UNIVERSITI PUTRA MALAYSIA

HISTOLOGY AND IMMUNOHISTOCHEMISTRY STUDIES ON HEAVY METALS POLLUTION IN DIFFERENT SIZES OF CATFISH IN KUALA GULA AND BAGAN LALANG, MALAYSIA

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By

AMANI MOHAMMED NASSER AHMED

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

October 2019

DEDICATION

Father& Mother

Who they gave me all things in this life

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

HISTOLOGY AND IMMUNOHISTOCHEMISTRY STUDIES ON HEAVY METALS POLLUTION IN DIFFERENT SIZES OF CATFISH IN KUALA GULA AND BAGAN LALANG, MALAYSIA

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Chairman : Professor Ahmad bin Ismail, PhD
Faculty : Science

Heavy metals pollution is a serious issue globally, mainly as results of human activity. Although there are numerous studies on heavy metal pollution in Malaysia, the awareness of the public to the threat of heavy metals is still low. This could be due to most of the reports are solely based on metal concentration but not on the actual damage to the tissues and organ like lung damaged lung photo shown in anti-smoking campaign. The level of essential and non-essential heavy metal (Zn, Cu, Cd, and Pb) in the water; sediments; in two different species of fish of different sizes, and in different organs were studied. The objectives of this study are: to assess the heavy metals concentration in the water and sediment, to assess the level of heavy metals Zn, Cu, Pb and Cd in different sized and different organs of two catfish species *Arius thalassinus* and *Plotosus anguillaris*; to study impact of heavy metal pollution using histopathological changes and finally to demonstrate the expression stress of metallothionein3 in some tissues of the fish using immunohistochemistry technique. Thirty samples of water and sediments samples were obtained from two river plus thirty samples of each species catfish *Arius thalassinus* and *Plotosus anguillaris* of small, medium and large sizes caught by fisherman from Kuala Gula and Sepang Besar from April 2015 to December 2017. Water, sediments and fish samples were handled following the standard method for analyzing heavy metals. Heavy metals were determined using AAS. The records of fish weigh and length were taken and graded according to small, medium and large fish. Muscle, stomach, gills, liver, and kidney were separated to determine heavy metals concentration, histology and immunohistochemistry analysis. Tissues were processed following standard histological procedures and immunohistochemistry technique. The results showed the concentration of heavy metals in water and sediment was still with the range. The mean concentration of metals in water of Kuala Gula and Sepang Besar were Zn: (0.3 & 0.02 mg/L); Cu: (0.1 & 0.04 mg/L); Pb: (0.32 & 0.30 mg/L) and Cd: (0.40 & 0.01 mg/L). The concentration of metals in sediment of Kuala Gula and

Sepang Besar were Zn: (39.20 & 39.90 µg/g dry.wt); Cu: (20.10 & 13.10 µg/g dry.wt); Pb: (18.90 & 15.20 µg/g dry.wt) and Cd: (1.40 & 1.80 µg/g dry.wt) in two rivers respectively. The findings in this study recorded that *Arius thalassinus* and *Plotosus anguillaris* were contaminated with Zn, Cu, Pb, and Cd. Large catfish showed higher accumulation for heavy metals in two species catfish followed medium and small fish. Kidney in large *Arius thalassinus* showed a high score in Zn with mean 284.0±29.7µg/g dry.wt and liver showed high score of accumulation of heavy metals in Zn also in large fish *Plotosus anguillaris* 120.30±2.4µg/g dry.wt. The muscles had the lowest metals concentration in both species catfish. Muscle recorded the lowest Cd accumulation with mean (0.61±0.05& 0.51±0.03µg/g dry.wt.) in *Arius thalassinus* and *Plotosus anguillaris* respectively. Histopathological alterations were observed including degeneration with necrosis in liver, and kidney, proliferation in the epithelium of gill filaments and fusion of secondary lamellae. The severity of histopathological alteration corresponds to increasing metals concentration in the gill, liver, and kidney.

Antibodies showed a positive cross reactivity with MT3 proteins. MT3 immune reaction was high for Zn, Cu, Pb and Cd in kidney, liver, and gills. MT3 localization/induction expression upon exposure to Zn, Cu, Pb, and Cd can be demonstrated using immunohistochemistry in the cytoplasm, nuclei, renal tubules, melano-macrophage centres; hepatocytes, cytoplasm, nuclei melano-macrophages centre; filaments, cartilage, and basal membrane in the gills. There were positive relationship between MT3 and concentration of Zn, Cu, Pb, and Cd in this study. This study concluded that the high concentration of heavy metals in tissues of *Arius thalassinus* and *Plotosus anguillaris* affect them negatively. The different MT3 expressions in different sized fish and different polluted place showed the potential use MT3 as biomonitoring biomarker for heavy metals exposure in an aquatic environment. Present study showed that the two species of catfish *Arius thalassinus* and *Plotosus anguillaris* are good candidate for biological indicator and have ability to accumulate heavy metals. Histopathology techniques and immunohistochemistry with MT3 gave clearer picture on the impact of heavy metal on tissues and cells. The non parametric collective rank and score analysis are able to revealed the trend of heavy metal accumulation and impact of heavy metals concentrations based on metals species, different location, fish species, size of fish and different organs.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KAJIAN HISTOLOGI DAN IMUNOHISTOKIMIA TERHADAP PENCEMARAN LOGAM BERAT DALAM IKAN DURI YANG BERBEZA SAIZ DARI KUALA GULA DAN BAGAN LALANG, MALAYSIA

Oleh

AMANI MOHAMMED NASSER AHMED

Oktober 2019

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Pencemaran logam berat adalah isu yang serius di seluruh dunia, terutamanya akibat hasil aktiviti manusia. Walaupun terdapat banyak kajian mengenai pencemaran logam berat di Malaysia, kesedaran orang ramai terhadap ancaman logam berat masih rendah. Ini mungkin disebabkan kebanyakan laporan yang hanya berasaskan kepekatan logam tetapi tidak pada kerosakan sebenar pada tisu dan organ seperti foto paru-paru yang rosak yang ditunjukkan dalam kempen anti-merokok. Tahap logam berat penting dan tidak penting (Zn, Cu, Cd, dan Pb) di dalam air, sedimen, dalam dua spesies ikan yang berlainan dengan saiz yang berbeza, dan dalam organ-organ yang berbeza telah dikaji. Objektif kajian ini adalah untuk menilai kepekatan logam berat di dalam air dan sedimen, untuk menilai tahap logam berat (Zn, Cu, Pb dan Cd) dalam organ yang berlainan, dan saiz berbeza dari spesies ikan *Arius thalassinus* dan *Plotosus anguillaris*, untuk mengkaji kesan pencemaran logam berat menggunakan perubahan histopatologi dan akhirnya menunjukkan tahap ekspressi metallothionein 3 pada tisu ikan yang berlainan menggunakan teknik imunohistokimia. Sampel air dan sedimen diperoleh dari dua sungai bersama tiga puluh sampel setiap spesies ikan (*Arius thalassinus* dan *Plotosus anguillaris*) ukuran kecil, sederhana dan besar yang ditangkap oleh nelayan dari Kuala Gula dan Sepang Besar dari April 2015 hingga Disember 2017. Sampel air, sedimen dan sampel ikan dikaji mengikut kaedah piawai untuk menganalisis logam berat. Logam berat ditentukan menggunakan AAS. Rekod berat ikan dan panjang telah diambil dan digredkan mengikut saiz ikan; kecil, sederhana dan besar. Otot, perut, insang, hepar, dan ginjal diasingkan untuk menentukan kepekatan logam berat, histologi dan analisis imunohistokimia. Tisu diproses mengikut prosedur piawai histologi dan teknik imunohistokimia. Hasilnya menunjukkan kepekatan logam berat di dalam air dan sedimen masih berada dalam aras yang tidak membahayakan di Malaysia. Purata kepekatan logam dalam air Kuala Gula dan Sepang Besar adalah Zn: (0.3 & 0.2 mg / L); Cu: (0.1 & 0.04 mg / L); Pb (0.32 & 0; 30 mg / L) dan Cd: (0.4-0.01 mg / L). Kepekatan logam dalam

sedimen di Kuala Gula dan Sepang Besar bagi Zn: (39.20 & 39.90 μg / g berat kering); Cu: (20.10 & 13.10 μg / g berat kering); Pb (18.90 & 15.20 μg / g berat kering) dan Cd :(1.40-1.80 μg / g berat kering). Keputusan menunjukkan bahawa *Arius thalassinus* dan *Plotosus anguillaris* dicemari dengan Zn, Cu, Pb, dan Cd. Ikan bersaiz besar menunjukkan pengumpulan logam berat yang lebih tinggi untuk dua spesies ikan tersebut diikuti ikan bersaiz sederhana dan bersaiz kecil. Ginjal *Arius thalassinus* yang besar mempunyai skor tinggi untuk Zn dengan purata 284.0±29.7μg/ g dry.wt dan hepar menunjukkan skor pengumpulan Zn yang tinggi dalam *Plotosus anguillaris* bersaiz besar 120.30 ± 2.4 μg/g berat kering. Otot mempunyai kepekatan logam terendah bagi kedua-dua spesies ikan tersebut. Otot mencatatkan pengumpulan Cd terendah dengan min (0.61 ± 0.05 & 0.51 ± 0.03μg/g berat kering) untuk *Arius thalassinus* dan *Plotosus anguillaris* masing-masing. Kelainan histopatologi dapat dicamkan termasuk degenerasi dengan nekrosis dalam hepar, dan ginjak, pertambahan sel-sel dalam epitelium filamen insang dan gabungan lamellae sekunder. Peningkatan perubahan histopatologi sesuai dengan peningkatan kepekatan logam dalam insang, hati, dan ginjal.

Antibodi menunjukkan reaktiviti bersilang positif dengan protein MT3. Reaksi imun MT3 adalah tinggi untuk Zn, Cu, Pb dan Cd dalam ginjal, hepar, dan insang. Teknik imunohistokimia boleh menunjukkan ekspresi lokalisasi / induksi MT3 yang terbentuk apabila didedah kepada Zn, Cu, Pb dan Cd disitoplasma, nukleus, tubulus ginjal, pusat melano-macrophage; hepatosit, sitoplasma, pusat melano-makrofag nukleus; filamen, rawan, dan membran basal di insang.Terdapat korelasi positif antara MT3 dengan kepekatan Zn, Cu, Pb, dan Cd dalam kajian ini. Kajian ini menyimpulkan bahawa kepekatan logam berat yang tinggi dalam tisu *Arius thalassinus* dan *Plotosus anguillaris* memberi kesan negatif kepada mereka. Ekspresi MT3 yang berbeza mengikut saiz ikan berbeza dan tempat tercemar yang berlainan. Kajian ini menunjukkan potensi penggunaan MT3 sebagai "biomonitoring biomarker" untuk pencemaran logam berat dalam persekitaran akuatik. Kajian ini menunjukkan bahawa kedua-dua spesies *Arius thalassinus* dan *Plotosus anguillaris* adalah calon yang baik sebagai penunjuk biologi dan mempunyai kemampuan untuk mengumpul logam berat. Teknik histopatologi dan imunohistokimia serta MT3 dapat memberi gambaran yang lebih jelas mengenai kesan logam berat pada tisu dan sel. Pendekatan analisis aras dan skor secara kollektif bukan parametrik dapat mengenalpasti trend pengumpulan logam berat dan kesan kepekatan logam berat berdasarkan spesies logam, lokasi yang berbeza, spesies ikan, saiz ikan dan organ yang berlainan.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	Percentage
ºC	Degree Celsius
µg/g	Microgram per gram
AAS	Atomic absorption Spectrophotometer
ANOVA	Analysis of variance
cm	centimetre
g	gram
CRM	certified material
d.w	dry weight
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
NHO_3	Nitric acid
HClO4	Perchloric acid
mg/L	Milligram per liter
Cd	Cadmium
Cu	Copper
Zn	Zinc
Pb	Lead
No	Number
N	Number of sample
SD	Standard Deviation
Temp	Temperature
UPM	Universiti Putra Malaysia
ERL	Effect range low
EMR	Effect median range
G	Gula
SB	Sepang Besar

hrs	Hours
min	Minutes
MT3	Metallothionein 3
IHC	Immunohistochemistry
Cr	Chrome
Ni	Nickel
mg/kg	Mailgram per kilogram
HRP	Horseradish peroxides
SET	Sequential extraction technique
A.t	*Arius thalassinus*
P.a	*Plotosus anguillaris*
SRMs	Standard reference materials
Fe	Iron
Hg	Mercury
Mn	Manganese
DO	Dissolved oxygen
$CdCl_2$	Cadmium chloride
Tl	Thallium
CVD	Cardiovascular disease
BFA	Bioaccumulation Factor
EFLE	Easily or freely, leachable and exchangeable
Ig	Immunoglobulins
kDa	Kilo daltone
TOM	Total organic matter
ND	Not detect
BAF	Bioaccumulation Factor
WHO	World Health organization
DAB	Diaminobenzidine

CHAPTER 1

INTRODUCTION

1.1 General

The rapid industrialization and uncontrolled urbanization around the towns and released to the surrounding and later pollute the aquatic environment coastal areas have caused an alarming phenomenal level of pollutants (Naji et al., 2010). The Malaysian rivers have often been used as dumping spots for heavy metal effluent lawfully or unlawfully (Yap et al., 2005; Zulkifli et al., 2015; Saed et al., 2002; Yap et al., 2002a). The major route by which heavy metals get into the rivers are via terrestrial overflow, rainwater, and industrial effluent disposal. The metals resulting from human activities (anthropogenic) may regularly be absorbed by the aquatic system or are directly ingested by aquatic biotas such as plankton, benthic organisms and fish (Ahmad et al., 2009).

The pollution of heavy metals in water, sediment, and biota becomes an important focal point in scientific research in different coastal regions related to the water quality and concern of public health (Chen et al., 2004). The input of natural or anthropogenic metals has consequences for their biochemical cycles, temporal and spatial distribution patterns, their bioavailability to organisms; and eventually to human health (García et al., 2008).

1.2 Problem statement

Aquatic pollution has caused acute reductions in the number of aquatic species while some other aquatic species may become abundant. One of the most important pollutant heavy metals pollution. Heavy metals such as Zn, Cu, Pb, and Cd are the most common polluting elements in aquatic Malaysia environment (Shazili et al., 2006). Hence, the presence of hazardous heavy metals in the environment are of great concern.

The Increase of population living along the coastlines and rivers has intensified pollution in the water bodies. Water resources are affected by man-made pollution worldwide to such a degree that restoration to pristine conditions is not achievable (Yin, 2016). These chemicals are known to cause acute or chronic effects on living organisms (fishes, wildlife) by disrupting the reproductive systems and internal organs. Additionally, heavy metals can cause many damages in the tissues of the fish, such as bones, muscles, liver, and kidney. Many studies have reported on the toxicity effects of heavy metals on fish and their tissues. In one of these studies by Jaishankar et al. (2014); Arantes et al. (2016); Jan et al. (2015) mentioned that heavy metals can cause many damages in tissues of the such as bones, muscles, liver, kidney and spleen. Serious damages in gill, liver, and kidney have been found to be caused by chronic exposure to heavy metals such as Zn, Pb and Cd

(Abalaka, 2015). Yusof (2005) reported that the accumulation of non-essential heavy metals in different organs of fish

The present approach of identifying and labelling polluted area based on concentration of heavy metals in abiotic and biotic component of the environment are no longer adequate. To some people, the heavy metals report is like hearsay that offer no prove that heavy metal actually harmed the living organism particularly human. There is a need to provide visual evidence and biological marker that directly indicate the harm caused by heavy metals using histopathology to detect tissue damage and immunohistochemistry to detect cellular damage and correlate it with existing heavy metal concentration.

1.3 Significance of the study

The west coast of Peninsular Malaysia is characterized by high population and densed industrial areas. The location is liable to contamination through the discharge of pollutants from land-based sources (Ismail et al., 1993; Saed et al., 2002). The significance of the west coast of Peninsular Malaysia is related to the high number of industrial areas, receiving heavy metal contamination from the flow of the most riverine systems, densely population and finally offshore area of the west coast of Peninsular Malaysia is a vital shipping lane in the world (Yap et al., 2002a). Many sites have gained importance in this area for instance, Kuala Gula Bird's sanctuary in the west coast of Peninsular Malaysia is an important stopover site for migratory shorebirds of the East Asian-Australasian Flyway (Lomoljo et al., 2009). But, in term of pollutants condition Lomoljo et al. (2010), reported that the status of concentrations of metals Zn, Cu, Pb, and Cd in this area ten years before was still within the ranges of normal chemical concentrations in marine and estuarine sediments and can be comparable to the non-polluted coastal waters. While in a study by Rahman et al. (2013) founded that there is an elevation of the same metals in the sediment from Kuala Gula and had caused environmental degradation. Milky Stork is one of the organisms which, once had a scattered distribution, they were affects by pollution, these species later underwent a constant decline and there are now has less than ten individuals recorded (Ismail and Rahman, 2012).

Sepang river estuary is an economically important area to the local people, but it is one of the most polluted rivers in Bagan Lalng in Malaysia. It supports commercial fish, crabs, and bivalves such as clams and oysters. This river is regarded as being an important breeding ground and nursery for estuarine organism species and marine flora and fauna (Saed et al., 2002). This river showed an increase in the concentration of heavy metals due to discharges from pig farms, which have been the second-largest contributors to water pollution (Saed et al., 2004). Food consumption is often regarded as one of the most important pathways of human exposure to heavy metals. Many reported studies have showed that contamination of heavy metals via the food chain can cause human health risk (Taweel et al., 2013; Mohamat-Yusuff et al., 2015; Baharom and Ishak, 2015) and it is effectively increased as they long persistence, bioaccumulated, and biomagnificated (Yap et al., 2015).

Catfish are one of the many species of fish commonly found in Kuala Gula and Bagan Lalng. It is counted as a valuable food and appears very important component in the food chain. On the other hand, it is found to can accumulate heavy metals in their tissues. This catfish is a bottom feeder and feeding on small fishes, crustacean, gastropods, and mollusc (Bruton, 1996). This fish is a secondary or tertiary consumer in the food chain, Saleh and Marie. (2016) thus justified its selection as bio-indicator. In terms heavy metals monitoring Yi and Zhang. (2012) justified its selection as bio-indicator as a results of heavy metals accumulation in their tissues. Body sizes of fish can also have effect from heavy metals pollution and their accumulated in the organs.

A conventional monitoring system of environmental metal pollution includes measuring the level of selected metals in the whole organism or in respective organs. Aqua regia is one of the methods that provides a rapid, safe and effective digestion and low losses of volatile metals (Relić et al., 2011). Another method is Sequential Extraction Technique (SET). However, measuring only the metal content in particular organs does not give information about its effect at the subcellular level (Yap et al., 2002b). However, these methods are not enough to show the damage effects from toxicity impacts of heavy metals on tissues of fish. Therefore, there is a need to show how the high concentration of heavy metal damage the tissues, vital organ and at the cellular level. Therefore, this project was initiated to show the damage caused by heavy metals from the polluted river and less severely polluted river using fish susceptible to heavy metal exposure and fish less susceptible to heavy metals. New ways must be developed to complement existing knowledge of heavy metal contamination, to provide graphic presentation of the damage caused by heavy metal at tissue and cellular levels. Hence, the evaluation of biochemical biomarker such as histological study is constantly used as indicator of the overall health and pollutants on the environment (Sia Su et al., 2013), and may be useful in assessing metal exposure and the prediction of potential detrimental effects induced by metal contamination (Shariati and Shariati, 2011).

Immunohistochemistry involves the process of detecting antigens such as proteins in the cells by the use of antibodies binding specifically with these antigens (Ramos-Vara and Miller, 2014). Thirumoorth et al (2007) employed Immunohistochemistry (IHC) detection of MT proteins in liver, kidney and gills tissues between two different sites. The impact of heavy metals is assessed in different tissues by immunohistochemistry using primary antibody (mouse monoclonal antibody) and secondary antibody (HRP conjugated antibody) for expression of stress protein. Studying metallothionein as biomarker to evaluate contamination and bioaccumulation of heavy metals may be useful in assessing the prediction of potential detrimental effects induced by metal contamination.

Hopefully the knowledge gain from this study can minimize periodic field sampling thus saving time and money. Furthermore, in spite of the many and varied studies of heavy metals in Malaysia, there is no study on the effect of heavy metals on different size of catfish using histopathological and immunohistochemistry approaches. Thus, the current study shows the influence of heavy metals on catfish and describes histological alteration, localization and expression of metallothionein in different size and parts of the fish sampled from Kuala Gula and Bagan Lalang using immunohistochemistry method. Ecologically, the findings will help us determine an appropriate bio-indicator between the two species studied based on their niches.

1.4 Research objectives

i. To determine heavy metals in water, sediment and different organs of different size and species of fish from two locations Kuala Gula and Bagan Lalang in peninsular Malaysia.
ii. To assessthe impact of heavy metals, Zn, Cu, Pb and Cd on different tissues of different size of the catfish species, *Arius thalassinus* and *Plotosus anguillaris* catfish from Kuala Gula and Bagan Lalang by using histological technique.
iii. To determine the impacts of heavy metals on the cellular damage from different tissues of different sizes of catfish by the expression of metallothioneins 3 in the organs of fish stress responses to pollution exposure by the use of immunohistochemistry method.

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