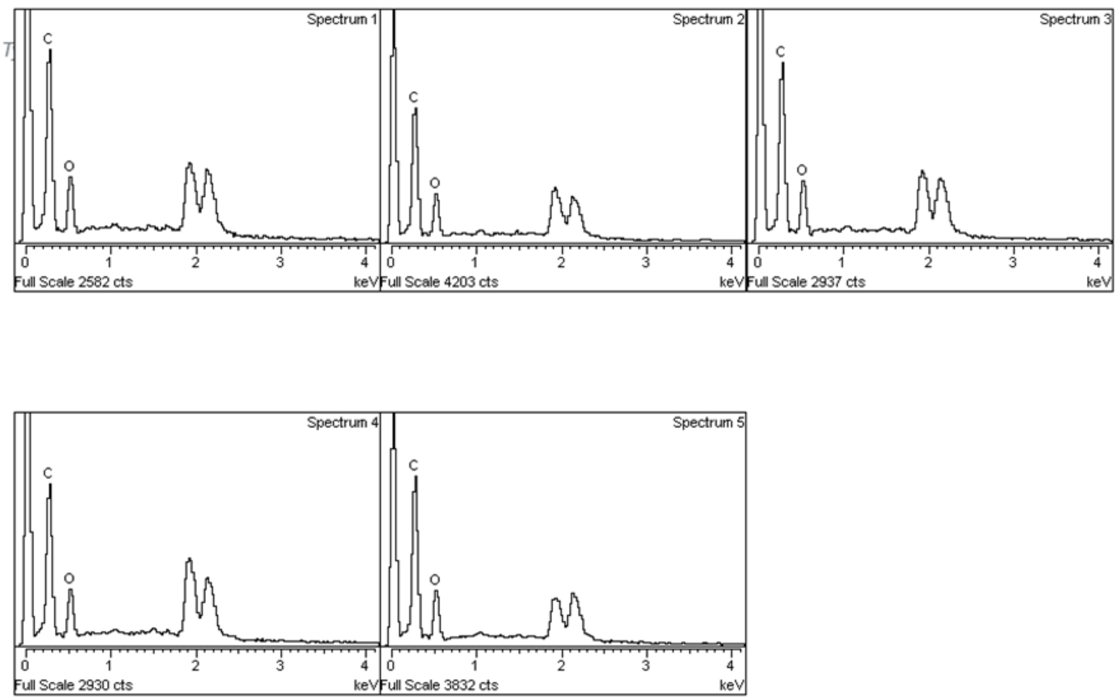

Figure 9: Result of the EDX analysis of the liver of a juvenile giant mudskipper. No traces of heavy metals were observed.

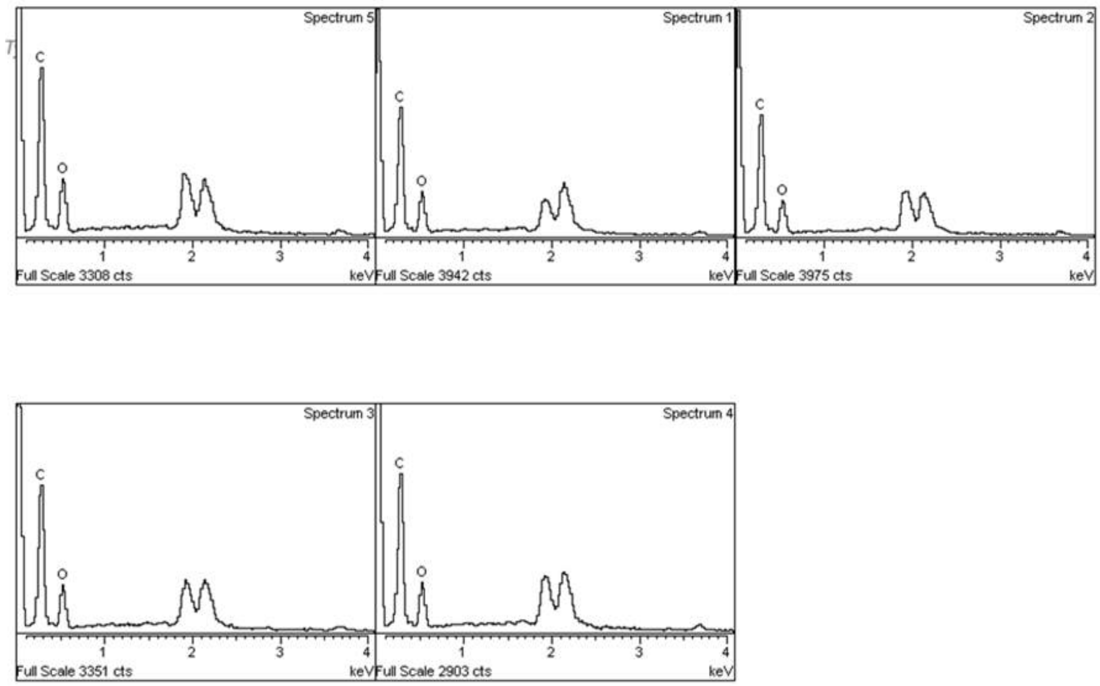

Figure 10: Result of the EDX analysis of the liver of an adult giant mudskipper. No traces of heavy metals were observed.

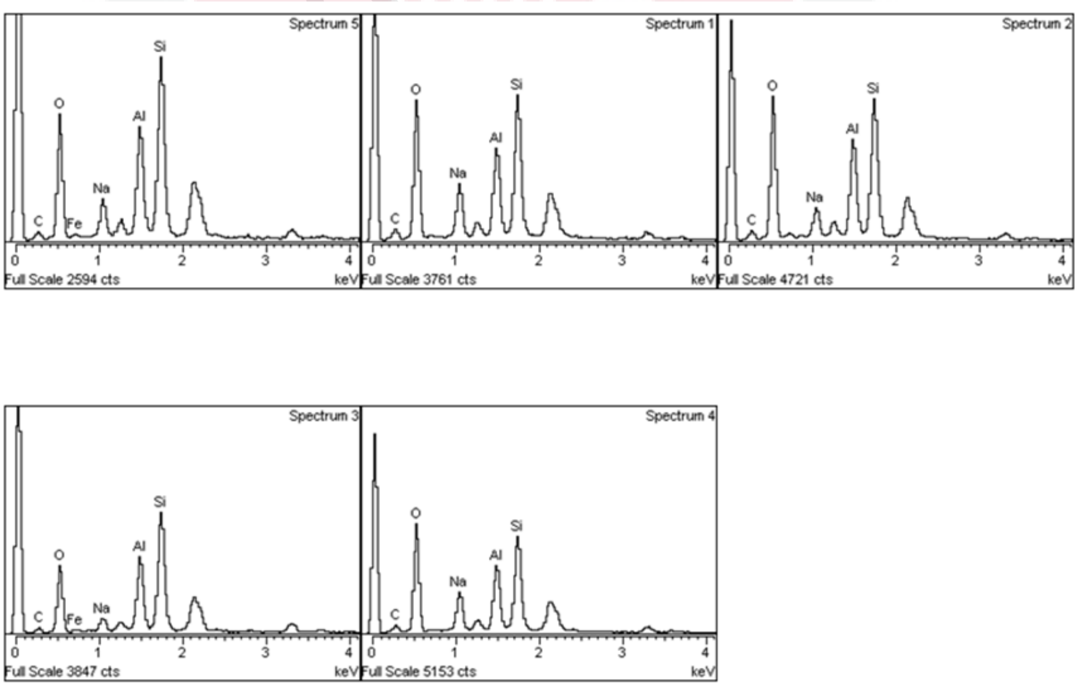

Figure 11: Result of the EDX analysis of soil sample. There were traces of Sodium (Na), Silica (Si) and Aluminium (Al). Note that Silica and Aluminium are normal compositions of soil.

4.5 Statistical Analysis Result

From statistical analysis of the histopathological changes by the Mann-Whitney U test, it revealed that there is a significant difference ($p<0.005$) in the score for MMC between the large and small groups, while only the score for lymphocytic infiltration is not significantly different ($p>0.005$) (See table 3 in Appendix A).

5.0 DISCUSSION

The histological study of the liver is vital in diagnosing the effects of heavy metals on fish. Since the liver is involved in detoxification and biotransformation processes, it is the organ that has the highest risk of damage from toxins (Camargo & Martinez, 2007). Due to its functions, we may see changes in the tissue of the liver, which is caused by toxins, for example, inflammatory reactions such as inflammatory cell infiltrations and melanomacrophage centre formation.

Melanomacrophage centre (MMC) may resemble the mammalian germinal centre structurally, according to preliminary histological examinations, which supports the idea that the MMC contributes to the adaptive immune response (Steinel & Bolnick, 2017). Inflammatory cell infiltration indicates that there is an ongoing inflammatory response in the liver. In most of the samples, it was discovered that the MMC was present together, or in close association with the lymphocyte aggregates. Steinel & Bolnick (2017) also mentioned that MMC also functions in antigen retention, while inflammatory cells react to the antigen. The MMC will present the antigen to the inflammatory cells as a part of the immune response. Steinel & Bolnick (2017) also observed that lymphoid cells exist in close proximity to teleost MMC.

Although there are other lesions mentioned in other literature, for example, vacuolation, hepatocyte degeneration and congestion (Anyiamuka-Chinedu *et al*., 2022), MMC and ICI lesions were the only parameters observed since these were the most prominent in the samples. Polymorphonuclear (PMN) cells consist of necrosed liver cells and inflammatory cells that came in to remove the dead cells. This lesion was graded together with ICI since it also contained inflammatory cells.

Although congestion was very severe and generalised in all the liver samples, it was suspected to be due to handling procedures, where the fish were anaesthetised by submerging them in ice water. When the fish is in a very cold environment, similar to other animals, the blood will be redirected away from the peripheries into the visceral to conserve heat. This will cause physiologic congestion of the visceral organs, including the liver. Hence, congestion was not graded as part of the histopathological lesion.

As mentioned before, the hepatocytes of mudskippers store fat, causing the cells to become rounded. In the cases of very high lipid content, the nuclei may even be pushed to the side and become flattened. One unique feature of the liver of fish is that they are non-lobulated, and in mudskippers, they do not have portal triad. Thus, the sinusoids are scattered since they do not have parallel arrangements as in mammalian or some other fish livers. There is no difference between the liver of adult and juvenile giant mudskippers, grossly and histologically.

From the result, it was proven that bigger fish have higher scores of lesions compared to the smaller ones. Mature fish accumulated higher levels of heavy metals in comparison to juvenile fish. It is since fish with consistent growth rates that live in permanently polluted habitats maintain the build-up of heavy metals (Ahmad & Suhaimi-Othman, 2010). Even so, the lesions present may not be due to the heavy metals, or any environmental pollution after all. Numerous other conditions may induce the lesions observed, as long as it is able to provoke the adaptive immune response, such as an ongoing infection.

Although MMC reacts to environmental contamination, it can also be present in response to chronic infection/inflammation (Agius & Roberts, 2003) or other physiological or environmental factors (Steinel & Bolnick, 2017). One example of a physiological process causing an increase in MMC is ageing. Lipofuscin, one of the pigments in MMC, increases with age, thus directly increasing the size of MMC. Other than that, it can be said that MMC size increases with any kind of stress since stress increases the susceptibility of the fish to infection (Agius & Roberts, 2003).

For the total scoring of both MMC and ICI, the most observed scoring from all the fields were mostly only scores 1 and 2. This can be correlated with the level of heavy metal traces found in the mud and liver samples that were analysed using EDX analysis. There were no significant levels of heavy metals detected at all in the liver samples of both adult and juvenile groups, and only sodium, silica and aluminium were found in the mud sample, with sodium and silica being normal components of soil. Thus, aluminium was the only heavy metal presence detected in the mud sample. Nevertheless, it still cannot be confirmed that aluminium is the cause of the lesions observed.

6.0 CONCLUSION

From the gross observation, there were no gross morphological changes specifically due to pollution observed, while histopathological observations identified that there was a significant difference in MMC, but no significant difference in ICI between adult and juvenile groups. However, it cannot be concluded that the livers of giant mudskippers can be used as environmental pollution bioindicators in this study since there were no heavy metal elements traced in the liver samples, while only aluminium was found in the mud sample.

7.0 RECOMMENDATIONS

To improve the accuracy of the results for future studies, it is recommended that samples from other organs are also analysed to support the findings on the gross and histopathological changes of the liver. This is because other than the liver, heavy metals can also accumulate inside other organs such as skin, gills, and muscles (Sangur *et al.*, 2021). Plasma enzyme levels may also be recorded since according to Ansari *et al.* (2020), mudskippers inhabiting polluted areas have a higher level of plasma enzymes.

Besides that, it is also recommended to use atomic absorption spectroscopy (AAS) for heavy metal levels evaluation. AAS is better than EDX since it has the ability to quantify the level of heavy metals, whereas EDX can only detect the presence of heavy metals without accurately measuring their levels.

Another recommendation is to obtain samples from areas that are proven to be polluted. This is so that the results could then be compared with the results from this study, which was done in a less polluted area.

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APPENDIX

APPENDIX A

Test Statistics[a,b]		
	Melanomacrophage centre	Inflammatory cells infiltration
Wilcoxon W	621.00	684.50
df	4	4
Asymptotic Sig.	0.002	0.093
a. Mann-Whitney U Test		
b. Grouping Variable: Group		

*Significant level at $p<0.05$ (Mann-Whitney U Test)

Table 3: Results of Mann-Whitney U Test