

**TOXICITY ASSESSMENT AND BIOACCUMULATION OF HEAVY
METALS IN RED TILAPIA**

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ABSTRACT

The aims of this study are to evaluate the acute toxicity of different exposure concentrations of Cd, Cu, Hg, Mg, Pb, and Zn on survival, histopathological effects and to assess accumulation level in the tissues of muscles, gills and liver of red tilapia *Oreochromis* sp. As well as to measure heavy metals concentration in aquaculture ponds fish at selected different sites in Malaysia (Serendah, Kampar, Bistari Jaya and Bukit Tinggi). In this work, red tilapia was chosen because it has become a significant food source for human being and is considered as a commercially important fish of the aquacultures in Malaysia. In the method of acute toxicity test, the healthy fingerlings were collected from a commercial aquaculture in Serendah, Selangor Kuala Lumpur, Malaysia. Thereafter, the fish were acclimatized in laboratory; the tilapias were semi-statically exposed to different concentrations of heavy metals during 96 hours in order to determine the median lethal concentration (LC_{50}) which was estimated by the probit transformed concentration response curves. The fish were dissected into gills, liver and muscles and then digested by adding 6 ml nitric acid (65 %) and 1ml H_2O_2 (35%) in microwave oven; the concentration of heavy metals in fish samples was determined by ICP-OES (Perkin Elmer AA Analyst) and mercury analysis was performed by Flameless Atomic Absorption spectrophotometer. Histopathological characteristics and lesions were conducted on gills and liver from fishes which were exposed to sub-lethal concentration $96hLC_{50}/2$ over 96 hours. The digital images were obtained by using a light microscope Nikon type Eclipse E200, equipped with a Dino eye camera Ø30mm. Morphological analysis on gills of experimental fish was carried out and the weight percent mineral contents through the cross-section of gills were quantified by energy dispersive X-ray (EDX) spectroscopy analysis using a Scanning Electron Microscope equipped with EDX. In toxicity assay, the results showed that tilapia fish had a higher

sensitivity to Hg and Cu followed by Cd and Zn but poor response to Mg and Pb. Among the tested metals, Mg had a lower impact on fish survival and it was accumulated in higher level. Toxic metals accumulation levels were in the following order: $Mg > Zn > Cu > Pb > Cd$ in all organs. These elements caused severe tissue damage which led to alterations of histopathological aspects represented in proliferation of filamentary epithelium with fusion of adjacent secondary lamellae and an increase in chloride cell density of gills. The liver of tested fish showed disorganization of hepatic cells, hypertrophy of hepatocytes severe degradation of the liver parenchyma, and necrosis. The scanning electron micrographic images gave more details about the effect of the elements on the gills which showed disappearance and fusion of microridges in pavement cells. In addition, EDX microanalyses showed an increase in the weight percentage of element in primary and secondary lamellae of gills in experimental fish. Current study provided useful information and a baseline for future along with continuous studies on the heavy metals concentrations in red tilapia fish of aquaculture ponds. The detected metals concentrations varied significantly ($p < 0.05$) among different tissues and the lack of significant variation between the tested sites. The heavy metals concentrations were found to be lower than the recommended maximum level allowed in food by Malaysian Food Act 1983 and Food Regulations 1985. These findings confirmed that tilapia fish from all studied aquaculture ponds are safe for human consumption.

ABSTRAK

Tujuan kajian ini adalah untuk menilai ketoksikan akut pada pendedahan kepekatan Cd, Cu, Hg, Mg, Pb dan Zn yang berbeza untuk kelangsungan hidup, kesan histopatologi dan untuk menilai tahap pengumpulan dalam tisu otot, insang dan hati ikan tilapia merah *Oreochromis sp.* Di samping itu untuk mengukur kepekatan logam berat dalam ikan di kolam akuakultur terpilih yang berbeza di Malaysia (Serendah, Kampar, Bistari Jaya dan Bukit Tinggi). Dalam kajian ini, tilapia merah dipilih kerana ia telah menjadi sumber makanan penting bagi manusia dan juga dianggap sebagai ikan komersial yang penting dalam akuakultur Malaysia. Dalam kaedah ujian ketoksikan akut, ikan sihat bersaiz jari telah dikumpulkan daripada akuakultur komersial di Serendah, Selangor 48200 Kuala Lumpur, Malaysia. Selepas ikan tersebut telah menyesuaikan diri di dalam makmal; ikan tilapia didedahkan secara separa statik kepada kepekatan logam berat yang berbeza dalam 96 jam untuk menentukan kepekatan median maut (LC50) yang dianggarkan melalui lengkung probit berubah tindakbalas kepekatan. Ikan itu telah dibedah di bahagian insang, hati dan otot kemudian dicerna dengan menambah 6 ml asid nitrik (65%) dan 1ml H₂O₂ (35%) di dalam ketuhar gelombang mikro; kepekatan logam berat dalam sampel ikan yang ditentukan oleh ICP-OES (Perkin Elmer AA Analyst) dan analisis merkuri telah dilakukan menggunakan Spektrofotometer Flameless Atomic Absorption. Ciri histopatologi dan luka-luka telah dijalankan ke atas insang dan hati dari ikan yang telah didedahkan kepekatan sub-maut 96hLC50 / 2 lebih 96 jam. Imej-imej digital telah diperolehi dengan menggunakan mikroskop cahaya Nikon jenis Eclipse E200, dilengkapi dengan kamera Dino Ø30mm. Analisis morfologi pada insang ikan eksperimen telah dikaji dan kandungan peratus berat mineral diperolchi melalui keratan rentas insang telah dinilai oleh tenaga serakan sinar-X (EDX) analisis spektroskopi menggunakan Mikroskop Imbasan Elektron dilengkapi dengan

EDX. Dalam esei toksisiti, keputusan menunjukkan bahawa ikan tilapia mempunyai sensitiviti yang lebih tinggi terhadap Hg dan Cu diikuti oleh Cd dan Zn tetapi tindakbalas yang lemah terhadap Mg dan Pb. Antara logam yang diuji, Mg mempunyai kesan yang lebih rendah terhadap kelangsungan hidup ikan dan ia terkumpul di tahap yang lebih tinggi. Peringkat pengumpulan logam toksik adalah mengikut susunan yang berikut: $Mg > Zn > Cu > Pb > Cd$ dalam semua organ-organ. Unsur-unsur ini menyebabkan kerosakan tisu yang teruk yang membawa kepada perubahan ke arah aspek histopatologi diwakili dalam percambahan epitelium berfilamen dengan gabungan lamela sekunder bersebelahan dan peningkatan dalam kepadatan klorida sel insang. Hati ikan yang diuji menunjukkan sel-sel hati tidak teratur, hipertropi hepatosit menyebabkan kemusnahan teruk parenkima hati, dan nekrosis. Imej mikrofotik pengimbas elektron memberikan maklumat terperinci tentang kesan elemen pada insang yang menunjukkan kehilangan dan gabungan microridges dalam sel pavements. Selain EDX mikroanalisis telah menunjukkan peningkatan dalam peratusan berat elemen dalam lamela primer dan sekunder insang dalam ikan eksperimen. Kajian ini telah menyediakan maklumat yang berguna dan menjadi data asas untuk masa depan kajian ke atas logam berat dalam ikan tilapia merah kolam akuakultur. Kepekatan yang dikesan berubah dengan signifikan ($p < 0.05$) antara tisu yang berbeza dan kekurangan perbezaan yang ketara di antara kawasan yang diuji. Kepekatan logam berat didapati lebih rendah daripada tahap maksimum yang disyorkan dibenarkan dalam makanan oleh Akta Makanan Malaysia 1983 dan Peraturan-Peraturan Makanan 1985. Penemuan ini mengesahkan bahawa ikan tilapia dari semua kolam akuakultur yang dikaji adalah selamat untuk kegunaan manusia.

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To them I dedicate this thesis

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LIST OF SYMBOLS AND ABBREVIATIONS

&	and
<	less than
>	greater than
°C	degree Celsius
%	percent
<i>et al.</i>	et alia (“and others”)
i.e.	id est (“that is”)
WHO	World Health Organization
GIFT	Genetic Improvement of Farmed Tilapias
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
ATP	adenosine triphosphate
ADP	adenosine diphosphate
LC ₅₀	50% lethal concentration
LT ₅₀	50% lethal time
pH	potential of hydrogen
TDS	total dissolved solids
DO	dissolved oxygen
μs/cm	micro Siemens per centimeter
ppt	part per thousand
ppm	part per million
ppb	part per billion
wt	weight
m	meter
mg/l	milligram per liter
μg/l	microgram per liter
cm	centimeter
ml	milliliter
h	hour
ANOVA	analysis of variance
r	correlation of coefficient
S.D.	standard deviation
S.E.	standard error
±	plus-minus
sp.	Species (singular)
spp.	Species (plural)
Conc.	Concentration
kg	Kilogram
mg	milligram

U.V.	ultraviolet
SH	sulphydryl group
ICP-OES	Inductive Coupled Plasma – Optical Emission Spectroscopy
DORM 2	Dog fish muscle certified reference material
MT	Metallothionein
SEM	Scanning electron microscope
EDX	Energy dispersive x ray microanalysis
TEM	Transmission electron microscope
PCs	Pavement Cells
CCs	Chloride Cells
INWQS	Interim National Water Quality Standards

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Toxicity and Effect of Heavy Metals

Toxic water pollutants are not easily biodegradable and are a serious health problem of highly industrialized countries. Like illnesses, exposures may also be subdivided into acute and chronic types. Acute exposures are those that occur over short periods of time, often to high concentrations of hazardous substance. Chronic exposures, which are much more common among the general public, involve longer periods of time and for the most part, lower concentrations (Rand, 2003). The median lethal concentration (LC50) is one of the parameters more commonly used to refer to the acute lethal toxicity of pollutants. This parameter is often estimated in the more sensitive species and/or stage of a particular community and is a potential tool to establish safe concentrations of pollutants in the environment (Ayotunde et al. 2011). Several toxic elements, such as As, Sb, cadmium (Cd), mercury (Hg), lead (Pb), uranium (U), and bismuth (Bi), are often included in heavy metals.

1.1.1 Toxicity of Cadmium

In laboratory animals, Cd produces reduced growth, kidney and liver damage, brain hemorrhages, skeletal decalcification, and testicular necrosis; moreover, the effect on the activities of several enzymes, enhanced activity of δ aminolevulinic acid dehydratase, pyruvate dehydrogenase, and pyruvate decarboxylase have been noted,

while depressed activity of δ aminolevulinic acid synthetase, alcohol dehydrogenase, arylsulfatase, and lipoamide dehydrogenase result from Cd intoxication (Rana, 2006).

In addition, Cadmium has the ability to cause severe damage to the hepatic tissue represented by the loss of characteristic architecture with increased vacuolation in hepatocytes as well as increase of hemorrhage, and infiltration of sinusoids with leukocytes. On the other hand, the intestinal tissue suffered from a large number of inflammatory leukocytes and disturbance of the longitudinal muscularis (Younis et al., 2013).

1.1.2 Toxicity of Mercury

Hg^{+2} has no known role in biological systems. It is considered as inessential, imperishable and lasting heavy metal, and moreover the amalgamations of Hg are extremely poisonous. Additionally, constant low-level exposure towards Hg might result in serious health complications, which are categorized as carcinogenic and mutagen (Di Francesco and Robert, 2002; Zahir et al., 2005).

There is no known nutritional requirement for Hg and most of the Hg present in foods results from environmental contamination. Because it has many uses, there are numerous opportunities for contamination of food, air, and water with Hg.

Mercury can be seen in different chemical states:

- a) Elemental or metallic mercury which is liquid and volatilizes readily at room temperature, is the major form in air and is scarcely soluble in water. It is symbolized as Hg^0 .
- b) Divalent inorganic mercury, symbolized as Hg^{+2} , forms salts with various anions and ionizes readily. Mercuric salts are sparingly soluble in water and in the atmosphere Hg^{+2} associates readily with particles and water.

- c) Methylmercury is the most important organic form of mercury (Friberg and Vostal, 1974).

Mercury compounds are highly reactive and can interact with various chemical groupings of proteins and nucleic acids. The binding of Hg to sulfhydryl groups (SH) of membrane proteins causes an inactivation of membrane ATPase and blockage of glucose transport into the cell. Mercury also reacts with phosphoryl groups of membranes, sulfhydryl, amino, and carboxyl groups of enzymes and phosphoryle groups and bases of nucleic acids (Rema and Philip, 2012). Chronic exposure can lead to symptoms of central nervous system involvement. Hg in the body is inhibition of enzyme activity and cell damage. Inhibition of a large number of enzyme systems by Hg react with the SH group has been reported (Vieira et al., 2009).

1.1.3 Toxicity of Lead

The usual valence state of Pb is (II), in which it forms inorganic compounds. Lead can also exist as Pb (IV). The atmosphere is the major vector for the transport of Pb to living receptors as well as to other media.

Aquatic biota is potentially at risk from exposure to Pb from indirect and direct releases into their environment. Toxic effects of Pb to aquatic life via the aqueous phase are probably quite unusual in the real world and are likely to occur mainly when there is direct release into water, such as in industrial effluents or from accidental spills, in combination with water of low pH and low hardness (Wright and Welbourn, 2002). Acute toxicity of Pb to fish in laboratory experiments has been recorded at concentration from 0.1 to 500mg/L. However, in laboratory tests, death by asphyxia may result from Pb induced production of mucilage, which has adverse effects on gill

function. Even insoluble Pb in colloidal form can have harmful effects on fish gills (Wright and Wolbourn, 2002).

Symptoms of Pb poisoning include abdominal pain, anemia, and lesions of the central and peripheral nervous systems. The principal biochemical effect of Pb toxicity in humans and animals is defective hemoglobin synthesis. Lead inhibits Fe incorporation into protoporphyrin, which results in lower heme concentrations and higher protoporphyrin concentrations in erythrocytes. Excretion of protoporphyrin increases, and the Fe content of the blood plasma and bone marrow is elevated (McFree and Leikin, 2007).

1.1.4 Toxicity of Copper

Copper has two valence states Cu^{+1} , cuprous, and Cu^{+2} , cupric. In the lithosphere, it occurs in trace quantities in metallic form and as Cu compounds in ores, including copper pyrites (CuFeS_2) and malachite [$\text{CuCO}_3\text{Cu}(\text{OH})_2$]. It has long been used in pure metallic form or alloyed with other metals, as bronze (copper-tin) and brass (copper-zinc).

The major processes that result in the mobilization of Cu into environment are extraction from its ore (mining, milling, and smelting), agriculture, and waste disposal. Soils have become contaminated with Cu by deposition of dust from local sources such as foundries and smelters, as well as from the application of fungicides and sewage sludge. Aquatic systems similarly receive Cu from the atmosphere, as well as from agricultural runoff, deliberate additions of copper sulphate to control algal blooms, and direct discharge from industrial processes. The major concern for environmental impacts of copper concerns the aquatic system. Fish and crustaceans are generally 10-100 times more sensitive to Cu than mammals (Wright and Welbourn, 2002).

Copper cause histopathological changes in gills which are exposed to the highest concentration represented by damage in edema, lifting of lamellar epithelia and an intense vasodilatation of the lamellar vascular. Furthermore, lamellar fusion with some lamellar aneurisms and also the liver has been impacted by vacuolation and necrosis, especially evident in fish exposed to 1.0 and 2.5mg L⁻¹ copper concentrations. (Figueiredo-Fernandes et al., 2007)

1.2 Background of Target Species

Commercial tilapia farming is mostly developed in Malaysia and other countries in the near region are also interested in this activity. Tilapia is one of the best researched species for aquaculture, and there is a wealth of experience in their husbandry. Tilapia are tough and tolerate a wide range of environmental conditions: little environmental modification is needed, so aquaculture systems can be low-tech. Earthen ponds of appropriate design in non-flood-prone areas will be sufficient for tilapia farming. Concrete tanks or raceways can be used, but are more expensive to build and usually cannot be justified in rural areas.

Tilapia belongs to a group of fish called cichlids and is native to Africa. Tilapia are shaped like snapper but can be identified by an interrupted lateral line, which is a characteristic of the cichlid family of fishes. They are laterally compressed (flat-sided) and deep-bodied with long dorsal fins. The front portion of the dorsal fin is spiny and the rear is soft rayed. Spines are also found in the pelvic and anal fins. The tilapia group consists of three important genera, *Oreochromis*, *Sarotherodon* and *Tilapia*. Several characteristics distinguish these three genera, but the most important one relates to reproductive behavior, the genus *Oreochromis*, of which there are three main species used for farming. These are Nile tilapia (*Oreochromis niloticus*), Mozambique tilapia

(*Oreochromis mossambicus*) and blue tilapia (*Oreochromis aureus*) (Nandlal and Pickering, 2004).

Tilapia and other cichlids along with top ten species groups for 91 % of the total aquaculture contribution to fisheries food supply because of faster production rate, tolerance with salinity and meeting the market needs (FAO, 2007).

Aquaculture grows rapidly especially for cichlid/tilapia in many parts of Asia and some pacific Island countries. Furthermore, tilapia fish have excellent characteristics for farming due to faster growth, large size, ability to survive in different water conditions, adaptability to a wide range of food types, and easy reproduction which does not need any exclusive hatching technologies. As a result, the production of farmed tilapia has significantly increased in respect of commercial production (Nandlal and Pickering, 2004b). On top of that, tilapia can tolerate a wide range of environmental conditions, especially low dissolved oxygen, high ammonia level, and a wide range of pH (5-11) (Watanabe et al., 1997).

Tilapia is the third group of the most important farmed fish in the world, after carps and salmonids. Its culture is also one of the fastest aqua cultural growths with an average annual growth rate of 13.4% (FAO, 2004).

Target samples are hybrid tilapia, which has become popular through collaborative program with world fish centre on Genetic Improvement of Farmed Tilapias as the GIFT. The hybrid tilapia has been selected due to the following reasons: high productivity, significant improvements in growth rate in successive generations, as well as remarkable survival rates in the Malaysian aquacultures, which became an important food source for human beings (Ponzoni et al., 2005; Ponzoni et al., 2010); in addition,

the ability to respond against environmental pollution is also another reason for the selection (Mokhtar, 2009; Low, 2011).

The hybrid tilapia has been selected due to the following reasons:

- a) Red tilapia have high productivity and significant improvements in growth rate in successive generations
- b) Remarkable survival rates in the Malaysian aquacultures, which became an important food source for human beings (Ponzoni et al., 2005; Ponzoni et al., 2010).
- c) The hybrid tilapia, which has become popular through collaborative program with world fish centre on Genetic Improvement of Farmed Tilapias as the GIFT.
- d) In addition, the ability to respond against environmental pollution is also another reason for the selection (Mokhtar, 2009; Low, 2011).

1.3 Significance of Research

The research study could provide information on the toxicity and effects of different heavy metals which may damage aquatic organisms at the cellular level and this can be considered risk factors for several diseases and possibly affect the ecological balance. Further, this study would also be a review of the histological changes which occur earlier than any other evidence. Bio-markers can offer additional biologically and ecologically relevant information a valuable tool for the establishment of guidelines for effective environmental management. This study would be beneficial to provide a better assessment technique of fish health and to the effects of pollution on each biochemical parameter.

To the future researchers, this study can provide baseline information on the recent status of bio-accumulation of metals in artificial aquacultured fish which adversely affect the health of fish that may affect human health as well as negative effects on production.

1.4 Objectives of Research

- a) To investigate the acute toxicity effect of different exposure concentrations of Cd, Cu, Hg, Mg, Pb, and Zn on the survival of red tilapia and accumulation level in the fish tissues of muscles, gills, liver.
- b) To determine the histopathological effect of toxic metals in tilapia fish.
- c) To examine effect of heavy metals on the gills via scanning electron microscope. Furthermore, to utilize energy dispersive X-ray analysis (EDX) to quantify the concentrations and weight percent of metals content within gills.
- d) To measure heavy metals concentration in fish at different ponds.

CHAPTER 2

LITERATURE REVIEW

2.1 Heavy Metals

The term “heavy metal” has become entrenched in the literature of environmental pollution. One of the most common definitions of “heavy metal” is a metal with a density greater than 5g/cm^3 (i.e., specific gravity >5). Heavy metals are perhaps the most common of all metabolic poisons. The mechanism of metal toxicity is different from other metabolic poisons. Metal toxicity can affect the enzymes which are cellular proteins regulating many important chemical reactions. Furthermore, heavy metals are toxic primarily because they react with and inhibit sulfhydryl (SH) enzyme systems, such as those involved in the production of cellular energy (Csuros and Csuros, 2002; Rana, 2006).

2.1.1. Sources of Metals and Their Compounds

2.1.1.1- Metal pollution from mining and processing ores

The environmental damages are mostly resulted from mine digging and ore removing as well as minerals extraction and processing. For instance, the processing of mining may cause habitation, homes and farmlands damage and generate the erosion for soil and eventually make the waterways contaminated by the means of toxic drainage. Moreover, it can be observed that the release of toxic materials from sulfur oxides, smelters arsenic (As), cadmium (Cd), selenium (Se), and lead (Pb) as compared to others can make the air more polluted. On the other hands, comparing the surface mining and underground mining, it can be said that the surface mining can make wastes eight times more than the latter one, though deep mining is even more problematic and can cause problems like earthquakes. In case that the underground mines collapse, it can give fatal damages to the miners as well as making surface subsidence which shapes holes which results in the

collapse of roads and houses. While the near-surface minerals are empty, miners are obliged to go deeper in order to explore minerals (Lottermoser, 2012).

Dangerous water quality problems are caused by the release of acid-mine drainage from abandoned and mines specifically coal mines in which the toxic materials are dissolved from soils and tailings and moved to groundwater and waterways. The challenges of water quality are associated with approximately high degrees of metal like cobalt (Co), iron (Fe), nickel (Ni), manganese (Mn), copper (Cu) as well as zinc (Zn) (Mukti, 2014). Ore processing, smelting, and refining may result in trace metals deposition if they are in plethora, like silver (Ag), lead (Pb), cadmium (Cd), zinc (Zn) and arsenic (As) into sewage basins or direct discharge into aquatic atmosphere (Mc Geer et al., 2011).

2.1.1.2- Domestic waste water effluents

The drainage of domestic waste water have large quantity of trace metals from household products like arsenic (As), detergents iron (Fe), nickel (Ni), manganese (Mn), zinc (Zn), chromium (Cr), cobalt (Co), boron (B), and metabolic waste materials, the corrosion of water pipes copper (Cu), cadmium (Cd), lead (Pb) and zinc (Zn). Lower than 50% of the metal of the influent is normally erased by the wastewater treatment, abandoning the drainage with remarkable metal loading. The mud made by wastewater treatment is as well rich in metals. Moreover, the domestic wastewater as well as the dumping of industrial and domestic mud are the main artificial sources of iron (Fe), cadmium (Cd), mercury (Hg), chromium (Cr), lead (Pb) and copper (Cu) pollution (Mara, 2004).

2.1.1.3- Storm water runoff

Storm water runoff from urbanized areas is a significant source of metal pollution in the receiving waters. Metal composition of urban runoff water is dependent on many factors, such as city planning, traffic, road construction, land use, and the physical characteristics and climatology of the watershed (Goodwin et al., 2003).

2.1.1.4- Industrial wastes and discharges

Metals and their concentrations in industrial sewage and releases are distinguishing and based on the history of a certain industry, such as Cr effluents from tannery industries; Al from textile industries.

2.1.1.5- Sanitary landfills

The metal essence and typical concentration of sanitary-landfill leachates are Cu (5 ppm), Hg (60 ppb) Zn (50ppm) and Pb (0.3 ppm) (Galarpe and Parilla, 2014).

2.1.1.6- Agricultural runoff

The metal substance of agricultural runoff starts in soils and sediments saturated by plant debris and animals, certain herbicides and fungicides, fertilizers as well as employing waste and mud as plant nourishment.

2.1.1.7- Fossil fuel ignition

Fossil fuel ignition is a main source of airborne metal pollution of natural waters.

2.1.2. Metallic Substances Essential to Life

Minerals, including some metals, constitute about 4% of total body weight and are concentrated most heavily in the skeleton (Couture and Pyle, 2011). Minerals known to perform functions essential to life include potassium, sodium, magnesium, calcium, manganese, cobalt, copper, selenium, zinc, chromium, chloride, iodine, and phosphorus. Other minerals, such as aluminum, silicon, arsenic, and nickel are present in the body, but their exact functions have not yet been determined. Calcium and phosphorus form a part of the structure of bone, but because minerals do not form long-chain compounds, they are otherwise poor building materials. Their chief role is to help regulate body processes. Calcium, iron, magnesium, and manganese are constituents of some coenzymes. Magnesium also serves as a catalyst for the conversion of ADP (adenosine diphosphate) to ATP (adenosine triphosphate). Without these minerals, metabolism halts and the body dies. Generally, the body uses mineral ions rather than nonionized forms (Csuros and Csuros, 2002).

2.1.2.1 Copper (Cu)

The element was discovered in prehistoric times and has its name from the island of Cyprus (Latin: cuprum). Copper is an essential trace nutrient for all the known living organisms. It is required for the functioning of more than 30 enzymes involved in electron transfer (cytochrome oxidase), free radical defense (catalase, superoxide dismutase) and it is a component of the enzyme necessary for melanin pigment formation (tyrosinase) or deoxygen carriers like haemocyanin. Copper is also essential for the utilization of iron and synthesis of hemoglobin (Prasad, 2008).

2.1.2.2 Magnesium (Mg)

Magnesium as an important constituent of many coenzymes, is vital to many basic metabolic functions, and also aids in bone growth and the function of nerves, bones, and

muscles, including heart rhythm regulation. It is nontoxic for humans, except in large doses. Magnesium does not constitute a public health hazard; before toxic levels occur in drinking water, the taste cannot be tolerated (Raskin, 2007).

2.1.2.3 Zinc (Zn)

Zinc is an important part of many enzymes that are necessary for normal tissue growth and healing of wounds and the sense of taste and appetite. As a part of peptidase, zinc is important in protein digestion. Zinc is also necessary for prostate gland function. Next to iron, zinc is the second most abundant trace mineral in the body (Prasad, 2008).

2.1.3. Common Plant Nutrients

Of the 18 elemental essential plant nutrients, 15 are minerals. Of the 15 minerals, 11 are metals, including potassium, calcium, magnesium, boron, copper, iron, manganese, molybdenum, sodium, vanadium, and zinc (Marschner, 2012).

2.1.4. Nonessential metals

Many metals found in our environment are nutritionally nonessential. There is a group of metallic elements that exhibits certain chemical and electrical properties and are generally those having a density greater than 5 g/cm^3 . These metals exceed the atomic mass of calcium. Most of the heavy metals are extremely toxic because as ions or in certain compounds, they are soluble in water and may be readily absorbed into plant or animal tissue. After absorption, the metals tend to combine with biomolecules, such as proteins and nucleic acids, impairing their functions; particularly, Cd, Pb, and Hg are generally considered as the most toxic to humans and animals (Atta et al., 2012; Raldua et al., 2007 and Greenfield et al., 2008)

2.1.4.1 Lead

Lead has a low melting point of 327°C. It is extremely stable in compound forms. Therefore, dangerous forms may remain in the environment for a long time. Although Pb intake from paints, water pipes, tin, cans, and insecticides has markedly declined, the exposure to other forms of Pb such as in motor vehicle exhausts and tobacco smoke has either stabilized or increased, and therefore, Pb is still a potential problem in aquatic systems because of its industrial importance.

Once emitted into the atmosphere or soil, Pb can find its way into aquatic systems. Surface and ground waters may contain significant amounts of Pb derived from these sources (Wright and Welbourn, 2002).

Plant can absorb and accumulate Pb directly from ambient air and soil. Lead toxicity to plants varies with species and the other trace metals present. Then, up to the animal and humans through the food chain.

2.1.4.2 Cadmium

Cadmium is a nonessential trace element and is present in air, water, and food. It is a silver white metal with an atomic weight of 112.4, and a low melting point of 321°C. As a metal, Cd is rare and not found in a pure state in nature. It is constituent of smithsonite (ZnCO_3) and is obtained as a byproduct from the some Cu ores.

A unique characteristic of Cd is that it is malleable and can be rolled into sheets. The metal combines with the majority of other heavy metals to form alloys. It is readily oxidized to the +2 oxidation state, resulting in the colorless Cd^{2+} ion. Cadmium has an electronic configuration similar to that of Zn, which is an essential mineral element for living organisms. However, Cd has a greater affinity for thiol ligands than does Zn. It binds to sulfur containing ligands more tightly than the first row transition metals other than Cu, but Hg and Pb both form more stable sulfur complexes than does Cd. The Cd^{2+} ion is similar to the Ca^{2+} ion in size and charge density (Mc Geer et al., 2011)

In natural fresh water, Cd usually occurs at very low concentrations ($<0.01\mu\text{g/L}$). However, the concentration varies by area and environmental pollution. Many Cd containing wastes end up in lakes and marine water. Wastes from Pb mines, motor oils, rubber tires, has many industrial uses, such as in electroplating, in low melting alloys, in low friction, fatigue resistant bearing alloys, in solders, in batteries, in pigments, and as a barrier in atomic fission control. Therefore, it is to be expected that low to moderate Cd content of the environment is widespread (Lee, 2015).

2.1.4.3 Mercury

Mercury is the only common metal that is liquid at room temperature. It has a high specific gravity, 13.6 times that of water. Its boiling point is 357°C , which is relatively low. Mercury has a long liquid range of 396°C , and it expands uniformly over this range. This linear expansion, in addition to the fact that Hg does not wet glass, makes the metal useful in thermometers. Mercury has the highest volatility of any metal. Its good electrical conductivity makes it exceptionally useful in electrical switches and sealed relays. Many metals dissolve in Hg to form amalgams (alloys).

Mercury has no known biological role and is an industrial health hazard, because of its diversity of usage. It is a bio-accumulative metal that is fat soluble and has many hazardous effects on living organisms (Rand, 2003; Rana, 2006).

2.1.4.3.1 Source of mercury pollution

Basically, mercury is released into the atmosphere through a number of sources, such as, surface water and soil from pulp and paper, chlorine factories, electrical industries, combustion of fossil fuels; apart from these, human activities are also considered as responsible for the mercury contamination (Friberg and Vostal, 1974).

Mercury contamination of the environment is caused by both natural and anthropogenic sources. Natural sources include volcanic action, erosion of Hg containing sediments, and gaseous emissions from the earth's crust. The majority of Hg comes from anthropogenic sources. Mining, combustion of fossil fuels in municipalities and hospitals, transporting Hg ores, processing pulp and paper, incineration, use of Hg compounds as seed dressings in agriculture, and exhaust from metal smelters are some examples. In addition, Hg waste is found as a byproduct of chlorine manufacturing plants, used batteries, light bulbs, and gold recovery processes (Yu et al., 2011).

Elemental Hg is used in thermometers, barometers, diffusion pumps, Hg vapor lamps, electrical switches, dental fillings, paints, batteries, catalysts, and the manufacture of chlorine. Mercury salts are used as medicine, paint pigments, explosive detonators, and in the manufacture of paper. Organic Hg compounds are used as fungicides for seed treatment and in the manufacture of certain types of plastic. Generally, industries are one of the main sources of releasing inorganic mercury into the atmosphere, which create an intensive impact on fish tissues as opposed to the organic form of mercury (Sunderland and Chmura, 2000; Oliveira-Ribeiro et al., 2002).

2.1.4.3.2 Effects of mercury on animals

Fresh water, marine organisms and their predator normally contain more Hg than terrestrial animals. Levels in top predatory fish are higher. Fish may accumulate Hg in excess of the 0.5mg/g. This accumulation is part of a dynamic process in which an organism strives to maintain equilibrium between intake and excretion. The mercury accumulated in fish comes primarily from the absorption of water across the gill or through the food chain, although some higher species may convert inorganic Hg into MeHg. Some Hg is also taken up through the mucous layer and or skin (Di Francesco and Robert, 2002).

2.2. Acute Toxicity and Bioaccumulation of Heavy Metals

Progresses in all branches of sciences have brought a lot of impressive effects on lives; on the other these progresses had also created huge negative impacts on all aspects of environment. The aquatic environment in particular has been continually subjected to numerous contaminants. These contaminants include chemicals, such as heavy metals that have significantly polluted both marine and freshwater sources. This resulted in what is seemed be a substantial obstacle and a severe danger. Rana (2006) declared that about 75 % of all known chemical elements are metals, a cluster of them are toxic elements, such as arsenic (As), mercury (Hg), cadmium (Cd), antimony (Sb), lead (Pb), bismuth (Bi) and uranium (U).

Rainbow (2002) reported that toxicity by a certain metal happens when the amount of its intake in the body overtakes both the shared rate of its excretion and detoxification of available metal in metabolism and ultimately accumulates in soft tissues. So, even nutritionally required elements, such as iodine (I), selenium (Se), iron (Fe), cobalt (Co), molybdenum (Mo), copper (Cu), manganese (Mn), and zinc (Zn), may cause negative impacts on aquatic fauna, depending on the species when taken in higher amounts (Sloman, 2007; Qiu, et al., 2011); with the severity depending on its direct toxicity at the specific trophic level (Couture and Pyle, 2011).

Heavy metals are also widespread in industrial applications such as in the manufacture of pesticides, alloys, batteries, textile dyes, electroplated metal parts, steel, and so forth (IOSHIC, 1999). Generally, acute toxicity occurs as result of a sudden or unexpected exposure to a relatively high concentration of chemicals in a short period of exposure, consequently, acute effects symptoms can appear after such exposure (Ahmed et al.,

2013). Concentrations of toxic metals ions are exponentially increasing due to their anthropogenic and ecological influence on aquatic ecosystems in different reservoirs, including those used for aquaculture. Contamination by heavy metals in aquatic environments is increasing globally and it is described as one of the most critical environmental risks (Nriagu et al., 1998).

Acute toxicity of heavy metals can cause damage to circulatory system, gastrointestinal tract, the nervous system and other vital organs, especially the liver (Naughton et al., 2011 and Pohl et al., 2011). Sometimes, changes in growth, behaviour and reproduction that may be conducive to death of fresh water organisms (Rand et al., 2003).

In bioaccumulation process in the tissues of a living organism can absorb toxic metals if their availability is very high in environment or food. In addition they have tendency to move up the food chain as one species consumes another, becoming increase in concentration of a substance than as they go, is called biomagnification (Rana, 2006). The level of heavy metals in fish tissues are influenced by biotic, abiotic and environmental factors such as fish species, habitat, fish age, concentration of metal in water, exposure period, water temperature, pH in water, dissolved oxygen (DO) concentration, water salinity and other physiological conditions of fish (Scott et al., 2004; Tsai and Liao, 2006; Has-Schon et al., 2006; Uysal *et al.*, 2008; Vinodhini and Narayanan, 2008; Ling et al., 2009; Rema and Philip, 2012). A number of studies in Malaysia have been focused on the toxicity and bioaccumulation of heavy metals on tilapia and other commercially important fish species (Mokhtar et al., 2009; Taweel et al., 2011; Low et al., 2011; Ashraf et al., 2012). Cuvin-Aralar (1994) studied survival and heavy metal accumulation in two strains of Nile tilapia *Oreochromis niloticus* (L) by exposing samples of fish to mixtures of zinc, cadmium and mercury to test

differences in resistance or tolerance to heavy metals as well as evaluating the bioaccumulation of these metals after short –term exposure.

Guardiola et al., (2013) demonstrated that when *Sparus aurata* was exposed to waterborne Cd, the metal had accumulated at high concentrations in tissues; besides producing skeletal deformities. In another study by Wu et al., (2007) in hybrid tilapia *Oreochromis* sp showed that toxicological stress of cadmium can changed physiological parameter and ion regulation. In more details , Da Silva and Martinez, (2014) investigated toxic effects of cadmium on osmoregulation by exposing juveniles of neotropical fish *Prochilodus lineatus* to two different concentrations of the metal (1 and 10 g/L) for 24 and 96 h respectively. They found that the metal resulted in a decrease in Ca^{2+} ATPase , Na^{+} K^{+} ATPase activities and carbonic anhydrase level in gills with 24 and 96 h exposure to this metal.

Biochemical and physiological changes induced by acute exposure to cadmium have asserted that fish are highly susceptible to high concentration; the toxic effects of this metal on fish was found to be constant and can be revealed within a few hours of exposure (De La Torre et al., 2000). Furthermore, it had been demonstrated that Cd contamination results in the metal entering the body through the blood circulation, then consequently access to other organs including kidney and liver (Pretto et al., 2011 and Mc Geer et al., 2012).

Also, when *Tanichthys albonubes* were exposed to acute concentrations $\frac{1}{2}$ and $\frac{1}{4}$ 96 h-LC₅₀ (0.027 mg/ L), (0.0135 mg /L) of copper and (2.31 mg/ L),(1.15 mg/ L) of cadmium for 96 h, it was concluded that gene expression patterns in the fish liver were

dose and time dependent furthermore, it had a negative impact on the genomic DNA structure (Jing et al., 2013)..

In a study by Leonard et al., (2014) on rainbow trout (*Oncorhynchus mykiss*) and round goby fish (*Neogobius melanostomus*) exposed to waterborne and dietary Ni for 30 days; the investigators observed that goby fish was found to be more sensitive to exposure to the metal than trout, this higher sensitivity was attributed to the pre-exposure of goby fish samples to contaminants at their collection site; so it was concluded that persistent mortality in goby fish due to high Ni bioaccumulation in gills than in the gut during the experimental exposure.

Schmidt et al., 2011 reported that short term metal bioaccumulation can be used to predict longer term toxicity; as well as, they described Tissue Residue Approach (TRA) that the bioaccumulation of heavy metals in tissues leads to adverse biological effects such as mortality which can be applied to predict the toxicity cross and within species, these methods may be used separately or together as tools for evaluating toxicity in aquatic organisms. However, Adams et al., (2011) noted that accumulation of metals changes with passage of time. There could be organizing absorption and elimination or subcellular partitioning, both of which can affect on the metal to make more or less toxic

Some of studies highlighted the different accumulation patterns of metals in different fish species such as study of Wang, And Rainbow, (2008) found that both physiological and biochemical responses on one hand and metal geochemistry on another hand had impacts on the manner metal accumulations observed in different populations of aquatic

species. Wood (2001) explained that many cationic metals cause toxicity as a result of their inhibitory effects on ion transport functions in fish gills. Oost et al., (2003) noted that biological and biochemical effects of nutritionally important metals may be due to the correlation between bioavailability and concentration of these compounds at target organs and intrinsic toxicity. Yilmaz et al., (2004) demonstrated effect of acute toxicity of cadmium ions on behavioural changes in the guppy fish (*Poecilia reticulata*, Pallas, 1859) in a static bioassay test system and they found 96-h LC₅₀ value was (30.4 mg/l).

In the short term contaminations, the non essential metals such as mercury (Hg) conventionally received exclusive attention. Buhl, (1997) asserted this element has been classified the most toxic metal introduced into the natural environment by anthropogenic sources. As well as study by Sindhe et al. (2002) in fish *Notopterus notopterus* exposed to sub-lethal concentrations of HgCl₂ found that lipid, protein, and cholesterol content of liver and ovary were reduced, and Hg was more toxic than Cd.

On studying the effects of cadmium accumulation and antioxidant defenses, study of Qu et al. (2014) on goldfish *Carassius auratus* as an experimental organism, exposed the fish to 0.1 mg/L Cd throughout the trial period, they observed a continuous accumulation of Cd which occurred in tissues in the following order: gill > liver > muscle on the third day and liver > gill > muscle on the 12th day.

2.3. Histopathological Study

A lot of researchers have considered histopathological lesions of natural water fish as typical signs of toxic damage which may affect fish quality. Subjection to mercury contaminants or other metals, under similar conditions, showed different histological changes in aquatic organisms with difference in severity, depending on the type of

organism and concentration of the chemicals (Oliveiro-Ribeiro et al., 2002; Greenfield et al., 2008; Triebskorn et al., 2008; Gehringer et al., 2013). Kaewamatawong et al., 2013 conducted laboratory studies on Hg toxicology using tilapia, *Oreochromis niloticus*, as a model animal; the animals were treated with lethal or sublethal doses and structural damage of liver were observed significant microscopic lesions with Hg accumulation. Jalaludeen et al. (2012) noted that low level cadmium exposure may have a higher gross biological impact comparable to that of repeated exposures of much greater intensity, furthermore short term cadmium exposure causes pathological conditions in various tissues including gills, liver and kidney of the freshwater fish *Tilapia mossambica*.

Fish gills perform indispensable functions like respiration, osmoregulation, nitrogenous waste elimination and acid–base balance (Evans, 2005). With exception of the skin, gills are the first organs which come in close contact with environmental contaminants; therefore, they are often used in the evaluation of the impact of water contaminants in freshwater habitats. The available data indicate that the vast majority of studies on the gills of fish based on the evaluation of the effects of exposure to single metal mostly for a shorter period (Athikesavan et al., 2006; Pandey et al., 2008). Heavy metal ions such as Cu^{2+} and Zn^{2+} have an effect on the antioxidants of fish tissues including gills (Craig et al., 2007; Hansen et al., 2007).

When fish environments were contaminated by Hg, they suffer histopathological alterations including gills damage which are considered as the most affected organ. These alterations include hypersecretion of mucus succeeded by mortality are related to secondary physiological respiratory disturbance (Sharma et al., 2001; Silva et al., 2012). Such histopathological alterations were showed in the gills and muscle of a lot of fish as

a result of exposure to different toxic substances (Camargo and Martinez, 2006; Abbas and Ali, 2007). Thus, gills were proved to be outstanding indicators of environmental contamination because it presented many histological alterations as a result of acute exposure of the median lethal concentration 96hLC₅₀ of Zn (10 mg/L) on yellow tail lambari (*Astyanax aff. bimaculatus*) and these alterations in the gill epithelium are the result of a combination of pollutants exposure with the severity of the changes that depend on the concentration of pollutants and duration of exposure (Santos et al., 2012). Pereira et al. (2013) evaluated the main histological changes and oxidative stress responses in gills of three native fish species, samples of the northwestern Portuguese rivers (chub *Squalius carolitertii*, barbell *Luciobarbus bocagei* and nase *Pseudochondrostoma* sp.); their results recorded epithelium proliferation of filament, lamellar fusion, aneurisms and necrosis. In addition histopathological study by Oliveira Riberiro et al. (2005) on different organ of the eel *Anguilla anguilla* found unexpected lesions in gills and livers with high concentration of heavy metals.

Pathological changes in fish are documented as biomarkers of environmental contamination and are extensively used in programs of water quality monitoring in numerous countries. They are especially valuable in the detection of biomarkers for histopathology from the toxic effects of contaminants is the investigation in liver pathology. Previous literature indicated that fish exposed to contaminants (industrial, agricultural, and sewage) suffer from many of the pathological changes in the liver, as the most important organ in metabolism (Triebkorn et al. 2008; Syasina et al., 2012). Ebrahimi and Taherianfard, (2011) declared that exposure to heavy metals can result pathological alterations in liver of two species *Capoeta* sp. and *Cyprinus carpio* in highly polluted areas. Greenfield et al. (2008) emphasized that histopathology of fish liver is a monitoring tool which can give an assessment of the impacts induced by

environmental stressors on fish populations, and they suggested it to be one of the most reliable indicators of the weakness of aquatic animal health by human activities. Study of Fernandes et al. (2008) which recorded hepatic alterations include general diagnostic categories like foci of necrosis, vacuolization and also suggested that Cu and Zn content which accumulated in higher concentrations in liver of fish *Liza saliens* could trigger alteration represented by heterogeneous parenchyma. Study of Abdel-Warith et al. (2011) have investigated the effect of zinc on the histological structure of liver in Nile tilapia, *Oreochromis niloticus* to determine the toxic effect of Zn on liver of this fish species, after exposing to 2, 4, and 6 mg/L over both short and longterm exposure periods, so as to assess the damage and get an insight in its functional consequences. Furthermore, short- term exposure periods may provide an indication of the time elapse necessary for the onset of cellular damage and who found a marked difference in these changes among the different Zn concentrations and the extent of exposure period. As well as study of Van Dyk et al. (2007) noted that the effect of zinc (Zn) and cadmium (Cd) on the liver histology of the fresh water fish *Oreochromis mossambicus* exposed to 5 and 10% of lethal concentrations over both short and long term exposure.

Low, (2003) provided an overview of the use of small fish models in toxicologic pathology to evaluate the aquatic system along with some historical perspective. Furthermore, the status of fish health as reviewed by Zeitoun and Mehana(2014) who highlighted the impact of the bioaccumulation of heavy metals in different organs of fish with histopathological studies which can play an important role in the diagnosis of fish diseases caused by the heavy metals. Histopathology of Nile tilapia *Oreochromis niloticus* studied by Figueiredo-Fernandes et al., (2007); Osman, (2012) who evaluated the health of aquatic systems and their biological responses.

2.4. Investigation of heavy metals on tilapia fish via Scanning Electron Microscope techniques

Previous literature showed the use of scanning electron microscope techniques with energy dispersive X-ray analysis EDX used in the investigation of heavy metals in aquatic organisms in general such as effects of lead acetate on the freshwater amphipod *Gammarus pulex*. The study suggested that the exposure to the lead acetate may cause some ultrastructural changes on hepatopancreatic ceca of digestive system (Kutlu et al., 2002). The chemical composition of the precipitate formed on fish gills of perch *Perca fluviatilis* L. which exposed to heavy metals described by means of scanning electron microscopy and energy dispersive X-ray microanalysis revealed that the deposits of the gills contain high amounts of iron and titanium, in addition, considerable peaks of sulphur, phosphorus, potassium and calcium were detected (Lehtinen and Klingstedt, 1983).

Previous study used electron microscopy coupled with X-ray in investigation of many species of other aquatic animals such as study of Khalil et al, (2009) used microanalysis of electron microscopic X-ray in parasite eggs of *Bothriocephalus acheilognathi* which exposed to sub lethal and lethal concentrations of cadmium (1 and 10 mg/L) indicated that cadmium accumulates on the surface of egg and does not penetrate detectably into the enclosed coracidium. Adams and Shorey, (1998) also used microanalysis of energy dispersive X-Ray spectroscopy to analyze content of metal ion of granular concretions in the mantle of the Australian freshwater mussel *Hyridella depressa*.

Other researchers adopted a method of electron microscope to examine fish, such as study conducted by da Silva et al., (2012) in neotropical predator fish *Hoplias malabaricus* (traíra) from Amazon basin, Northern Brazil; they found the high incidence

of histological changes in the liver and gills with mercury bioaccumulation and they suggested that during continuous exposure to this metal ions is posing potential risks to the species. Review by Lobinski et al. (2006) mentioned about recent progress in techniques for biological trace element imaging as well as identified and quantified of chemical species in the biological environment and this distribution of elements in cell or tissue which became possible with radiation X ray fluorescence microprobes.

Song et al., (2010) utilized an electron microscopy coupled with Energy Dispersive X ray to characterize the quantitative microstructure as weight percent mineral content of the armor of stickleback *Gasterosteus aculeatus*.

Pandey et al. (2008) examined the gills of *Channa punctata* via scanning electron microscopy to describe morphological characteristics after exposure to four metals (Cu, Cd, Fe and Ni) their results showed that metal exposure induced alterations which include raising the density of the chloride cells. Furthermore, study by Kaddissi et al. (2011) on histopathological effects and metal distribution by using Transmission Electron Microscopy coupled with Energy Dispersive X-ray (TEM-EDX), they assessed as uranium (U) endpoints which examined on adult male crayfish *Procambarus clarkii* during 4 and 10 days of exposure and they concluded that increasing waterborne U concentration lead to increasing bioaccumulation in organs and then increasing in histological damages.

Sauer and Watabe, (1989) utilized X-ray microanalysis system with scanning electron microscopy to measure zinc/calcium ratios in scales of *Fundulus heteroclitus* with different periods of exposure to heavy metals which included Cd, Cu, Pb, and Zn. And also Brraich and Jangu, (2012) reported heavy metal pollutants on scales of freshwater

fish *Cyprinus carpio* by using Scanning Electron Microscopy (SEM) and Energy dispersive X-ray Microanalysis (EDX). Ikoma et al., (2003) also examined the microstructure of fish scales extracted from sea bream *Pagrus major*, in addition study of ultra structurally by Atta et al. (2012) in the effect of heavy metals on the regenerating tail fin of the teleost fish, *Oreochromis niloticus*, that revealed in the tail fins of the specimens treated with lead (Pb) at 0.005 mg/l, at the fifth day post amputation.

Oliveira Ribeiro et al., (2000) studied tropical fish, namely nordic species, *Salvelinus alpinus* and *Trichomycterus zonatus*; they noted presence of some changes in the gills of several fish induced by exposure to different toxic substances including heavy metals. Carmona et al., (2004) examined the morphological and ultrastructural modification in the pavement cells and chloride of gill of *Acipenser naccarii* which effected by hypertonic environmental conditions (salinity 35); they found that marked variability in the morphology of the apical surface membrane of chloride cells which had a distinctive appearance that distinguishes them from adjacent pavement cells. Wu et al. (2008) reported that morphological alterations of mitochondria-rich (MR) cells occur when exposure to copper in the gills of *Oreochromis mossambicus*.

Recent studies proposed modern and accurate diagnostic methods to explain the element effects analysis by using scanning electron microscopy technology with energy dispersive X-ray spectroscopy microanalysis to examine the effect of heavy metals contaminants in different organ tissues of fish such as study conducted by Barillet et al. (2010) whose tested histopathological impacts resulted in gill, gonadal, and muscle tissues of adult zebrafish (*Danio rerio*) exposed to waterborne uranium, they found major pathological symptoms took place in the gill alterations including hyperplasia of

chloride cells. In other study accomplished by Vasanthi, (2013) on *Mugil cephalus* which reported noticeable alterations observed in liver like large lipid droplets and effects on gills represented as in increasing in mucus cells. In addition study of Hassanain et al., (2012) noticed deformities in gills and spinal column of *Oreochromis niloticus* after exposure to sub lethal concentration of lead acetate.

2.5. Bioaccumulation of Heavy Metals in Tilapia Fish at Different Ponds

An important biological characteristic of fish is their tendency of bioaccumulation of metals in their tissues. Bioaccumulation is a very important aspect in hazard evaluation strategies; furthermore, fishes have the ability to collect elements from water environment to the highest level, and therefore bioaccumulation of metals is considered as an evidence of metal pollution index (Osman, 2012). As humans are on the top of food chain, it is highly possible for them to get contaminated with high levels of heavy metals by consumption of polluted foods (WHO, 2012). The level of a metal in water and the period of subjection, are the main aspects for the accumulation of heavy metals in the tissues of aquatic organisms. Apart from these, salinity, pH, hardness and temperature of water are few other factors that have affect the collection of metals (Carvalho and Fernandes, 2006; Costa et al., 2009; Alhashemi et al., 2012; Mohan et al., 2012).

Fish is a significant bio-indicator in fresh water system for the estimation of metal pollution level because it is optimum size for analysis and easy to be obtaining in large quantity, liable to accumulate metals, long life span, moreover, fish can quickly response to environmental changes (Batvari et al., 2007). So a lot of global studies have focused on the issue of the use of fish as biomarker to examine the existing pollution level by these environments such as study of El-Sadaawy et al. (2013) in *Tilapia*

niloticus to evaluate the accumulation of heavy metals in different tissues (gills, liver, heart, bone, brain, muscle and skin) in order to assess their potential risk to consumers and fishermen in Egypt. Abdel-Baki et al., 2011) had also evaluated the level of certain heavy metals (Cd, Cu, Pb, Hg, and Cr) in water, sediment and organs of *Tilapia nilotica* from Wadi Hanifah in Riyadh, Saudi Arabia. Authman et al.(2012) have examined status of concentrations of (Cd, Al, Hg, Pb and Ni) in water and some organs (spleen, kidney and muscles) of *Oreochromis niloticus* fish collected from illegal fish farms, Egypt; as well as comparing their findings with the maximum permissible international limits in fish for human consumption.

Previous studies in Malaysia focused on the measurement of heavy elements in organs of gills, liver, and muscles in different fish including tilapia. For instance Mokhtar et al. (2009) conducted a study on heavy metals concentrations (Cd, Cu, Fe, Cr, Ni, Pb, Mn and Zn) in tilapia fish (*Oreochromis* spp.) collected from highly stocked aquaculture ponds in Bandar and Jugra, nearby the Langat estuary Malaysia; they evaluated bioaccumulation of these metals based on the studied Index of Metal Pollution; their findings showed that the index was lower than their maximum levels in food. Low et al. (2011) also measured levels of heavy metals (Cd, Cu, Mn, Co, Zn, Fe, Se, As, Pb, and V) in muscles, liver, and gills of (*Oreochromis* spp) collected from three different aquaculture production sites in Jelebu, Malaysia; in general, they found level of Cu in liver was higher than those in muscles and gills, whereas Mn and Pb were higher in gills while high As was detected in muscles. Taweel et al. (2011) have determined concentrations of (Cu, Cd, Ni, Cr, Zn and Pb) in three different organs gills, liver, and muscles of tilapia fish *Oreochromis niloticus* in natural habitat and cultured ponds sites at the Bangi area, Selangor, Malaysia; their results showed that heavy metal concentrations in the tissues varied significantly depending on the locations of sampling

as well as, detecting higher concentrations of heavy metals in the liver followed by the gills and muscles. Taweel et al., (2012) have evaluated metals levels which compared within and between liver, gill and muscle tissues of tilapia fish *Oreochromis niloticus* collected from four local markets; as well as Taweel et al., (2013b) examined distribution of metal concentrations (Cd, Cu, Zn, Ni and Pb) in three parts (gills, liver and muscles) of tilapia fish *Oreochromis niloticus* from the Langat river and engineering lake in Bangi, Selangor; in addition, they estimated the health risk from these heavy metals, they concluded that muscle samples were classified in one of the safest level for human consumption. Furthermore, different studies on other fish species such as Ashraf et al. (2012) determined the levels of As, Cu, Pb, Sn, and Zn in different tissues of commercially important fish species that included Cyprinidae family collected from former tin mining catchment/Bestari Jaya; they showed high levels of tin, lead and zinc, while copper and arsenic at lower concentrations in most of their samples. Ismail and Saleh, (2012) concerted only on the accumulation of heavy metals in the edible part muscle of *Tilapia* sp., in view of the fact that it is the main fish part that is consumed by human beings. And study of Yin et al. (2012) whose estimated the level of heavy metals in selected organs gill, liver, muscle, kidney, bone, skin and gonads of Asian swamp eel *Monopterus albus* from paddy fields in Kelantan, Malaysia; they showed that gill had the highest level of lead, cadmium and nickel, furthermore zinc was the highest concentration in the liver, kidney, gill, skin and muscle.

Hishamunda et al. (2009) mentioned that studying of fish in Malaysia is very important in view of the fact that the consumption of seafood in Malaysia is more than triple that of the world average. Idriss and Ahmad, (2012) reported that sediments of Langat (Johor straits) and Juru (Penang) Rivers in Malaysia are polluted by Cd, Zn and Pb; the concentrations of the latter two metals are double and triple that of the international

limits.

CHAPTER 3

ACUTE TOXICITY AND BIOACCUMULATION OF HEAVY METALS ON RED TILAPIA FISH *OREOCHROMIS* SP.

3.1. INTRODUCTION

Metal pollution has been an environmental issue in many developed and developing countries for decades, and there is a substantial need to understand the bioaccumulation and toxicity of metals in aquatic organisms (Wang and Rainbow 2008). Heavy metals are hazardous pollutants largely found in waste water of industries. They have a

significant ecological impact on the ecosystem and can modify the physical and chemical properties of water affecting the aquatic flora and fauna (Körbahti et al., 2011; Tovar-Gómez et al., 2012)

Among the metals, mercury (Hg), arsenic (As), lead (Pb) and cadmium (Cd) are classified as potentially toxic heavy metals because they are very harmful, even at low concentrations, when ingested over a long time period. Metals are non- biodegradable and are considered as major environmental pollutants causing cytotoxic, mutagenic and carcinogenic effects on animals (Sana et al., 2009 and Ahmed et al., 2013); as well as accumulation and immunotoxicological effects on fish (Guardiola et al., 2013). Heavy metals such as Cu and Zn are essential for fish metabolism but in elevated levels, they tend to accumulate in fish body and later forms threats to human health through food chain. Some others such as Pb and Cd dos not play a role in biological systems (Moraes et al., 2003).

Among various metals, due to the possible dangers posed to aquatic organisms, few heavy metals such as mercury (Hg) has gained exclusive consideration. This element is classified as one of the most toxic metals, which are introduced into the natural environment by anthropogenic sources (Buhl, 1997). Basically, mercury is released into the atmosphere through a number of sources such as surface water and soil from pulp and paper, chlorine factories, electrical industries, combustion of fossil fuels. Apart from these human activities are also considered responsible for the mercury contamination (Friberg and Vostal, 1974). Hg^{+2} has no known role in biological systems. It is considered as an inessential, imperishable and lasting heavy metal and the amalgamations of Hg are extremely poisonous. Additionally, constant low-level exposure towards Hg, might result in serious health complications, which is categorized as carcinogenic and mutagen (DiFrancesco and Robert, 2002; Zahir et al., 2005).

Generally, industries are one of the main sources of releasing inorganic mercury into the atmosphere, which creates an intensive impact on fish tissues as opposed to the organic form of mercury (Sunderland and Chmura, 2000; Oliveira-Ribeiro et al., 2002).

The toxicity of metal is a stress factor for fish, but the physiological responses including ion changes, osmoregulation, water balance, and growth inhibition in fish during short-term exposure to high dosages can be alleviated by a variety of acclimation mechanisms. The level of stress depends on the concentration and the exposure time to heavy metals (Wu et al., 2003). Toxic substances dissolved in water often increase the sensitivity of aquatic organisms to temperature variations and make changes in the dissolved O₂ (da Silva and Martinez 2014). In acute tests experiments, one of the commonly used measures is the lethal median concentration that causes mortality in 50% of the test organisms abbreviated LC₅₀ (Straus, 2003; Chen et al., 2012).

Heavy metals have potential threat to organisms which attribute to high toxicity. Aquatic organisms including farmed fish have the ability to accumulate these metals in tissues directly from the ambient water or by ingestion of food that become potentially toxic when the accumulation increases to considerably high level (Tsai et al., 2013; Leonard et al., 2014). The level of accumulation in distinct organs depends on uptake and elimination rates which are different from one tissue type to other; subsequently, metal accumulation in fish has produced damage to organ structure (Giari et al., 2007). Fresh water fish mainly absorb waterborne metal through their gill epithelia. Hence, gills are the first target organs of xenobiotics. Once inside the organism, then the metal enters the blood circulation to reach other organs and significantly accumulates in kidney, followed by liver and gills (Pretto et al., 2011). Accumulation of heavy metals in a tissue is mainly dependent on water concentrations of metals and exposure period; in

addition, some other environmental factors such as salinity, pH, hardness and temperature play significant roles in metal accumulation (Has-Schon et al., 2007).

The objectives of this study are to investigate the acute toxicity effect of different concentrations of Cd, Cu, Mg, Hg, Pb, and Zn on the survival of hybrid tilapia in laboratory environmental conditions and to quantify the accumulation levels in the fish tissues of muscles, gills, liver with short exposure period.

3.2. MATERIAL AND METHODS

3.2.1. Acute Toxicity Assay

The study was carried out with hybrid tilapia (*Oreochromis* sp.) under different concentrations of heavy metals. The healthy fingerlings (7 ± 1 g body weight and 7.5 ± 2 cm total length) were collected from a commercial aquaculture in Serendah, Selangor 48200 Kuala Lumpur, Malaysia. Acclimatization was done in groups of 25 in a 50-L glass aquarium (60 L capacity; 60 cm×35 cm×40 cm) system containing UV sterilized (EHK-UVC) filled with de-chlorinated tap water for one week; with a pH of 7.6 ± 0.06 , and maintained at a temperature of 26.5 ± 2 C°, salinity (0.085 ± 0.022 g/L). Water was kept oxygen saturated by aeration at dissolved oxygen 7.0 mg/L. A dry commercial food pellets with 25% of crude protein was provided to feed fish during this period. Thereafter, fingerlings were transferred to 5-L (20 x 20 x 40 cm) test containers/glass aquarium for toxicity assay. Air pumps and individual air stone diffusers were provided for well aeration. The stock solution (1000 mg L^{-1}) of Cd, Cu, Mg, Pb, and Zn was prepared from analar grade of CdSO₄, CuSO₄, HgCl₂, MgSO₄, Pb(NO₃)₂, and ZnCl₂. Cadmium, Cu and Zn was used at rates of 0, 0.5, 1, 3 and 5 mg L⁻¹ while Mg was used at the rate of 0, 1, 3, 5 and 10 mg L⁻¹ and Pb was used at the rate of 0, 11, 13 and 15 mg

L⁻¹. In addition, a series of six concentrations were prepared by adding a calculated volume from the stock solution with local tap water into test containers.

The Tilapias were semi-statically exposed to different concentrations (control (0), 0.1, 0.3, 0.5, 0.7, 0.9 and 1.2 ppm) of mercury metal during 96 hours (range determined by preliminary tests) with three simultaneous replicates. Each metal was prepared by adding a calculated volume from the stock solution into test containers considering an equivalent of respective heavy metals. The experiments were conducted at light: dark condition of 16:8h and 26±2°C for 24, 48, 72 and 96h. Individual experiment has been conducted for each metal with different concentration and exposure period. A stocking density of 10 fish per aquarium/container was used against each metal.

The experiment was carried out under a completely randomized design with three replications. No food was supplied for fish during experimental period. Test solutions were replaced by fresh ones of the same respective concentration at every 24h interval until 96h exposure (APHA *et al.*, 1999). Fish mortalities were recorded at 6, 12, 24, 48, 72, and 96h exposure, and dead organisms were regularly removed from the test solutions. The aim of the test was to determine the median lethal concentration (LC₅₀) which was estimated by the probit transformed concentration response curves (USEPA, 2002).

3.2.2. Bioaccumulation Test

Juvenile hybrid tilapia fish was exposed to various concentrations of Cu, Cd, Zn, Mg, Pb and Hg. The median lethal time (LT₅₀) was determined through higher concentrations of each toxic metal with different exposures at 15, 18, 53, 48, 96 and 21h, respectively. The active fish was collected and dissected into gills, liver and muscles (dorsal surface of the fish) by using stainless steel knife (scalpels). The tissues of fish organs were then

dried in an oven at 105°C for 24 hour to be consistent in using . The dry samples of each organ were grounded using a porcelain mortar and pestle. From each sample, muscles, gills and liver tissues were digested by using closed vessel (Nguyen *et al.*, 2005, Uysal *et al.*, 2008) in a microwave oven (Milestone model Start D, Italy) for analysis. The samples were digested by adding 6 ml nitric acid (65 %) and 1ml H₂O₂ (35%). A ramped temperature control program was applied at 150°C during 15 minutes followed by 15 minutes at 150°C and 10 minutes cooling down in the microwave until they reached to room temperature. The residues were then dissolved and diluted to 50 ml for muscle and gill and 25 ml for liver sample in deionized water. Then, Whatman filter paper (0.45 µm) was used to filter the samples. The concentration of heavy metals in fish samples were determined by ICP–OES (Perkin Elmer AA Analyst). All glassware were soaked in nitric acid for 3 days and rinsed with deionized water before being used (Csuros and Csuros, 2002). The instrument was calibrated with chemicals standard solution prepared from commercially available chemicals. Standard stock solutions of Cd, Cu, Pb, Mg and Zn were prepared from titrasol (1000 mg/L) and mercury analysis was performed by Flameless Atomic Absorption spectrophotometer (AOAC 1998). Standard stock solutions of mercury Hg were prepared from Titrasol 1000 mg/L. The working solution was freshly prepared by diluting an appropriate aliquot of the stock solution. The certified reference materials DORM-2 was used as quality control samples.

3.2.3. Data Analysis

LC₅₀ at 24, 48, 72, 96 hour exposure values were calculated by probit analysis (USEPA, 2002). Data was statistically analyzed and carried out to variance analysis (ANOVA) using SPSS Package Program. Further statistical validity of the differences among treatment means was evaluated at $P < 0.05$ levels.

3.3. RESULTS AND DISCUSSION

3.3.1. Median Lethal Time and Median Lethal Concentration:

Clinical signs of tilapia, affected by mercury exposure were observed in the first experimental session, mainly at the higher concentrations (0.7, 1.2 and 1.4 mg Hg L⁻¹). The following aspects were identified hyperactivity and aggressiveness followed by respiratory distress and death. Similar behaviours have also been reported by Ishikawa et al. (2007) in *Oreochromis niloticus* exposed to HgCl₂ (0.370, 0.740 and 0.925 mg Hg L⁻¹).

The LC₅₀ values of Hg within 24, 48, 72, and 96 hour recorded for *Oreochromis* sp. in the present study, with 95% confidence limits were 1.09 (0.92 - 1.40 mg/L), 0.75 (0.47 - 1.32 mg/L), 0.54 (0.12- 0.96 mg/L), and 0.30 (0.17- 0.44 mg/L), respectively (Table 3.1). Furthermore, the results show that the tolerance to mercury decreases with the increased time of exposure. The 96h LC₅₀ 0.30mgL⁻¹ was very similar to those estimated with Nile tilapia *Oreochromis niloticus* (0.24 mg Hg/L) (Kaoud and Mekawy, 2011); while it had higher value (1.15mg/L) in airbreathing fish *Channa punctatus* reported by Pandey et al. (2005), due to some differences in type of species. Moreover, older and larger aquatic organisms were more resistant to toxicants.

A safe concentration estimated in the present study (LC₅₀-96h × 0.01) was 0.003 mg L⁻¹. This value is very similar to those recommended by Malaysian National Water Quality Standards (DOE-UM, 1986), which has considered Hg level (0.0001 mg L⁻¹) as safe water quality requirement for fish. However, the recommended level is lower than the safe mercury concentration in this study.

The higher LC₅₀ values were recorded with Pb at rates of 17.7, 14.3, 13 and 11 mg L⁻¹ under 24, 48, 72 and 96 h exposure, respectively (Table 3.5). The lower LC₅₀ values were recorded with Cu at rates of 1.85, 0.9, 0.55 and 0.45 mg L⁻¹ (Table 3.2). The LT₅₀ and LC₅₀ values decreased with higher levels of toxic metal concentrations and exposure, respectively (Table 3.1 -3. 6). This corresponds with the study results which were obtained by Taweel et al. (2013) revealing that the LT₅₀ for measured Cu concentration 0.46, 0.96, 2.14 and 3.5 mg/L were 170, 146, 46, and 20 h, respectively. On the other hand, Pb LT₅₀ for 0.12, 0.71, 3.3, 5.4 and 9.81mg/L were 177, 144, 79, 52, and 24, respectively. The results also have shown an opposite relationship between LC₅₀ and exposure time; increase in the concentration reduces the time to kill 50% of tilapia fish (*Oreochromis niloticus*) and it was reported that the LC₅₀-96h values 1.5 for Pb, 1.09 for Cu and 16.17 mg/L for Zn.

Table 3.1. Median lethal concentration (LC₅₀) and median lethal time (LT₅₀) of Mercury in red tilapia, *Oreochromis* sp.

Exposure Time (hour)	LC ₅₀ (mg Hg L ⁻¹)	95% Confidence Limit (mg Hg L ⁻¹)	Concentration mg.L ⁻¹	LT ₅₀ (hour)
24	1.09	0.92 - 1.40	0.3	>96
48	0.75	0.47 - 1.50	0.7	58
72	0.54	0.12- 1.56	1.2	21
96	0.30	0.17- 0.44	1.4	13

Table 3.2. Median lethal concentration (LC₅₀) and median lethal time (LT₅₀) of Copper in red tilapia, *Oreochromis* sp.

Exposure Time (hour)	LC ₅₀ (mg Cu L ⁻¹)	95% Confidence Limit (mg Cu L ⁻¹)	Concentration mg.L ⁻¹	LT ₅₀ (hour)
24	1.85	0.77-1.99	0.5	79
48	0.9	0.3-1.2	1	44
72	0.55	0.4-0.8	3	15
96	0.45	0.09-0.7	5	10

Table 3.3. Median lethal concentration (LC₅₀) and median lethal time (LT₅₀) of Cadmium in red tilapia, *Oreochromis* sp.

Exposure Time (hour)	LC ₅₀ (mg Cd L ⁻¹)	95% Confidence Limit (mg Cd L ⁻¹)	Concentration mg.L ⁻¹	LT ₅₀ (hour)
24	3.8	2.1-4.2	0.5	170
48	1.58	1.0-2.4	1	83
72	0.9	0.84-1.54	3	31
96	0.7	0.4-0.81	5	18

Table 3.4. Median lethal concentration (LC₅₀) and median lethal time (LT₅₀) of Magnesium in red tilapia, *Oreochromis* sp.

Exposure Time (hour)	LC ₅₀ (mg Mg L ⁻¹)	95% Confidence Limit (mg Mg L ⁻¹)	Concentration mg.L ⁻¹	LT ₅₀ (hour)
24	22	16-26	1	>96
48	11.3	8.2-13.5	3	114
72	5	4.3-7.0	5	74
96	3.74	1.9-4.8	10	48

Table 3.5. Median lethal concentration (LC₅₀) and median lethal time (LT₅₀) of Lead in red tilapia, *Oreochromis* sp.

Exposure Time (hour)	LC ₅₀ (mg Pb L ⁻¹)	95% Confidence Limit (mg Pb L ⁻¹)	Concentration mg.L ⁻¹	LT ₅₀ (hour)
24	17.7	15.6-30.2	11	96
48	14.3	12.4-18.4	13	75
72	13	8.1-14.4	15	46
96	11.6	7.4-13.1		

Table 3.6. Median lethal concentration (LC₅₀) and median lethal time (LT₅₀) of Zinc in red tilapia, *Oreochromis* sp.

Exposure Time (hour)	LC ₅₀ (mg Zn L ⁻¹)	95% Confidence Limit (mg Zn L ⁻¹)	Concentration mg.L ⁻¹	LT ₅₀ (hour)
24	17.3	10.4-25.0	0.5	>96
48	11	8.6-13.9	1	215
72	5.62	4.1-7.5	3	94
96	2.10	1.8-2.9	5	53

Our observed LC₅₀ values at 96 h exposure are in agreement with the findings of Subathra and Karuppasamy (2008). They reported that heavy metals toxicity of *Mystus vittatus* fingerlings LC₅₀ for Cu was 18.6 ppm under 96-h exposure whereas Othman *et al.*, (2010) found that *Rasbora sumatrana* (cyprinidae) was (0.005ppm) for Cu and (0.101ppm) for Cd. Cu²⁺ is an essential element in a variety of cellular processes but is toxic at excess levels and it was found that acute or chronic exposure to Cu²⁺ has an effect on ion balance via a decline in ion uptake of Na⁺, K⁺, Ca²⁺, and Cl⁻ in juvenile or adult fish of *Oreochromis mossambicus* (Wu et al., 2003).

The fish toxicity in these studies was in the following order: Hg> Cu > Cd > Zn > Mg > Pb. The toxicity of heavy metal differed among test organisms, which was attributed to

several factors such as the mechanism action of different metals, chemical characteristics of the test solution and sensitivity or the tolerance of test organism (Otitolaju and Don-Pedrok, 2002; Straus et al., 2003). The LC₅₀ values indicated that Hg and Cu ranked most hazardous among tested heavy metals and caused significant mortality followed by Cd. Similar results were reported by Grosell et al. (2002) showing that acute toxicity effect of Cu on rainbow trout gills inhibited branchial Na⁺ and Cl⁻ uptake that leads to mortality. Gundogdu (2008) found that Cu ion concentrations were more toxic than Zn for rainbow trout fish.

Cadmium LC₅₀ was very little and it relies on the type of fish while it was higher in the study of Yilmaz et al. (2004) on the guppy (*Poecilia reticulata*) exposed to different cadmium chloride concentrations, they found that 96- h LC₅₀ value with 95% confidence limits as 30.4 (29.3-31.7) mg/l. Cd can rapidly cause physiological changes in the gills and kidneys of freshwater fish (*Prochilodus lineatus*) specially inhibits the active uptake of Ca²⁺ which is necessary to maintain plasma homeostasis and essential for the growth of young individuals through the gill epithelia and interferes with the metabolism of this ion, with the loss of the fish ability to regulate the levels of calcium in the blood (da Silva and Martinez, 2014). Al-asgah et al. (2015) mentioned that the effect of exposure to cadmium caused an increased activities of antioxidant enzymes in fish (*Oreochromis niloticus*); and when the metal concentration increased more than the capacity of natural detoxifying systems a various adverse effects were appeared which led to mortality.

Tilapia fish had a higher sensitivity to Zn during 96-h (LC₅₀ at 2.10 ppm) (Table 3.6). These results are consistent with the study of Rema and Philip (2012) and they found

4.2 ppm in *Oreochromis mossambicus* while Firat and Kargin (2010) on *Oreochromis niloticus* was more tolerant to Zn under 96-h at LC₅₀ with 60 ppm.

Gündoğdu (2008) reported various effects of zinc in rainbow trout at different hardness, pH and temperature levels, and noted a low level toxicity at high hardness and low pH values. He illustrated that LT₅₀ and LC₅₀ values varied according to water conditions, such as temperature, pH, hardness, dissolved O₂, the size and species of fish as well as the type of heavy metals. The LT₅₀ values in 15 and 27 mg/l Zn concentrations were reported at 96 and 10 h, respectively. In addition, the LT₅₀ values in 0.1 and 2 mg/l Cu concentrations were 96 and 22 h, respectively. LC₅₀ -96 h Zn was 12.8 (9.81 – 15.94) and Cu was 0.094 (0.05 – 0.13) (mg/l). This difference in LC₅₀ values might be caused by the different metal compounds used in the studies and environmental conditions and may also be due to different ratios of carbonate hardness to total hardness; the effects in hard water were found to be lower than that of soft water.

Magnesium showed a significant effect of mortality, although it is an important constituent of many enzymes and also supports the bone growth, nerves function, the high concentration of salts especially magnesium sulfate and magnesium citrate is not completely absorbed in the intestine which creates a hypertonic condition that causes diarrhea and dehydration (Csuros and Csuros, 2002).

The relationship between LC₅₀s and exposure times for red tilapia fingerlings was shown in Table (3.7). It is clear that the Pb gives the highest concentration among all metals. This reflects that Pb has less toxicity as compared with other metals, whereas mercury has the strongest toxicity effect on red tilapia. In the present study, tilapia showed a low sensitivity to Pb ion which could be due to a function of metallothionein

(MT) synthesis. Cheung et al., (2005) explained a protective role by metallothionein against toxic effect of metal in fishes. Furthermore, the low toxicity of Pb ion can be observed. Otitolaju and Don Pedrok (2002) reported that free inorganic ion Pb^{+2} is not lipid soluble which causes transfers across membranes. However, Pb with higher

Metals	LC ₅₀ (mg/L) after different exposure			
	24h	48h	72h	96h
Hg	1.09	0.75	0.54	0.30
Cu	1.85	0.9	0.55	0.45
Cd	3.8	1.58	0.9	0.7
Zn	>5	> 5	5	2.10
Mg	>10	10	5	3.74
Pb	>15	14.3	13	11

concentration can create toxic effect by binding to the thiol (SH-) chemical group in the enzymes which are necessary for respiration (Csuros and Csuros, 2002).

LC₅₀ values depend on environmental factors such as pH, total alkalinity and total hardness Straus (2003) demonstrated an increase in typical acute toxicity response of blue tilapia fingerlings (*Oreochromis aureus*) which were exposed to copper (Cu) in a series of static toxicity tests with the decreases of pH, total alkalinity and total hardness; and estimated that the LC₅₀-96h values for Cu in different values of alkalinities in water (225, 112, 57, and 16 mg/L CaCo) were (43.06, 6.06, 0.69 and 0.18 mg/L Cu), respectively.

Table 3.7.Median lethal concentrations (LC₅₀) of heavy metals in red tilapia fish under different exposure time

Fish mortality increased with higher concentration and exposure of heavy metals. This was possibly due to the fact that these toxic metals have direct influence on respiration process of tilapia fish. Similar results were demonstrated by Chen et al., (2012). The heavy metal toxicity is ascribed to the fall in the diffusing capacity of the gill, the decrease of oxygen tension and consumption, the physiological imbalance, restlessness, the fall in blood pH, the increased gill ventilation, the opercular movement, the breathing rate and the concentration of metabolic products. Furthermore, smaller-sized species are more sensitive to acute toxicity of heavy metals than the larger ones (Grosell et al., 2002). The metals concentrate in the cell membranes and causing lysis as well as biotic concentrations activate certain enzymes which participate in metabolic synthesis of the organic compounds in fishes may influence the survival (Tan et al., 2008; Fidan et al., 2008).

3.3.2. Bioaccumulation of Toxic Metals

Bioaccumulation of metals in red tilapia fingerlings increases with the additions of metal concentrations in water and recorded significant differences (ANOVA, $P < 0.05$) in all studied metals as compared to control fish. Accumulation of toxic metals in fish tissues was significantly affected by treatment variations (Table 3.8). Regardless of fish organ, the highest accumulation was recorded from higher concentration of metal except Cd and Mg. Toxic metal concentrations was higher in liver than gill followed by muscle for the fish exposed to heavy metals. Among different organs liver obtained the highest accumulation of Cu and Zn. Gill tissues were recorded as higher levels of Cd, Mg and Pb at rates of 1, 3 and 5 mg L⁻¹, respectively. The metal accumulation among fish organs were in the following order: liver > gills > muscles for Cd, Cu, and Zn while gills >

liver > muscles for Mg and Pb. The maximum level of toxic metals accumulation was observed in liver (72 mg kg⁻¹ for Cd 136 mg kg⁻¹ for Cu and 423 mg kg⁻¹ for Zn) as compared to other organs (Table 3.8). The levels of metal increased with higher exposure among all heavy metals except Mg and Cd.

Tilapia fish has a greater capacity for metal bioaccumulation due to low sensitivity to some heavy metals (Mokhtar et al., 2009). This is similar to the findings of the study of Taweel et al. (2013) who have reported that Zn and Pb were the most accumulated and Cu was the least accumulated to tilapia fish (*Oreochromis niloticus*) they also found that bioaccumulation factor of Cu, Cd, Pb, and Zn by this fish which appeared within a wide range was 79, 774, 374 and 26 times more than control concentration, respectively, after 96 h exposure for maximum metals concentration used.

Table 3.8. Bioaccumulation (mg kg⁻¹ dry wt.) in muscles, gill and liver of red tilapia *Oreochromis* sp. under different concentrations of heavy metals and exposure

Toxic metals	Fish organs	Concentration (mg L ⁻¹)				
		0.0	0.5	1.0	3.0	5.0
Cd	muscle	0.05e	0.34e	0.91e	1.36e	2.38e
	gill	0.33e	2.82e	22.06c	120.80a	32.59c
	liver	0.53e	20.58d	20.97d	35.70c	71.80b
Cu	muscle	1.35f	4.58f	2.50f	4.00f	--
	gill	6.33f	10.50e	6.35f	38.40d	--
	liver	19.38e	78.33c	117.50b	135.98a	--
Zn	muscle	11.50g	16.08g	18.09g	16.90g	30.80f
	gill	49.50f	40.26f	72.18e	105.38d	98.10d
	liver	93.92d	177.26c	184.96c	217.57b	422.78a
		Concentration (mg L ⁻¹)				
		0.0	1.0	3.0	5.0	10.0
Mg	muscle	508.00h	1370.22d	1370.56d	1486.67d	1248.52e
	gill	1259.33e	2101.21b	2143.47b	2789.37a	1695.88c
	liver	379.38h	836.93g	1043.55f	1129.93ef	833.41g
		Concentration (mg L ⁻¹)				
		0	11	13	15	
Pb	muscle	1.60f	24.62e	21.90e	44.75e	
	gill	3.21f	144.83cd	152.36c	268.32a	
	liver	6.68f	51.33e	106.03d	207.92b	

		^c (0)	^b 0.3	^a 0.7	^b 1.2
Hg	^b muscle	0.0008± 0.0002	0.055±0.057	0.164±0.018	0.024±0.018
	^a gills	0.004± 0.0002	0.357±0.023	0.679±0.141	0.526±0.082
	^a liver	0.004± 0.003	0.371±0.207	0.799±0.126	0.335±0.172

Values with same letter differ non-significantly (P>0.05)

The toxic metal accumulation in fish tissues depends on the concentration and exposure as well as other factors, such as interaction with other metals, water chemistry, and metabolic activity of fish (Heath, 1995). The results of the present research demonstrated that the exposure to heavy metals has an effect on bioaccumulation levels. Among the tested metals, Mg showed a lower effect on fish survival and was accumulated in higher levels. Toxic metals accumulation levels were in the following order: Mg > Zn > Cu > Pb > Cd in all organs. The results showed that the concentrations of most metallic ions were accumulated in lipid tissues especially in liver. Similar study was reported by Wong *et al.* (1981). They found that the accumulation of tetramethyl lead by rainbow trout could be due to the lipophilic properties of metallic compounds and were likely to be found partitioned into fish especially in the lipid tissue. The results of the present study are in agreement to the findings of Subathra and Karuppasam (2008). They reported that the accumulation of Cu in liver of control and tested fish was 12.36 and 82.12 mg/kg, respectively. Liver appears to be one of the most important sites for Zn accumulation in channel punctuates and principal site which represent storage of metal in the fish while the metal levels in the gills reflect the concentrations of element in the ambient water (Senthil et al., 2008). The high levels of accumulated heavy metals in liver may be attributed to the sequestering and binding of this metal by metallothionein (MT) (Montaser et al., 2010). Some of essential elements such as Cu are found in fish under homeostatic regulatory control and usually the normal range of Cu concentration is below 50 mg kg⁻¹ dry weight (Couture and Rajotte, 2003). But any impact or loss to mechanism of

homeostatic control will be over loaded; hence, Cu concentration in liver can increase (Subathra and Karuppasamy, 2008). Furthermore, the higher accumulation in liver may alter the level of various biochemical parameters and may also cause severe liver damage (Abdel-Warith et al., 2011).

Our results showed that the gills have ability to accumulate heavy metals (Table 3.8). This results support the findings of Karuppasamy (2004) who suggested that the accumulation of heavy metal in gills may be highly attributed to the large amount of water that passes through the gills to supply oxygen under stress of toxicity. The post-exposure results showed that Pb and Mg accumulations were relatively higher level in gills than other organs (Table 3.8). The possible reason could be due to the fact that Mg and Pb uptake was mainly from water rather than diet. Similar results were reported by Karatas and Kalay (2002) and showing that Pb in the gills tissues were higher than the liver or muscles of *Tilapia zilli*. Heavy metal obstructs gill epithelium absorption and allocates distribution of the element in blood (Firat and Kargin, 2010). Consequently the accumulation of Mg and Pb in gills tissues are expected to be directly correlated with the concentration of elements in surrounding water.

The present study showed that Cd at the rate of 3 mg L⁻¹ accumulated more in gill tissues than liver tissues. The gill accumulated more concentration of heavy metal than liver followed by muscle for short time exposure. Nevertheless, the heavy metal level was in liver > gills for long term exposure (Bervoets et al., 2001). Gills may be the first target for Cd accumulation before its distribution to other organs; because aqueous Cd ions which are in direct contact with gills may bind in a non-specific manner to the mucopoly saccharides (constituents of mucoproteins, which are glycoproteins) present on the outside of the gills and then, the metal is probably transferred to storage organs such as the liver or kidney at longer exposure periods (Qu et al., 2014).

Metal accumulation in the tissues of fish varies based on the rates of uptake, storage and elimination. Suggesting that the metals with high uptake and low elimination rates in the tissues of fish are expected to be accumulated to higher levels. Metal uptake is dependent upon the exposure concentration and period as well as other factors such as salinity and temperature.

The study by Kalay and Canly (2000) on *Tilapia zilli* showed that the accumulation of cadmium after 10 days exposure to the metal produced tissue cadmium concentrations in the order: gill > liver > muscle > brain. The gills were the only tissue that showed significant elimination of cadmium, copper and lead. The gill tissue is also exposed to environmental metals to a greater extent as compared to the other tissues and this might cause more accumulation and adsorption of the metals in or on the gill surface. Different accumulation levels of metals in different tissues may be primarily due to different metabolic activities. Tissues like liver, spleen, kidney and gills are highly active in fish metabolism and therefore, they may accumulate metals to higher levels than other tissues like the muscle. This may result in having greater metal accumulations than metal eliminations. Thus, it was suggested that once metals accumulate in tissues, it is difficult to eliminate them from the body, especially the non-essential metals.

The results in the present study revealed that the fish muscles contained lower concentration of metals as compared to other organs. This is in agreement with what was found by Guardiola et al., (2013) indicating that Cd concentration in muscle of sea bream (*Sparus aurata*) was very low to undetectable. Moreover, these levels in muscle decrease over the time suggesting that the Cd directly enters by the skin and not from the inside by the blood route.

The concentrations of mercury in different tissues (muscle, gills, and liver) of fish exposed to 0.3, 0.7, and 1.2 mgHg/L are given in Table (3.8); in *Oreochromis* sp., the mercury concentrations in muscle, gills and liver of control fish were 0.0008, 0.004, and 0.004 mg/kg, respectively. Relatively, similar levels have been found in the same tissues of the *Oreochromis niloticus* (0.0005, 0.001, and 0.005mg/kg) (Osman, 2012); and in goliath grouper *Epinephelus itajara* maximum mercury concentration have been recorded, ranging (22.68 µg/g) in liver with a mean of (0.63 µg/g) in muscle (Adams and Sonne, 2013).

After exposure to mercury (0.7 mg/l), the results showed that, the concentration of mercury increased in different tissues. The highest mercury accumulation was observed in liver (0.799 mg/kg), followed by the gills (0.679mg/kg), and the muscle (0.164mg/kg). A similar hierarchy of accumulation was observed in *Oreochromis niloticus*: in muscle (3.21mg/kg), gills (20 mg/kg), and visceral organs in abdomen (45.75 mg/kg, after exposing to 1 mg Hg/L for 3 days (Kaewamatawong et al., 2013). Hg concentration in control fish muscles was within the approved limits for human consumption and lower maximum level limit, which has been reported by Malaysian Food Act 1983 and Food Regulations 1985 (Ministry of Health Malaysia 2012); whereas, the metal content in muscles tissues of treated fish has significantly varied ($p < 0.05$) and is higher than in the control groups.

Metal concentration in the liver might originate from a progressive transfer of mercury from the gills to the liver via the blood (Firat and Kargin, 2010) However, the higher mercury concentration has been observed in the liver of post-exposure fish, with levels reaching up to 10 times higher than the values measured for control group due to their

strong binding with cystine residues of metallothionein (MT), where the lower molecular weight protein has high affinities for heavy metals, and its storage as a constituent of hepatic cytoplasm, trigger increased the accumulation of metal in the liver (Montaser et al., 2010; Yacoup, 2007). Similar finding was also obtained in Hg-contaminated fish *Gymnotus carapo*, after acute exposure to Hg^{+2} ; the highest mercury level was found in liver, followed by gills, and lowest concentration was observed in muscle (Vergilio et al., 2012). Muscle was found to accumulate small amounts of all the heavy metals, and might have received them through circulation. It is suggested that, the low accumulation of metals in muscle may be due to lack of binding affinity of these metals with the proteins of muscle. This is particularly important, because muscles contribute the greatest mass of the flesh that is consumed as food (Osman, 2012).

The toxic effects and accumulation of all forms of inorganic mercury are ascribed to the action of ionic mercury, because elemental mercury (Hg^0) cannot form chemical bonds. Nevertheless, ionic mercury exists in mercurous (Hg^{2+}) (Hg_2Cl_2) and mercuric (Hg^{2+}) (HgCl_2) forms. The mercurous ion is unstable and dissociates further into the mercuric ion and ionic mercury gradually forms complexes with SH group and other ligands in the tissues of the body. Only a very small fraction exists in the free form (Friberg and Vostal, 1974).

The toxic metal concentration in control fish muscles were within the approved limits for human consumption and lower maximum level limit which has been reported by Malaysian Food Act 1983 and Food Regulations 1985 (Ministry of Health Malaysia 2012) whereas, the metal content in muscles tissues of treated fish was significantly varied ($p < 0.05$) and higher than in the control groups. These results are similar to findings of Vinodhini and Narayanan (2008). They reported that heavy metals were

uniformly spread over the body muscles in lower ratios and this information can be used to estimate the biochemical measurements alteration in fish metabolism.

3.4. CONCLUSIONS

The results revealed that tilapia fish had a higher sensitivity to Hg and Cu are considered as the most hazardous among tested toxic metals followed by Cd and Zn. The red tilapia fish showed poor response to Mg and the least sensitivity to Pb. The juvenile hybrid tilapia fish is capable of accumulating heavy metals in their tissues from an aquatic environment and the ability of fish is another important factor to be considered for future studies. In addition these data constitute an important reference to assess the hazard of metal accumulation in fish tissues in the ecotoxicological testing scheme.

CHAPTER 4

HISTOPATHOLOGICAL CHANGES INDUCED BY TOXICITY OF HEAVY METALS IN RED TILAPIA FISH *OREOCHROMIS* SP.

4.1. INTRODUCTION

The aquatic environment has been continually subjected to numerous chemical contaminants such as heavy metals which have significantly polluted the water sources. Toxicity of metal happens when the amount of metal intake into the body surpasses the combined rate of excretion and detoxification of metabolically available metal (Rainbow, 2002). Aquatic animals have different capabilities to maintain their internal chemical composition, depending on the type of species and the physiological function of trace elements (Sloman, 2007; Qiu, et al., 2011). Generally fish populations are indirectly affected either negatively or positively, based on the direct metal toxicity at any trophic levels (Couture and Pyle, 2011).

Increasing the contamination of aquatic ecosystems by metals has caused various morphological, physiological and biochemical changes in aquatic organisms. Generally, heavy metals are potent toxins and their bioaccumulation in tissues leads to several damages including cellular and tissue damage, cell death and dysfunction of a variety of

organs. Therefore, the cellular and tissue damages as well as those related to the histopathology of the considered organs play a significant role in evaluating the toxic potential of contaminants regarding to the living bodies. These damages depend on various factor including the environmental conditions, levels of contaminants, exposure time and type of subjected organism (Oliveira Ribeiro et al., 2005; dos Santos et al., 2012). Metal pollution make damage in aquatic organisms at the cellular level and possibly affect the ecological balance. Histological changes are more sensitive and occur earlier than any other changes. They provide a better assessment technique of fish health (Zeitoun and Mehana, 2014)

Fish pathological changes are considered as the environmental pollution biomarkers which have been extensively applied in the programs of water quality monitoring; It should be noticed that in sensing the toxic influences of histopathological biomarkers, gills and liver pathologies analysis play an important role (Syasina et al., 2012). Generally the fishes contaminated by heavy metals suffer from histopathological alterations, with consequent inhibition of metabolic processes, including gills damage, which are considered as the most affected member, such as, hyper secretion of mucus. Moreover, ensuing mortalities are related to secondary physiological respiratory disturbance (Silva et al., 2012).

Exposure to heavy metals may cause histological changes in liver which plays an important role in vital functions of the metabolism. Furthermore, it is the major organ of accumulation, biotransformation and excretion of xenobiotic compounds with morphological alterations occurring in some toxic conditions (Younis et al., 2013; Figueiredo-Fernandes et al., 2007). The clinical histological alteration of the *Tilapia nilotica*'s liver which can be observed in the places polluted heavy metals are vacuolar, cloudy swelling as well as hydropic alteration of the hepatocytes along with significant

coagulative necrosis and serious congestion and hemorrhage (Abdullah et al., 2008). It can be also added that the side effects are degeneration of cytoplasm in hepatocytes as well as vacuolar hydropic that are lastly necrotic and infiltrated with inflammatory cells (Velcheva et al., 2010). However, under the same conditions, the histological changes of exposed aquatic organisms differ in severity, based on the type of organism and the concentration of chemicals (Greenfield et al., 2008; Triebskorn et al., 2008; Gehringer et al., 2013). Thus, it was found necessary to investigate the structural damage of gills and liver of the studied fish induced by acute toxicity of heavy metals in this research.

4.2. MATERIAL AND METHODS

Fingerling tilapias 7 ± 1 g in mean weight and 7.5 ± 2 cm in length were collected from a commercial aquaculture facility in Serendah, Selangor 48200 Kuala Lumpur, Malaysia. The fishes were divided in groups of 25 in 50 L glass aquarium (60 L capacity; 60 cm \times 35 cm \times 40 cm) system containing UV sterilized (EHK-UVC) filled with de-chlorinated tap water for one week; with a pH of 7.6 ± 0.06 , and maintained at a temperature of 26.5 ± 2 C°, salinity (0.085 ± 0.022 g/L). Water was kept oxygen saturated by aeration at dissolved oxygen 7.0 mg/L. Tilapia fish was acclimatized to laboratory conditions at a photoperiod of 12 h light and 12 h darkness for 14 days with daily feeding (once per day) of a dry commercial food (pellets with 25% of crude protein). Feeding was stopped 24 h before and during the actual experiment. Aquarium water was replaced every 24 h to reduce contamination from metabolic wastes. Fingerlings were then transferred to assay aquaria (20 x 20 x 40 cm) 5 L glass aquaria, which provided aeration via air pumps and air stone diffusers. Each group was at a stocking density of 10 fish /aquarium.

The chemicals product used in this study were purchased from analar grade of CdSO_4 , CuSO_4 , MgSO_4 , $\text{Pb}(\text{NO}_3)_2$, and ZnCl_2 and inorganic mercury chloride (HgCl_2) Analar BDH chemicals with 99.5% purity dissolved in double deionized water, to prepare the

stock solution (1000 mg.L^{-1}) of Cd, Cu, Mg, Pb, Zn and Hg, respectively. This stock solution was diluted to the desired concentrations with local tap water. The mentioned solutions were substituted with new solutions of the identical corresponding concentration at every 24h interval until 96h exposure (APHA et al., 1999).

Histopathological analysis was conducted on liver and gills of post-exposure fish to 96hLC_{50} . The median lethal concentration (LC_{50}) during 96 h of exposure was determined from the probit transformed concentration - response curves (U.S. EPA, 2002); The LC_{50} values of heavy metals within 96h recorded for *Oreochromis* sp. in the present study with 95% confidence limits as shown in Table (3.1-3.6) in the previous chapter determined by preliminary test (0.7 for Cd, 0.45 for Cu, 3.74 for Mg, 0.3 for Hg, 11 for Pb, and 2.1 for Zn). The tested concentrations chosen were 50% of the 96-h LC_{50} value from the acute toxicity test (Thophon et al., 2003) which were (0.35, 0.23, 1.87, 0.15, 5.5, and 1.05 mg/L) for metals, respectively; and fishes were exposed to sub-lethal concentration of $96\text{hLC}_{50}/10$ (0.03ppm) for 21 days for Hg metal. The test procedure was a semi-static system with continuous aeration over 96 h. No food was supplied during the experiment. Three replicates were performed for test concentration and control. Fish mortalities were observed daily. Three fish from each aquarium were sampled at 24, 48, 72 and 96 h of exposure.

Histopathological analysis was conducted on gills and liver from fishes which were exposed to sub-lethal concentration $96\text{hLC}_{50}/2$ over 96 hours. The fish anesthetized in ice cold water and sacrificed by cervical decapitation and then gill filaments treatments and liver uptake and being fixed in neutrally buffered formalin for 48hrs, afterward dried out in a graded ethanol series and inlayed in paraffin. Each block of tissue has been cut in to serial sections ($6\mu\text{m}$ thick) and stained with hematoxylin and eosin

(H&E) (Triebskorn et al., 2008). Later the tissues were tested for wide range of histopathological characteristics and lesions. After examining the tissues the digital images were obtained by using a light microscope Nikon type Eclipse E200, equipped with a Dino eye camera Ø30mm, employing 10x, 20x and 40x objectives.

4.3. RESULTS AND DISCUSSION

4.3.1. Effects of Exposure of Red Telapia to Hg Metal

The untreated gills showed a characteristic arrangement (see Fig. 4.1A), the gill comprised of four sets of gill lamellae, whose both sides had been reinforced by bony structure and primary lamellae. While viewed in vertical section, the secondary lamellae comprised several blood capillaries, which were segmented by single layered pillar cells. The laminar epithelium was thicker and accompanied by basement membrane, underneath the pillar cells which had enclosed the blood spaces. Many mucous cells on the epithelial gill rakers also observed; in contrast, the primary lamellae had relatively smaller and lesser number of mucous cells.

At the 24-96h Hg exposure points, the changes were seen in the mentioned organ under concentration (0.3ppm), demonstrating that few parts with focal proliferation like what can be seen in (Fig. 4.1B), infrequently lead in fusion of the neighboring secondary lamellas (Fig. 4.1C). Based on the epithelial cells, vacuolization after 96 h of exposure was observed. The fusion of the secondary lamellae may work to protect the affected gills, and thus helps to reduce the entry of the toxic substance, which increases suffocation and death of fish. This is in consistent with the study carried out by Silva et al. (2012) regarding predator fish *Hoplias malabaricus*.

The histopathology of experimented fish gill, treated with sub-lethal concentration (0.03ppm) has showed slight damage in the 10th day of mercury chloride exposure. Nevertheless, we have observed that the gill had sore in the epithelial layer due to the existence of mucous cells and vacuolation in gill membrane.

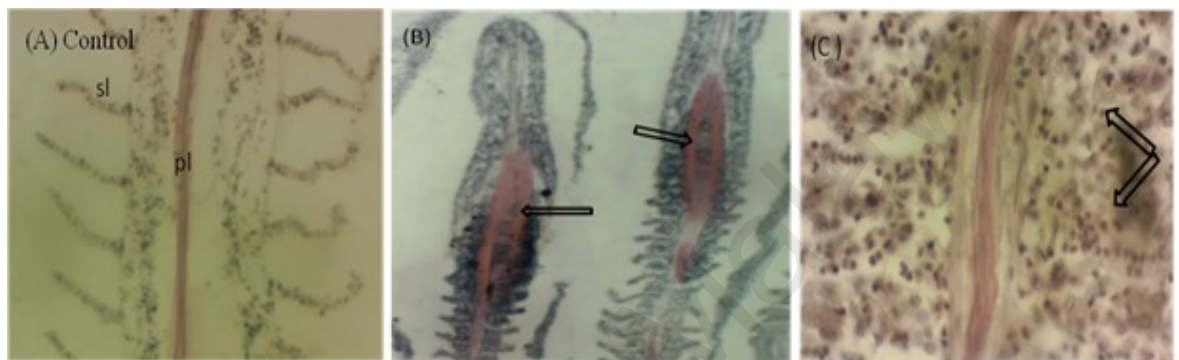


Figure 4.1: (A) Regular shape of primary lamellae (pl) and secondary lamella (sl) in gills of controlled *Oreochromis* sp. (B) some areas with focal proliferation in primary lamella (arrows) (C) fusion of adjacent secondary lamellas (arrows).

As can be observed from the Fig. (4.2A), the histopathology of fish gill which has been exposed to mercury for 3 weeks (21 days) shows a remarkable edema and influential secretion of mucous with an increased size though decrease in number. It was also observed that most of them were either vacuolated or almost empty and it was confirmed by the secondary lamellae that the harm of epithelial cells or lamellae have curled that makes blockage and gills hemorrhage (Fig. 4.2B). The gills of the experimented fish turned out to be reddish. This is consistent with the observations of Cerqueira and Fernandes (2002), in which it was shown that the gill of the tropical fish *Prochilodus scrofa* exposed to different concentrations of heavy elements, has become coated with a mucosa layer, due to a defensive reaction of the fish, against the presence of contaminants, which reduces the absorption of these pollutants through gills; and

consequently causes an increased amount of mucus, which affects the breathing process. Some of the observed histological and healthy changes in this study have also been similar with alterations that have been reported with contamination with other metals (Jalaludeen, 2012).

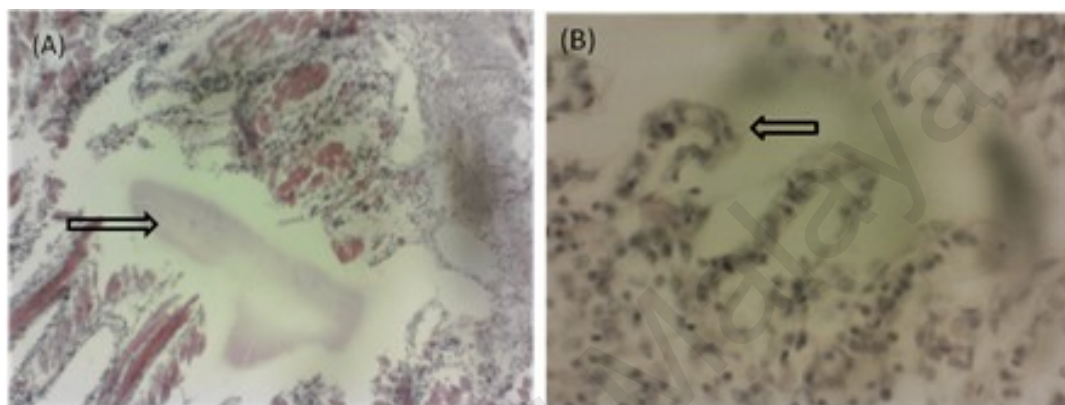


Figure 4.2: (A) the gill shows lesion in the epithelial layer with marked edema and active secretion of mucous in treatment with sub lethal concentration (0.03ppm). (B) Showing curled in secondary gill lamellae during 21 days in sublethal concentration (0.03 mgHg/L) (arrow X400).

Morphological changes regarding the secondary lamellae of the studies fish were effortlessly sensed even at the initial stages of the test. Therefore, while the exposure reached the time of 24 h, hypertrophied cells and changes on the surface of the secondary lamellae were observed. The existence of squamous epithelium and broad epithelial hyperplasia which was observed after 96 h, led to modifications of the structure of the secondary lamellae represented in the formation of an interlamellar bridge; this is similar to a study by Oliveira Ribeiro et al. (2000) who attributed this bridge to the fusion in the adjacent lamellae that caused reduction of the water space. According to Oliveira Ribeiro et al. (2002), dissolved inorganic mercury at $0.015 \text{ mgHgCl}_2 \cdot \text{L}^{-1}$ resulted in main morphological changes on respiratory lamellae lowering the capacity of the exchange of gas with the environment. The gills of fishes play vital

activities including respiratory, osmoregulation, and excretion functions. Furthermore the gills have close contact with the surrounding environment, and predominantly delicate to changes in the quality of the water, therefore, they are regarded as the primary target of the contaminants (Pereira et al., 2013).

Damage in epithelial membranes is the primary reaction of gills with variant pollutants, whereas mercury element pick-up charge Hg^{+2} , which is similar to many of ions charges Ca^{+2} and Mg^{+2} , and competing on the union and transit through the chloride cells, which are important in the process of ion balance, causing damage to those cells, affecting the process of osmotic regulation of the fish, and results in multiple damages, such as, electrolytic imbalances, disruption and necrosis in gill tissues, and thus resulting in a lack of oxygen uptake, and ultimately suffocation and death (Olivera-Ribeiro et al., 1996; Chang et al., 2003; Wu et al., 2008).

After 24 h, the changes in liver morphology were seen as exposed to intense concentration of 0.3mg/L Hg. Based on the observation of the untreated liver, typical compact structure in which the hepatocytes showed a feature cytoplasmic allocation as well as nuclear morphology were observed (Fig. 4.3A). The 24 h Hg treatment caused the mix-up of the hepatic tissue; moreover, serious lipid loss was distinguished through low-fat vacuolation in cytoplasm paired with cytoplasmic alterations and nuclear morphology (Fig. 4.3B). The treatments with 72h and 96h have shown that the vessels will be widened and congested. Those regions which have serious deterioration of liver parenchyma have been seen closer to the blood circulation, as well as, lymphocytic and macrophage infiltration in the liver (Fig. 4.3C). Furthermore, necrosis has occurred in the liver over 96h (Fig. 4.3D). Advanced micronecrosis was observed after 10 days of

exposure of sub-lethal concentration 0.03mgHg/L (Fig. 4.3E), moreover, small regions of necrosis under 21days exposure have also been observed (Fig. 4.3F).

When the fishes were subjected to severe exposure of inorganic Hg, the metabolism of those fishes has increased, due to the loss of the stored lipid substances in hepatocytes; furthermore alarming quick and primary response of the cells were observed, as well as, the liver alterations, including multiple necrotic sites; and these conditions are considered as potential biomarkers. However, histological alterations observed in the liver cannot be regarded as a distinguishing biomarker of mercury exposition though they are mainly related to the reaction of hepatocytes to toxicants. The mentioned system most probably shows that the liver is a fragile body part for assessing the harm after being exposed to pollutants (Senthamilselvan et al., 2011; Velcheva et al., 2010). In addition, these induced alterations are in agreement with other studies related to Hg contamination in fish liver (Oliveira Ribeiro et al., 2002, Raldu'a et al., 2007). Researchers focuses on toxicology have revealed that, the accumulations of contaminants might affect the plasma blood biochemistry, including activities of plasma enzyme, and directly cause cell damage in particular tissues (Yang and Chen, 2003; Fernandes et al., 2008).

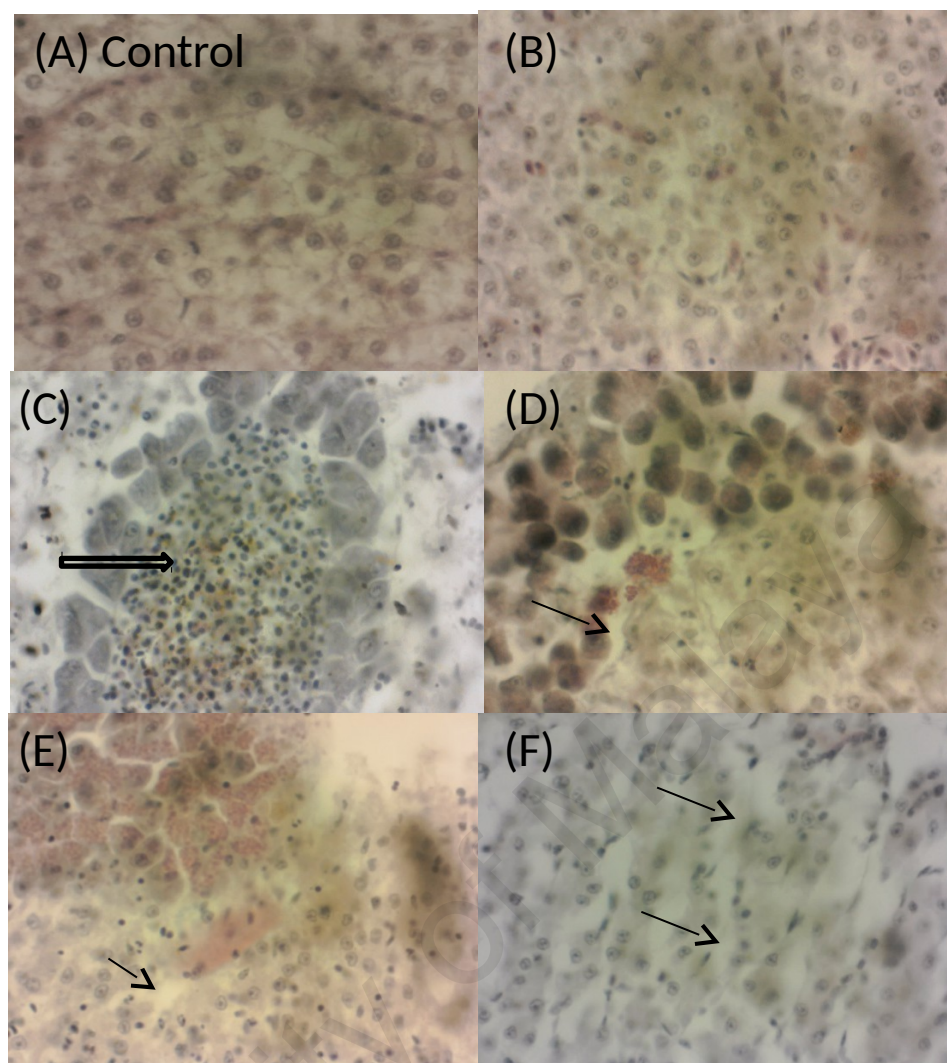


Figure 4.3 (A) Normal liver showing the normal location and morphology of the nucleus and the cytoplasm of the hepatocytes (X400). (B) The 24 h Hg treatment induced disorganization of hepatic cells; (C) The 72 h and 96 h treatments, showing areas with severe degradation of the liver parenchyma, leucocytic infiltration (arrows) (X400); (D) necrosis occurred in the liver over 96h; (E) micronecrosis after 10 days of exposure of sublethal concentration 0.03mgHg/L; (F) small regions of necrosis under 21 days exposure (arrows X400).(H&E)

This study focused on enhancing the knowledge of tissue damage of the organs of tilapia *Oreochromis* sp., such as, gills and liver, due to lethal and sub-lethal concentration exposure of waterborne mercury chloride. These results are highly influential factors in assessing the seriousness of toxicity, and are also important for the references of future studies.

4.3.2. Effects of Exposure of Red Telapia to Pb Metal

In the present study the untreated gills indicated carrying four pairs of gill lamellae whose sides were backed up with bony system of gill arch. The characteristic arrangement of primary and secondary lamellae is demonstrated in Fig.4.4 (A1, and B1). The secondary lamellae showed numerous channels of blood capillaries each separated by single layer pillar cells; chloride cells and mucous cells were located (Fig. 4.4C1). The gills of the fishes exposed after 72 and 96 h Pb shows proliferation and hypertrophy of epithelial cells which occasionally results in fusion in adjacent secondary lamellae (Fig. 4.4 A2). Bulb shape of the large pavement cells was found at 72h (Fig. 4.4B2) at the tips of the secondary lamellae as well as, increase in chloride cell density at 96 h exposure (Fig. 4.4C2).

These results are in agreement with the study of Tribskorn et al. (2008) which showed the increased large mucocytes at the tips of the secondary gill lamellae, cellular necrosis, cellular hypertrophy in *Leuciscus cephalus* exposed to heavy metals including lead element. Also, are in agreement with the study of da Silva et al., (2012); dos Santos et al., (2012) and Pereira et al., (2013) confirmed that gills are sensitive subjects for identifying under the effect of heavy metals on it by various histopathological alterations including hypertrophy and hyperplasia of epithelial cells , lamellar fusion, hyper secretion of mucous, and lamellar aneurysm.

The level of accumulation in distinct organs depending on uptake and elimination rates which are different from one tissue type to other; subsequently, metal accumulation in fish has produced damage to gill structure (Giari et al., 2007)

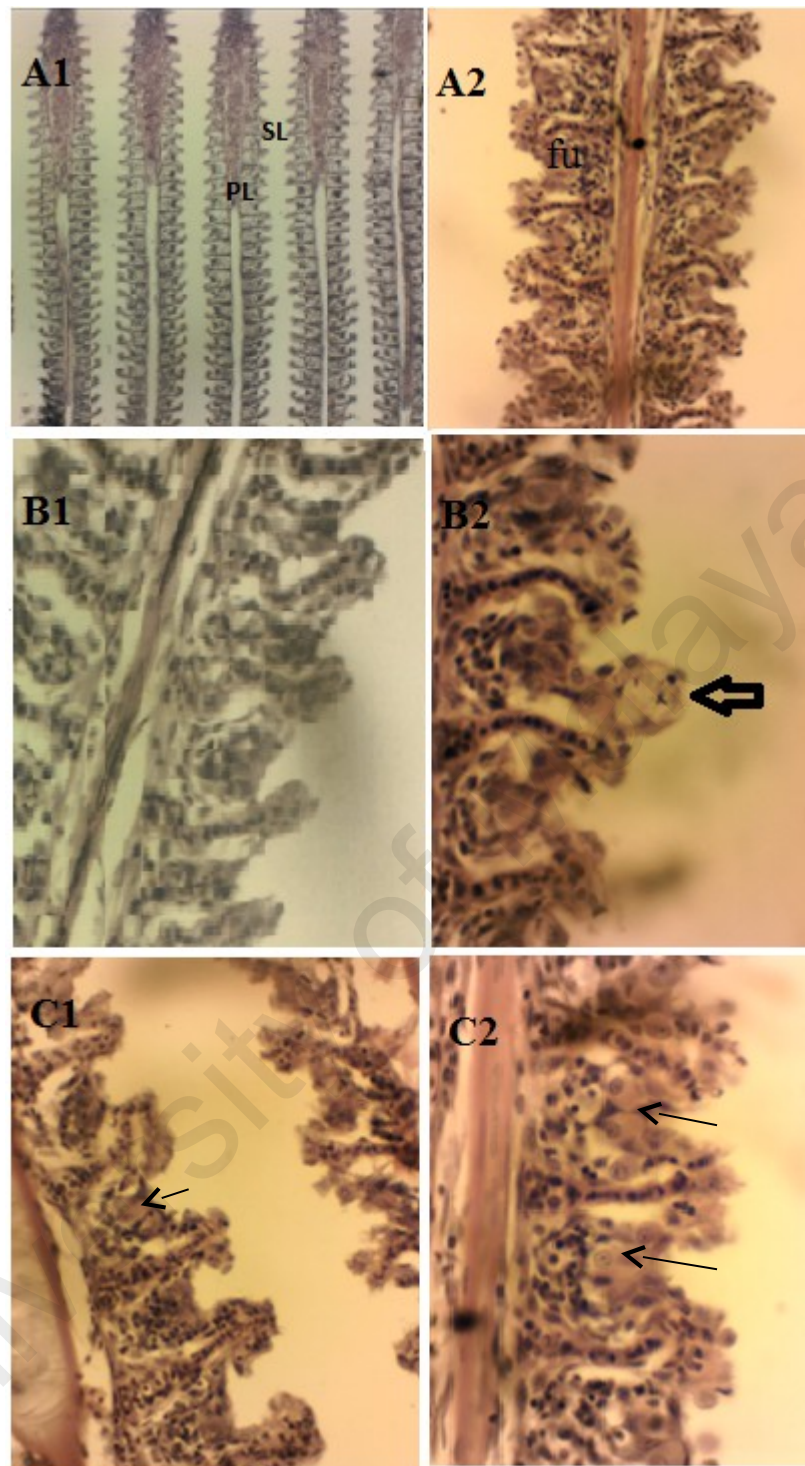


Figure 4.4. Microphotographs of the gill filaments of *Oreochromis* sp in control (A1, B1, and C1) and experiment (A2, B2, and C2). (A1) General view demonstrates the characteristic arrangement of primary (PL) and secondary lamellae (SL) in gills of control fish. (A2) Shows fusion of adjacent secondary lamellae (fu) in Pb exposed fish at 72 h (X200). (B2) Large pavement cells at the tips of the secondary gill lamellae at 72h (Arrow). (C2) shows presence an increase in chloride cell density (←) (x400) (H&E).

Liver tissue of treated fish showed deformities in tissue after 48 h exposure to lead ions.

The nucleus has shown an abnormal increase in the surface area of liver tissue with the

hypertrophy of hepatocytes. In addition necrosis occurred after 96 h exposure to lead (Fig. 4.5). This corresponds to the study of Olojo et al. (2005) in which they observed degeneration of the hepatocytes and focal necrosis in the liver of *Clarias gariepinus* exposed to lead that attributed to obstruct sinusoids lead to block the blood passage of the hepatic artery and interbiliary portal vein which has to pass through the sinusoids to the central vein; lack of blood's ability to gain access to the central vein on time makes the liver pump blood harder leading to liver stress.

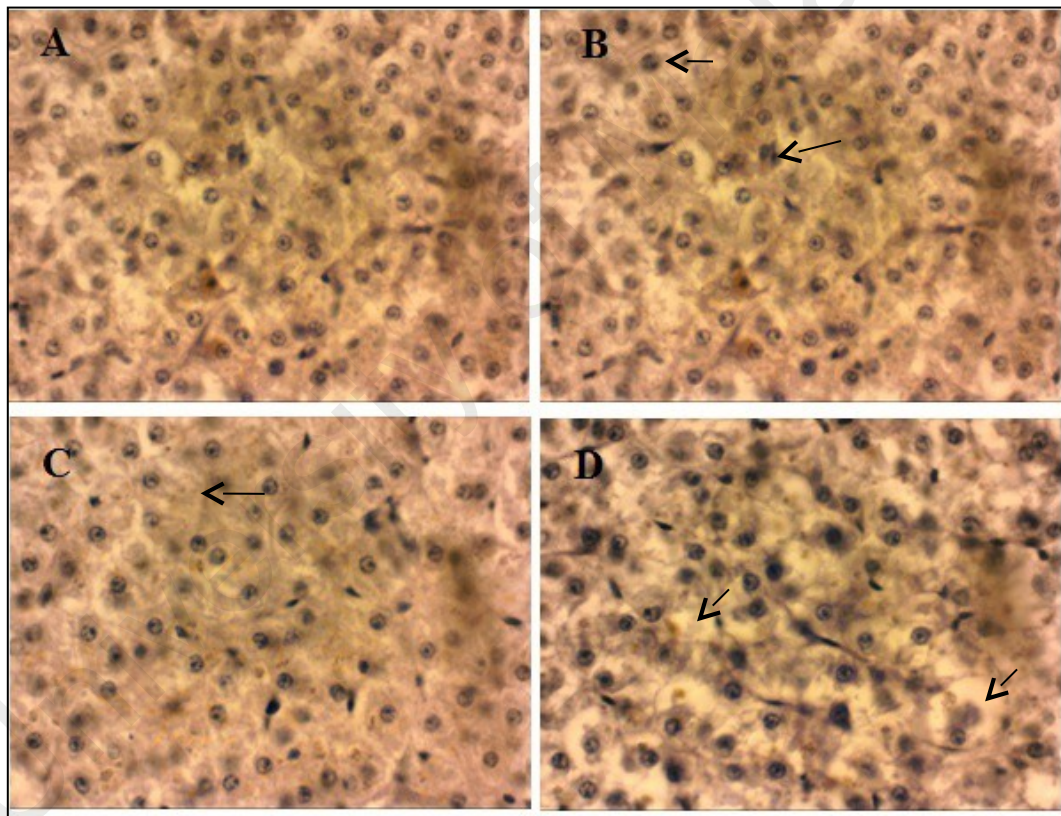


Figure 4.5. Liver tissue showing deformities in the tissue after 48 h exposure to lead (A). Nucleus and the nucleolus are shown conspicuously with an abnormal increase in the surface area of liver tissue (←) (B). Hypertrophy of hepatocytes has been occurred (←) (C) and necrosis in liver tissue after 96 h of exposure to lead (←) (D). X 400

This result also are in agreement with the study of Syasina et al (2012) in *Carassius auratus* reporting that liver histopathological changes in fish includes vacuolization and necrosis of hepatocytes and they demonstrated the relationship between the levels of pollution by heavy metals in the aquatic environment and the occurrence of toxicopathic damage to fish liver. Leads can be observed as putting its use whether biochemically or physiologically as a mimetic agent replacing for fundamental factors taking part in metabolism including zinc, calcium and iron. Particularly, it directly intervenes with iron and zinc in heme's biosynthesis, in the function sulfhydryl group rich protein enzymes or generally whether in direct or indirect way in synthesis of protein. Moreover, lead is able to bind to various types of transport proteins such as calmodulin, metallothionein, transferrin and calcium-ATPase. This will be resulted in metabolic function loss carries on as the main hypothesis which determines the detrimental effects of lead exposure (Corpas et al., 2002; Lewis and Cohen 2004; Zeitoun and Mehana, 2014). Thus, this may lead to many effects of the Pb element which appear on the internal and external structure of liver tissue.

The harshness of the harm relies on the toxic potentiality of a specific toxicant which has been grown in the tissue. Thus, the polluted water exposure might unfavorably influences different organs in fish that finally may result in general toxic effect on organs such as liver and gill. The vacuolization of hepatocytes was observed in liver that indicate an imbalance between the rate of synthesis of substances in the parenchyma cells and the rate of their release into the circulation (Osman, 2012; Parvathi et al., 2011).

4.3.3. Effects of Exposure of Red Tilapia to Cd Metal

The histopathology of experimental fish gill indicated a minor harm in the 48th h of procedure which has been carried out with the help of the sub lethal concentration of Cadmium ions. The lesion in the epithelial layer was observed in the gill as well as vacuolation in gill membrane and hypertrophy in mucous cells. In Fig (4.6), the histopathology of fish gill after reaching 96 h of being exposed to Cadmium can be found. The gill which has been exposed to Cadmium indicated active secretion of mucous and it should also be mentioned that the secondary lamellae indicated destruction of whether epithelial cell or some lamellae were curled as well. This results in excess and hemorrhage of gills which then leads to changing the color of gill to red. This in agreement with Karlsson-Norrgren et al. (2006) who reported showing severe gill filaments curling and necrotic in secondary lamellae of gill of zebrafish (*Brachydanio rerio*) and rainbow trout (*Salmo gairdneri*) after exposure to cadmium concentrations of $10\mu\text{g l}^{-1}$ and above.

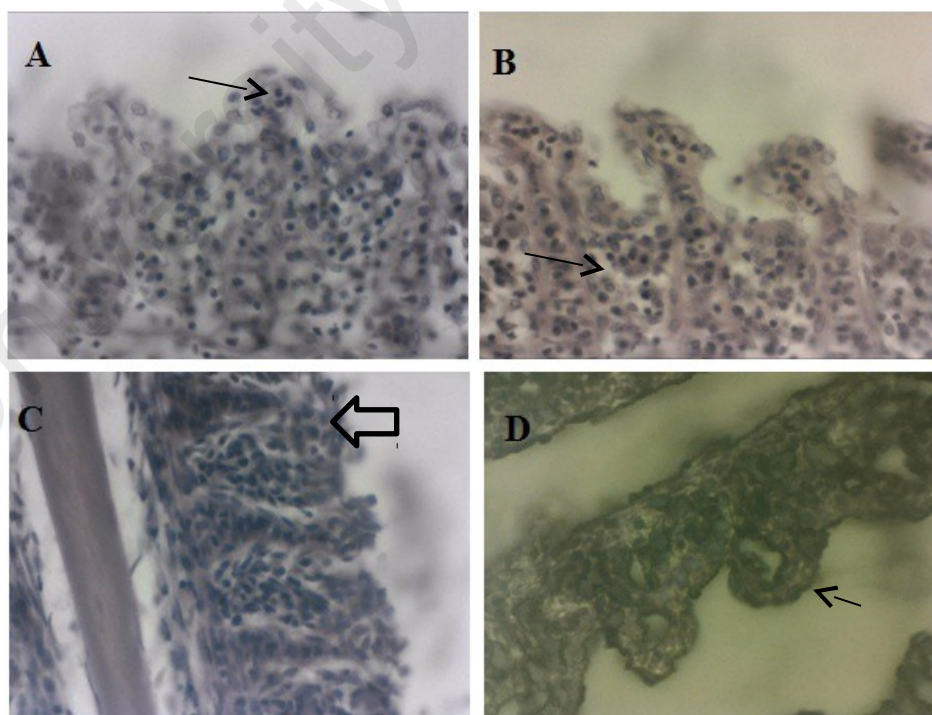


Figure 4.6. Histopathology of gill of cadmium (0.35mg/L) exposed red tilapia showing hypotrophy in mucous cells after 48 h exposure (\leftarrow) (A). Vacuolation and destruction of epithelial cells 48 h exposure (\leftarrow) (B). Fusion of adjacent of secondary lamellae during 96 h exposure (big arrow) (C). Lamellae curled after 96 h exposure (\leftarrow) (D).

The results of our study demonstrated that the exposure to sublethal concentrations of cadmium induces modifications in gills even following short-term exposure. The gill's damage and structural changes caused by water-borne cadmium have been reported for several fish species.

For example, regarding *Thalassoma pavo*, Brunelli et al., (2011) reported that histological lesions were hyperplasia of both primary and secondary lamellar epithelium, fusion of adjacent secondary lamellae, necrosis, telangiectasia, hyperplasia of chloride cells and metaplasia of goblet cells. Moreover, as reported by Wangsongsak et al. (2007) the hypertrophy and hyperplasia of primary and secondary lamellae in gills of Common silver barb *Puntius gonionotus* which exposed to 0.06 mg/L cadmium.

The first defense mechanism in gills against the exposure to heavy metals is the secretion of acidophilic mucus and the acidification of the mucus layer has been appeared due to the increase of the protective function of mucus. (Wu et al., 2007)

The cadmium is affect on calcium balance in fish and induces damage in gill structure because the cadmium taken up across the epithelial layer of fish gills via calcium channel; Calcium is known to exert considerable control over the permeability of the gills and displacement of calcium could stimulate ion loss and water uptake (da Silva et al., 2014; Thophon et al., 2003).

The results of the liver examination observed that many of the vacuolation in the cell cytoplasm and necrosis of the liver tissue (Fig. 4.7A). This was in agreement with data obtained by Bilal et al. (2011) who reported that the liver of catfish exposed to 4 and 8 ppm CdCl₂ has been affected by several histological alterations such as de-shaping of

hepatocytes, eccentric position of nuclei, enucleation, development of vacuoles in cell cytoplasm and necrosis of hepatic tissue.

Infiltration of red blood cells was observed in the liver tissue (Fig. 4.7B). These results are in agreement with data obtained by Younis et al. (2013) who studied the liver sections of *Oreochromis niloticus* exposed to sublethal concentration of Cd showed hyalinization and increased vacuolar degeneration in hepatocytes That have been related to factors such as blockage of blood vessels, excessive lipid as well as cellular swelling which therefore, result in the loss of characteristic architecture. Moreover, the hepatocytes' cytoplasm was identified with a rough, darkly stained and pink vacuoles and granules. Abundant erythrocytic infiltration was also observed in this group.

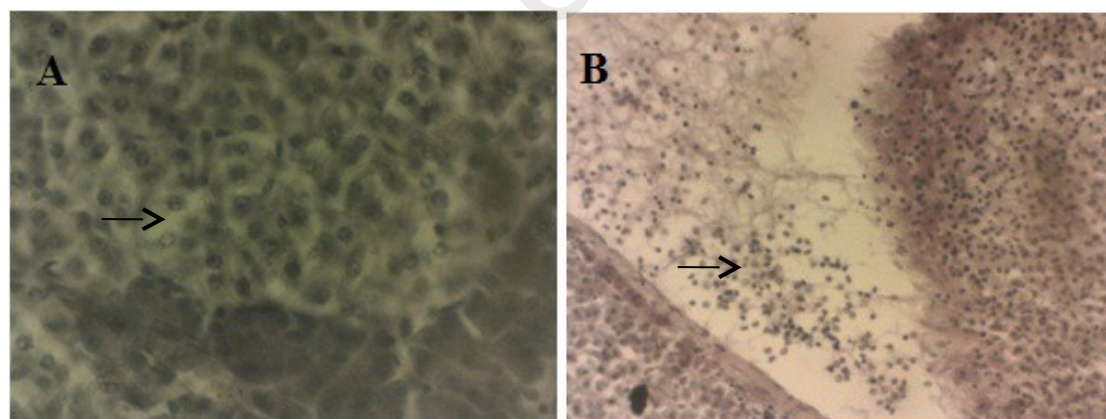


Figure 4.7. Light micrographs of liver tissue of cadmium (0.35mg/L) exposed red tilapia showing increased vacuolar degeneration in hepatocytes (←) (A). Infiltration of erythrocytes in liver tissues (←) (B).

In agreement with these results, Kaoud et al. (2011) reported that the liver of *Oreochromis niloticus* treated with cadmium showed hepatocyte degeneration, with nuclear pyknosis in the majority of the cells and the accumulation of metal binding proteins in their nuclei.

These changes may be attributed to the direct toxic effects of pollutants on hepatocytes and also the vacuolization of hepatocytes might indicate an imbalance between the rate of synthesis of substances in the parenchymal cells and the rate of their release into the circulatory system. Therefore, the histological changes identified in hepatocytes in this study may have been the result of various biochemical disruptions. (Van Dyk et al., 2007)

4.3.4. Effects of Exposure of Red Telapia to Cu Metal

Histological study of the gills on the unexposed fish shows a characteristic structural organization of the lamella (Fig. 4.1A). However, fish exposed to copper shows several histological alterations, specifically lifting of lamellar epithelium, proliferation of lamellar epithelial cells resulting in the fusion and necrosis of secondary lamellae; with increased exposure period produced more mucous secretions, and blood congestion in the neck of primary lamellae (Fig.4.8).

Similar results were observed in *Oreochromis niloticus* exposed to copper by Monteiro et al. (2005) who reported that the strong histological effects in the gills which are attributed to the exposure to relatively high values of Cu at sampling sites. Triebkorn et al. (2008) in their study on fish of chub *Leuciscus cephalus* showed that gill necrosis, cellular hypertrophy and increased mucous secretion do generally occur more frequently after heavy metal exposure including copper. These gill histological alterations has been observed by Figueiredo-Fernandes et al. (2007) in fish *Oreochromis niloticus* submitted to copper. They found histological changes including cell proliferation with thickening of gill filament epithelium which results in the mixture of few secondary lamellae that can be considered as an instance of defense mechanisms which decrease the branchial

superficial region exposed to the external environment. Furthermore, separation of lamellae epithelium helped to increase the distance in which waterborne pollutants diffuse to reach the bloodstream. Moreover, necrosis caused the degeneration of gills in fresh water fish through presence of high Cu concentrations in water, whereas apoptosis was observed in gill tissues exposed to lower concentrations of Cu (Vergolyas et al. 2010).

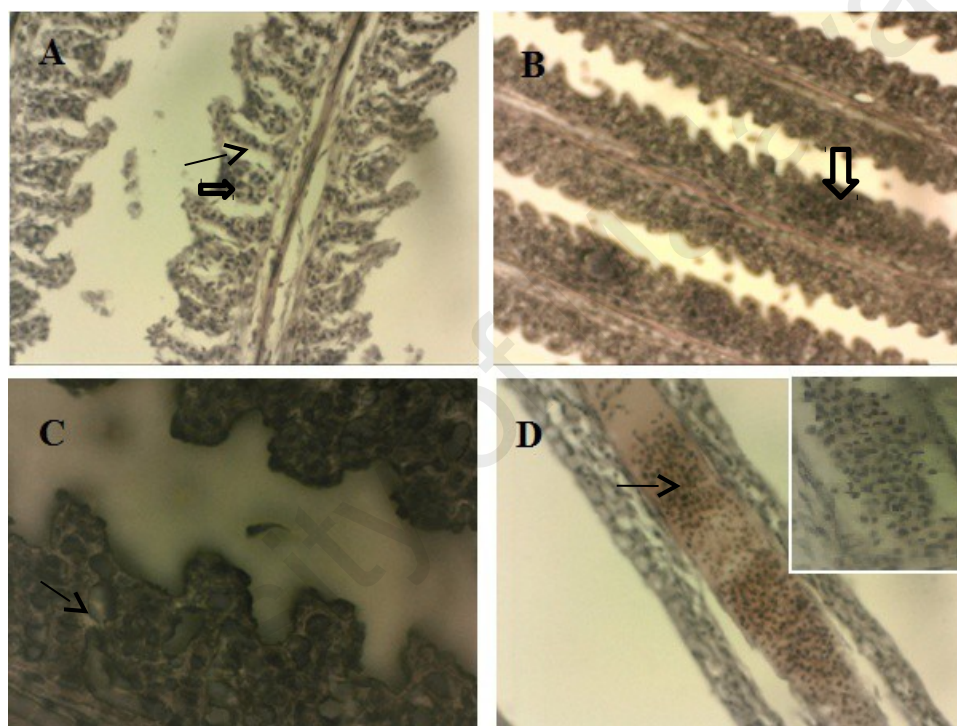


Figure 4.8. Light micrograph of gills in copper treated (0.23 mg/L CuSO₄, 96 h) red tilapia *Oreochromis* sp. Showing an intense lamellar epithelium lifting (←) with epithelium proliferation (big arrow) (A). Proliferation of filamentar epithelium with fusion of adjacent lamellae (big arrow) (B). Necrosis (←) (C). Gill filament of treated fish showing blood congestion in lamellar axis (←) (D). X400 (H&E).

The liver histology showed that copper caused some alterations to liver parenchyma, like vacuolization and necrosis (Fig. 4.9A). The liver histological changes observed were more evident in fish exposed to 96 h exposure. This is similar to the study by Figueiredo-Fernandes et al. (2007) showed that the cause of these alterations are often associated with a degenerative necrotic condition.

The present work also shows that picnotic nucleus and the number of hepatocytes nucleus in hepatic tissue decrease with the copper concentration (Fig. 4.9B). Several studies demonstrated that alterations in number, size and shape of the hepatocytes nucleus can be due to the existence of contaminants. Alterations in the size of nucleus have been previously noticed by Paris-Palacios et al. (2000) in *Brachydanio rerio* exposed to sublethal concentrations of copper sulphate.

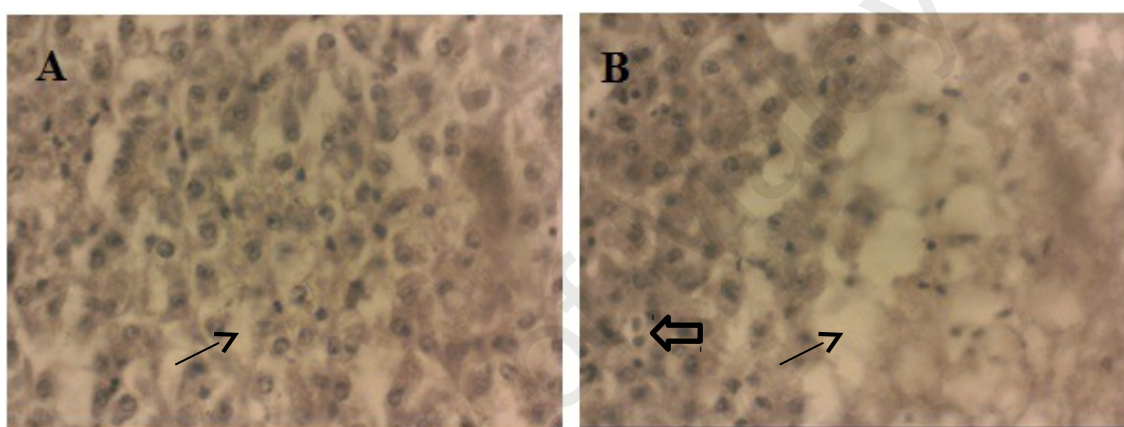


Figure 4.9. Light micrographs of liver tissue of red tilapia exposed to copper (0.23 mg/L96). Showing alterations in hepatocytes and vacuolation (←) (A). Vacuolation, necrosis area (←) and picnotic nucleus (big arrow) (B). X400 (H&E).

The observed inflammatory changes and increased numbers of macrophage aggregates in the red tilapia livers were seen to respond to metals. This corresponds with the study by Atamanalp *et al.*, (2008) on the exposure of *Oncorhynchus mykiss* to copper sulfate which induced degeneration of hepatocytes, sinusoidal dilation and congestion in the blood vessels of the liver. Metals can either increase or decrease hepatic enzyme activities and can lead to histopathological hepatic changes. The degree of these changes depends on the metal type and concentration, fish species, length of exposure and other factors (Paris-Palacios et al. 2000).

4.3.5. Effects of Exposure of Red Telapia to Zn Metal

Hyperplasia, lamellar epithelium annihilation, remove of secondary lamellar epithelium, membrane split and lamellar fusion are among the histological changes observe on the Zn-exposed gills of red tilapia fish, then detachment some of secondary lamellae, which presented more severity after 96 h of exposure (Fig. 4.10). This result is in agreement with dos Santos et al (2012) who studied the histological changes after acute exposure of Zn for *Astyanax aff. bimaculatus*. They reported that a sectional or complete fusion of secondary lamellae in exposure concentration (3mg/L) resulted from the increase in lamellar epithelium. Under the concentration of 5 mg/L, the fusion of lamellae have completely raised up to 100% showing that those fish which have been affected by the intrusion in their respiratory system made by metal exposure. In addition, a research work carried out by Nero et al. (2006) on histological alterations of the gills in goldfish (*Carassius auratus*) and yellow perch (*Perca flavescens*) in which the lamellar epithelium annihilation, lamellar fusion, cellular rupture, hyperplasia and aneurysm as well as secondary lamellar disengagement followed by secondary lamellar epithelium removal with concentrations above 3mg/L; The exposure to Zn is the essential reason for the mentioned structural alterations.

This fusion may be due to the proliferation of cells rich in mitochondria and stem cells, which causes partial or complete fusion of secondary lamellae. This alteration is considered as a defense mechanism which decreased the exposure area of secondary lamellae to the toxic metal (Cengiz and Unlu, 2002; Cruz, 2005).

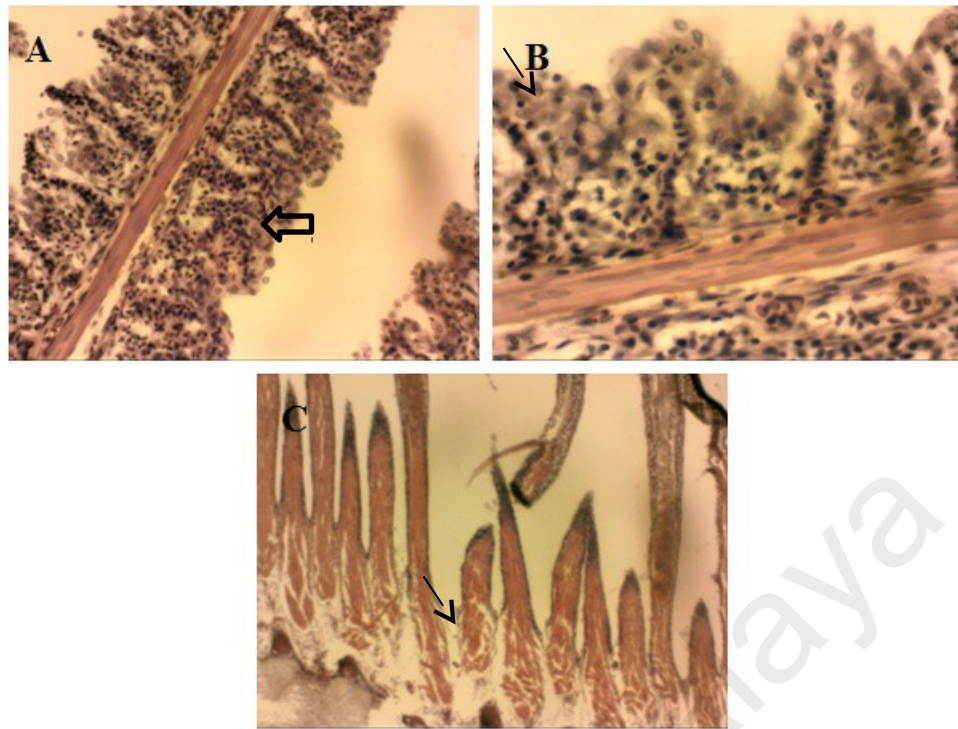


Figure 4.10. Light micrographs of gill of zinc (1.05 mg/L) exposed red tilapia fish showing lamellar fusion (big arrow) (A). Hyperplasia of epithelial cells (\leftarrow) (B). Lamellar destruction with detachment of secondary lamellae after 96 h of exposure (\leftarrow) (C).

Hogstrand and Wood (1995) observed that the calcium absorption (Ca^{2+}) restriction is the most remarkable sub-lethal influences of Zn^{2+} in fish. This is due to the fact that Zn tries to achieve the same absorption regions in gills. As a result, zinc excess is probably results in hypocalcaemia. Thus, if the concentration Ca^{2+} gets lower, it may damage the organism, because this factor is important for the integrity of the cell membrane and branchial permeability stabilization. Thus, histological changes were induced by rupture of membrane with destruction of the lamellar epithelium cells in gill of the studied red tilapia fish which were exposed to Zn.

Light microscopic study of the fish liver exposed to Zn for 96 h showed several changes including the degenerated liver cells effect on the normal architecture of liver which became markedly disorganized, as well as, hypertrophy of hepatocytes and congested blood vessel, as well as hypertrophy and hyperplasia of bile duct cells (Fig. 4.11). The

liver is associated with detoxification and biotransformation processes, and it combined with its location and access to the blood supply due to these functions. Thus, this organ was affected by water contaminants (Camargo and Martinez, 2007). This may be led to the histological changes in liver of the studied red tilapia fish.

These results are in agreement with the study of Abdel-Warith et al. (2011) who investigated the accumulative effect of low and high concentrations (2 and 6 mg/L) of zinc on the histology of the liver. They explained that the degree and nature of histological changes in the liver of exposed fish *Oreochromis niloticus* was affected by the zinc exposure period. It was mainly observed in fish exposed over the short-term periods while regenerative responses were noted in fish exposed over the long-term period and their results showed a histological response in exposed specimens with the most prevalent histological characteristics identified being congestion of blood vessels, and cellular swelling. Moreover, in a study by Senthil et al. (2008), it was noted that the liver appears to be one of the most important sites for Zn accumulation in *Channel punctatus*. In addition, findings by Loganathan et al., (2006) namely were severe necrosis, haemorrhage nuclear pyknosis and degeneration of hepatocytes observed in the liver tissue of *Labeo rohita* exposed to zinc.

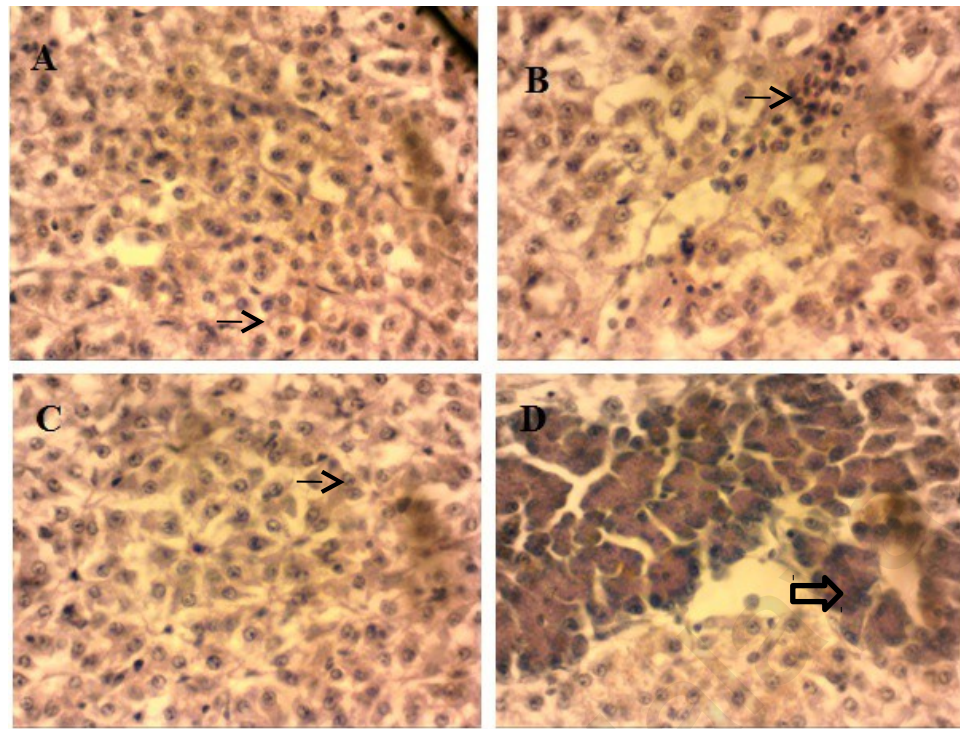


Figure 4.11. Photomicrograph of liver of red tilapia fish exposed to (1.05 mg/L Zn) showing hypertrophied hepatocytes (←) (A) congested blood vessel (←) (B). Pycnotic nuclei (←) (C). Hypertrophy and hyperplasia of bile duct cells (big arrow) (D).

4.3.6. Effects of Exposure of Red Tilapia to Mg Metal

The gill morphology of red tilapia showed some signs of epithelial lesions when exposed to magnesium concentration (1.87mg/L). The main changes observed after 96 h of exposure were lifting of the lamellar epithelium as well as epithelium proliferation on top of filament. Moreover, few aneurisms were also observed at secondary lamellae (Fig. 4.12).

Morphological findings described here are corroborated by field studies of contaminant exposure (Poleksic et al., 2010 and Kaur and Dua, 2015)

The cellular harms seen in the gills regarding epithelial lifting and necrosis are able to unfavorably influence the exchange of gas and ionic regulation, due to the existence of the increased gap between blood and water because of epithelial lifting. Defective oxygen uptake was observed.

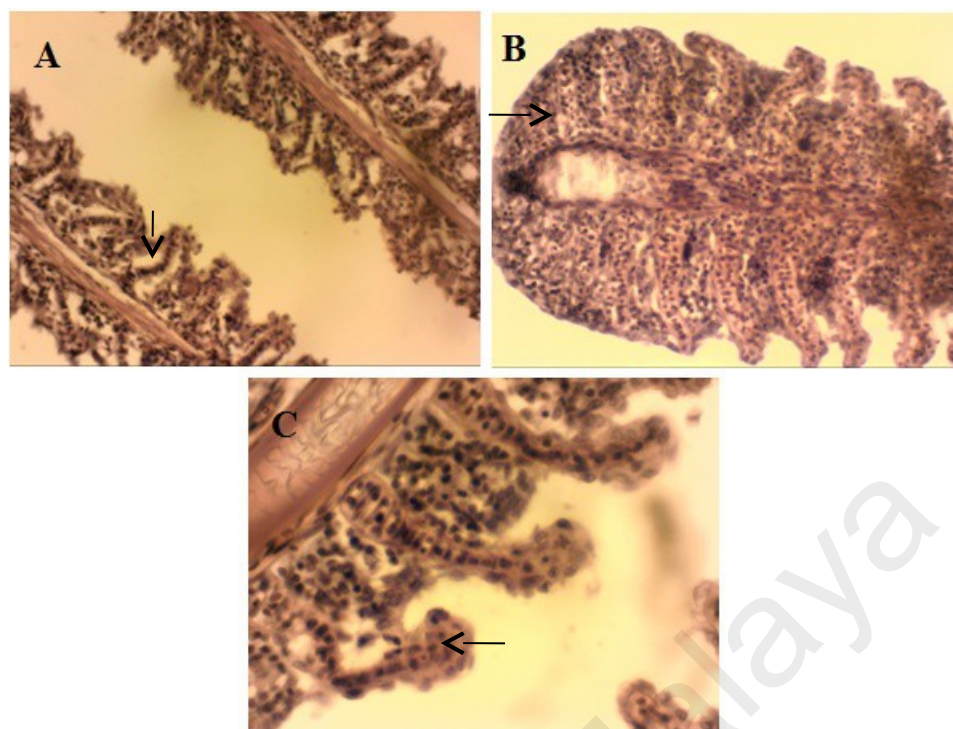


Figure 4.12. Photomicrographs of gill of magnesium (1.87 mg/L after 96 h) exposed red tilapia showing lifting of lamellar epithelium (←) (A). Proliferation of epithelial cells on top of filament (←) (B). Aneurism in secondary lamellae (←) (C).

Liver histology from the exposed red tilapia showed alterations and the increase on the size of hepatocytes as well as vacuolation and necrosis area (Fig. 4.13). This is similar to study by Oliveira Ribeiro et al. (2005) who described that the liver was the most important target organ for heavy metals including magnesium which have been accumulated in range of (646mg/kg) in *Anguilla anguilla*.

Necrosis is strongly associated with oxidative stress where lipid peroxidation is a clear source of membrane bilayer susceptibility (Li et al., 2000; Avci et al., 2005) These oxidative forms may increase programmed cell death or disturbed cell homeostasis and cellular necrosis.

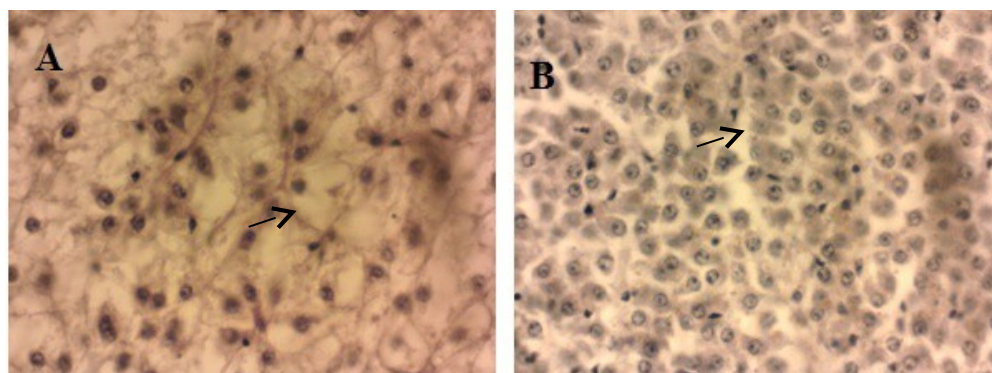


Figure 4.13. Photomicrograph of liver of red tilapia exposed to magnesium (1.87mg/L after 96 h). Showing alterations and increase in size of hepatocytes (←) (A). Vacuolation and necrosis in hepatocytes (←) (B).

4.4. CONCLUSIONS

The present study had showed that, lethal and sub-lethal concentration exposure of Hg, Cu, Cd, Pb, Zn, and Mg for short periods of time could cause severe tissue damage which leads to alterations towards histopathological aspects in the gill and liver of red tilapia *Oreochromis* sp. These results are a very important factor in assessing the potential damage from metal exposure, and are also important for the references of future studies.

CHAPTER 5

INVESTIGATION VIA TECHNIQUES OF SCANNING ELECTRON

MICROSCOPE WITH ENERGY DISPERSIVE X RAY

5.1. INTRODUCTION

Metal ions chemistry is considered as an essential area in biochemical evolution. Trace elements such as Fe, Zn, Mo, Co and Cu are necessary and favorable for human health, plant growth and nutrition. The other elements such as Hg, Cd, Pb, etc have possible toxicity and homostatic mechanisms are necessary to control the intracellular stages. An organism's life is highly rely on the suitable control of assimilation, uptake, intercellular compartmentation and intercellular translocation of trace metals (Guerinot and Salt, 2001).

Heavy metals accumulate in the tissues of aquatic animals and may become toxic when the accumulation reaches a substantially high level (Kalay and Canli 2000). Toxicity of these elements are because of their ability to oxidative stress and damage the living tissues which includes damage mainly to central nervous system (neurotoxicity), liver (hepatotoxicity), kidney (nephrotoxicity) and DNA (genotoxicity) in humans and animals. Furthermore, the accumulation of these elements can cause severe damage to mucus tissues and intestinal tract and skeletal (Sharma et al., 2014). Thus, heavy metals can provoke problem with fish health and pathological conditions of the fish tissues which includes various histopathological lesions in kidney, spleen and muscle (Authman, 2012).

Image formation with Scanning Electron Microscope gives easily comprehensible for biological research, because of the quasi-three dimensional representations of objects

studied. Moreover, at the wide range of magnifications, this leads to better understanding as well as reveals unsuspected detail (Heywood, 1971). This device gives morphological information, as well as the X ray microanalysis presenting analytical information about the specimen. Image formation processes through the basic scanning action which is used for the construction of an image in addition to the origin of the commonly encountered contrast mechanisms that arise from the electron – specimen interaction. Whereas by calculating the energy and intensity distribution of the X- ray signal which created by a focused electron beam, the chemical analysis is carried out. These signals are detected and measured and then converted into a useful form for qualitative and quantitative analysis (Goldstein et al., 1981)

Scanning electron microscope has been carried out with secondary electrons or backscattered electrons at an accelerating voltage of 10-20 kV, as well as, lower accelerating voltage about (1kV) is also utilized (Shindo and Oikawa, 2002).

Histopathological test has been increasingly distinguished as a valuable tool for field assessment of the impact of environmental pollutants on fish (Teh et al., 1997). Histopathological changes have already been examined via electron microscope in the observing of fish health and environmental pollution in natural water.

Electron microscopic studies have showed obvious variability among various teleost species in the morphology. Goldstein et al., (1992) presented a detailed explanations of electron microprobe analysis and showed that the electron probe X-ray microanalysis permits the elemental composition determination in a specimen; when a specimen is irritated by an electron beam, consequently, X rays are created due to the rearrangement of outer shell electrons after an inner shell electron has been emitted from the atom. The

ejected X-rays give valuable information in the irradiated region about the elemental composition of the specimen.

Electron microprobe analysis has usually used to analyze the heavy metal in the environment and to quantitative measurement of major mineral elements (P, Cl, S, Na, Mg, Ca and K) (Zierold, 2000; Roomans2002)

The previous study reported that the pollutants of agriculture, industries and sewage induced several pathological alterations in different tissues of fish (Abbas and Ali, 2007) The gills, liver, kidney, and muscle are the tissues most frequently studied in bioaccumulation researches; and X-ray microanalysis studies have shown that a variety of heavy metals can be accumulated by fish. Recently, some techniques have been developed for the element analysis of tissues using X-ray microanalysis equipped with scanning electron microscope.

5.2. MATERIALS AND METHODS

5.2.1. Fish Specimen

Red tilapia fish with an average standard length of 7 ± 0.5 cm and an average weight of 7.2 ± 1 g were collected from a commercial aquaculture facility in Serendah, Selangor 48200 Kuala Lumpur, Malaysia. Upon the arrival, the fishes were stocked in group of 25 in 50L in a semi-static glass aquaria (60 L capacity; 60 cm×35 cm×40 cm) system containing UV sterilized (EHK-UVC) de-chlorinated tap water with a pH 7.6 ± 0.06 , and maintained at a temperature of 26.5 ± 2 C° as the water kept oxygen saturated by aeration at dissolved oxygen 7.0 mg/L. Tilapia fish were acclimatized to laboratory conditions at a photoperiod of 12 h light and 12 h darkness for 14 days with daily

feeding (once per day) of a dry commercial food (pellets with 25% of crude protein). Feeding was stopped 24 h before and during the actual experiment. In order to reduce metabolic wastes contamination, aquarium water was changed every 24 hours.

5.2.2. Exposure

Fish were transferred to assay aquaria (20 x 20 x 40 cm) 5L glass aquaria, which provided aeration via air pumps and air stone diffusers. Fish were separated into four categories (stocking density of each category was 10 fish /aquarium). One group was set as control and the others as exposed groups.

Completely dehydrated Analar grade BDH chemicals with 99.5% purity of CdSO_4 , CuSO_4 , HgCl_2 , MgSO_4 , $\text{Pb}(\text{NO}_3)_2$, and ZnCl_2 were dissolved in double-deionized water to prepare the stock solution (1000 mg L^{-1}) of Cd, Cu, Hg, Mg, Pb, and Zn. This stock solution was diluted to the desired concentrations with local tap water. Each metal with various concentrations was considered as an individual experiment. Solutions were replaced by the same specific concentration at every 24h interval until 96h exposure (APHA et al., 1999). In preliminary experiments the medium which caused 50% mortality during 96 h of exposure (LC_{50}) and was estimated by the probit transformed concentration - response curves (USEPA, 2002), were (0.7 Cd, 0.45 Cu, 11Pb, 0.3 Hg, 3.74 Mg, 2.10 Zn) mg/L. The test concentrations chosen were 50% of the 96-h LC_{50} value from the acute toxicity test (Thophon et al., 2003) which were (0.35 Cd, 0.225 Cu, 5.5Pb, 0.15Hg, 1.87Mg, 1.05Zn) mg/L. Experiment was a semi-static system with continuous aeration over 96 h. For test concentration and control, three replicates were carried out. The water quality characteristics were: dissolved oxygen (DO) $7.25 \pm 0.4 \text{ mg/l}$, temperature $26 \pm 2^\circ\text{C}$ and pH 7.65 ± 0.5 . Fish mortalities were monitored every day. At 24, 48, 72 and 96 h of exposure time, two fish from each aquarium were sampled.

5.2.3. Scanning Electron Microscopy

For SEM, Fish from the experimental and control groups ($n = 3$) were anesthetized in ice cold water and sacrificed by cervical decapitation. The fish abdomens were opened and the opercular covers were removed for improved tissue fixation (Al-Zaidan et al., 2013). The gill filament and liver treatment was as described by Pandey et al. (2008). Tissues were fixed at 4°C in phosphate-buffered 8% gluteraldehyde (at pH 7.2) for 1h. The samples were then washed three times in the same buffer for 15 min and then post-fixed in 4% osmium tetroxide OsO_4 in the same buffer overnight to increase electron density, and then twice using deionised water, Tissues dehydrated in ascending series concentrations of ethanol from 10% to absolute. Samples were then dehydrated in a grade series of ethanol acetone mixture solutions until achieving pure acetone at room temperature. Afterwards, they were dried in a critical point-drying apparatus (CPD 030, LEICA EM) with liquid CO_2 for 30–60 min while specimens were mounted onto aluminum stubs and coated with gold by a coating machine (SCD005 Sputter Coater, LEICA EM). Morphological analysis was carried out using a JEOL JSM- 7001F, Japan Scanning Electron Microscope at an accelerating voltage of 15 kV.

5.2.4. Energy Dispersive X-ray Analysis (EDX)

Energy dispersive X-ray (EDX) spectroscopy analysis using a scanning electron microscope (JEOL JSM- 7001F, Japan) equipped with EDX (OXFORD Instrument X-Max) were carried out for quantifying the weight percent mineral contents through the cross-section of gills. All the specimens were analyzed under the same conditions in order to minimize the matrix effects. The data were collected at a 15 kV accelerating voltage with a 10l A operating current and a 15 cm working distance. In order to compare with the results obtained from the SEM image analysis, the same samples were used for the EDX analysis.

5.3. RESULTS AND DISCUSSION

5.3.1. SEM and Energy Dispersive X-ray Studies on Control Gills Specimens

Scanning electron micrographs confirmed the results obtained by light microscopy. In control fish, four gill arches on each side of the body have observed on the gills. Each one supports several gill filaments (primary lamellae) arranged in two rows called hemibranches, organized arrangements of primary and secondary lamellae with uniform interlamellar space. On the upper and lower surface of each filament, there is a row of secondary lamellae that in which there were gas exchanges and other physiological phenomena, such as acid-base balance and osmoregulation occur.

SEM observations showed data obtained by light microscopy. A normal arrangement of gill filaments and lamellae was observed at low resolution. In filament epithelium, pavement cells (PCs) were the most plentiful cell types, while chloride cells and mucous cells were almost scarce located mainly on the trailing edge of the filament and at the bases of lamellae. At high resolution, characteristic surface pattern of pavement cells was created by long microridges and concave apical surface of the CCs covered by microvilli (Fig. 5.1. A, B, C, D).

X-Ray microanalysis with energy dispersive spectroscopy was used to scan primary and secondary lamellae of gills to determine their metal composition. Eight elements were predetermined for analysis: Ca, P, Cd, Cu, Mg, Hg, Pb and Zn and their abundance was recorded as raw X-ray counts. The EDX analysis of the normal gills of red tilapia fish has indicated that there are five elements of viz. Calcium, Phosphorous, Magnesium, Copper, and Zinc present in the gills. Among these elements, Ca and P have the maximum percentage while Hg, Cd, Pb were not detected (Fig. 5.2).

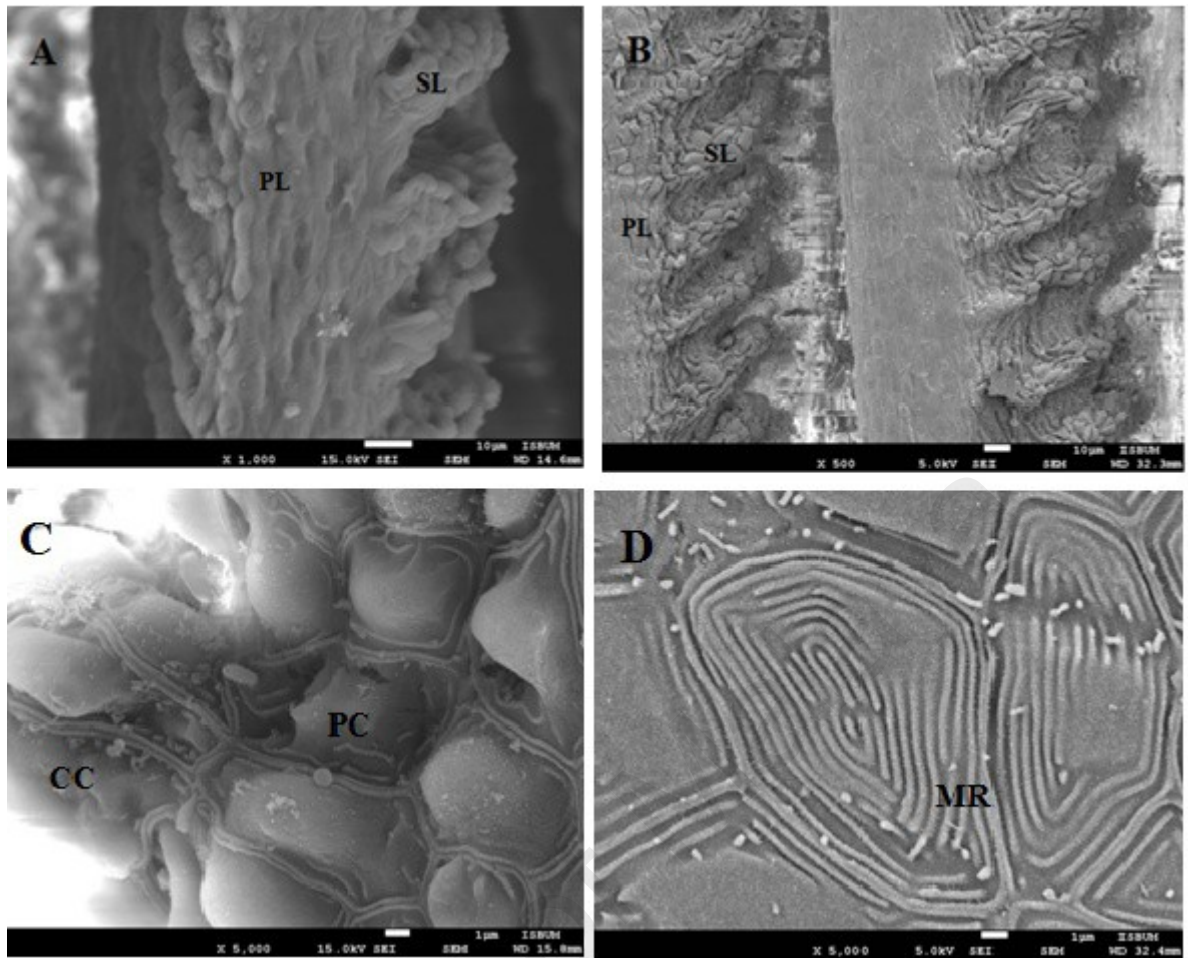


Figure 5.1. Scanning electron microscopic micrographs of gills *Oreochromis* sp. (A) General view of control fish gill filaments and lamellae showing normal morphological features. (B) Primary lamellae (PL) and secondary lamellae (SL). (C) Note well-organized pavement cells (PC), chloride cells (CC) in base edge of secondary lamellae. (D) Organized microridges in pavement cells.

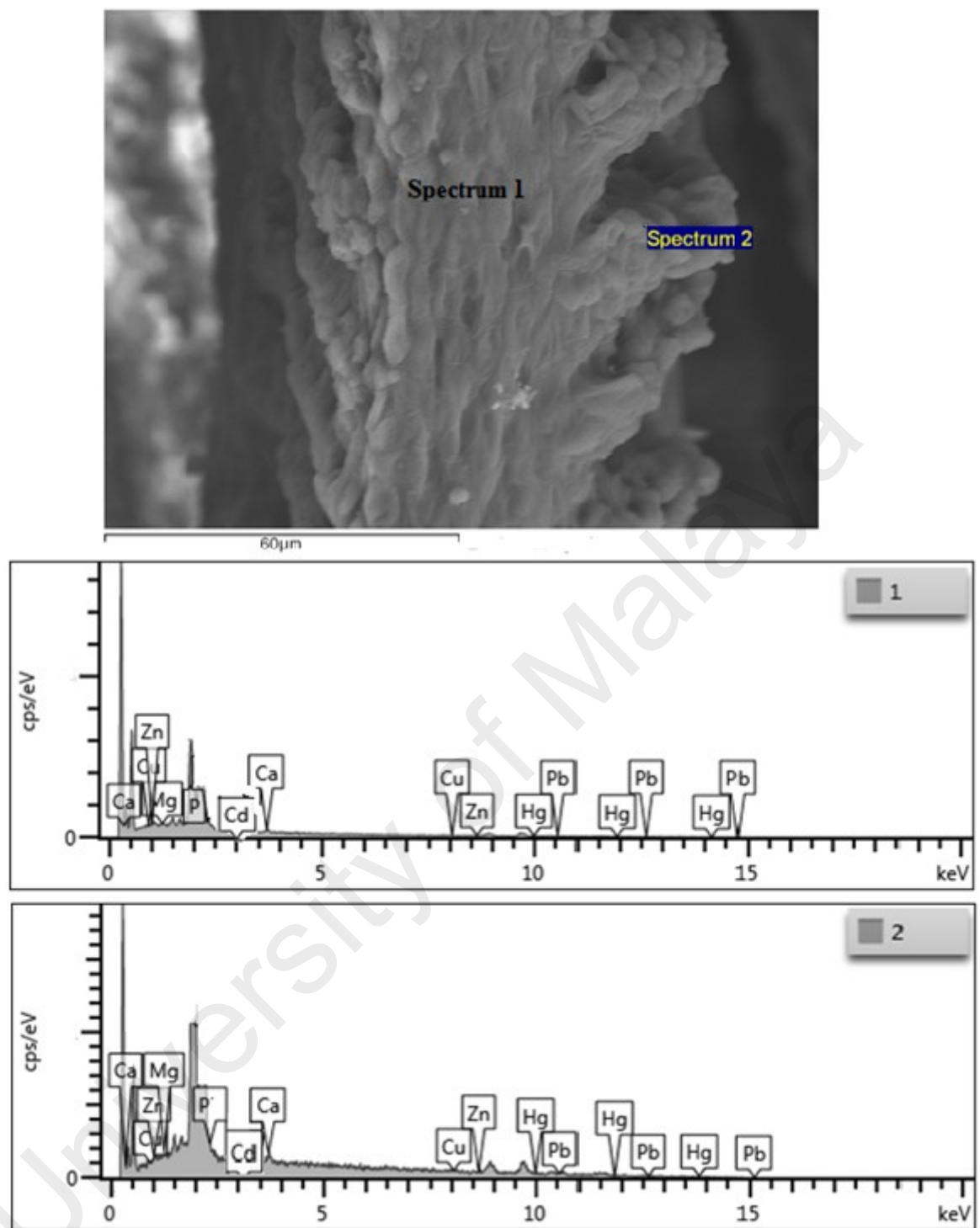


Figure 5.2. Scanning electronic micrograph and energy dispersive X-ray spectroscopy microanalysis of the control gill tissue. X-ray spectrum shows only essential elements usually present in biological specimens Ca, P, Cu, Zn, and Mg and not detecting of Cd, Hg, Pb. In primary lamellae area (1) and secondary lamellae area (2)

5.3.2. SEM and Energy Dispersive X-ray Studies on Gills of Fish Exposed to Cadmium Metal

In light microscopic examination, chloride cells are recognized as large epithelial cells with light cytoplasm, which is generally demonstrated at the base of lamellae. At the base of lamellae, pavement cells and mucus cells also exist in the epithelium of the filament, but they lack the light cytoplasm and are smaller than chloride cells.

The breakdown of pillar cell system was observed in the SEM examination. Additionally, SEM also revealed the harsh enlargement of many secondary lamellae at 96 h (Fig. 5.3). The gills of a lot of fish presented huge hypertrophy (increase in size) and hyperplasia (an increase in the reproduction rate) of chloride cells and mucus cells at the base of the gill filaments and secondary lamellae. SEM examination has been showed complete fusion of secondary lamellae in numerous areas which were caused by epithelial hyperplasia and/or hypertrophy. The disappearance and the lack of clarity of microridges of pavement cells in gills filaments are due to the secretion of mucus that leads to the fully covered (Fig. 5.4)

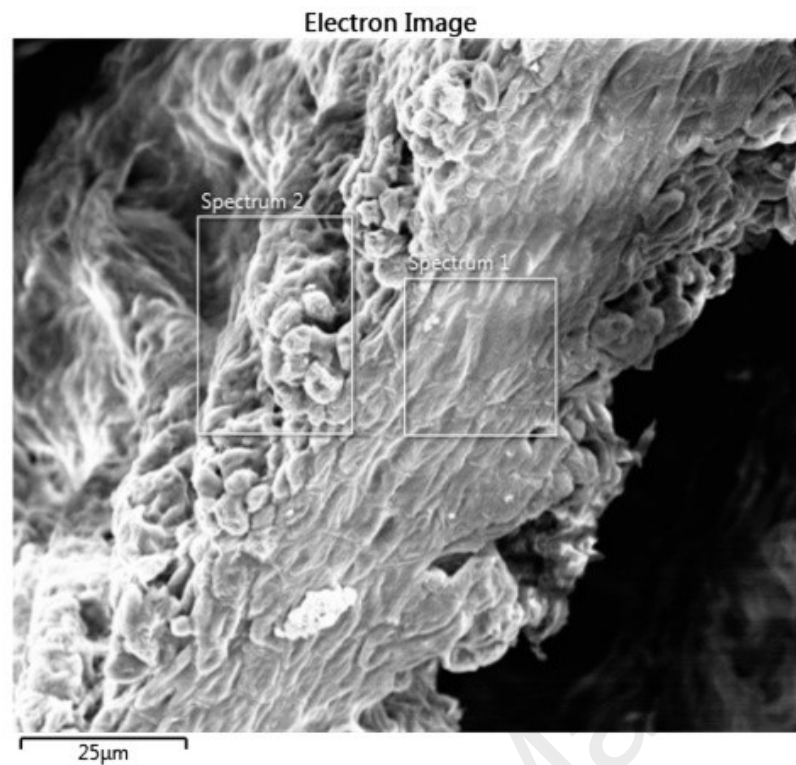


Figure 5.3. SEM image of gill showed severe enlargement of secondary lamellae, extensive hypertrophy and hyperplasia of epithelial cells and chloride cells with complete fusion at 96 h exposure to Cadmium ions.

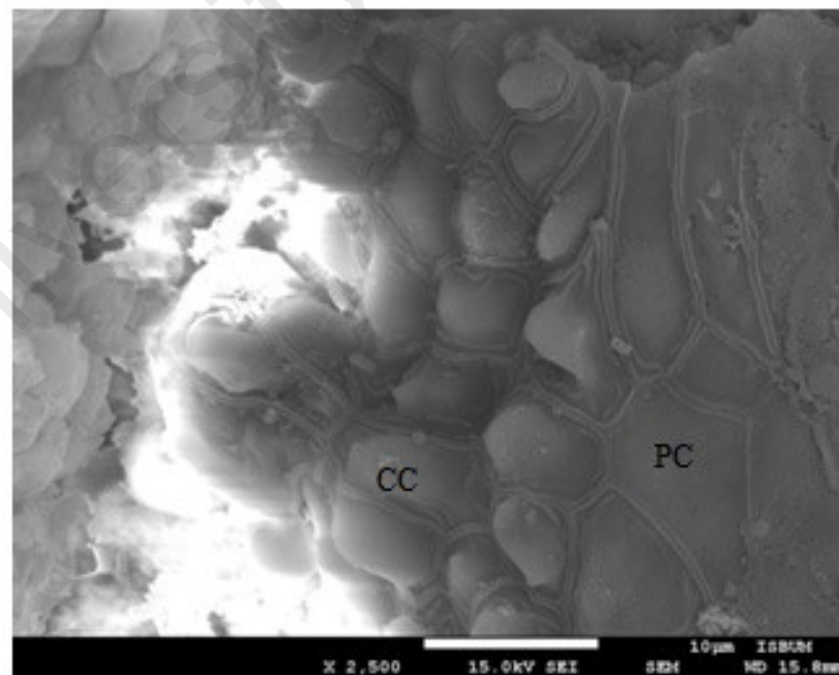


Figure 5.4. Scanning electron micrographic image showing disappear of microridges in pavement cells after 96h exposure to Cd ions.

Rauf et al. (2009) observed high concentration of Cadmium in the various body parts of the commercial carp fishes because of its capability of receiving high load of toxicants. The effect of cadmium on gills in *Oreochromis* sp. was specifically serious since they serve as a major organ for ion regulation and respiration and osmotic regulation, due to the gills having direct contact with cadmium. Therefore, they stored the metal and then transmitted to the internal compartments by blood transport. As it has been reported in previous studies, cadmium was taken up across the epithelial layer of fish gills via calcium channels (Wicklund Glynn et al., 1994). Therefore, cadmium affect on calcium balance and induces damage in gill structure of tilapia fish *Oreochromis mossambicus* (Pratap and Wendelaar Bonga, 1993)

Thophon et al. (2003) revealed that the chloride cell hyperplasia took place meeting the need for ejecting the Cd^{2+} absorbed by the gills of white sea bass (*Lates calcarifer*) under intense cadmium entering the chloride cells in the gills through calcium channels and interact with cytoplasmic compartments such as enzymes and metallothionein (MT); followed by metal bound to the external body surface and consecutively eliminated by sloughing of dead cellular materials.

Elemental composition of red tilapia' gill was assessed via dispersive X-ray microanalysis (EDX). Cadmium recorded a slight increase of the weight percentage 15.29 % and 27.73%. In primary and secondary lamellae of gills respectively (Fig. 5.5). The present study can be supported by the fact that the differences in chemical composition of the gills depends upon the ambient element exposure. Hence the stress conditions caused by heavy metal pollutants disturb the elemental composition of the gills and therefore the percentage composition in the gills of red tilapia fish can be considered as a reliable pollution indicator with authenticity.

Wong and Wong et al. (2000) suggested a two-phase adaptational change of chloride cells, involving first a rapid process in which an increase in these cells apical exposure could be due to the retraction of neighboring pavement cells or swelling of the chloride cells itself ; and also suggested that the effect of Cd in increasing the number of exposed apical Ca^{+2} channels, which could increase Ca^{+2} uptake that in turn might attenuate the hypocalcemic effect stimulating chloride cells proliferation.

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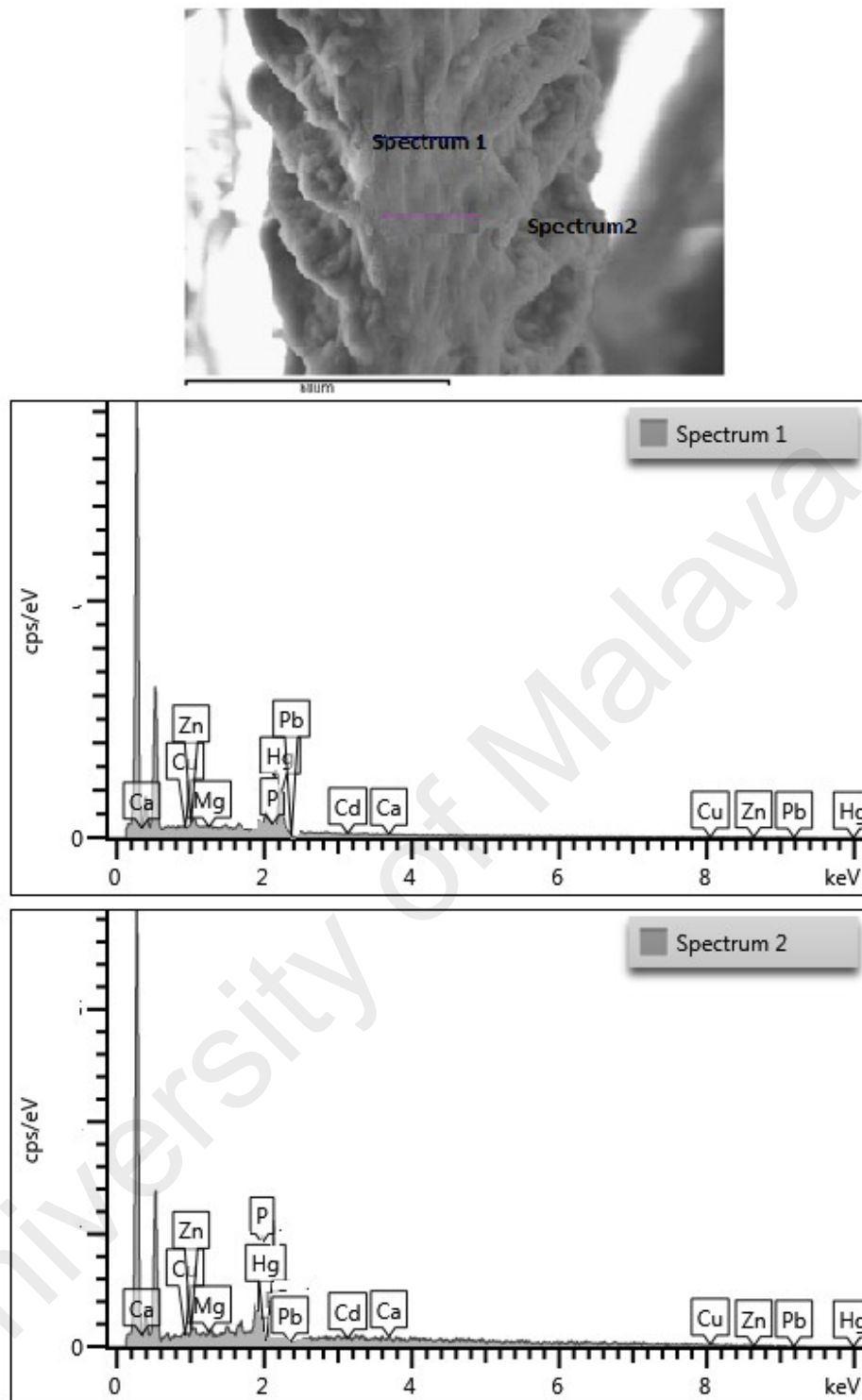


Figure 5.5. Shows the scanning electron micrograph and EDX microanalysis of gill. X-ray spectrum taken in raster mode of primary lamellae shows peaks of P, Ca, Mg, Zn, and Cu with slight increase in the weight percentage of Cd (15.29%) in primary lamellae (1) and (27.73 %) in secondary lamellae (2) after 96 h exposure to Cd ions. Hg and Pb not detected.

5.3.3. SEM and Energy Dispersive X-ray Studies on Gills of Fish Exposed to Copper Metal

Upon the exposure to different heavy metal pollutants present in water, in particular copper ions, some alterations were observed in the structure of gills by employing Scanning Electron Microscopic technique. The effect of waterborne copper resulted in hypertrophy and hyperplasia in epithelial cells of the primary and secondary lamellae resulted in complete fusion of secondary lamellae (Fig. 5.6). Extensive damage sometimes involves the necrosis in secondary lamellae (Fig. 5.7).

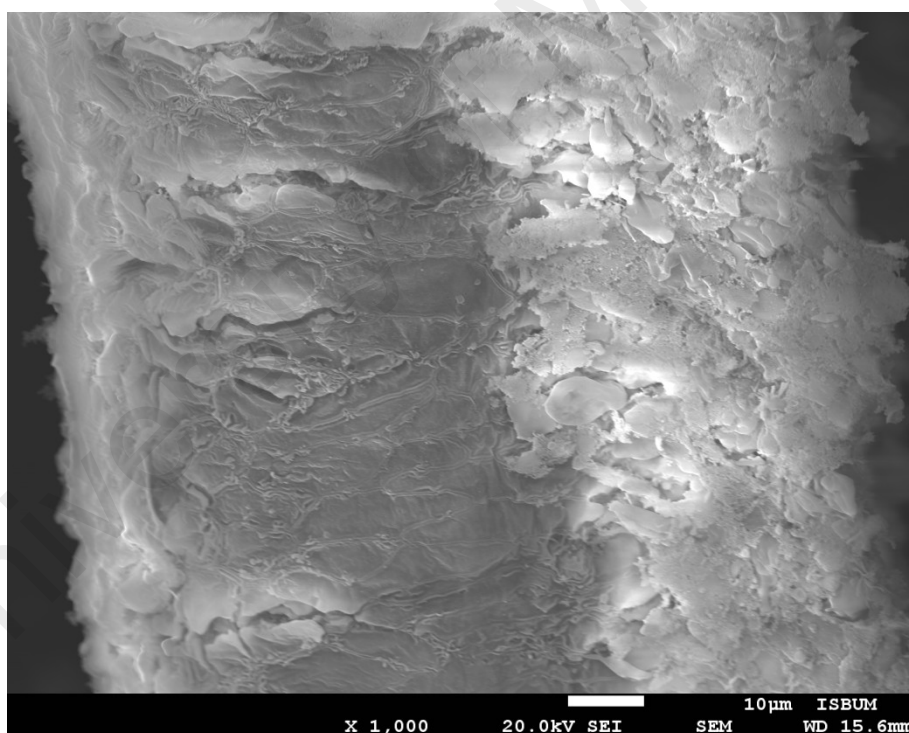


Figure 5.6. SEM examination shows complete fusion of secondary lamellae of gills from *Oreochromis* sp. at 72 h exposure to Cu ions.

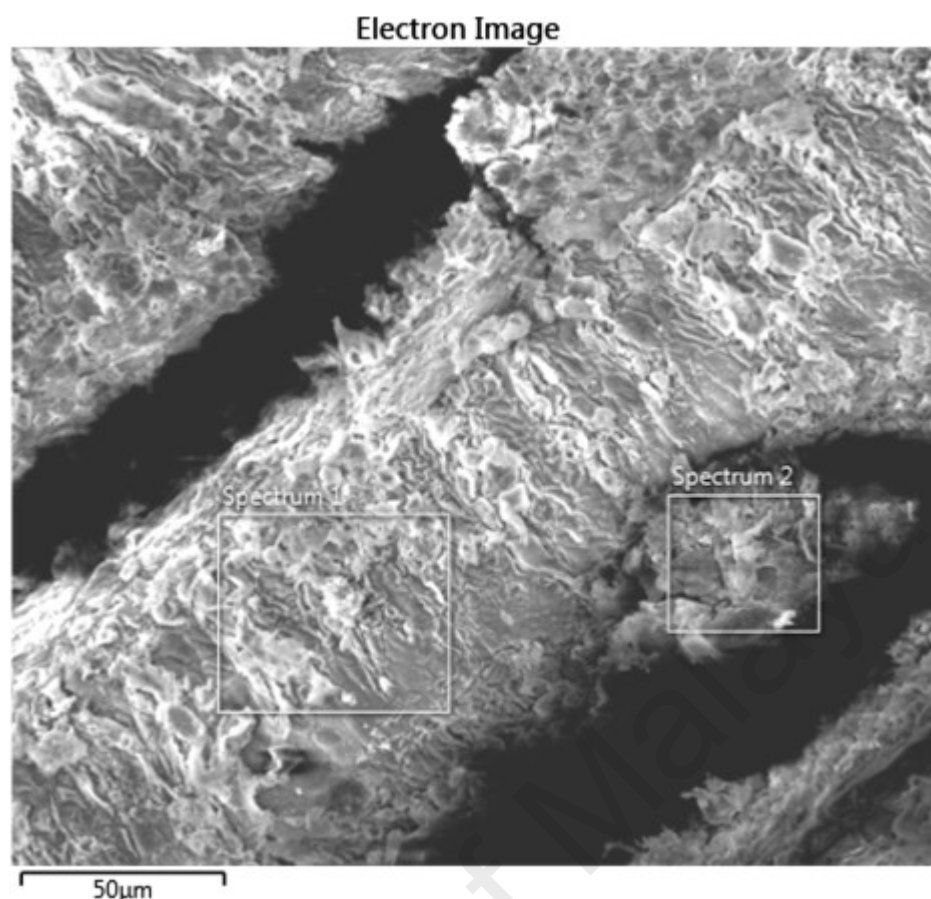


Figure 5.7. Scanning electron micrographic image shows necrosis in secondary lamellae of gill of *Oreochromis* sp. at 96 h exposure to Cu ions.

These results correspond to a study Thophon et al., (2003) and Barillet et al. (2010) whose reported hyperplasia of secondary lamellae in organisms which were exposed to environmental pollutants (pesticides and heavy metals) is often related to the full fusion of two adjacent secondary lamellae; Such symptoms result in an increase in the distance between water and blood. So, they can be regarded as defense mechanisms opposite to surrounding toxicants, but they also may result in lacking of the oxygen supply of the blood.

Logically, it can be predicted that fish would change their observed mucus secretion with copper exposure which is in agreement with Alazemi et al. (1996) observations on freshwater fish *Gnathonemus petersii*. A critical physiological operation of mucus

secreted by gill cells is actually the protection of the sensitive and thin gill epithelium from environmental effects. As a result, hyper secretion of mucus at the same time causes a full mucus cover of the gill epithelium impeding gas exchange (Pawert et al., 1998). Hypertrophy and hyperplasia of chloride cell have been reported have occurred in response to the need to eject heavy metal that absorbed by the gill (Barillet et al., 2010).

The mitochondrial metabolism impaired by intake of heavy metals in the cell and also it was accompanied by alterations in the expression levels of genes that involved in responses to oxidative stress responses and which caused histological damages (Al Kaddissi et al., 2011). Wu et al., (2008) revealed that after 48–72 h of sub-lethal Cu^{+2} exposure (2mg/L), the cortisol is raised and the Mitochondria Rich (MR) cell morphology altered. They proposed that the primary cortisol induction might be associated with MR cell transformation due to the increase of ion uptake and keep against Cu-induced necrosis of MR cells. They demonstrated that morphological alterations in fish gills as two different responses: compensation and defense via cell proliferation or mucus secretion. These two responses assist in decreasing the entry of toxicants and avoid damage due to the direct effects of Cu. Therefore, it is expected that the histopathological responses to Cu result in respiratory disturbances and electrolytic imbalances because the gills have a function in gas exchange, ionic and osmotic regulation, and acid–base equilibrium.

Mitochondria-rich chloride cells show particular ultrastructural characteristics that are features of cells engaged in ionic transport. The huge growth of the basolateral tubular system was one of the most effective ultrastructural alterations related to the enhance in their size, relating an enhanced activity of $\text{Na}^{+}/\text{K}^{+}$ -ATPase. The various mitochondria

observed in relation to the elements of this network demonstrate the high-energy requirements of ionic exchange reflected that they particularly associated with one type of chloride cell with two different localizations, on the lamellae and on the filament and also the pavement cells indicate a high metabolic activity (Carmona et al., 2004)

The results recorded an increase in the proportion of copper percentage weight in the gills which have been exposed to the same element ions as 35.36% and 25.56% for primary and secondary lamellae respectively (Fig. 5.8). These results are in agreement with the study by Rauf et al. (2009) who observed that fish have the ability to accumulate high concentrations of heavy elements in the various body parts of the commercial fishes of major carps (*Catla catla*, *Labeo rohita* and *Cirrhina mrigala*), because it receives high load of toxicants including copper.

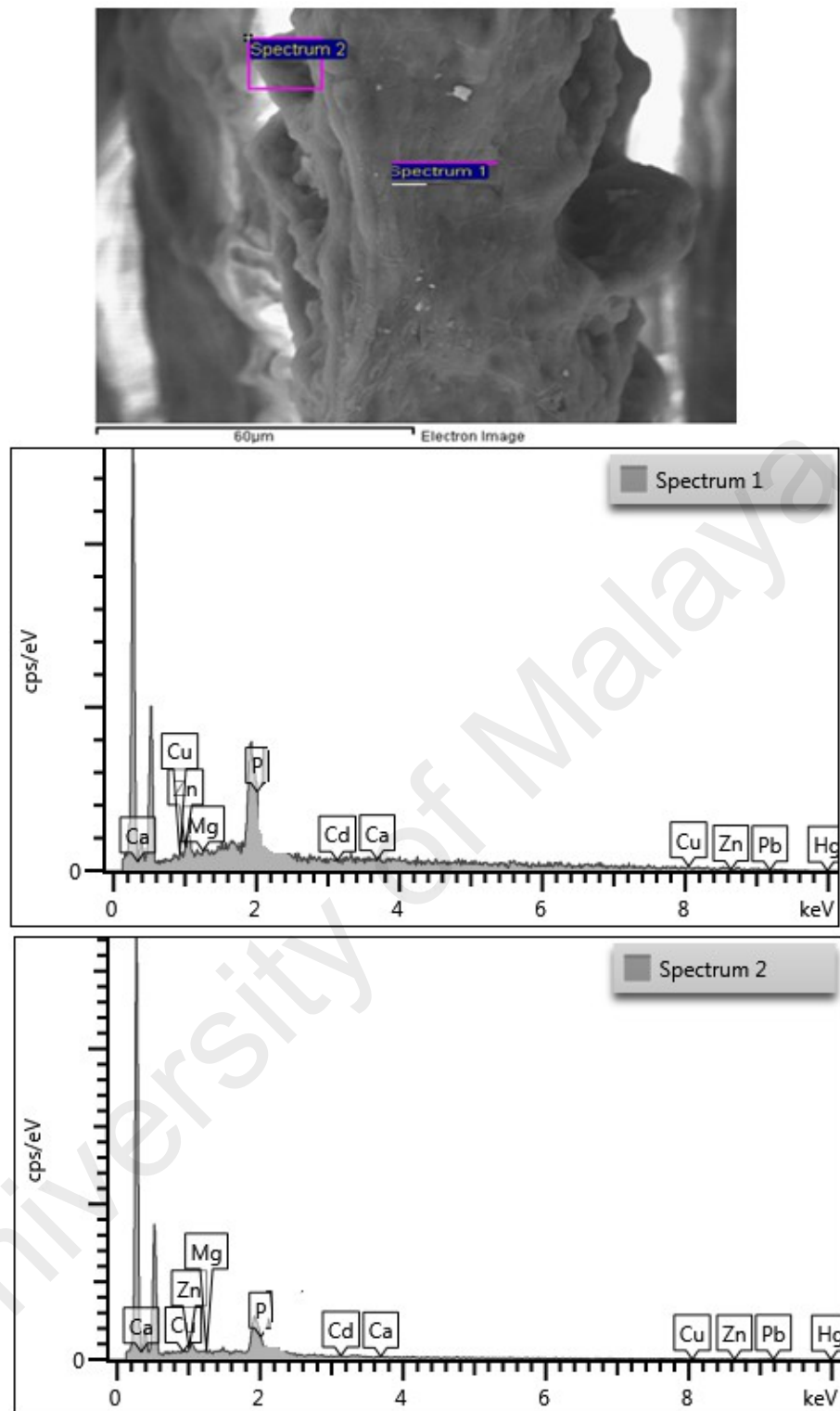


Figure 5.8. SEM and EDX microanalysis gill from exposed fish. X-ray spectrum shows peaks of slight increase in the weight percentage of Cu (35%) (1) in primary lamellae and (8%) secondary lamellae (2) at 96h exposure to Cu ions.

5.3.4. SEM and Energy Dispersive X-ray Studies on Gills of Fish Exposed to Magnesium Metal

SEM-study confirmed that at low resolution, a common arrangement of lamellae and gill filaments in control specimens were observed. Pavement cells (PCs) were the most plentiful cell type in filament epithelium; however mucous cells and chloride cells were almost rare located chiefly on the trailing edge of the filament and at the bases of lamellae. At high resolution, characteristic surface pattern of PCs created by enlarge microridges and concave apical surface of the chloride cells (CCs) protected by microvilli (Fig. 5.1).

Some of the noticeable changes are induced by Mg metal ions exposure involving gradual fusion of secondary lamellae which results in transformation of filaments and loss of normal architecture which leads to increase enhance harshness of morphological alterations after 96 h of exposure to Mg ions (Fig. 5.9). Generally, SEM observations at low and high resolutions confirmed that Mg metal ions exposure induced alterations which were observed at light microscopic level. This corresponds with the results obtained by Pandey et al., (2008) that recognized quantitative and qualitative changes in the surface morphology of chloride cell, and pavement cells in the gills of the exposed fish. Metal exposure caused an increase in chloride cells density and apical surface area, over the same in control fish, and they also explained that an increase in apical membrane area and chloride cells density could be considered as an adaptive response against metal exposure, especially in the fish exposed for a longer duration.

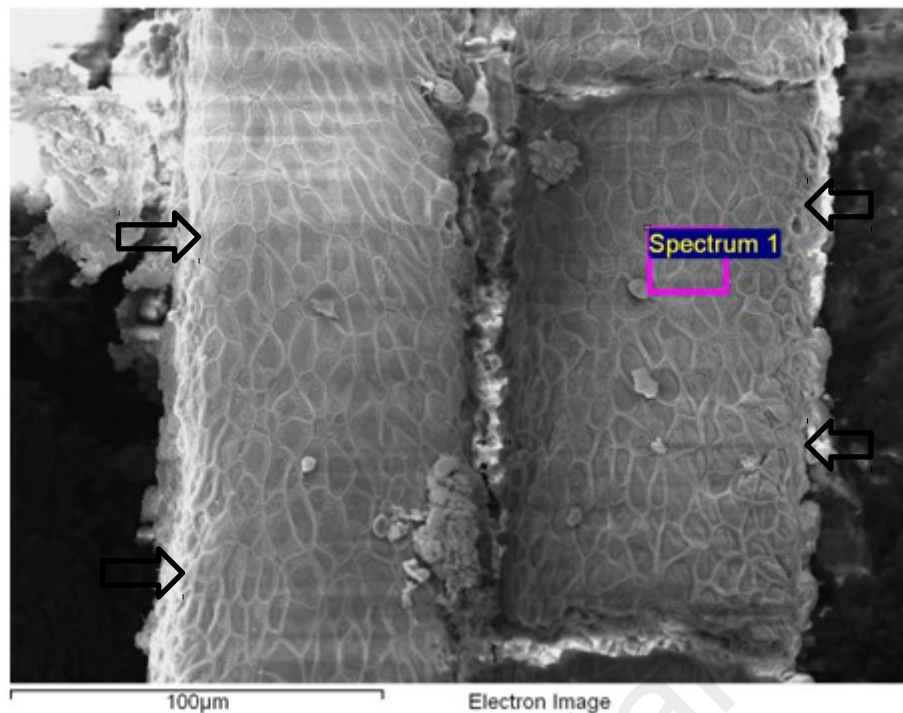


Figure 5.9. Scanning electron microscopic micrograph of gill from *Oreochromis* sp. showing fusion of secondary lamellae (big arrow) with increased severity of morphological changes after 96h exposure to Mg ions.

Enhance in CC apical membrane area and CC density in the fish exposed to trace metal mixture demonstrates a flexible response of CC. A fast response of enhance in the CC apical surface area could be because of the retraction of adjacent PC or swelling of the CC itself which gave an indirect clue of function of CC in discharge of element (Wong and Wong, 2000).

The microridges of pavement cells in exposed group were dilated and the swelling led to fusion of microridges at few places (Fig. 5.10 A, B, C). In addition, our results reported increased amount of magnesium weight percentage (8-20 %) in microanalysis of EDX (Fig. 5.11 and 5.12). The ultrastructural differences that were detected are consequence of their exposure to Mg ions which are in agreement with Carmon et al. (2004) who mentioned that the function of apical microridges may mechanically enhance the adhesion of water molecules, thus helps to attract the diffusion of

respiratory gases from water to blood and vice versa. This direct contact with the surrounding water containing metal ions may affect immediately with microridges of pavement and chloride cells. The study of Pandey et al. (2008) confirmed that the microridges of PCs in exposed group were dilated and at swelling led to fusion of these microridges a few places.

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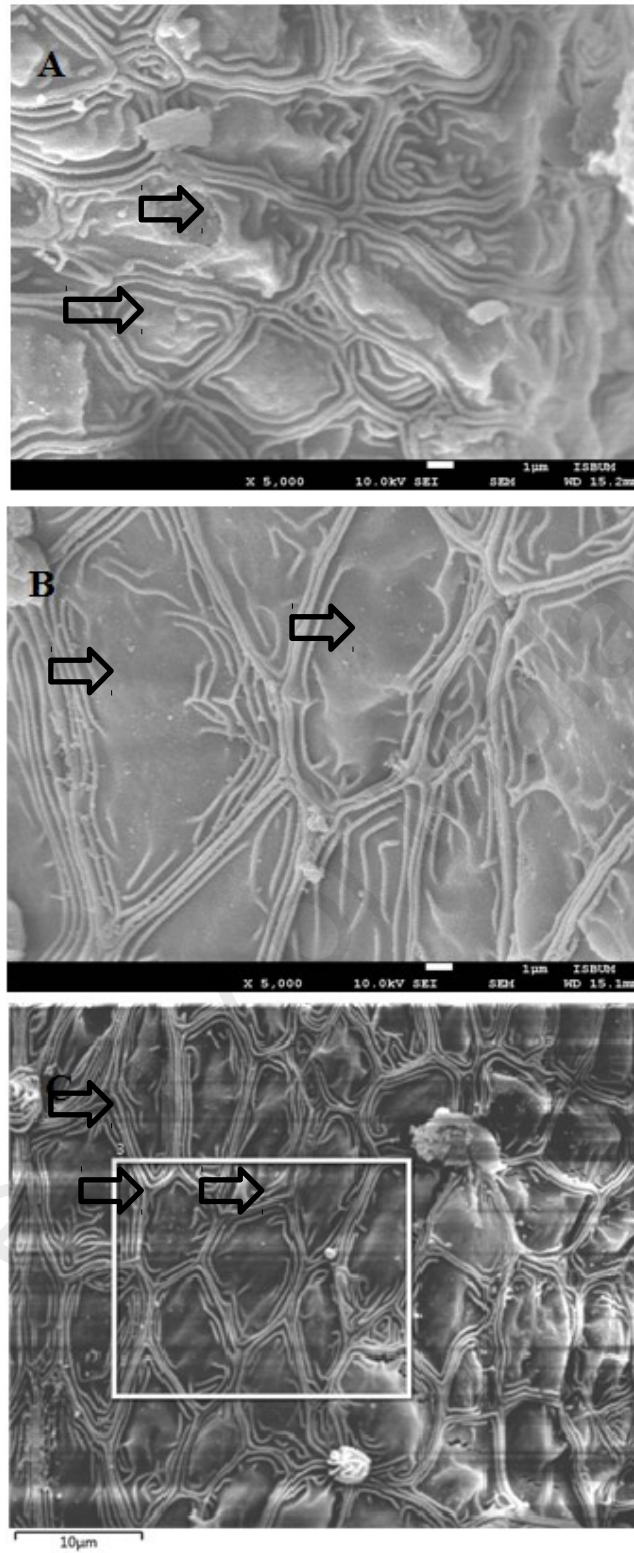


Figure 5.10. SEM images of gill lamellae from exposed fish to 96h Mg ions showing increased chloride cells density and apical surface (arrow) (A and B) and fusion of microridges (arrow) (C).

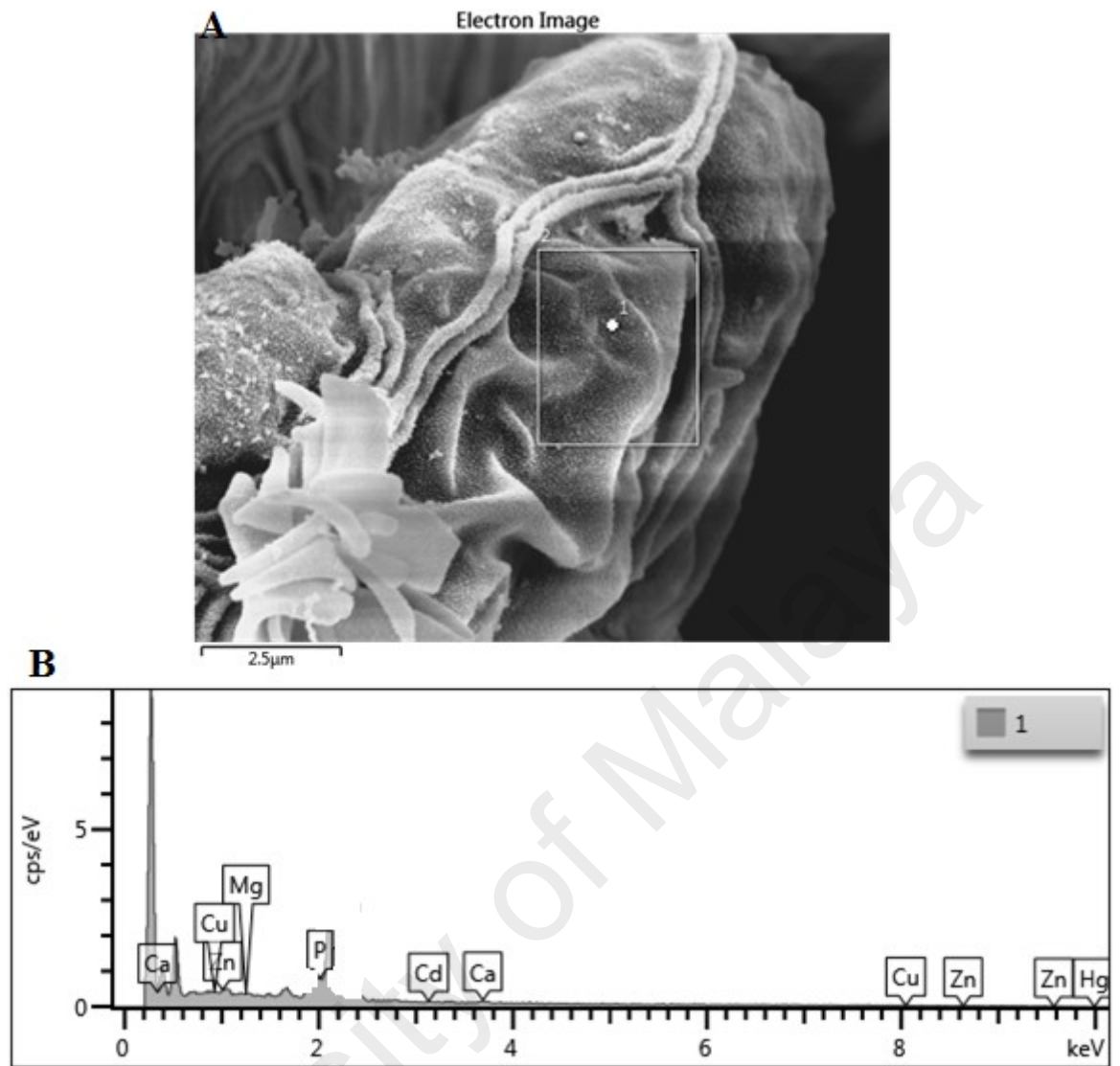


Figure 5.11. SEM and EDX spectroscopy microanalysis of the secondary lamellae of gill from *Oreochromis* sp. (A). X-ray spectrum revealing slight amount of Mg weight percentage (8%) with present essential element (Ca, P, Cu and Zn) and not detected Cd, Hg, and Pb.

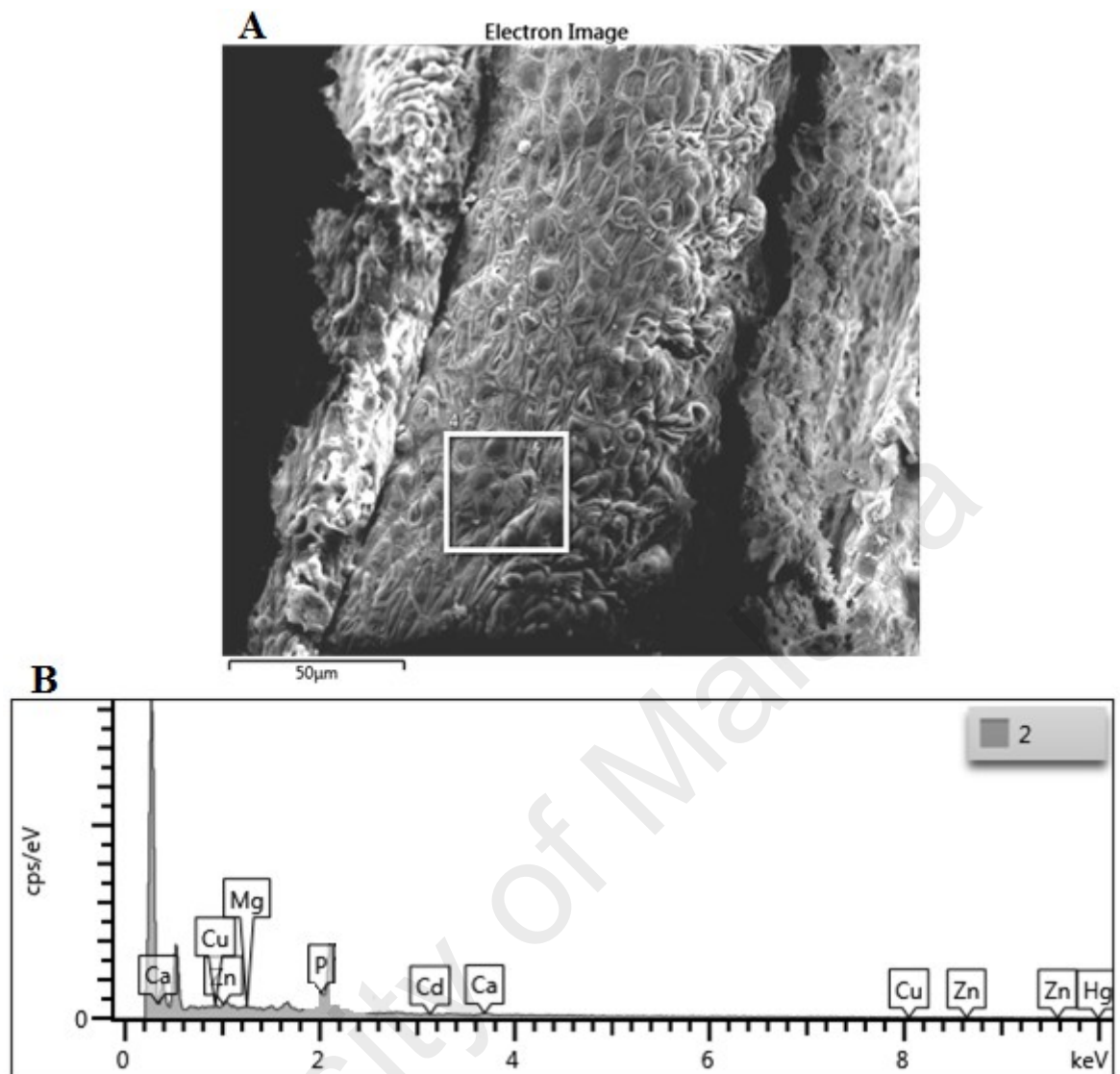
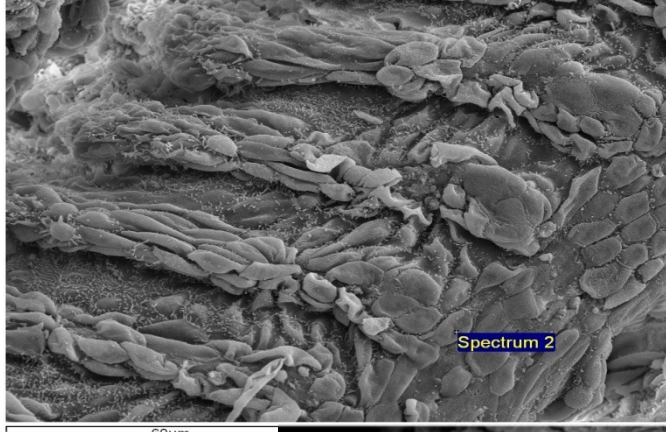


Figure 5.12. SEM and EDX spectroscopy microanalysis of the primary lamellae of gill from *Oreochromis* sp. (A). X-ray spectrum revealing increased amount of Mg weight percentage (20.04%) with present essential element (Ca, P, Cu and Zn) and not detected Cd, Hg, and Pb.

5.3.5. SEM and Energy Dispersive X-ray Studies on Gills of Fish Exposed to Mercury Metal

In experimental fish, damages were much earlier. As a matter of fact, morphological changes on the secondary lamellae of the experimental fish were simply discovered even from the start of the experiment. Inorganic mercury affected secondary lamellae



can be distinguished from the show proliferation
secondary lamellae (Fig. 5.13)

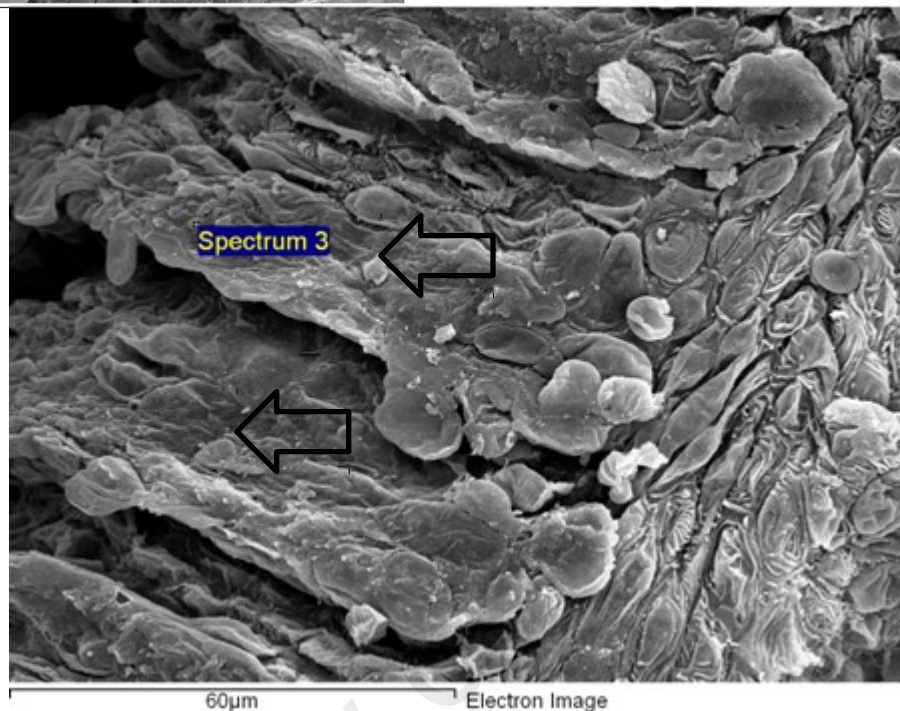


Figure 5.13. Scanning electron micrographic image of the gill from *Oreochromis* sp. showing hyperplasia and proliferation in secondary lamellae (arrow) at 96 h exposure to Hg ions.

Scanning electron micrograph illustrated morphological effects by the presence of extensive epithelial hyperplasia resulting in the formation of an interlamellar bridge which causes a reduction of the water space (Fig. 5.14A).

After 96 h, the number of interlamellar bridges between the secondary lamellae of red fish has increased attributed to the fusion of the adjacent lamellae and subsequently, the water space is completely lost (Fig. 5.14B). In addition, there was a high increase in the availability of mercury in spectrum shape of EDX analysis showing a percentage (53.10%) (Fig. 5.15) compared with intangible ratio in the control gill sample (Fig. 5.2).

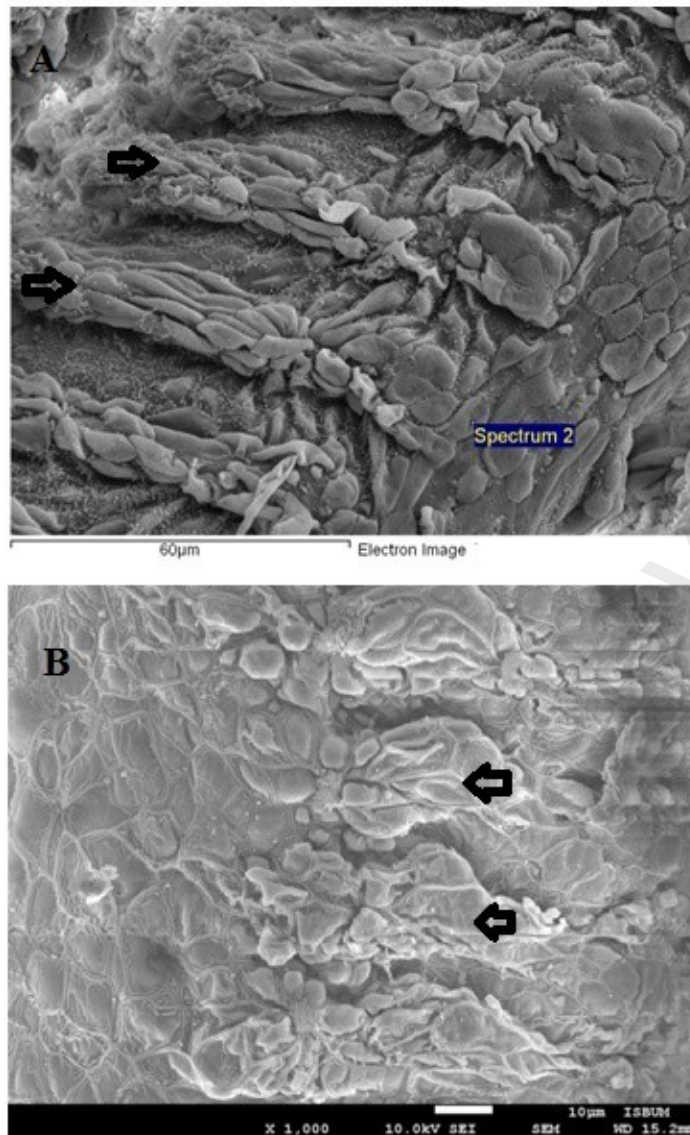


Figure 5.14. Scanning electron micrographic images of the gill from exposed *Oreochromis* sp. to Hg ions showing extensive epithelial hyperplasia and lamellae fusion (arrow) at 72 h (A); and increasing in number of interlamellar bridges (arrow) at 96 h (B).

This corresponds to the study of da Silva et al. (2012) on the gills of *Hoplias malabaricus* exposed to mercury ions revealing that the structural alterations like aneurisms and lamellar fusion and this morphological alteration reinforce the potential of natural mercury levels to impair physiological processes over time in systems that have high background levels.

When mercury accumulates in amounts that are more than acceptable in the crucially essential organs of fish, it must certainly affect different features of their vital behavior. Previously, our data indicate substantial differences in the accumulation of inorganic Hg in gills which resulted in significant damages to the gills of the treatment fish.

Mercury causes strong toxicological effects on the cell membrane and many aspects of its toxic action have been attributed to its ability to cross the cell membrane and to disrupt cellular ion transport processes (Oliveira Ribeiro, 2000). Due to this fact that gills have crucial functions, such as acid base balance, respiration, osmoregulation and excretion, and as long as they are the most influential way for the uptake of inorganic mercury in fish. As reported by Oliveira Ribeiro et al. (1996), dissolved inorganic mercury at 0.1mg/L HgCl₂ led to considerable morphological changes on respiratory lamellae that reduces their gas exchange capacity with the environment. The gill damage caused by the impact of mercury is similar to the effects which occurred by the influence of other heavy elements (cadmium, chromium, and copper). In the study of Alazemi et al. (1996) on gills of rainbow trout *Gnathonemus paersii*, they reported changes in brachial structures of the gills, a significant reduction in the diffusion capacity and a drastic diminish in the water space between adjacent secondary lamellae, both of which were assigned to the raise in the volume of the swelling secondary lamellae, as well as the fusion of the secondary lamellae was the most important lesion in gills of fish which were exposed to Cr , and also epithelial hyperplasia. The mentioned study demonstrates that these alterations in gills are strictly related to metal exposure which is like those mentioned here for inorganic mercury on tilapia fish.

Under the same experimental conditions as those described in present work, Oliveira Ribeiro et al. (2002) showed that the effect of waterborne inorganic mercury on gill of arctic charr (*Salvelinus alpinus*) after 96 h exposure was represented in blood

congestion (aneurysms) with the changes in red blood cell shape in the capillaries as well as proliferation of epithelial cells with lamellar fusion and then the secretion of excessive mucus which led to reduce gas exchange. In addition, they confirmed the intervention of chemical and physical conditions with the bioavailability of mercury ions to biological membranes.

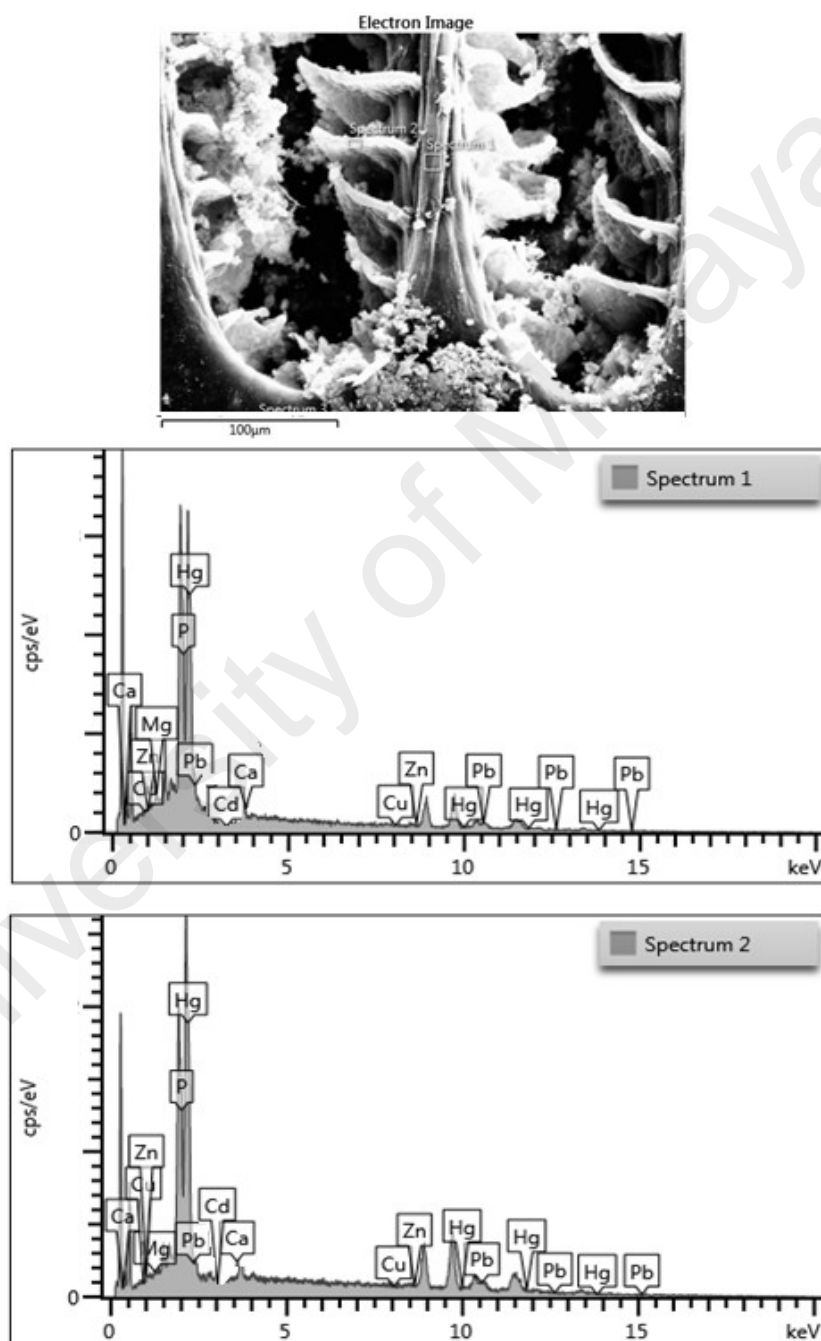


Figure 5.15. SEM and EDX spectroscopy microanalysis of gill from *Oreochromis* sp. after exposure to Hg ions over 96h. X-ray spectrum revealing high amount of Hg (53.10%) (1); and (51.10 %) (2); and additionally presence of amount of P with peaks of Ca, Cu, Zn, and Mg.

5.3.6. SEM and Energy Dispersive X-ray Studies on Gills of Fish Exposed to Lead Metal

Heavy metals pollution of the aquatic environment is a subject of considerable concern. These metals tend to accumulate in organisms and have been found to have a variety of adverse effects on fishes. Higher concentrations of lead, cadmium and mercury were toxic to the fishes; even lower concentration is also considered toxic to the fishes (Atta et al., 2012)

SEM of gills from treated red tilapia with Pb ions presented in the present study revealed impairment and disturbance of bony ossification of gill filament and lamellae and also have shown abnormalities as well as changes in architectural formality in gill filaments (Fig. 5.16) and coagulate necrosis in pavements cells with the disappearance of its architecture to gather with its microridges because of the influence of the toxicity of lead ions which produced changes in the ultrastructure and chemical composition of gill filaments (Fig. 5.17). This is in agreement with the review by Jezierska et al. (2009) about the disturbance of heavy metals on early development of fish may be caused by metal toxicity which reduces gill calcium uptake and resulted in changes in gill filament properties as they become flexible.

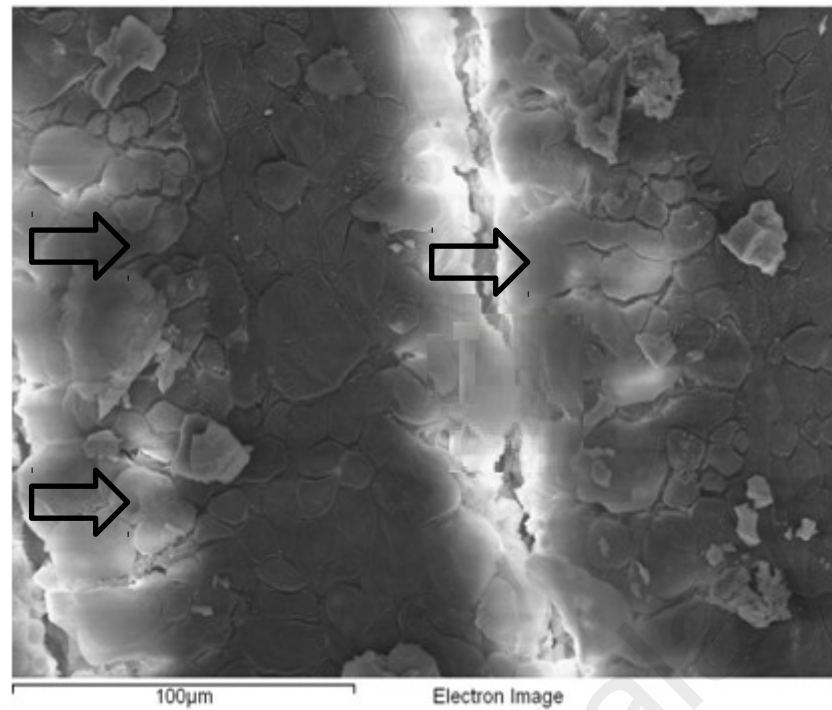


Figure 5.16. Scanning electron micrographic image of gill from *Oreochromis* sp. showing fusion of secondary lamellae (arrow) and loss of normal architecture and increased severity of morphological changes at 96 h exposure to Pb ions.

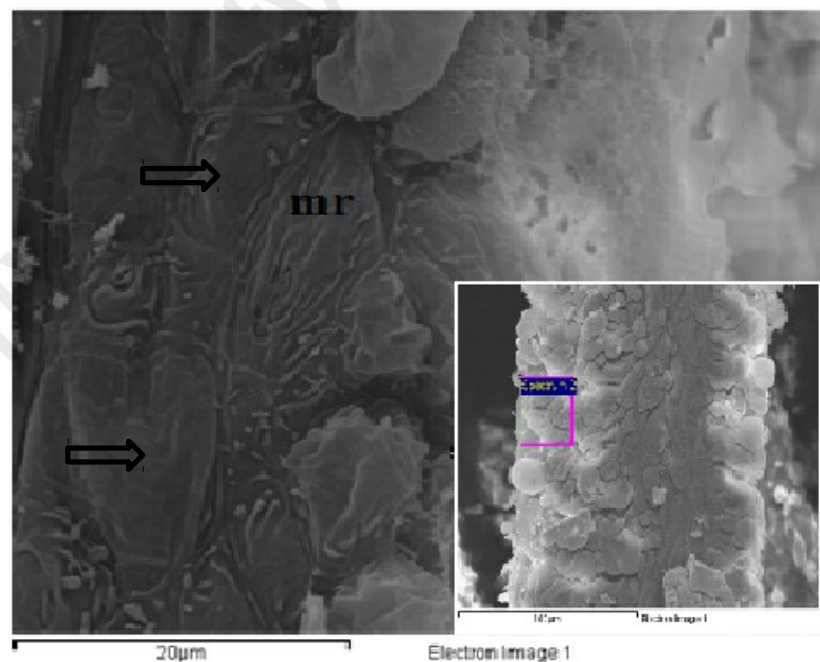


Figure 5.17. Scanning electron micrographic image of gill from *Oreochromis* sp. showing disappear of microridges (mr) (arrow) in pavements cells at 96 h exposure to Pb ions.

In addition, the presence of lobulated areas with the deep dark inter lamellar space of gills may be due to the mixing of the increased mucus secretion with inflammatory fluid (Fig. 5.18). This is similar to the study by Hassanain et al., (2012) who explained the Pb element analysis via SEM with EDX technique on gills of Nile fish *Oreochromis nilotica* which have been treated by the lead acetate (14.6 mg/L). Their results indicated that gill filament and pavement cells have distinct degeneration. It also revealed impairment and disturbance of bony ossification of gill lamellae and filaments that is due to bony proliferation changes. Moreover, the pavement cells showed coagulated necrosis with the disappearance of its architecture and microridges. They also observed the deep dark inter lamellar space with lobulated areas that may be due to the organization of the increased mucous mixed with the inflammatory fluid.

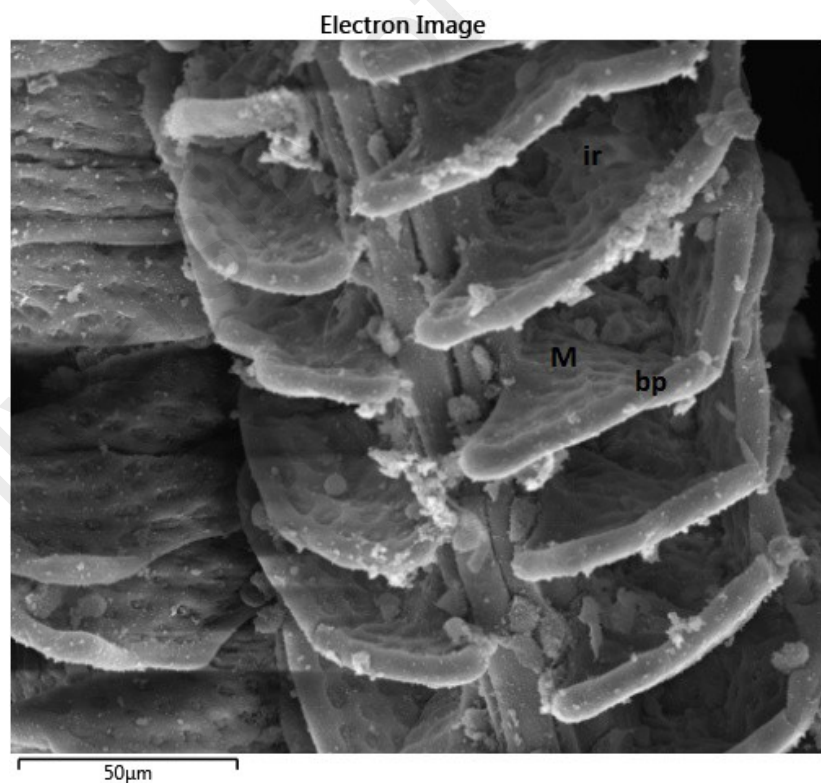


Figure 5.18. Scanning electron micrographic image of gill from *Oreochromis* sp. showing presence of mucus secretion (M) in interlamellar region (ir) and bony projection (bp) appeared on the lamellae surface at 96 h exposure to Pb ions.

The fish gill is an extremely specific organ with several important functions. In addition to the function of a respiratory gas exchange area, it also acts as a location for clearance of waste products of nitrogenous metabolism along with maintenance of acid-base and mineral balances. The osmotic issue of fish living in hypotonic situation is to keep a higher osmotic pressure in the extracellular fluids as compared with the neighboring environment. This is possible due to an active ion uptake via the gills and by preventing ion loss over the membranes and water inflow to the tissues. So, heavy metals influence on both osmoregulation and oxygen uptake in fish. Consequently, it will meet different pathological changes in gill tissues (Lehtinen and Klingsted, 1983).

The examination by EDX in scanning electron microscope showed that the spectrum increasing in the lead element percentage and this is an evidence of a cumulative susceptibility to the Pb metal in the fish gill of *Oreochromis* sp. At the same time, this metal has an effect on to reducing the calcium and phosphorus as in Fig. (5.19, 5.20).

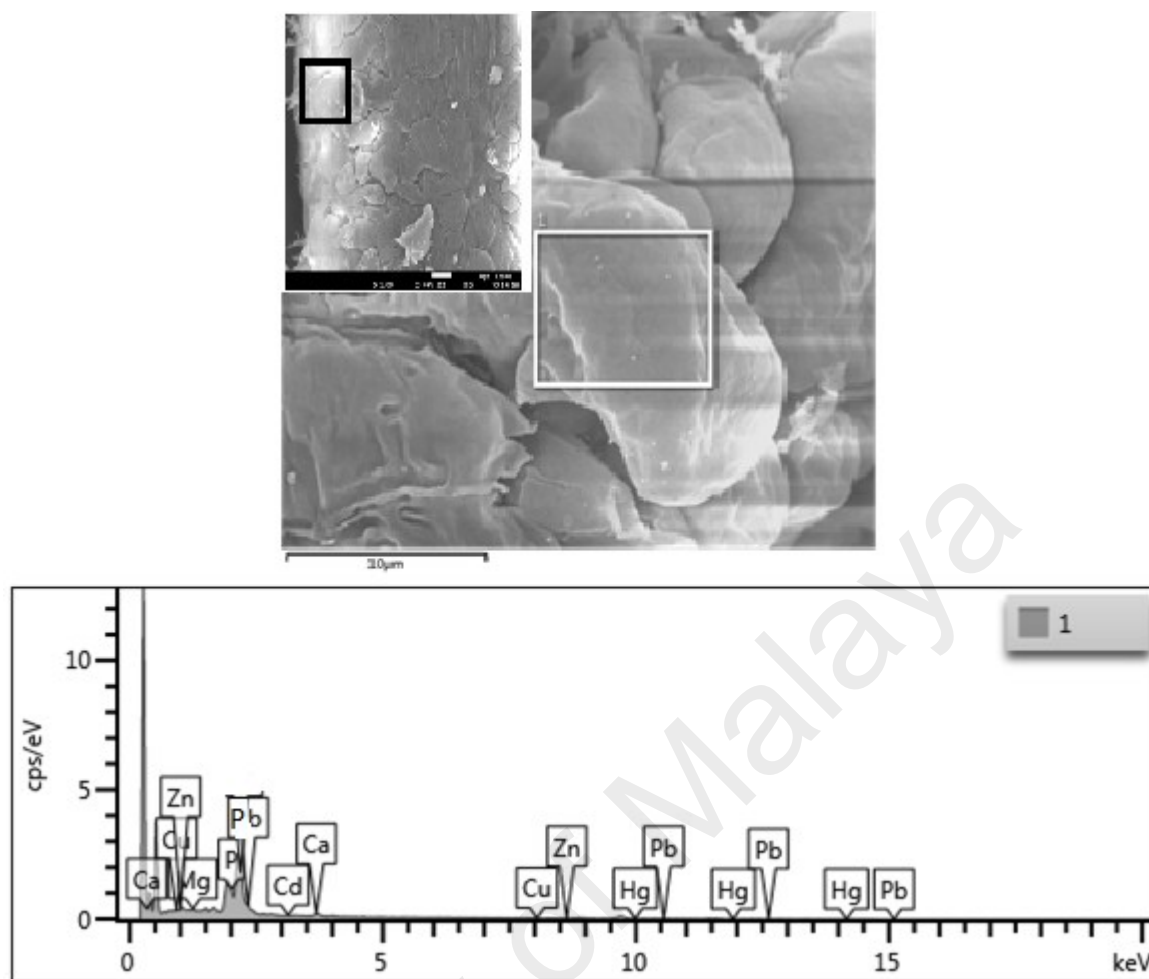


Figure 5.19. SEM and EDX microanalysis of the gill lamellae from *Oreochromis* sp. after 96 h exposure to Pb ions. Elemental analysis spectrum shows appearance of Pb in weight percentage (25.27%) with low peaks of essential elements (Ca, P, Zn, Cu and Mg) and not detecting of Cd and Hg.

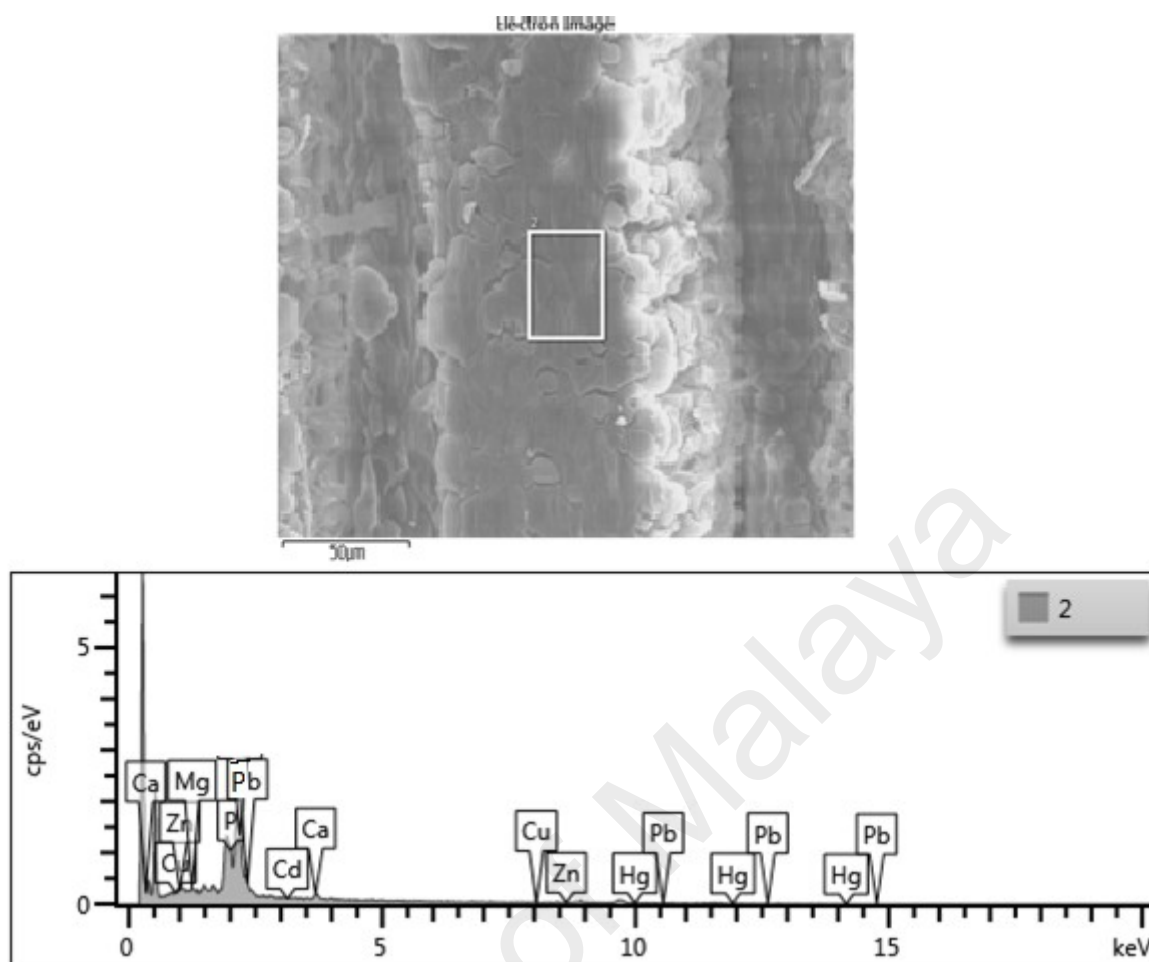


Figure 5.20. SEM and EDX microanalysis of the gill filament from *Oreochromis* sp. after 96 h exposure to Pb ions. Elemental analysis spectrum shows appearance of Pb in weight percentage (27.47 %) with low peaks of essential elements (Ca, P, Zn, Cu and Mg) and not detecting of Cd and Hg.

The presence of lead in exposed fish gills in high concentration is due to that Pb ion from waterborne bind with the mucus layer which exists on general body surface and particularly on gills of the fish (Tao et al., 2000). It should be also mentioned that lead has affinity with fish biomass which is considered a potential biomass to remove Pb^{+2} ions from synthetic solutions with lead contaminated water (Ashraf et al., 2012). Nevertheless, the concentrations of metals in the tissues of fish gill reflect the presence of these concentrations in the ambient water, whereas increasing the concentrations in the liver indicates the metal storage in longer period (Rao and Padmaja, 2000).

In the present study, the lead had the impact on the histopathological changes in gill filaments including necrosis, fusion and proliferation in epithelial cells. This is consistent with the research Olojo et al. (2005) studying cat fish *Clarias gariepinus* exposed to environmental pollution such as lead and noticed breakdown of pillar cells system which then resulted in capillary congestion.

It also corresponds with the study by Vasanthi et al. (2013) in which they found that the accumulation of heavy metals including lead, iron and zinc was high concentrations in the gill tissues due to the defense mechanism of body and this organ is the main way for the entry of pollutants from the water that resulted in several histological lesions observed such as slight malformation of the gill lamellae. In addition, the fusion of adjacent lamellae was more obvious and more prevalent in the fish *Mugil cephalus* which was found in polluted environment; and this alteration could be a protective effect for minimization the quantity of surface area in susceptible gill.

Heavy metals have effects on the regenerating or degenerations of the cells as recorded by Atta et al. (2012) who found that the cytoplasm of the cells is vacuolated with multi-nucleoli in treated fishes *Oreochromis niloticus* with Pb at 0.025 mg/l; and this may be irregularly led to cell proliferation.

5.3.7. SEM and Energy Dispersive X-ray Studies on Gills of Fish Exposed to Zinc Metal

The deformation in gill lamellae was observed through the results of the current study, due to the expansion and proliferation of epithelial cells with a complete fusion of the secondary filaments in addition to disappear of microridges in pavement cells with an increase in the secretion of mucus in the samples exposed to the element zinc during the

period of 96 hours (Fig. 5.21). This indicated that red tilapia has a high susceptibility in phenotypic changes during the raise of exposure periods.

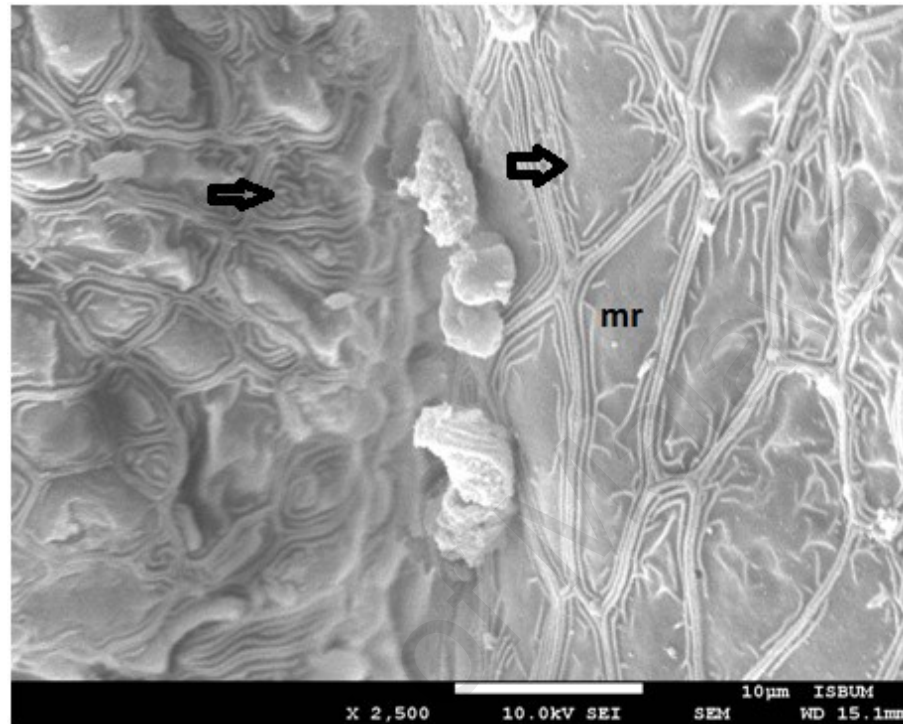


Figure 5.21. Scanning electron microscopic micrograph of gills from *Oreochromis* sp. after 96 h exposure to Zn ions shows necrosis and disappearance of microridges (mr) (arrow) in pavement cells.

An examination by SEM- EDX noticed a small increase in the availability of zinc by watching the spectrum shape that increases the percentage (15-39%). These increases are similar to the flaks as can be seen in Fig. (5.22). This indicated the presence of cumulative susceptibility to zinc metal in gill of *Oreochromis* sp. and at the same time, this metal affected the reduction of the calcium and phosphorus. These results are in agreement with Sauer and Watabe, (1984) in the analysis of zinc in *Fundulus heteroclitus* L. showing that high environmental Zn concentrations have an apparent affect on the calcified regions of the scales which caused a reduction in the Calcium and Phosphorus ratios.

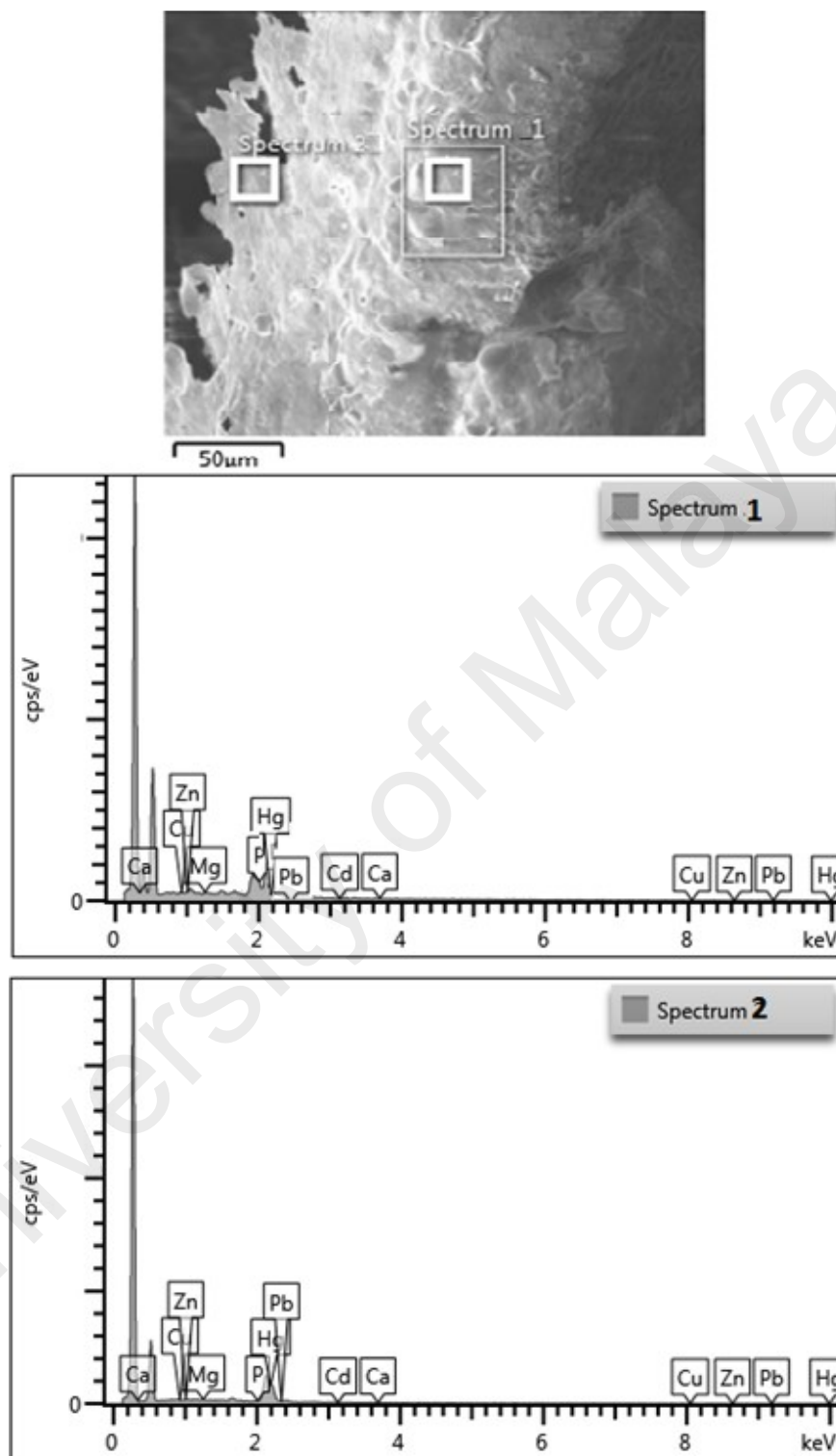


Figure 5.22. SEM and EDX microanalysis of gill from *Oreochromis* spp after 96 h exposure to Zn ions. Elemental analysis spectrum shows appearance of Zn in weight percentage (15%) in primary lamellae area (Spectrum 1) and (39.48 %) in secondary lamellae area (Spectrum 2) with low peaks of essential elements (Ca, P, Cu and Mg) and not detecting of Cd, Pb and Hg.

Similar results were observed in gills of *Astyanax aff bimaculatus* after exposed to acute exposure concentration of 5 mg/L of Zn by dos Santos et al. (2012) indicated that fish suffered interference in the respiratory system represented in the fusion of lamellae was 100% in gills, proliferation of cells rich in mitochondria and stem cells resulting in thickening in epithelial tissues of secondary lamellae; all of these effects can be considered protection mechanism because it decreases the exposure area of secondary lamellae to the toxic elements.

Zinc is necessary to perform a cellular function and classified as a micronutrient. Nevertheless, it may turn into toxic to fish and other aquatic organisms in high concentrations (Celik and Oehlenschläger, 2004). The morphological alterations may present the most affected target organs and discover the sensitivity of the organism to the levels of contaminants to which they were exposed (Fernandes and Mazon, 2003).

After exposure to metals, huge number of mucus was seen over the gills (data not shown). This is in agreement with the study by Pandey et al. (2008) who confirmed that variations in the environmental conditions lead to stress inducing the proliferation of mucus cells and consequently enhancing mucous secretion; in addition, the histopathological alterations in the gill such as epithelial necrosis, edema, hemorrhage at filaments, fusion of secondary lamellae, hypertrophy of epithelial cells and sloughing off of epithelial surface are the main effects stated in gills from the fish exposed to different kinds of pollutant. It is also suggested that by adapting to apparently pathological symptoms such as lifting of the epithelium and lamellar fusion, fish may be able to survive the pollution effect (Evans et al., 2005).

5.4 CONCLUSION

Findings of scanning electron micrographs gave more details of elements effects on gills of red tilapia fish. Energy dispersive X ray proved the existence of the accumulation of heavy metals in the surface of the exposed fish gill. This work advances a new knowledge as influence of heavy metals in the gill histology of red tilapia fish and confirmed that their effects could be observed at different exposure periods; in addition, supporting environmental watch over aquatic systems when polluted by heavy metals.

CHAPTER 6

BIOACCUMULATION OF HEAVY METALS IN RED TILAPIA CULTURED AT FOUR DIFFERENT SITES

6.1. INTRODUCTION

Fish is considered as an essential food resource which serves as an important factor in several food chains. Fish is high in protein content, omega-3 fatty acids, amino acids and fats as well as vitamins. Moreover, fish has been shown to have minerals such as Ca, Fe, Cu and Zn that can improve and counteract the advantages of omega-3 and protein (Mary and Adeniyi, 2012). Tilapia/cichlidae is a hardy fish which grows very rapidly and has been a major source of protein food in several developing countries and extensively popular in several developed countries (WWF, 2011). Generally, this kind of fish is among the top ten types which possess high rate of expansion in production quantity as well as straightforward breeding with no specific hatchery systems. It should also be mentioned that after carp, tilapia takes the place of the most frequently consumed farmed fish. (Nandlal and Pickering 2004).

Comparing the prices in Malaysia with global pricing, it was observed that they are 10% higher than the global import prices. This situation leads to the fact that market for tilapia inside the country is richer than the export market. As a result, the business has included several commercial companies that expand the scope of aquaculture and production system-low tech earthen ponds to more expensive concrete tanks (Josupeit 2008).

Generally in Malaysia, aquaculture has a rapid growing, notably for red tilapia (*Oreochromis* sp.); Heretofore, red tilapia has been considered as the most remarkable

aquaculture products due to the fact their big size and tolerance with the salinity extent. Moreover, the species has been selected for their level of aquaculture organism's sensitivity in terms of heavy metals contamination and accumulation (Mokhtar et al., 2009).

The increased level of pollutants in fish can lead to health dangers. Thus, recognizing heavy metals' bioaccumulation capacity is remarkably noticeable to evaluate the probable danger to human health and efficiently step in order to support the public care particularly the metals that pose serious health hazards to humans (e.g. Hg, Cd, and Pb). The aquatic environment has been prevented biological deterioration and identified the sources which threaten ecological equilibrium (Otitoloju, 2002; Ambedkar, and Muniyan, 2011).

Fish can respond to environmental changes that can be used as good bioindicator for pollution studies because it has the potential to accumulate metals, easy to be obtained in large quantity, long lifespan, optimum size for analysis and easy to be sampled (Batvari et al., 2007).

Furthermore, contaminant debris in the body of the fish might finally get to a high concentration hundreds or thousands of times more than what has been assessed in the water. Therefore, monitoring fish tissue contamination serves as an early warning biomarkers of aquatic environmental contaminants. Eventually, dietary intake of these biomagnified species poses risk to human health (Türkmen et al. 2005, Osman, and Kloas, 2010). Furthermore, the absorbed heavy metals with the tiny lot are whether kept in a metabolically accessible form for fundamental biochemical procedures or to be detoxified in metabolically neutral forms and kept in the tissues whether for short time or forever (Hashmi et al. 2002).

Aquaculture in Malaysia is suffering from water pollution (Ahmad and Shuhaimi-Othman, 2010). Some of the coastal sediments of the Juru River in Penang and Johor Strait are contaminated by Pb, Cd and Zn about two and three times higher than the international limits. Consequently these sediments become the main carrier of most metals (Idriss and Ahmad, 2012). Therefore, many pond, lake, and river systems in the developed world have been subjected to regular monitoring of their contaminant levels (Shinn et al., 2009). The main purpose of the present study was to determine the levels of heavy metals: Mg, Cu, Zn, Cd, and Pb in three different organs namely gills, liver and muscle of red tilapia (*Oreochromis* sp) collected from four different aquaculture production ponds sites and to make a comparison of their concentration among these cultured sites.

It should be mentioned that the present research has been induced by the fact that red tilapia has turned to be the most important fish especially the highest income yields that is achieved with producing 80% of the total tilapia output (Department of Fisheries et al. 2008). Moreover, fish consumption and seafood in Malaysia is three times higher than the global consumption (Hishamunda et al. 2009).

6.2. MATERIALS AND METHODS

6.2.1. Sampling Locations

Red tilapia samples were collected from four different aquaculture ponds located in Selangor and Pahang, Malaysia. The sampling sites were concrete tanks in Serendah (1) situated at 03° 21 northern latitude and 101° 36 eastern longitude; Kampar in Perak(2) at N and E; Bistarijaya (3) at 03° 22 N and 101° 20 E; and Bukit tinggi (4) in Pahang at 03° 23N and 101° 50 E (Figure 6.1). Specimens were collected during the sampling period in November 2013 and January 2014.

6.2.2. Sample Collection and Preparation

Red tilapias (*Oreochromis* sp.) were randomly caught from each site (10 fish from each sampling site) using fishing net to catch the fish. The fish length ranged between 21 to 23 cm and the weight ranged between 210 to 250 g. The fish were washed using deionized water and inserted in a plastic bag that was also waterproof, and later sealed and labeled. Then, ice packets in a closed container were used to cool the packaged samples instantly prior to moving to the processing lab on the same day. In the processing lab, the samples were kept in the temperature of -20°C to the time that dissection is carried out.

6.2.3. Water Physicochemical Parameters

The water samples were filtered through Whitman 541 filter paper immediately after the samples have been transported to the laboratory. The filtered samples were acidified with HNO₃ and were kept at 4 °C prior to the analysis. Water Physicochemical Parameters including the pH, electrical conductivity (mScm⁻¹), temperature (°C) and total solid (ppm) were measured at four sampling sites by using the instrument of water

checker HANNA model HI9828. For indicating the nature and the sources of the polluting substances, heavy metals measured the total cadmium (Cd), copper (Cu), magnesium (Mg), lead (Pb), and zinc (Zn) after digestion using ICP–OES (Perkin Elmer AA Analyst). Data on the selected sites are shown in Table (6.1).

6.2.4. Sample Preparation and Analysis

After unfreezing the fish sample to room temperature, body parts samples including liver, gill and muscle were moved to have metal test that was performed by stainless steel knives which have been homogenized and scaled. Individual samples were washed with tap water followed by bi-distilled water and then oven-dried to constant weight at 105°C for 24 h. The dried samples were crushed and powdered in an agate porcelain mortar and pestle. The samples were 0.5g in dry form for powdered gill and muscle but for powdered liver the weight is 0.1g in dry form. The sample went under process for three times and later digested by the help of a closed vessel microwave digestion (Milestone model Start D, Italy) with an ultrapure nitric acid (65%) hydrogen peroxide (35%) mixture at a 3: 1 ratio at a temperature of 150 C for 20 min. after this stage, a 35 min cooling period was performed at room temperature in the microwave (Durali et al., 2010). Due to the fact that hydrogen peroxide can lower nitrous vapors' level and accelerate the digestion of organic substances by raising their action temperature, it was added to the samples with nitric acid (Dig-Acids, 2001). Blanks were simultaneously used in each batch of analysis to authenticate the analytical quality. The number of specimens of each organ for each location was three. The digested samples were diluted with deionized water to a total volume of 25 ml for liver and 50 ml for gills and muscles and then filtered through 0.45 µm What man filter paper (Germany). The analysis was conducted using inductively coupled plasma mass spectrometry (ICP-OES) (model 5300DV Perkin Elmer, USA). It included the assessment of concentrations of the

following five heavy metals: cadmium (Cd), copper (Cu), magnesium (Mg), lead (Pb) and zinc (Zn). Element standard solutions from the Merck Company used for calibration were prepared by diluting stock. The results were calculated in milligram per kilogram dry weight (mg/kg dry wt). All of the glassware and plastics were soaked overnight in 10% (v/v) nitric acid, rinsed with distilled and deionized water and dried before being used (Csuros, and Csuros. 2002).

6.2.5. Statistical Analysis

All values from chemical analyses are presented as mean \pm SD. Data obtained from the experiment were subjected to two-way analysis of variance (ANOVA) test using a computer program SPSS version 20. In all cases, the accepted significance was reported at $P < 0.05$ levels.

Table 6.1. Physical and chemical parameters of water samples collected from different locations

Sampling Sites	Serendah	Bestarijaya	Bukit tinggi	Kampar	Permissible limit*
	Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD	
Parameter (unit)					
PH (Unit)	7.5 \pm 0.4	7.1 \pm 0.3	7.6 \pm 0.1	7.6 \pm 0.4	7 - 8.5
Conductivity (mS/cm)	0.07 \pm 0.01	0.10 \pm 0.02	0.10 \pm 0.01	0.09 \pm 0.01	-
Temperature (°C)	27 \pm 1.2	29 \pm 1.4	28 \pm 1.3	28 \pm 1.7	Over 5°C
Total solid (ppm)	28.5 \pm 2.5	30 \pm 3	27 \pm 2	29 \pm 3	500
Cd (ppm)	0.004 \pm 0.001	0.002 \pm 0.001	0.001 \pm 0.0	0.001 \pm 0.001	0.1
Cu (ppm)	0.094 \pm 0.002	0.024 \pm 0.012	0.081 \pm 0.009	0.025 \pm 0.017	1
Mg (ppm)	1.841 \pm 0.036	ND	1.880 \pm 0.020	0.003 \pm 0.001	-
Pb (ppm)	0.013 \pm 0.017	ND	ND	0.007 \pm 0.001	0.05
Zn (ppm)	0.061 \pm 0.006	0.058 \pm 0.002	0.081 \pm 0.003	0.038 \pm 0.009	1

*Canadian environmental quality guidelines.

Results shown as mean \pm SD

6.3. RESULTS AND DISCUSSION

Thorough analysis on the level of chemical and physical factors in chosen regions water has been carried out (Table 6.1). Mean value of conductivity, total solid, temperature, pH in addition of heavy metals parameters. According to the present results the pH seems to be constant all over the sites. All the pH values were in the alkaline side (7-7.9). However, it can be concluded that the physical and chemical parameters including the concentrations of heavy metals in water from all different artificial ponds are within acceptable levels and lower than the Canadian Environmental Quality Guidelines (CCME 2014)

Many sources and factors contribute to the entry of heavy metals to aquaculture organisms including oceanic sources (upwelling), continental sources (river runoff and atmospheric transport) in addition to the diagenetic exchanges at water sediment interface, fertilizers, sewage sludge and anthropogenic atmospheric inputs (Hashmi et al., 2002). Subsequently, it was the presence of concentrations of heavy elements Pb, Cd, Fe, Cu in high average level in water samples collected from the Langat Basin rivers reported by Yusuf and Nordin (2003) who found (0.100, 0.032, 4.87, and 0.201 mg/l respectively). These concentrations are higher than the level standardized by the Ministry of Health Malaysia, Interim National Water Quality Standards (INWQS). The standard concentrations were 0.05, 0.01, 1.0 and 0.20 mg/l respectively. The level of Zn ranged from 14.63 to 91.56 µg/l which was under the permitted level of INWQS 5.0 mg/l. Besides that, Pb metal was also reported to be higher in the locations near industrial areas (Praveena et al., 2008)

Generally, physicochemical analysis gave useful information on the levels of contamination in water along the studied different production ponds, but did not give information on the effects of the contaminants on biological systems. The knowledge of

metal concentration in fish is important in pollution control strategies. The majority of fish takes the highest position in aquatic food chain and is capable of accumulating high content of metal even in gentle polluted conditions. Hence, the concentration of metal in fish can be applied as an index to scale the pollution level particularly in aquatic bodies (Karadede-Akin & Unlu, 2007). Therefore, applying biomarker reactions in fishes is highly important. Subsequently, data of bioaccumulation in red tilapia (*Oreochromis* sp.) is regarded as the indicator of pollution in ponds.

In Table (6.2) the average concentration of the heavy metals in muscle, gill and liver samples of red tilapia can be found with great variety among different tissues as well as the dearth of meaningful deviation between the selected sites. The highest concentrations of all the heavy metals were recorded in tissues samples collected from Bestari Jaya and the lowest ones were detected in the samples collected from Kampar/Perak. Liver concentrated higher levels of Cd, Cu, and Zn than the other organs for all the four sites (Table 6.2).

The difference in the accumulation of heavy metals is due to several reasons, Sankar *et al.*, (2006) recommended the metal accumulation by the fish relies on the region, feeding conduct, trophic degree, exposure time to metals, size, age as well as homeostatic procedure activities of fish has considered several points that affect the accumulation of metals in fish. These factors can be named as chemical and physical property of water as well as season. This attribute to freshwater fishes are known to regulate constant internal metal concentration. However, the concentrations of metal in a tissue rely on the special metabolism of metal species in the tissue along with the accessibility of the metal species in ambient water.

Table 6.2. Heavy metals concentrations (mg/kg) in different tissues of red tilapia *Oreochromis* sp. collected from four different locations of production ponds

Parameters	Organs	Bestarijaya	Serendah	Kampar/Perak	Bukit tinggi
		Mean \pm SD	Mean \pm SD	Mean \pm SD	Mean \pm SD
Cd	Gills (G)	0.0060 \pm 0.001	0.005 \pm 0.001	0.002 \pm 0.002	0.007 \pm 0.000
	Liver (L)	0.008 \pm 0.001	0.007 \pm 0.001	0.004 \pm 0.004	0.009 \pm 0.002
	Muscles (M)	0.004 \pm 0.000	0.005 \pm 0.001	0.001 \pm 0.001	0.005 \pm 0.001
Pattern		L > G > M	L > G = M	L > G > M	L > G > M
Cu	Gills (G)	0.081 \pm 0.010	0.050 \pm 0.012	0.038 \pm 0.008	0.099 \pm 0.011
	Liver (L)	7.464 \pm 1.367	0.453 \pm 0.160	0.060 \pm 0.035	7.133 \pm 4.082
	Muscles (M)	0.074 \pm 0.024	0.041 \pm 0.014	0.028 \pm 0.002	0.082 \pm 0.006
Pattern		L > G > M	L > G > M	L > G > M	L > G > M
Mg	Gills (G)	18.261 \pm 5.713	12.034 \pm 0.499	3.741 \pm 0.136	23.764 \pm 2.886
	Liver (L)	12.567 \pm 7.855	2.988 \pm 0.484	0.649 \pm 0.054	9.196 \pm 6.917
	Muscles (M)	42.775 \pm 9.944	24.347 \pm 1.380	11.052 \pm 0.922	39.427 \pm 6.368
Pattern		M > G > L	M > G > L	M > G > L	M > G > L
Pb	Gills (G)	ND	0.187 \pm 0.019	0.070 \pm 0.005	ND
	Liver (L)	ND	0.101 \pm 0.043	ND	ND
	Muscles (M)	ND	0.069 \pm 0.012	0.008 \pm 0.001	ND
Pattern		--	G > L > M	G > M > L	--
Zn	Gills (G)	0.793 \pm 0.189	0.790 \pm 0.111	0.504 \pm 0.206	0.942 \pm 0.019
	Liver (L)	1.396 \pm 0.891	1.045 \pm 0.220	0.892 \pm 0.050	0.980 \pm 0.236
	Muscles (M)	0.480 \pm 0.191	0.650 \pm 0.121	0.440 \pm 0.053	0.550 \pm 0.092
Pattern		L > G > M	L > G > M	L > G > M	L > G > M

ND: not detected

Results shown as mean \pm SD. Values significantly different at $P < 0.05$

Generally, heavy metal concentrations were measured in three different organs of fish: gills, liver and muscles, because of the affinity between each of them and also the fact that the metal uptake in fish is via water and diet exposures and then transferred to other target organs; specially, the concentration of metals in gills effected by level of heavy metals in the ambient water (Uysal et al., 2008).

Liver is considered the site of metal metabolism, redistribution, detoxification, or transformation. Moreover, liver is a principal organ which plays a crucial role in

storage, tend to concentrate metals, as well as, reflecting the longer-term accumulation and also an active site of pathological effects induced by toxic metals. Therefore, it is a good indicator of chronic exposure to heavy metals. Meanwhile, the metal content in the fish dorsal muscle was estimated because of its significance as food for human consumption (Usero et al., 2004; Omar et al., 2013).

In the present work, heavy metal residues in the tissues of red tilapia exhibited different patterns of accumulation and distribution among the selected tissues and localities. According to the current results, Table (6.2). Generally, the concentrations of heavy metals in the investigated parts of tilapia fish showed that the liver and then the gill have the highest degree of the investigated metals. The mentioned heavy metals distributions is as liver > gill > muscle. The mentioned metals indicated meaningful gap between organs ($P < 0.001$). According to the previous researches, the metal concentrations are higher in liver than in gill and muscle tissues without considering the sample origin. Higher binding tendency of the metals with amino groups, oxygen carboxylate, nitrogen, cystine residues and/or sulfur of the mercapto group in the metallothionein protein (MT) which has high affinities for heavy metals and consequently, liver can concentrate and regulate these metals. So, it serves as a detoxification mechanism (Al-Yousuf et al. 2000).

The present study's results were in a good agreement with the literatures. Low et al., (2011) noticed that liver samples possesses more metal accumulation specially in Cu. Due to a high disparity in Cu and Cd levels, the liver samples are more outstanding compared to other samples. Copper can be found more in the liver followed by zinc. Contrary to copper, Cd and lead can be found present at the lowest concentrations in all the tested fish organs. These results are compatible with the findings by Al-Yousuf et al.

(2000) in which they have shown that the main elements around 95% of the whole body Cu accumulation is found in liver as tetrahedral metallothionein and metalloenzymes complex species. Moreover, a constant five or six ring chelates via zinc metalloenzymes and Zn^{2+} protonated enzymes can be made by Zn. Regarding the nonessential metals, Cd indicates special closeness to the liver, due to the fact that Cd is moved through circulatory system or the entero hepatic circulation to liver in which xenobiotic detoxification happens (Wu et al. 2007). This involves the removal of oxidative damage induced by Cd via selenium dependent glutathione peroxidase at the selenocysteine site (Zirong and Shijun 2007). Pb concentration in liver at Serendah was lower ($0.101 \pm 0.043 \text{ mg/kg}$) while they are not detected in fish liver collected from other sites. The Pb content seen in fish liver is lower than the results reported by other (Low et al., 2011) which was (0.61 mg/kg).

Tilapia fish from different sites exhibited higher level of studied heavy metals which were detected in the liver and the lowest level was seen in muscle tissue. These results are consistent with what has been reported by others (Yilmaz et al., 2007). In this study, the level of Cu in the liver was found to be higher than the muscle. This is consistent with the other study by Ozturk et al (2009) in which they determined the level of Cu in various tissues of the *Cyprinus carpio* species. They reported the highest level of Cu in the liver and the lowest level of Cu in the muscle. And other study by Alam et al. (2002) reported the metal concentrations were lowest in muscle as compared with liver, kidney, intestine and gonads of cultured and wild carps, and did not exceed the established quality standards for fish.

Fish have the ability to concentrate a component from the water higher in level compared to its level in the place it was taken. Hence, metals bioaccumulation in fish is

regarded as an index of metal pollution in aquatic bodies (Javed, 2005; Karadede Akin and Unlu, 2007). It should be mentioned that few species of fish are able to live in places with high level of pollutants and accumulate higher amount of heavy metals as compared to those fish that survive at areas with less pollution (Bahnasawy et al., 2009). Among the myriad pollutants that are emitted to water settings and are possibly have high levels of accumulation and biomagnified in the food chain, heavy metals have caught a huge interest because of some factors including their toxicity, bioaccumulation, persistence for a long time and biomagnification at several trophic levels (Ololade et al. 2008). Fish may absorb dissolved elements and trace metals and then accumulate them in various tissues in significant amounts above those found in their environment, thus exhibiting elicited sublethal effects or death in local fish populations (Xu et al. 2004)

The metals' lower binding affinity on the surface of gill is the reason for the lower concentration of almost all of the observed metals in gills comparing to liver. The other reason may be the evolution of some defense mechanisms, for example superfluous mucous secretion and gill's clogging. Results have recorded that the concentration of Pb in red tilapia gills at Serendah site was (0.187 mg/L). This is in agreement with Mokhtar et al., (1994) who recorded accumulation of these metals in aquatic organisms in Sabah, Malaysia (Borneo) such as Phenomenon like urbanization and industrialization highly affect the tilapia fish. Moreover, applying phosphate fertilizers in agriculture is another factor affecting this kind of fish. Thus, small industries as well as workshops in the neighborhood of aquaculture ponds can have a negative impact on their water quality and as a result contaminated by heavy metals like Pb.

The obtained finding have shown that the gill tissues included higher level of Pb concentrations compared to other tissues in samples collected from Serendah and Kampar/Perak. It could be due to the availability of Pb in the surrounding medium,

where only 0.013 mgL⁻¹ of total Pb was detected in the ambient water for site of Serendah and 0.007 mgL⁻¹ for Kampar /Perak while it was not detected in ambient water of both Bistarijaya and Bukit tinggi. This suggests that Pb uptake mode is mainly via water, not diet. The observations are consistent with the findings of Kebede and Wondimu (2004) that showed higher concentration of Pb in the gill tissues than in the liver and muscles of *Tilapia zilli* and *Oreochromis niloticus*. The considerable amount of Pb observed in the gill tissues are due to binding capacity of mucous layer that covers the organs and their close contact with the surrounding environment (Palaniappan et al. 2008). Consequently, the accumulation of Pb in red tilapia is expected to be directly correlated with the concentration of metals in ambient water which makes origin tracking feasible.

It is argued that metal levels in gills rather than in liver are noteworthy for production sites investigation due to direct exposure of gills to ambient water.

In the present research work, the lower accumulations of metals are allocated to muscles as compared to other tissues which may have hold them by the way of circulation. The dearth of metals' binding affinity with the muscle's protein is the main reason of low accumulation of metals. This point is the matter of importance due to the fact that muscles help in having the greatest mass of flesh which is used up as food. The concentrations mentioned in this research work are in agreement with the previously recorded ones assessing red tilapia *Oreochromis* sp's metal concentration (Mokhtar et al., 2009). The highest concentration detected was from Mg in muscles tissues obtained from almost all the fields. The distribution of Mg was in the order of muscle > gill > liver and the metal in the following order Mg> Zn > Cu > Pb > Cd. This phenomenon has been previously observed by Begum et al. (2005) in three species of fish (*Tilapia nilotica*, *Cirrhina mrigala* and *Clarius batrachus*). They have reported that the average

concentrations of magnesium component were (2090-2560 mg / kg) in muscle tissues due to the fact that magnesium is one of the major elements. Therefore, level of Mg in muscle tissues is considerably higher than that in other tissue, and because of not recorded or detected any concentration of magnesium in the water component of the four sites. Mg is indicated as highly absorbed through diet suggesting that the more Mg content in the feeding pellets, the higher the level of Mg in aquacultured red tilapias.

Various studies on metal concentrations in fish samples have been previously done. Ashraf et al. (2012) reported that fish muscle showed lowest metal concentrations as compared to bone, gill and liver. This study focused on metals in fish muscle, since people eat fish muscle and not others and they also found Cu and Zn concentrations almost as low as from unpolluted lake. Another study by Mokhtar et al.(2009) on (*Oreochromis* sp.) from aquaculture ponds in Bandar and Jugra nearby the Langat estuary of Peninsular Malaysia reported that concentration of the heavy metals Cu, Zn, Cr, Mn, Fe and Ni were higher in Jugra whereas Pb and Cd were higher in Bandar.

The impacts of land-based expansions and aquaculture projects on the aquatic settings are the main reason of the majority of the environmental issues. Moreover, with great extent, the expansion of agriculture in Malaysia is meeting many issues related to those associated with the environment. Decreasing the quality of water is known as the factor increasing disease epidermis as well as products' contamination which leads to drastic loss in economy. Moreover, this fish is highly in demand in many local markets in various districts. Aquaculture has shown more contribution to the fish market around the world, suggesting the importance of aquaculture as one of the most rapidly growing food parts in the glob (Kamaruzzaman et al. 2008; WHO, 1999). And Red tilapia fish has a greater capacity for metal bioaccumulation than other species. Although it has a low sensitivity and the performance score metal pollution index (MPI) was a low, it

provides a representative picture of the environmental state of aquaculture ponds or any environmental impacts on its aquaculture organisms (Adeniyi et al., 2008; and Mokhtar et al., 2009).

Mean concentration in the muscle of fish in culture ponds in all sites have recorded that concentrations of zinc were higher than the copper element followed by lead and cadmium. This is similar to the study of Taweel, et al. (2011) on tilapia (*Oreochromis nilotica*) in natural and industrial ponds in which the concentrations were as follows: $Zn > Cu > Pb > Cd$. And the concentration of heavy metals in fish collected from natural river and lakes is higher than that collected from cultured ponds which attributed to the fact that natural water sources are more exposed to contamination than the controlled artificial ponds.

According to the present result, muscles were discovered to concentrate low amount of the heavy metals under detection, Pb's average concentration seemed to be as low as not detected in all the tissues in site of Bestarijaya and Bukit tinggi. Also, the Pb concentration in the muscles was almost the same at Serendah site (0.069mg/kg) as compared to the study by Low et al. (2010) in which it was shown that the concentration was (0.09 mg/kg). While in the muscles of fish that collected from the pond of Kampar it was recorded (0.008 mg/kg) as less than the record level by Taweel et al. (2011) which was (0.10 – 0.11 mg/kg).

The results in the present study recorded the range of Cu value in muscle as (0.028-0.082 mg/kg dry wet); this is less than what recorded by Mohammed (2009) in (*Oreochromis niloticus*) as (3.06 mg/kg) dry wet.

According to the analysis of the results, the heavy metal levels in muscles showed that Mg had the highest and Cd had the lowest concentrations (Table 6.2). These results are in agreement with the study carried out by Karadede and Ünlü, (2000) on various species of fish including *Cyprinus carpio*, *Chondrostoma regium*, *Acanthobrama marmid*, *Chalcalburnus mossulensis*, *Carasobarbus luteus* and *Capoetta trutta* from the Atatürk Dam Lake, Turkey. In their study, they demonstrated that muscles cannot be considered as active tissue to concentrate heavy metals. These results are in agreement with the results of Uluturhan and Kucuksezgin (2007) studying that in the muscles, the lowest bioaccumulated heavy metals may be related to the amount of fat in the tissue. Other factors were mentioned as low fat affinity in order to mix with heavy metals as well as muscles' low metabolic activity. Mostly the reason for the discrepancy in organ's pattern related to the fish species under examination can be related to the way their feeding habit was different as well as their lifestyle.

In the studies by Watanabe et al. (2003) and Masoud et al. (2007), it was found that the metals' bioaccumulation in tissues are different in different metals and organisms or even in various organs of the same organism. Furthermore, Koca et al. (2005) showed that the two factors of elimination rate of contamination as well as the uptake determine the patterns of contaminants' concentrations. Maheswari et al. (2006) explained that bioaccumulation of trace metals in fish is dependent on both the bioavailable concentration and species specific physiological and ecological characteristics. Metal distribution between different tissues within an organism depends on the mode of exposure and can serve as a pollution indicator. In addition, the study of Omar et al., (2013) supported this result and confirmed that the bioaccumulation measuring of metals is a useful tool for studying the biological role of the metals present at the increased levels in fish as well as the assessment of public health risk. Therefore, it has been widely used as bioindicator of metal pollution.

The concentrations of heavy metals in red tilapia fish soft tissue of edible parts from aquaculture ponds were well below the permissible limits for human consumption. All the results were found to be lower than the recommended maximum level allowed in food as recommended by the European Communities (EC, 2006), and which has been reported by Malaysian Food Act 1983 and Food Regulations 1985 (Ministry of Health Malaysia 2012).

6.4. CONCLUSION

In current study has provides useful information and a baseline for future along with continuous studies on the heavy metals concentrations in red tilapia fish of aquaculture ponds. Generally, the heavy metals concentration (Cd and Cu) in red tilapia (*Oreochromis* sp.) were higher in Bukit tinggi while Mg and Zn were found to be higher in Bestarijaya. Only Pb was found to be higher in Serendah. Detected concentrations varied significantly ($p < 0.05$) between different tissues and the lack of significant variation between the tested sites. The heavy metals concentrations were found to be lower than the recommended maximum level allowed in food by the European Communities (EC, 2006), and which has been reported by Malaysian Food Act 1983 and Food Regulations 1985 (Ministry of Health Malaysia 2012). These findings showed that tilapia fish (*Oreochromis* sp.) from all studied aquaculture ponds were safe for human consumption.

CHAPTER 7

GENERAL DISCUSSION

Special attention is given to heavy metals existing in the aquatic environment because of their toxicity, long persistence and bioaccumulation and non biodegradable properties in the food chain. Heavy metals from manmade sources pose serious water pollution problem that are deposited into the aquatic environment may accumulate in the food chain resulting in environmental damage and also constitute a carcinogenic in addition biomagnifications over time cause unfavorable effects on human health (Jaric et al., 2011; Ebrahimi and Taherianfard, 2010). Heavy metals toxicity affected on behaviour characteristics of fishes including swimming action subsequently may be impacted on population level (Solman, 2007; Eissa, et al., 2010). Contamination of heavy metals in aquatic environments, whether as a result of acute or chronic proceedings, constitutes an added source of stress which cause changing in biochemical and physiologic parameters in fish blood and tissues (Oner et al., 2008 and Firat and Kargin, 2010).

The data of the present study has obviously demonstrated a extremely toxic effect of heavy metals in acute concentrations to red tilapia fish, the important finding indicated that Hg and Cu which considered most hazardous effect on survival of fish and toxicity in the following order $Hg > Cu > Cd > Zn > Mg > Pb$. This result is in agreement with experimental findings of many authors obtained for different species of fish under laboratory conditions (Sikorska and Wolnicki, 2010; Huang et al., 2010; Little et al., 2012).

Basically, gills are target organs for heavy metals, our findings could be partially explained the early physiological reaction related with high demand of oxygen, secondary to the respiratory surfaces damage, as well as interference with ion regulation and oxygen uptake. Firat and Kargin (2010) explained that metals taken up and released

from the gills to the blood via serum proteins which are as main participants in metal carry for move to the other organs including liver for use in metabolism in tilapia fish *Oreochromis niloticus* exposed to single and combined Cd and Zn resulting in increased synthesis of protein in the liver.

Defensive response in treated fish was an increase mucous secretion in the gills; this is in agreement with Al-Zaidan et al., (2013) who explained that the complex defense mechanisms in secretion of mucus layer in the gills that acts as a physical barrier and covers the epithelial cells of the gills and skin area. It is a key mechanism in the innate immune system and is the first line of defense versus external influences such as microorganisms and contaminants. Gupta and Kumar (2006) have described the deleterious effect of heavy metals and suggested that the death of fish in Hg acute poisoning was due to disruption of respiratory process caused by the damage of gill epithelium. More pathological lesions were found in samples from exposed fish by heavy metals, which is in line with the ICP findings.

Histopathological changes in fish are documented as biomarkers of the toxic effects of pollutants widely used in monitoring programs of water quality; to date, of special value in the detection of biomarkers for histopathology is the analysis of gill and liver pathologies (Feist et al. 2004; Triebkorn et al. 2008). The current findings indicate that sublethal concentration exposure of Hg, Cu, Cd, Zn, and Mg for short periods of time could cause severe tissue damage which lead to numerous histopathological changes in gill and liver of red tilapias that are harmful to the health of this fish, the essential kind of gill histopathology, hyperplasia, was revealed in the great number of treated fish. Gills of fish immediately come into contact with the surrounding water, and thus are

responsive to water quality; the resulting hyperplasia is considered as a defense response which is non specific to heavy metals and mixed pollution (Triebkorn et al. 2008; Vergolyas et al., 2010). Similar observation by Wu et al. (2008) in juvenile tilapia (*Oreochromis mossambicus*) exposed to sub-lethal concentration of an ambient copper ions attempted to acclimate by morphological alterations and the increase in density of mitochondria rich cells in gills such as cell proliferation or mucus secretion and also released stimulating cortisol; these alterations illustrated in two types of reaction: defense and compensation, both responses help to decrease the entry of toxic substances and put a stop to damage resulting from the direct effects of copper ions. Gills have an important role in the gas exchange in addition to the ionic regulation on the one hand and the maintenance of equilibrium acid-base on the other hand therefore, the histopathological responses to toxic metal leads to respiratory disorders and electrically imbalances, subsequently, these series of effects can leads to fish deaths (Greenfield et al., 2008 and Omar et al., 2013).

Study of the gill histology illustrated a characteristic structural organization of the lamella in the untreated, however, gills of treated fish showed progressive architectural distortion at the end of the exposure period. This corroborates with the observation of Patnaik (2011) who reported gill tissues damages in *Cyprinus carpio* treated by Pb ions were disintegration and fusion of primary lamellae, extensive vacuolization with disturbance of epithelial layer whereas on sublethal exposure to Cd ions, hyperplasia of branchial arch, vacuolization and congestion of blood vessels were well noticeable. In the present study, due to heavy metal exposure, the epithelium of gill lamellae gets degenerated and separates from the lamellar tissue. Therefore, its osmoregulation function gets disturbed to fish which may become hypoxic. Moreover, the gill of exposed fish showed several histological changes such as fusion of secondary lamellae

joined with hypertrophy, hyperplasia, necrosis, epithelial lifting, curved lamellae, gill bridging, and infiltration of lymphocytes. Similar histopathological lesions have been reported by Javed et al. (2015) in *Channa punctatus*, they mentioned that these structural damages in the gill reflect that the fish was under acute stress due to heavy metal accumulations as these are responsible for oxidative stress which could be explained as defense responses of the fish as these changes increase the distance across which the dissolved toxic metals must disperse to arrive at the blood stream.

Liver tissue of treated fish showed deformities in tissue included hypertrophy of hepatocytes, many vacuolation in cell cytoplasm and necrosis. The lesions indicated that exposure to heavy metals can occur histopathological changes in liver as previously pointed out in other researches due to exposure to Hg (Raldia et al. 2007), and to Pb (Goswami et al. 2005). The following histopathological alterations were revealed in liver of *Carassius auratus* which examined by Syasina et al. (2012) who showed hypertrophy, vacuolization of multiple hepatocytes, irregular shape of the nuclei, karyopyknosis, in addition to presence of necrosis in hepatocytes.

Some tissue lesions, such as macrophage aggregates, hepatic glycogen depletion, and hepatic lipidosis, are general indicators of toxic injury resulting from exposure to contaminants or other stressors (Greenfield et al., 2008). Abdel-Moneim, (2014) found that histopathological and ultrastructural changes in liver of tilapia *Oreochromis niloticus* were resulted from impact of heavy metals including Ni, Fe, Zn, Co, Ba, Pb, and Cd which were far exceeded the international permissible limits; and these changes occurred long before the growth and reproductive changes, and can provide a better indication of organism health than a single biochemical change. Hepatocellular necrosis

observed in tilapia liver was a typical response against hydrophobic toxins and metals (Oliveira Ribeiro et al. 2005; Fernandes et al. 2008; Miranda et al. 2008). Mela et al. (2007) and Mela et al. (2013) informed that necrosis represents a structural and functional damage which reflects a set of disorders such as disturbances of enzyme activities, loss of cell membrane integrity, alteration in protein synthetic machinery and carbohydrate metabolism, or strongly associated with oxidative stress.

Scanning electron micrographic images confirmed clear morphological signs of gill damage in red tilapia exposed to acute heavy metals concentrations a similar observation under the same experimental condition in different fish was reported by Oliveira Ribeiro et al. (2002) and Osorio et al. (2014). The findings obtained from SEM examination gave more details about the effect of heavy metals on the histopathological changes in chloride and mucus cells represented by extensive hypertrophy and hyperplasia. Furthermore it has been demonstrated complete fusion of secondary lamellae in addition the microridges of pavement cells were dilated and at few places swelling lead to fusion of microridges. The results of element composition of gill obtained from dispersive X ray microanalysis (EDX) recorded a slight increase of the weight percentage of metal in treated samples than control samples. This is indicated that metal able to accumulate in fish tissue surfaces.

Many literature confirmed that aquatic organisms have the ability to accumulation of heavy metals in their tissues several times higher than the surrounding levels through the absorption process by the gills or by using up of contaminated food and sediments, thus, metal bioaccumulation in fish tissues can be considered as metal pollution index in the aquatic organisms (Karadede Akan and Unlu, 2007 and Bahnasawy et al., 2009). In our data, there was a significant difference of metals concentrations in different tissues

($p < 0.05$) in red tilapia fish, liver and gills accumulated higher levels of heavy metals than muscles because liver work as a main organ which have higher metabolic activities for storage and act as final depository of metal as well as it can also be detoxification and removal of toxic substances in the blood whereas gills acted as a close relationship with the ambient environment.

Significantly increasing of the metals uptake in these organs, as was also measured by Malik et al. (2010) in *Labeo rohita* and *Ctenopharyngodon idella* and they found very low concentrations of metals in muscles compared with gills and liver. This is especially important in view of the fact that muscles contribute the greatest mass of the flesh that is consumed as food for humans. Wang et al., (2010) who reported that active organs, such as gill, liver, and kidney, often sensitively accumulate larger amounts of metals than muscle. As well as different tissues have varied accumulating capacities of metals, which may be due to the different metabolic roles of metals and functions of organs. Similar study by Gupta et al. (2009) in two cat fish species *Aorichthys aor* and *Channa punctatus*, they found that the bioaccumulation level of toxic metals in fish related to their food habitats of fish types from different aquatic systems. Furthermore, the accumulation could be related to the uptake pathway of heavy metals from water or the food, therefore, some types of metals have the highest proportions in the liver, while other types rates higher in the gills. This is corresponded with Dsikowitzky et al. (2013) who reported that values of selenium, arsenic and cadmium were highest in fish livers, while chromium and lead levels were highest in the gills of different fish including tilapia *Oreochromis niloticus*.

In the present work, among all the studied metals, the highest concentration of Cu was observed in the liver in contrast, Cd and Pb were present at the lowest concentration in

all of the studied fish organs. Other investigator has also mentioned the highest accumulation of Cu in the liver of fishes *Oreochromis* spp. (Low et al., 2011). On the other hand gills were widely susceptible organ for metal pollution. The absorption of metals on gill surface could effect on the total levels of metal in the gill. While muscles are the important part of fish because its consumption by human and to its lipophilic nature. If the metal concentration increases in muscle according to threshold level, then it can cause serious health effects (Malik et al., 2014). Exposure to sub-lethal concentrations of a toxic substance that may adversely affect the fitness of freshwater fish species, which confirms the importance of this type of information to evaluate the risk of environmental toxicity in the local aquatic ecosystems.

CHAPTER 8

CONCLUSION

- 1- The results revealed that tilapia fish had a higher sensitivity to Hg and Cu which considered the most hazardous among tested toxic metals followed by Cd and Zn. The hybrid tilapia fish showed poor response to Mg and the least sensitivity to Pb.

Fish mortality increased with higher concentration and exposure periods of the heavy metals.

- 2- The juvenile hybrid tilapia fish is capable of accumulates heavy metals in their tissues from an aquatic environment and the ability of fish is another important factor to consider for future study. Mg had lower impact on fish survival and it was accumulated in higher level in addition metal accumulated level depended on exposed metal concentration and exposure period. These data constitute an important reference to assess the hazard of metal accumulation in fish tissues in the ecotoxicological testing scheme.
- 3- The present study had focused on enhancing the knowledge of lethal and sub-lethal concentration exposure of Hg, Cu, Cd, Pb, Zn, and Mg for short periods of time could cause severe tissue damage which leads to alterations towards histopathological aspects in the gill and liver of red tilapia *Oreochromis* sp. histopathological alterations considered alarming and primary response of cells induced by heavy metals toxicity in gill and liver. These results are a very important factor in assessing the possible damage from metal exposure, and it can be use in potential biomarkers.
- 4- Scanning electron micrographs gave more details of finding of elements effects on gills of red tilapia fish as well as clarified the extra tissue changes further confirmed the toxic effects resulting from heavy elements. Analysis of energy dispersive X ray proved the existence of the accumulation of heavy metals in the surface of the exposed fish gill and it proved that the studied metals caused disturb in the elemental compositions of gills. This work advance a new knowledge as influence of heavy metals in the gill histology of red tilapia fish and confirmed that their effects could be observed at different exposure periods; in addition, supporting environmental watch over aquatic systems when polluted by heavy metals.

5- In current findings have gives useful information and a baseline for future along with unceasing studies on the heavy metals accumulations in red tilapia fish of different aquaculture ponds. In general, concentrations of the heavy metals (Cu and Cd) in red tilapia (*Oreochromis* sp.) were higher in Bukit tinggi while Mg and Zn were found to be higher in Bestarijaya. Only Pb was found to be higher in Serendah. Detected concentrations varied significantly ($p < 0.05$) between different tissues and the lack of significant variation between the tested sites. The heavy metals concentrations were found to be lower than the recommended maximum level allowed in food by the European Communities (EC, 2006), and which has been reported by Malaysian Food Act 1983 and Food Regulations 1985 (Ministry of Health Malaysia 2012). These findings showed that tilapia fish (*Oreochromis* sp.) from all studied aquaculture ponds were safe for human consumption.

FUTURE RESEARCH

It is must to protect the water and sediments nearby fish aquacultures from anthropogenic sources of pollution to reduce environmental risks and this study may provide preliminary database for future research. Parameters such as temperature, DO, pH and others should be monitored regularly to ensure the sustainable use of water for different activities in general and to maintain the aquatic life and water environment in particular. The potential sources of these heavy metals to the fish in the aquatic system should be identified and quantified, hence further research is recommended. The load of heavy metals input to the ponds from the probable sources should be quantified and a proper measure should be taken in order to keep the fish safe for consumption and to maintain the aquatic ecosystem.

Further work should be necessary to determine the form of storage of metals in the liver of the studied fish species. Metallothionein induction in fish liver, coupled with metal determination in this organ, may represent good biomarkers of metals present in the surrounding environment. Furthermore, future work needs to be done in order to reveal a better understanding the effect of metal concentration in fish organs on their histopathological alteration and EDX. And also we can be carried out on the toxic effect of metals on blood enzymes.

This research serves as a reference for future work on the assessment of the levels of toxic metals in fish and is integrated into future studies on pollution risk assessment studies of soil, water, sediment and plankton in the different areas in Malaysia, therefore it is important to continuously monitor heavy metals concentration in water and their accumulation in fish to know the current pollution status of the aquaculture water and focus on reducing the volume of heavy metals discharged from agriculture area as well as mined out ponds into the catchment

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PRESENTATION

Oral Presentation

Jasim Aldogachi, M.A., Sofian-Aziran, M., and Yusoff, I. (2012) Effect and bioaccumulation of heavy metals on survival of Tilapia sp. Seminar on Zoological & Ecological Research in Progress (ZERP 2012), 18 December 2012, Institute of Biological Sciences, Faculty of Science, University of Malaya, Malaysia.

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Poster Presentation

Jasim Aldogachi, M.A., Sofian-Aziran, M., and Yusoff, I. (2015). Heavy metals concentration in different organs of red tilapia fish (*Oreochromis* sp.) from selected aquaculture ponds in Malaysia 20th Biological Sciences Graduate Congress (20th BSGC), 9-11 December 2015, Chulalongkorn University, Bangkok, Thailand.

PUBLICATIONS

Aldogachi, M.A., Sofian-Aziran, M., Yusoff, I., and Ashraf, M.A. (2015). Ultrastructural effects on gill tissues induced in red tilapia *Oreochromis* sp. by a waterborne lead exposure. Saudi Journal of Biological Sciences, ARTICLE IN PRESS. <http://dx.doi.org/10.1016/j.sjbs.2015.08.004> **(ISI-Cited Publication)**

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Jasim Aldogachi, M.A., Sofian-Aziran, M., Yusoff, I. and Motior Rahman, M. (2016) Bioaccumulation and Histopathological Changes induced by Toxicity of Mercury (HgCl_2) to Tilapia Fish *Oreochromis* sp. Sains Malaysiana 45(1)(2016): 119–127 **(ISI-Cited Publication)**