METALLOTHIONEIN GENE EXPRESSION AND GENOTOXIC EFFECTS OF HEAVY METALS ON *OREOCHROMIS* SP.

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INSTITUTE OF BIOLOGICAL SCIENCES
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ABSTRACT

Metallothionein is a small, cysteine rich protein that aids in ion homeostasis in a cell. It binds naturally to zinc and also has the tendency to bind to other metals as well if present in the cell. This study was conducted in order to determine the effects of heavy metals exposure on metallothionein expression and other genotoxic effects on the tilapia fish as test subjects. *Oreochromis* sp. was chosen as the test subject because of the many advantages of its characteristics and it can be easily found in Malaysian rivers. Test subjects were exposed to two types of metals which were copper and lead. The concentrations of exposure were 0, 0.5, 1.0 and 1.5mg/L. Three approaches were selected to assess the effects of metal exposure which were gene expression analysis, micronucleus test and RAPD. For the gene expression analysis, lead at the highest concentration was able to induce the highest fold induction of metallothionein relative to the control sample at a 7.64-fold increase. Copper at 1.5mg/L and lead at 1.0mg/L were also able to significantly induce an increase in fold induction of 5.05 and 3.42-fold respectively. 1.5mg/L lead was able to induce the highest frequencies of micronucleus and nuclear abnormalities compared to the other samples. The banding patterns of RAPD bands were used to calculate the Jaccard distance of the exposed samples to the control sample. It was found that 1.5mg/L lead has the furthest Genetic Distance at 0.297. The sample that had the closest Genetic Distance to the control sample was copper at 0.5mg/L. The results of the micronucles test and RAPD were able to support the results of the gene expression study whereby lead created a bigger impact on the samples compared to copper at the same concentration.

ABSTRAK

Metallothionein merupakan sebuah protein bersaiz kecil dan kaya dengan *cystein* yang membantu proses homeostasis ion-ion dalam sel. Lazimnya, ia akan mengikat zink dan juga berupaya untuk mengikat logam lain sekiranya logam tersebut berada di dalam sel. Kajian ini dijalankan untuk menentukan kesan terhadap ekspresi *metallothionein* dan kesan kerosakan lain terhadap ikan tilapia setelah didedahkan kepada logam-logam berat. *Oreochromis* sp. telah digunakan sebagai subjek kajian kerana kelebihan yang ada pada ciri-cirinya dan boleh didapati dengan mudah dikebanyakan sungai di Malaysia. Subjek-subjek kajian telah didedahkan kepada dua jenis logam berat iaitu kuprum dan plumbum. Kepekatan yang digunakan ialah 0, 0.5, 1.0 and 1.5mg/L. Kajian telah dilakukan menggunakan tiga kaedah iaitu melaluli kajian ekspresi gen, ujian mikronukleus dan *RAPD***.** Bagi kajian ekspresi gen, plumbum pada kepekatan tertinggi menghasilkan data induksi signifikan tertinggi iaitu 7.64 kali ganda berbanding sampel kawalan. 1.5mg/L kuprum dan 1.0mg/L plumbum juga berjaya menghasilkan peningkatan induksi yang signifikan iaitu masing-masing pada 5.05 dan 3.42 kali ganda. 1.5mg/L plumbum telah menghasilkan frekuensi mikronukleus dan nukleus abnormal tertinggi berbanding sampel-sampel yang lain. Jarak Jaccard telah dikaji berdasarkan hasil produk PCR RAPD. 1.5mg/L plumbum mempunyai Jarak Jaccard yang paling jauh daripada sampel kawalan iaitu pada 0.297. Manakala sampel yang mempunyai Jarak Jaccard yang paling hampir kepada sampel kawalan ialah sampel yang terdedah kepada 0.5mg/L kuprum. Keputusan kajian mikronukelus dan RAPD adalah sejajar dan menyokong keputusan ekspresi gen yang telah diperolehi dimana plumbum menghasilkan impak yang lebih tinggi berbanding dengan sampel-sampel lain.

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LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviation	**Represents**
A	Adenine
Ag^{+}	Argentum ion
C	Cytosine
Cd^{2+}	Cadmium ion
Cr^{6+}	Chromium ion
Cu	Copper
Cu^{2+}	Copper ion
D	Genetic Distance
DNA	Deoxyribonucleic acid
g	Gram
G	Guanine
L	Litre
min	Minute
mg	Milligram
$MgCl_2$	Magnesium chloride
mL	Milliliter
MN	micronucleus
MT10	Metallothionein gene family - 10th
MT20	Metallothionein gene family - 20th
NA	Nuclear abnormalities
Pb	Lead
PCR	Polymerase Chain Reaction
qPCR	Real-time Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
sp.	Species
T	Thymine
UPGMA	Unweighted Pair-Group Method with Arithmetic Means
Zn^{2+}	Zinc ion

Symbols	**Represents**
^{o}C	Degree Celsius
x g	Times gravitational force
cm^3	Cubic centimeter
%	Percentage
x	times
*	Significant value
C_t	Threshold cycle

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Metallothionein is a low molecular weight protein lacking in aromatic amino acid residues. The main characteristics besides being rich with cysteine is that metallothionein is a metal-binding protein which can be found in many organisms (Roesijadi, 1996). Since it is able to bind to metals, metallothionein has also been known to detoxify excess metal in the cell. When an organism is being treated or exposed to heavy metals, theoretically the synthesis of metallothionein will increase. Thus, a polluted area with high level of heavy metals would induce an increase in metallothionein synthesis. In other words, metallothionein can also be used as a biomarker against heavy metal toxicity and pollution.

Fish is widely known as a good source of proteins which can be obtained easily in the market. Aquaculture activity is important in order to support the market demands of fresh water fishes. One of the most commonly cultured fish is tilapia (*Oreochromis* sp.). Tilapia has a high reproductive rate, good adaptability to the environment and tasty flesh. Most of the tilapias cultured in Malaysia are being widely marketed domestically compared to being exported out of the country (Low *et al*., 2011). Apart from being beneficial for aquaculture activity, tilapia can also be used as a biomarker to detect pollution in aquatic environment. This is due to its characteristic of being able to withstand harsh environmental conditions. Thus, it serves the purpose of being an

essential test subject in studying ecotoxicological effects upon exposure to certain chemicals or heavy metals (Cheung *et al*., 2004).

There are many ways of determining the effects of pollutants or heavy metals on an organism. Analyzing metallothionein in terms of its expression is one way. Gene expression study at the RNA level can be conducted using real-time PCR. Apart from using the metallothioein gene as a biomarker to indicate heavy metal pollution, other methods can also be used to substitute or support the results of the gene study. Micronucleus test at the cellular level is a fairly simple and cheap test that can be conducted to analyze the DNA damage of a cell after being exposed to toxicants. Observing variations of the banding pattern produced by RAPD primers between control sample and the exposed samples is another way to observe damage at the DNA level. Thus, combining the three different methods would complement one another to produce good and reliable data to assess the level of toxicity for each metal towards the test organism which is the tilapia, *Oreochromis* sp.

1.2 Objectives

1.2.1 To determine the gene expression of metallothionein in tilapia on exposure to copper and lead using real-time PCR (qPCR).

1.2.2 To determine the genotoxic effects in tilapia on exposure to copper and lead using the micronucleus test.

1.2.3 To detect the changes in the RAPD banding patterns, through the loss or gain of bands in tilapia exposed to copper and lead.

CHAPTER 2

LITERATURE REVIEW

2.1 *Oreochromis* sp.

Oreochromis sp. belongs to the family of Cichlidae, order Perciformes and in the subclass of Teleostei. The common name that refers to *Oreochromis* sp. is tilapia. Some of the most common tilapia species are *Oreochromis niloticus, Oreochromis aureus* and *Oreochromis mossambicus. Oreochromis* sp. is a mouth brooder where the females would only release the fingerlings from their mouth after several days of hatching (Pena-Mendoza *et al*., 2005). Tilapias have sharp and spiny fins (Popma *et al*., 1999).

Oreochromis sp. is one of the most common species of freshwater fish being cultured and farmed because of their adaptability to the environment, tasty and affordable selling price in the market (Olurin & Aderibigbe, 2006). The species can tolerate and adapt to different surroundings including poor water quality. The species is able to live in condition of temperature from 13.5^0C to 33^0C (Cheung *et al*., 2004). *Oreochromis niloticus* are able to sense environmental changes surrounding them and will react to the changes accordingly (Almeida *et al*., 2001). However with the high reproduction rate and a rapid growth rate, *Oreochromis* sp. can in turn be an invasive species and may pose a threat to other aquatic vertebrates as it is known to be a very dominant species. Because of its characteristic that is able to withstand harsh environmental condition, tilapia has a high potential to be a very good biomarker against pollution (Baysoy *et al*., 2012).

2.2 Heavy metals

Heavy metals are natural elements present in the environment (Wegwu *et al*., 2010). It is known that heavy metals have atomic density greater than 6 g/cm^3. Such heavy metals include antimony, arsenic, bismuth, cadmium, cerium, chromium, cobalt, copper, gallium, gold, iron, lead, manganese, mercury, nickel, platinum, silver, tellurium, thallium, tin, uranium, vanadium, and zinc (Alloway, 1995). Metals are usually required in various industries as raw materials and are major constituents of industrial effluents (Benjamin & Thatheyus, 2012). Biologically, trace metals in minute concentration are essential for the biochemical process and metabolic functions of an organism and also in the aquatic environment (Saeed & Shaker, 2008). Metals such as copper, zinc and iron in trace amounts are essential in cellular functions while others like lead, cadmium and mercury are not required for biological function (Çoğun & Kargin, 2004).

Since heavy metals are non-biodegradable, excessive exposure and concentration of metals could lead to toxicity in organism and could also pose a threat to the ecological system. The main source of heavy metals can be found near industrial, agricultural and other anthropogenic activities (Atli *et al*., 2006). Runoff from industrial or anthropogenic waste into water bodies will increase the toxicity of heavy metals. The heavy metals that accumulate in the aquatic organisms including fish will eventually enter the food chain (Saeed & Shaker, 2008). Other than pollution, the source of heavy metals can also be found excessively in certain fish feed which was formulated from feces of farmed pigs. The pigs that were given a high metal diet will produce feces with high metal concentration. Certain formulation of fish feed uses feces from farmed pigs

with high metal diet. Fishes that were given this particular fish feed will ingest high level of metals originated from the feces (Lima *et al*., 2006).

Heavy metals can cause oxidative damage because of the accumulation of highly reactive oxygen species. Heavy metals in general can also affect the growth rate, reproduction and mortality of a fish (Hayat *et al*., 2007). Exposure to copper has shown various damages and effects both in gills and liver of *Oreochromis niloticus* (Fernandes *et al*., 2007). There was an increase in oxidative stress on *Oreochromis niloticus* and also an increase of catalase activity in the liver, kidney, gill, brain and intestine after being exposed to Ag^{+}, Cd^{2+}, Cr^{6+}, Cu^{2+} and Zn^{2+} at different concentrations (Atli & Canli, 2008). According to Martins *et al*., (2011) and Çoğun & Kargin, (2004), liver has a higher accumulation of heavy metals compared to gills and muscles.

The level of toxicity differs between species, maturity and also size. Certain metals are dependent on size of the fish while others are not. Different species also has different correlation and relationship of size with metal exposure (Çoğun *et al*., 2003). Certain species are also resistant towards a certain metal compared to other species. Tilapia can tolerate a higher concentration of copper compared to carp (Lam *et al*., 1998).

2.3 Metallothionein

Metallothionein is known as a small, low molecular weight, and cysteine rich protein. It has non aromatic amino acids and helps in metal ion homeostasis in a cell (Shariati *et al*., 2011). The synthesis of the protein is induced by metal present in cell. Metallothionein are conserved throughout species and the highly conserved region of

cysteine in metallothionein serves its function in metal binding to nontoxic essential metal ions such as zinc and copper. Metallothionein is also able to bind to heavy metals such as cadmium and mercury (Cheung *et al*., 2004) which makes it as a good method in detoxifying and reducing toxicity in a cell. Because of the ability to be induced by both essential and nonessential metals, metallothionein has been used as a biomarker to detect heavy metal pollution and the bioavailability of any particular metal in the environment (Atli & Canli, 2007). The thiol group of metallothionein facilitates metal exchange in tissues because of its high affinity towards various metals (Thirumoorthy *et al*., 2007).

Even though there are many metals that are associated with metallothionein, only certain metal ions can replace zinc ions such as copper, cadmium, lead, argentums and mercury (Chan & Chan, 2008). However, metallothionein has a higher affinity towards copper, cadmium and zinc in teleosts (Wu *et al*., 2008). There was a positive effect upon copper exposures that was able to induce metallothionein in the liver of trout. Metallothionein acts specifically and has a stable structure which is also why it is a heat resistant protein (Baykan *et al*., 2007).

The metal binding property of metallothionein helps in metalloregulatory process in mammals including cell growth and multiplication. Furthermore, metallothionein can also act as anti-oxidants which protect the cell from hydroxyl free radicals (Thirumoorthy *et al*., 2007). Other than that, transcriptional activity of metallothionein can also be induced by hormones and it also has the ability to induce a redox reaction (Coyle *et al*., 2002). Retaining redox potential is one property of metallothionein which allows it to be an essential biomarker in toxicological studies (Schlenk & Rice, 1998). Increase expression of metallothionein can also reduce

apoptosis in a cell.

The state of an individual nutritional condition, pre-natal development and reaction to stress are determined by the expression level of metallothionein (Andrews, 1990). It was found that metallothionein is being rapidly stimulated in the liver of mammals. Other than liver, metallothionein can also be found in gills, kidney, brain and intestines of various fish species (Dang *et al*., 2000).

2.5 Real-time PCR (qPCR)

Reverse transcriptase real-time PCR is a technique that allows RNA to be amplified and reverse transcribed to its complementary DNA (cDNA) sequences using the enzyme reverse transcriptase. Apart from *Oreochromis* sp., various studies have been conducted on metallothionein using real-time PCR to detect the RNA transcriptional level and determine the potential of it to be a good biomarker against pollution (Tom *et al*., 2004). Reverse transcriptase PCR only requires a small number of purified RNA sample in order for it to be amplified. Reverse transcription has usually being coupled with real-time PCR for gene expression study (Livak & Schmittgen, 2001). The level of metallothionein's mRNA can be determined using this method.

Real-time PCR is able to perform detection and quantification of DNA simultaneously which can reduce the possibility of contamination to occur. The conventional PCR method uses agarose gel and staining technique to view the PCR product. However for qPCR, it allows quantification of the C_t value which is detected by a fluorescent molecule. It can be used for the analysis of gene expression study which the conventional PCR cannot do. Apart of having to quantify the result, qPCR

also allows us to determine results qualitatively by observing whether there's amplification or not based on the amplification graph. When there's no amplification taking place that would mean that the template lacks that particular sequence or gene of interest. An example of such study that could use both quantitative and qualitative results from qPCR is when doing gene silencing (Shlomo *et al*., 2007).

Using this technique, Dondero *et al*., (2005) was able to discover the differences in the expression of metallothionein between two molluscan. In fact, they did the analysis for two types of metallothionien gene and found out that the two genes have different profiles. MT10 was able to be induced by cadmium, zinc, copper and mercury. However MT20 was only successfully being induced by cadmium. Thus, this shows that qPCR was able to detect and analyze gene expression precisely. The mechanism of metallothionein gene activation by different types of metals can be determined by using qPCR. Other than that, the relationship between parent that was exposed to cadmium with its progeny can be determined with the use of qPCR (Wu *et al*., 2008).

2.4 Micronucleus Test

There are many studies that have been conducted to determine the toxicity effect of pollutants to living organisms and there are also many methods of choice depending on the objective of the research. One proven method in assessing the quality of a water body and their treatment strategies is by using micronucleus test (Hoshina *et al*., 2008). The genotixicity effect of a polluted water sample can also be determined using micronucleus test (Matsumoto *et al*., 2006).

Micronucleus rises from a part or a whole lagging chromosome that is left in the cytoplasm and separated from the main nucleus. Micronucleus test is a method which allows determination of whether a cell has damaged DNA or not. Damaged DNA would mean that the condition of the DNA strand is not normal and the stability of the double helix is being interrupted. This will lead to many irregularities and disruption to the normal condition of the cell function. Excessive DNA damaged on an organism will lead to death (Terradas *et al*., 2010).

For this method, samples are usually taken from erythrocytes. However, samples could also be taken from caudal fin epithelial cells and gill cells (Ergene *et al*., 2007). The exposure period also varies depending on the aim of the study. The micronucleus are indentified by size which is smaller than 1/3 of the main nucleus. It should be found in the cytoplasm, detached from the main nucleus. Cells that are overlapping with each other are not counted (Hoshina *et al*., 2008; Frieauff *et al*., 1998; Jiraungkoorskul *et al*., 2007). If mutation is present, chromatid gaps, sub-chromatid gaps, centromeric gaps, precocious separation of chromatids and polyploidy are some of the abnormalities that can be observed as what was discovered in *Channa punctatus* which was exposed to dichlorvos (Rishi & Grewal, 1995).

Channa punctata that was exposed to copper, arsenic and mercury shows increase in frequency of micronucleus when compared with the control sample (Yadav & Trivedi, 2009). Minissi & Lombi, (1997) used micronucleus test to determine the pollution level of Tiber river. There was no significant increase of the micronucleus frequency observed. However, the data is still important in comparing the results that they obtained with the test that they previously conducted. When comparing both data, it could be concluded that the pollution level has decreased from the first test. In another

study, the sample was exposed to three different types of heavy metals which were copper, cadmium and chromium. The study was conducted for 21 days. What they observed was is that there was a significant increase in frequencies of both micronucleus and binucleated cells on all heavy metals (Çavaş *et al*., 2005). Certain studies conducted perform data collection on a few different time intervals. Jagetia & Aruna, (1998) took data every 12, 24 and 36 hours after treatment. They have found out that there was an increase in the frequency of the micronucleus. However, it did not show a dose related response on the different concentrations of heavy metals.

2.6 Randomly Amplified Polymorphic DNA (RAPD)

RAPD is a PCR reaction using random single primers that anneal to its complementary sequences throughout the DNA template. The technique is usually selected as a mean to determine the genetic diversity or mutation of individual and systematic studies between species (Ahmed *et al*., 2004). However, the method can also be used to determine genetic variation or DNA damage among the same organism in different condition, environment or treatment. Many studies have opted for this method in ways to assess DNA damage as it is fairly simple, reliable and inexpensive. Another advantage of using RAPD method is that background information of the DNA sequence of the selected species are not required prior to testing. However, the results obtained using this method is not necessarily reproducible (Jones *et al*., 1997).

Cenkci *et al*., (2010) has used RAPD in assessing genotoxicity in seedlings of *Phaseolus vulgaris* L. exposed to two types of herbicide which were 2,4-D and Dicamba. This was done by comparing the banding pattern or the RAPD profiles of the control sample and other samples that were exposed to different concentration of the

two herbicides. Through this, it can be said that both herbicides were able to induce DNA damage dose-dependently to the seedling. In a certain study conducted, RAPD was used to assess the possibility of it being a potential biomarker to detect genotoxic effect of environmental pollution. Through the study, it can be concluded that RAPD was found out to be a good method in detecting genotoxic effect on the samples and has potential as a good biomarker to analyse DNA damage (Duman *et al*., 2011). *Danio rerio* that was exposed to several doses of cadmium shows a different RAPD profile from the ones that were not exposed to any cadmium. It was observed that the banding pattern of the exposed sample gained extra bands from the control sample (Cambier *et al*., 2010).

CHAPTER 3

METHODOLOGY

3.1 Sample Preparation

Oreochromis sp. weighing 106.65±10.89 were collected from Pusat Perkembangan Akuakultur Bukit Tinggi, Pahang. The fishes were reared in 100L tanks and were left to acclimatize with the new environment for 4 days. Each tank was provided with aerated, decholorinated and circulated tap water. The fishes were then transferred to a 20L tank individually before exposing them to heavy metals. The three fishes were exposed to 0.5, 1.0 and 1.5 mg/L of one heavy metal with an addition of one fish that was used as a control without the addition of any metal (0 mg/L). The study was conducted using two different heavy metals which were copper and lead. A total of three replicates of experiment were conducted for each heavy metal. After 96 hours of initial time of exposure to the heavy metals, the fishes were then sacrificed to obtain the blood for micronucleus test and the livers for molecular approaches.

3.2 Micronucleus Test

The blood obtained was smeared on a clean microscope slides. Three slides were made for each concentration of heavy metal used. Three replicates were made for both metal and all of its concentrations. The smeared slides were fixed in absolute ethanol for 20 minutes. Slides were left to dry at room temperature for 24 hours. The dried slides were then stained in 5% of Giemsa stain for 20 minutes. The excess stain

were removed and washed with distilled water. Slides are left to dry at room temperature for 10 minutes and them viewed under the light microscope.

3.3 DNA and RNA Extraction

Livers that were obtained were used to extract DNA and RNA of the fishes. Extraction was done using Trizol reagent which can yield both DNA and RNA with one time extraction process. Liver that were obtained from the fishes were grinded and homogenized in the Trizol reagent. 1ml of Trizol was added for 100mg liver used. The homogenized sample was incubated for 5 minutes at room temperature. 0.2mL chloroform were added, vortexed and incubated for 3 minutes at room temperature. Samples were then centrifuged at 12,000 x g for 15 minutes. The samples were then separated into three different phases. The upper phase contains RNA, the middle phase contains DNA and the lower phase contains protein. The upper phase was removed into a new tube. 0.5mL isopropyl alcohol was added to the upper phase to allow RNA to precipitate. The sample that contains RNA was left at room temperature for 10 minutes before being centrifuged for 10 minutes at 12,000 x g. RNA pellet was then washed with 1ml of 75% ethanol. After vortexing and centrifuging at 7,500 x g for 5 minutes, the pellet was dissolved in nuclease-free water. The remaining middle and lower phase were added with 0.3mL of 100% ethanol before being mixed and left for 3 minutes at room temperature. The mixture was then centrifuged at 7,500 x g for 5 minutes. Phenol-ethanol supernatant was removed and the DNA pellet was washed twice in 1ml of 0.1M sodium citrate in 10% ethanol. At each wash, the DNA pellet was stored in the washing solution for 30 minutes at room temperature and then centrifuged at 7,500 x g for 5 minutes. After the two washes, 1ml of 75% ethanol was added and stored for 20 minutes at room temperature. Sample was then centrifuged at 7,500 x g at room

temperature. The supernatant was removed and the pellet was dried before dissolving the pellet in nuclease-free water. The solution was centrifuged at 7,500 x g for 10 minutes and the supernatant which contains the DNA was then transferred into a new tube. Both quantity and quality of RNA and DNA were observed under gel electrophoresis and spectrophotometer.

3.4 Reverse Transcriptase (RT-PCR)

The purified RNA was used as template for reverse transcriptase PCR. Prior to real-time PCR, the RNA was reverse-transcribed into cDNA using Fermentas Revert Aid First Strand cDNA Synthesis Kit. The component of the PCR mixture includes purified RNA template, oligo primer, nuclease-free water, 5x reaction buffer, RiboLock RNase inhibitor, 10mM dNTP Mix and RevertAid M-MuLV Reverse Transcriptase. PCR cycle used is as tabulated in Table 3.4.

Table 3.4. The cycle for RT-PCR

Temperature (^{0}C)	**Time (min)**
42	60
70	5

3.5 Real time PCR, qPCR

Real time PCR was conducted using Sso Fast EvaGreen Supermix by Biorad. Component of the real time PCR master mix includes Sso Fast EvaGreen Supermix, both 0.5µM of forward and reverse primer, cDNA and nuclease free water. The concentration of the qPCR components are tabulated in Table 3.5 and the PCR cycle used is as tabulated in Table 3.5. The primer used was a pair of metallothionein primers with the forward primer's sequence of 5'-GCCAAGACTGGAACCTGC-3' and the reverse primer of 5'-GCACACGCAGCCAGAGGC-3' (Wu *et al*., 2008). Reference gene used was 18S rRNA.

Table 3.5 The component of qPCR mixture

COMPONENT	CONCENTRATION
Premix	1X
Forward primer	0.5µM
Reverse primer	0.5µM
cDNA	80ng/µL
Nuclease Free water	Up to total 20µL

Table 3.6 The cycle for qPCR

Temperature (^{0}C)	Time (min)	Cycle
98.0	2.00	1
98.0	0.02	40
61.5	0.30	
75.0-95.0	0.10	Melt curve (0.2^{o}C increment)

3.6 RAPD

Master mix for RAPD were made by using components by 1st BASE which were 10x PCR buffer, dNTP mix, $MgCl_2$, primer, DNA template, nuclease-free water and Taq Polymerase. PCR cycle used is as tabulated in Table 3.7. Components of the PCR master mix includes 10x PCR Buffer, 25mM $MgCl_2$, 10nM dNTP mix, 10µM random primers, 5u Taq Polymerase, DNA and nuclease-free water. The viewing of the PCR products were made in 1% agarose gel stained with ethidium bromide. The banding patterns of the RAPD were analyzed and scored based on the presence and absence of a band. From the scoring data, Jaccard similarity coefficient was used to calculate the Jaccard distance or the dissimilarity between the samples. The similarity coefficient was calculated using the equation:

$$\text{Jaccard similarity coefficient} = \frac{\text{The number of bands shared by sample A and B}}{\text{(The number of bands in sample A + the number of bands in sample B - the number of bands shared by sample A and B)}}$$

The distance between sample was calculated using the equation:

$$\text{Jaccard distance} = 1 - \text{Jaccard similarity coefficient}$$

From the Jaccard distance calculated, Unweighed Pair-Group Method with Arithmetic Means (UPGMA) tree was constructed using PHYLIP version 3.695 (Ge *et al.,2013*).

Table 3.7 The cycle for RAPD

Temperature (^{o}C)	**Time (min)**	**Cycle**
94.0	3	1
94.0	1	36
27.5	1	
72.0	2	
75.0	5	1

CHAPTER 4

RESULTS

4.1 Metallothionein Gene Expression

Samples that were used for testing consists of a control sample and samples that were exposed to three different concentrations of copper (Cu) and lead (Pb). The three concentrations were 0.5, 1.0 and 1.5mg/L for each metal. The liver of all samples were analyzed by reverse transcriptase real-time PCR. The percentage of amplification efficiency for target primers and internal control primers falls between the ranges of 90 to 105%, which was 92.25% for metallothionein primers and for internal control 18S rRNA was 104.55%.

Since the amplification frequencies for both control and target primers falls within 5% of each other, Livak method was the choice of calculation used for metallothionein gene expression. The calculation used the date of the exposed samples C_t values with C_t values of the control sample to produce a normalized expression ratio (see appendix I). Through the raw data of C_t values, it can be seen that the readings varies between samples. Calculations were made on the metallothionein gene expression and clearer data representation was graphed into a histogram as in Figure 4.1.

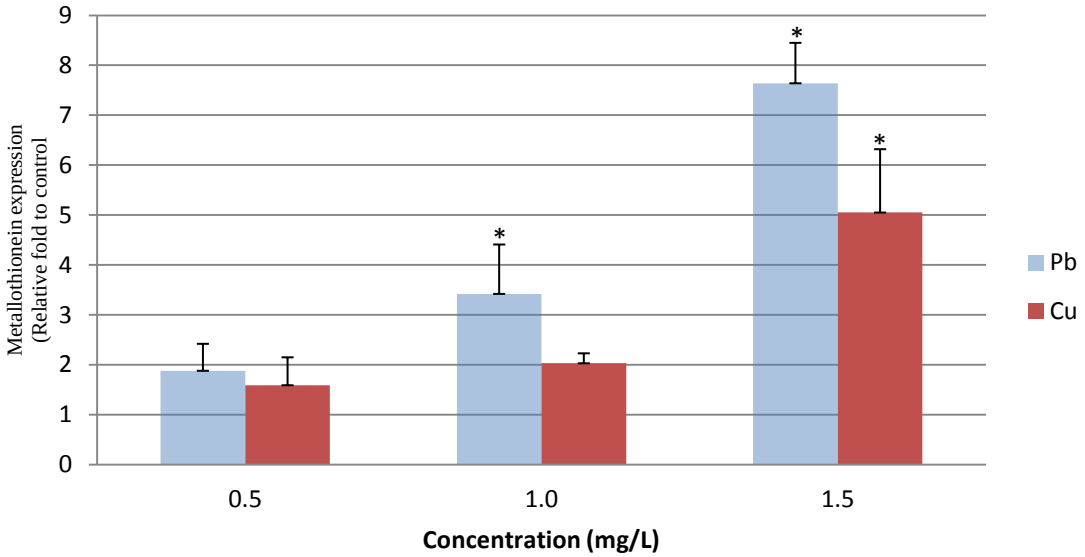
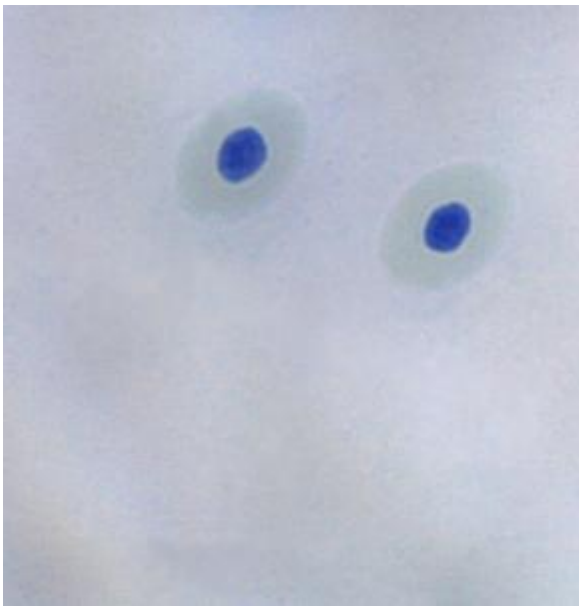


Figure 4.1. Histogram of metallothionein expressions relative to the control sample of samples that were exposed to lead and copper at three different concentrations. Means with significantly different values at $p<0.05$ are labeled with asterisk. The significant values were analyzed using Tukey's test. Standard deviations were indicated by error or T-bars.

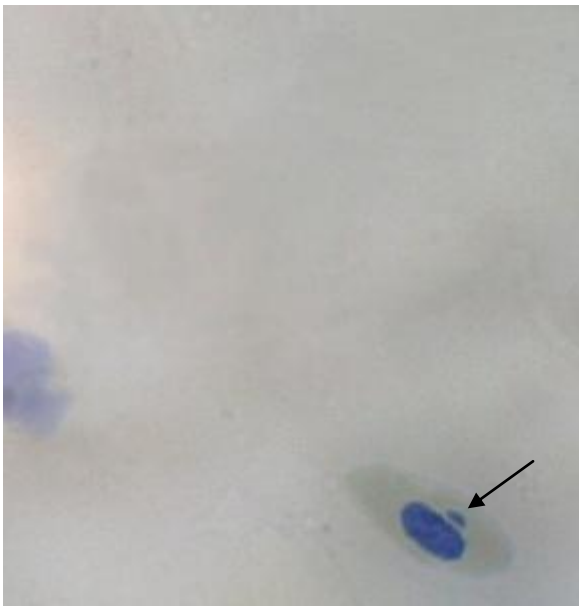
In general with the increase of metal concentration, the fold induction also increases. However only at a few concentrations for the metals the readings are significantly different. The sample treated with the highest concentration for copper and lead which is 1.5mg/L respectively shows a significant difference from the control sample. The expression in lead treatment had 7.64-fold increase and copper with 5.05-fold increase relative to the untreated sample. The highest gene expression which is lead at 1.5mg/L is significantly different from all other concentrations. In other words, lead was able to induce a higher fold increase compared to copper at the highest concentration. Other concentration that has a significant value was lead at 1.0 mg/L. At the same concentration, copper was not able to induce a significant fold increase. At 0.5mg/L, copper and lead were also unable to obtain a significant value of fold induction. It can be seen from the data obtained, overall, lead has a higher effect on the increase of gene expression for metallothionein on the samples.

4.2 Micronucleus and Nuclear Abnormalities

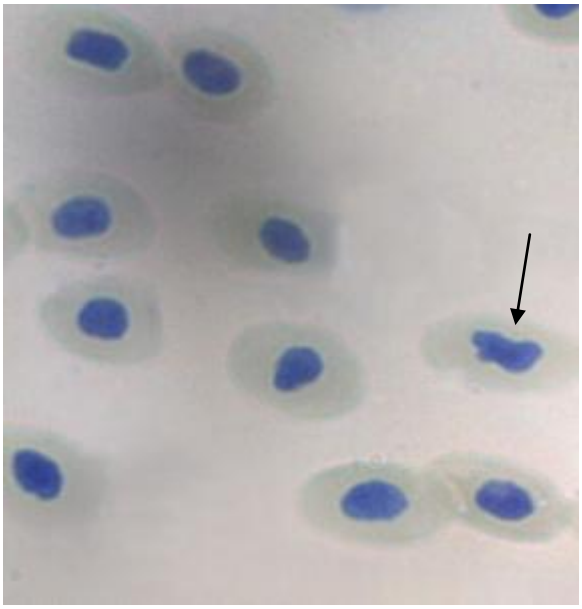
Erythrocytes were taken from each sample and used for micronucleus test. From the test, micronucleus (MN) and nuclear abnormalities (NA) were observed and counted for analysis. Example of the micronucleus and nuclear abnormalities that were observed under light microscope are presented in Figure 4.2.1. The comparison in frequency for micronucleus and nuclear abnormalities are presented in a histogram in Figure 4.2.2.



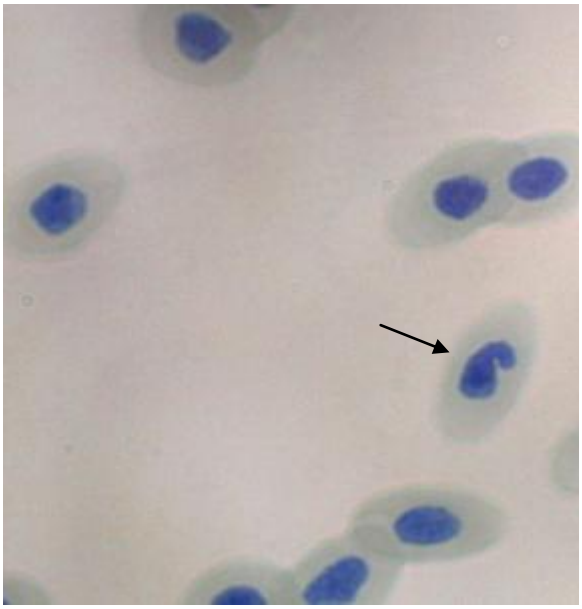
(A)



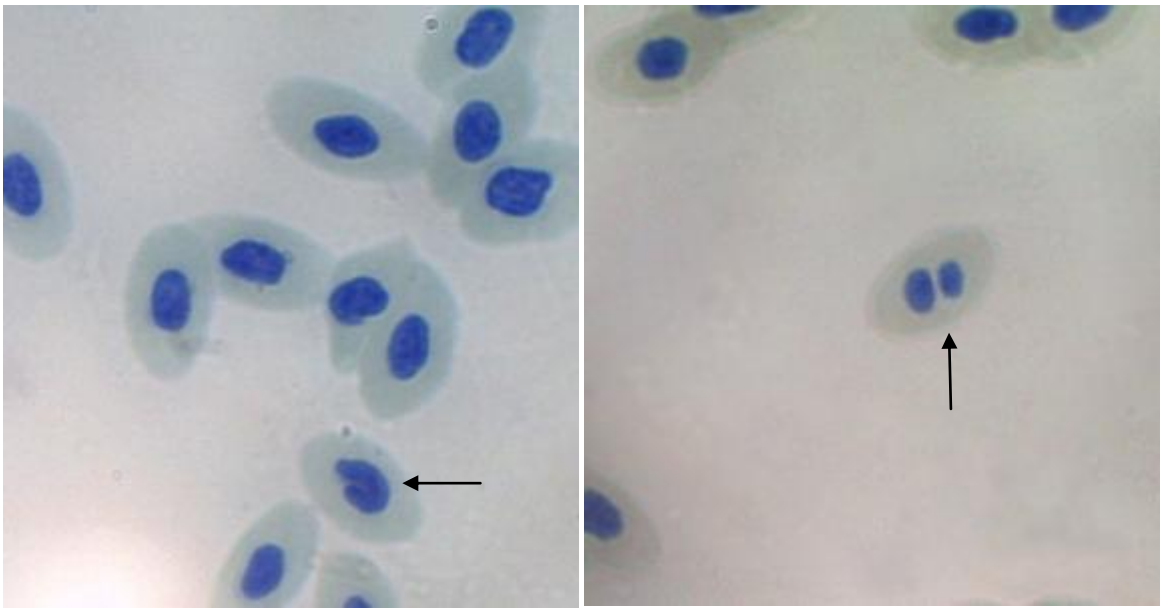
(B)



(C)



(D)



(E) (F)

Figure 4.2.1. (A) Normal cell that was observed by light microscope under 100x magnification. (B) is micronucleus that was observed under 100x magnification. Amongst the nuclei abnormalities that were observed under 100x magnification are (C) lobed nuclei, (D) blebbed nucleus, (E) notched and (F) binucleated cell.

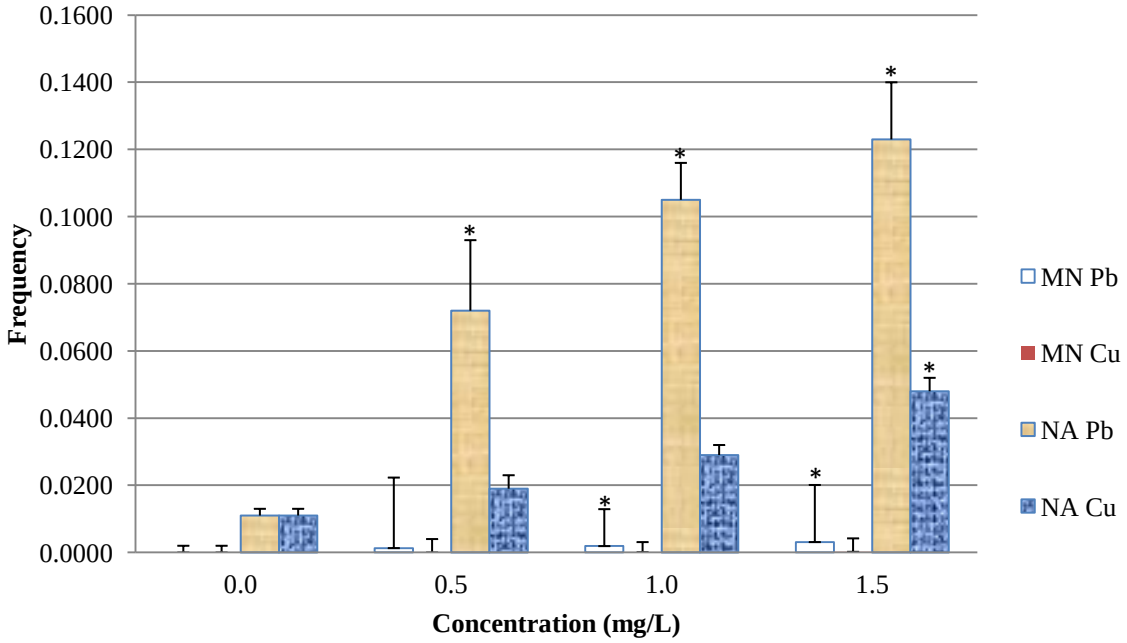
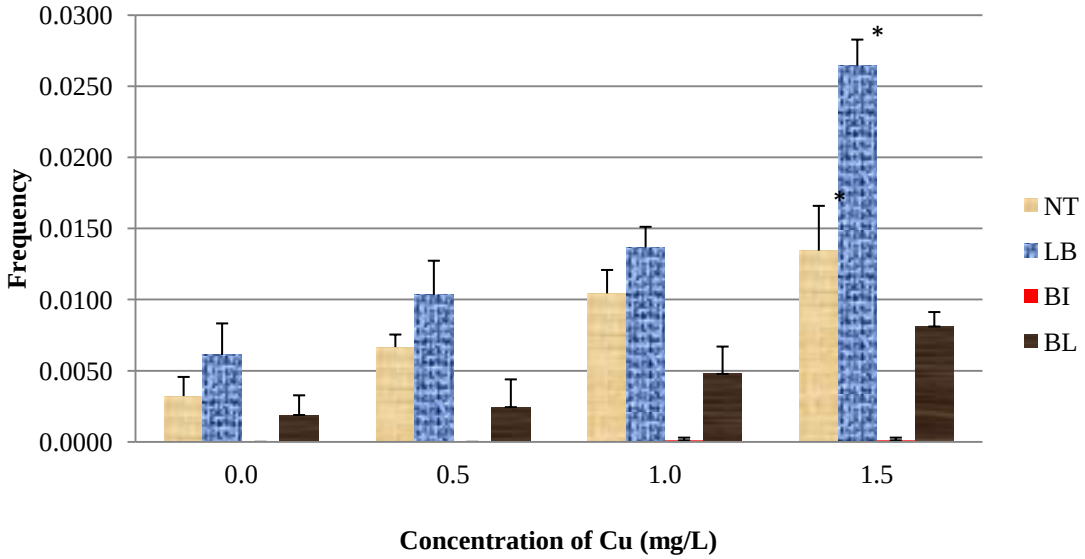


Figure 4.2.2. Comparison of micronucleus and nuclear abnormalities observed for control sample with samples exposed to copper and lead. Means with significantly different values of $p<0.05$ are labeled with asterisk. The significant values were analyzed using Tukey's test. Standard deviations were indicated by error or T-bars.

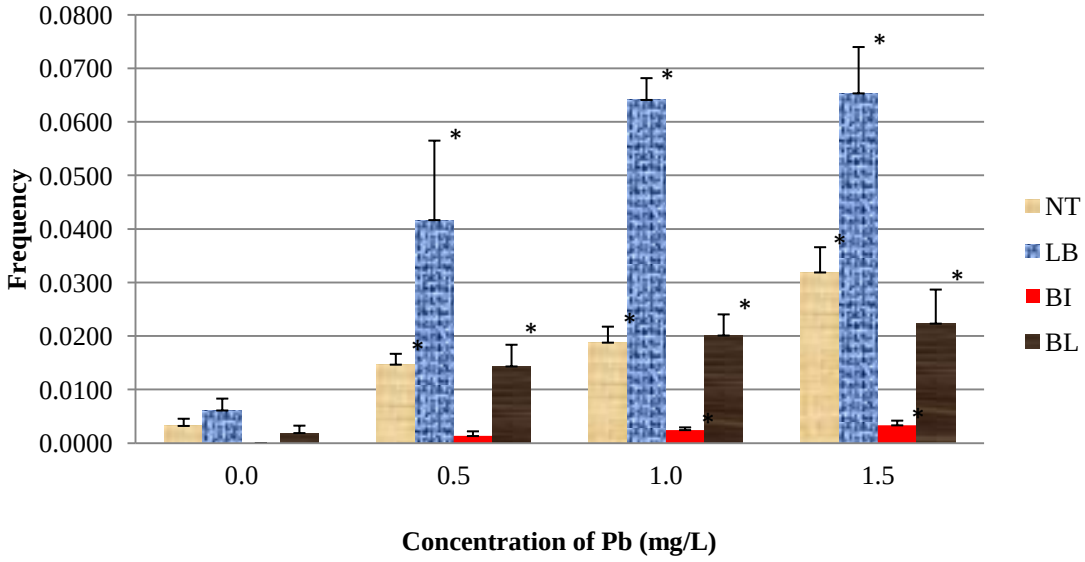
The frequency of the highest micronuclei was by the exposure of lead at 1.5mg/L which is at the highest concentration. The only significant figure is when sample was exposed to concentration of 1.0 and 1.5mg/L of lead and none for copper. Relatively, there was not much of a difference on frequencies of micronucleus with increasing concentration of copper. The frequencies of micronucleus for samples exposed to copper were also similar to the control sample.

Frequencies of nuclear abnormalities were tremendously higher than micronucleus observed. Nuclear abnormalities that were observed include notched (NT), binuclei (BN), blebbed (BL) and lobbed (LB) nuclei. Lead was able to induce nuclear abnormalities significantly for all concentrations which were 0.5, 1.0, and 1.5mg/L. However, copper was only able to significantly induce nuclear abnormalities at its highest concentration which was 1.5 mg/L. Comparison of different types of nuclear abnormalities are presented in Figure 4.2.3.

Copper was only able to significantly induce notched and lobbed nuclei at 1.5mg/L when compared to the control sample. However, lead was able to significantly induce notched, lobbed, binuclei and blebbed nuclei at all concentration except for binuclei at 0.5mg/L when comparing to the control sample. Lobed nuclei were found to be the highest frequency and binuclei have the lowest frequency out of all nuclear abnormalities for all samples.



(A)



(B)

Figure 4.2.3. Comparison of type of nuclear abnormalities observed for control sample with samples exposed to copper (A) and lead (B). Means with significantly different values at $p<0.05$ to the control sample are labeled with asterisk. The significant values were analyzed using Tukey's test. Standard deviations were indicated by error or T-bars.

4.3 Banding Pattern of RAPD

Out of the 10 random primers that were used for RAPD, only 7 were able to produce clear banding patterns. These primers were OPA-03, OPA-04, OPA-10, OPA-12, OPA-13, OPB-08 and OPC-11. These dominant markers do not require a set of 2 primers in order to conduct a test. One primer is sufficient enough to amplify the DNA fragments. The same RAPD primer can act both as a forward and a reverse primer. The primers attached randomly on the denatured DNA during the PCR cycle and the DNA bands can only be produced if two of the same primers are attached close to one another with the correct orientation. The random primers are not specific and thus the RAPD banding patterns are not necessarily reproducible from one run to the other.

Scoring of the banding patterns obtained was conducted in order to analyze the distance (Jaccard) between the control sample and the exposed samples. The similarity coefficient was calculated using the equation:

$$\text{Jaccard similarity coefficient} = \frac{\text{The number of bands shared by sample A and B}}{\text{(The number of bands in sample A + the number of bands in sample B - the number of bands shared by sample A and B)}}$$

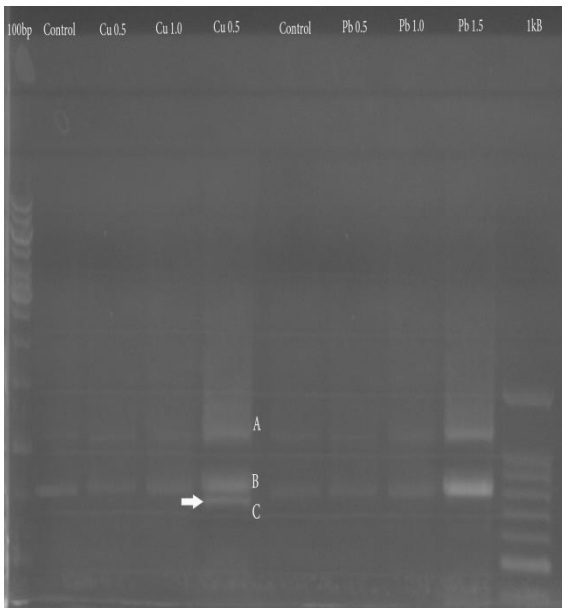
The distance between sample was calculated using the equation:

$$\text{Jaccard distance} = 1 - \text{Jaccard similarity coefficient}$$

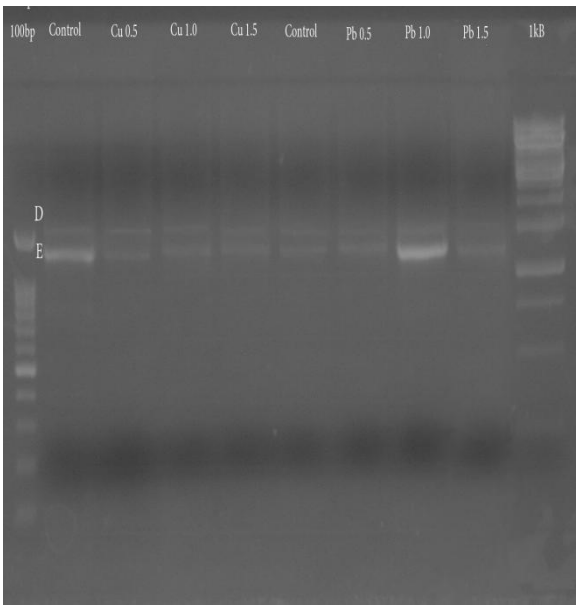
From the scoring done, 8 polymorphic bands can also be observed. The pair-wise comparison of the banding pattern was tabulated in Table 4.3.1 and Jaccard distance was tabulated in Table 4.3.2. Figures 4.3.1 and 4.3.2 show the pictures of the RAPD products on agarose gel. From the data obtained, a UPGMA tree was constructed using PHYLIP version 3.695 as in Figure 4.3.3.

After calculating the Jaccard distance for each exposed sample, it can be seen that the sample that had the closest distance to the control sample was 0.5mg/L of copper and the furthest sample was lead at 1.5mg/L. The arrangement of the distance from closest to furthest from the control is Cu 0.5mg/L < Cu 1.0mg/L < Pb 0.5mg/L < Pb 1.0mg/L < Cu 1.5mg/L < Pb 1.5mg/L. Samples with the highest concentration of exposure for both copper and lead which were at 1.5mg/L have the highest Jaccard distance for their own respective metals.

From the tree that was constructed in Figure 4.3.3, it shows that as the concentration of metal increases, the exposed samples with higher concentrations are not grouped together with the control sample. Generally from the tree generated, the samples can be grouped into 3 different clusters. Copper of 0.5 mg/L concentration is clustered together with the control sample as the distance between the samples are close to each other. The second cluster observed consists of most of the test samples which were lead (0.5mg/L, 1.0mg/L) and also copper (1.0mg/L, 1.5mg/L). Lead at 1.5mg/L is not clustered with any other sample.



(A)



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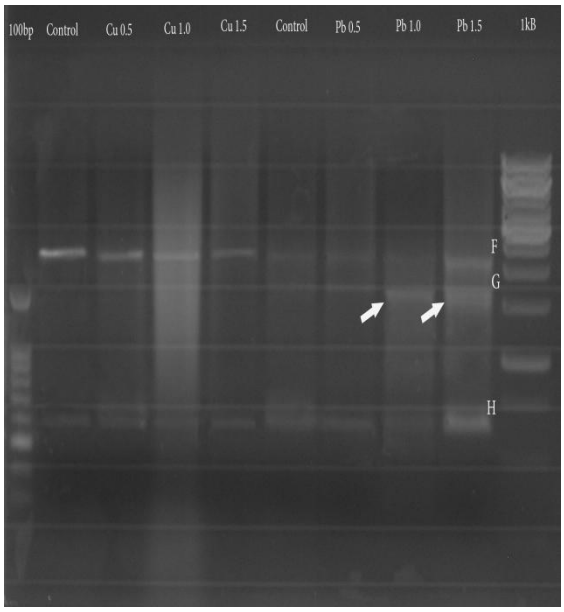

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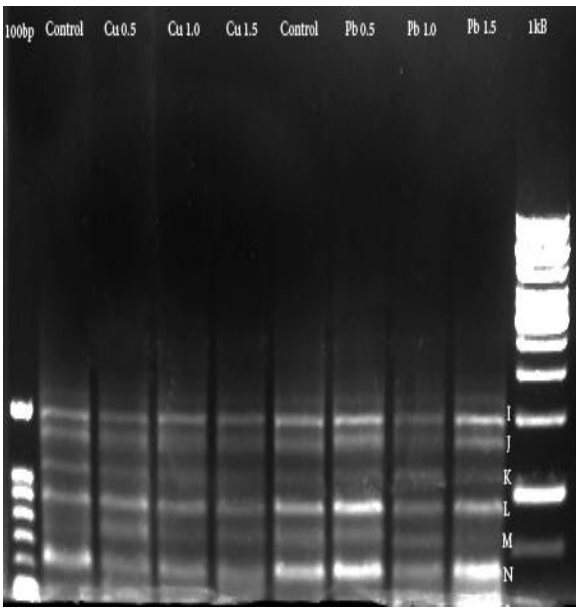
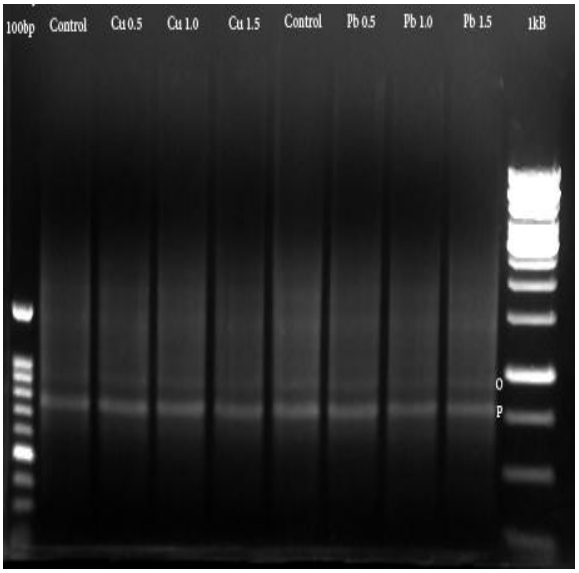
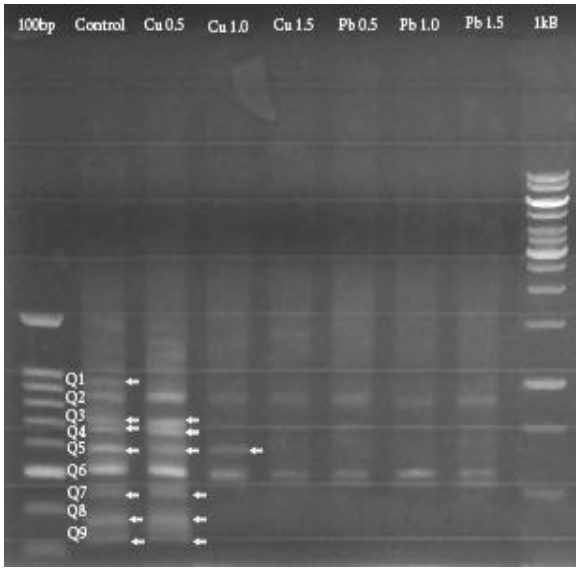


(D)

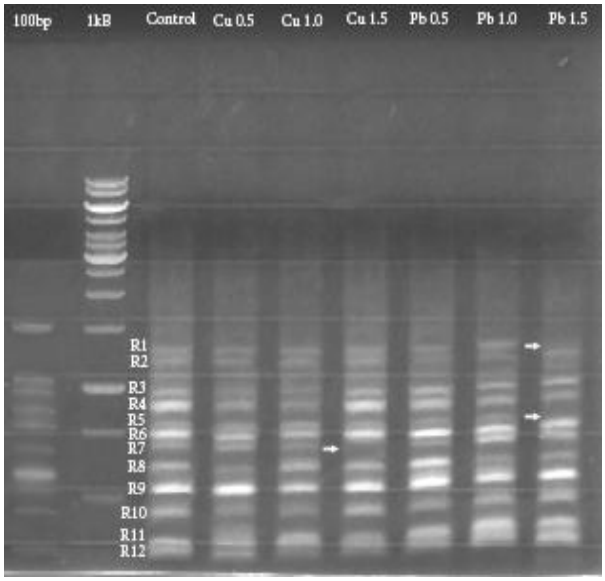
Figure 4.3.1. DNA banding pattern for RAPD PCR products on 1% agarose gel. Primer used was OPA-12 (A), OPB-08 (B), OPA-10 (C) and OPC-11 (D). The arrows show the differences of banding pattern amongst the samples.



(E)



(F)



(G)

Figure 4.3.2. DNA banding pattern for RAPD PCR products on 1% agarose gel. Primer used was OPA-03 (E), OPA-04 (F) and OPA-13 (G). The arrows show the differences of banding pattern amongst the samples.

Table 4.3.1. Pair-wise scoring on all RAPD PCR products for control sample and samples that were exposed to lead and copper.

BAND (kb)	Control	Pb 0.5	Pb 1.0	Pb 1.5	Cu 0.5	Cu 1.0	Cu 1.5
Control		28	28	26	34	29	27
Pb 0.5			28	26	28	28	27
Pb 1.0				27	28	28	27
Pb 1.5					26	26	25
Cu 0.5						29	27
Cu 1.0							27
CU 1.5							

Table 4.3.2. Jaccard distance of all exposed sample to the control sample.

Metal	**Jaccard Distance**		
	0.5 mg/L	**1.0 mg/L**	**1.5 mg/L**
Pb	0.200	0.222	0.297
Cu	0.029	0.171	0.229

CHAPTER 5

DISCUSSION

5.1 Metallothionein gene expression

Fish is an important source of protein for human consumption. Nowadays, the main source of freshwater fishes are being obtained from aquaculture practices. Aquaculture activities usually use the river as their main water source. Water run down from agricultural, industrial or other anthropogenic site may lead to the increase of toxicity level of the river as pollutants such as heavy metals are being introduced into the river. Thus, the levels of metal concentrations will increase above the permitted level. The purity of the water content used could be determined by taking samples and run appropriate tests on the samples. Aquatic organisms or fishes that are being exposed to the polluted water are faced with various threats such as neurological damage, decreased immunity, disruption in metabolic function, defect in reproduction and offspring (M'kandawire *et al*., 2012). The fact that the fish breathes polluted water will not only bring harm to those who consume it, if it turns out that it will cause death to the fishes, this will eventually leads to the disruption of the food chain. In the end, many organisms either human, animals or plants will be affected by disruption of the food chain. Thus, the need to monitor the aquatic environment at early stages before pollution gets worse is important in order to safeguard the aquatic organisms. One of the methods that can be used to indicate the condition of any aquatic bodies is by using biomarkers. A good biomarker will react to xenobiotics that are foreign and harmful to the surroundings.

Metal accumulation differs with different organs and different metals. Organs that usually accumulate xenobiotics that were introduced via the environment include liver, gills, kidney and muscle. Among all of them, the liver has the highest metal accumulation rate compared to other organs of fish as this corresponds to liver functions of metabolizing xenobiotics (Wu *et al*., 2006; Çoğun *et al*., 2003). It has been known that metals whether they are essential or non-essential can stimulate the synthesis of metallothionein. With essential metals, metallothionein functions in regulating the metal ions for cell function. Whereas for non-essential metal ions, metallothionein will bind to them to dispel the metal ions out of the system and prevents cellular toxicity (Li *et al*., 2007). The most common and highest levels of metals present in liver are copper, cadmium, lead and zinc. Metallothionein will bind naturally to zinc and copper at moderate concentrations since they are considered essential metal ions for cell function. However, non-essential metal ions such as lead and cadmium are foreign to the cellular environment. Mouse metallothionein that has been cloned and expressed in *E. coli* has shown resistance towards metals such as mercury, copper, cadmium and zinc by withdrawing the ions out of the cell (Hou *et al*., 1988). The severity of metal toxicity is different between species because it depends on what kinds of metal and the species dexterity to synthesize metallothionein naturally in their system (Alonso *et al*., 2005).

Real time PCR is a sensitive method to quantify the induction of metallothionein by exposure and treatment of heavy metals. The method allows for assessment of the result quantitatively by using several choices of quantification methods accurately. Many current studies have used real time PCR as a method of choice because of its accuracy and fast data representation. Tilapia's metallothionein gene promoter can be induced by zinc, cadmium and lead (Cheung *et al*., 2005). Although several metals are able to induce metallothionein, cadmium has been found to be the most effective metal

since it has a higher affinity to bind to metallothionein compared to the other metals (Dondero *et al*., 2005). However when comparing copper with lead, it has been found that copper has a higher ability to replace lead at the metallothionein binding site. The reason for this is because copper has a higher affinity towards metallothionein compared to lead and even zinc (Alonso *et al*., 2005).

According to a study conducted by Atli and Canli, (2008), copper cannot induce the increase of metallothionein expression level unless the basal level of copper is increased considerably. In other words, if the initial concentration of copper inside the cell is high to begin with, administration of slightly higher concentration of copper would not be sufficient enough to significantly induce additional production of metallothionein. Exposure of low concentration of copper from the environment would not pose a threat to the cell and would not induce over production of metallothionein. At a low and non-lethal concentration, copper is being regulated normally in tilapia. Thus, metallothionein synthesis will only increase as a defense mechanism if higher concentration of copper were being exposed above the basal level of the cell. Hence, metallothionein at basal level directly shows that the concentration of the essential metals are at a non-toxic level and are being regulated normally for extracellular and intracellular metabolism. (Atli & Canli, 2003). In other words, copper is only able to induce the increase of metallothionein synthesis when the test subject is being exposed or administered with large doses of copper that exceeded the current basal level concentration of copper regulated inside the cell.

For lead, on the other hand, might have a lower basal level prior to exposure than copper that cannot be detected in the control sample. Thus, the increase exposure of different concentrations of lead allows the elevated induction of metallothionein

synthesis on the exposed samples. Non essential metal such as lead and cadmium can exceed the metal uptake threshold level even with lower exposure or treatment concentration compared to those of essential metal such as copper and zinc. Even at low level of lead concentration, fishes are sensitive enough to be able to react to it (Monteiro *et al*., 2011).

Although level of expression among metals may be different, it has been discovered that copper and lead are two of the several metal ions that can act as primary inducers for metallothionein gene activities. Lead is one of the most potent inducer for metallothionein in livers of tilapia. At 24 hours of exposure, both copper and lead were able to increase metallothionein synthesis of the treated samples (Chan & Chan, 2008). They discovered that lead has a higher expression level than copper, which also corresponds to the result of this study. Apart from that, resistant level towards different metals differs within species. It has been reported that tilapia is a copper resistant species compared to carps (Lam *et al*., 1998). Metallothionein gene promoter of carps can be induced by many ions compared to tilapia. Other metals that were able to induce metallothionein gene promoter of carps are copper, mercury, nickel and cobalt (Cheung *et al*., 2005). In another study that was conducted, the concentration and accumulation of copper differed among fish species such as rainbow trout, common carp and gibel carp (De Boeck *et al*., 2003).

Under unfavorable condition, metallothionein can help to regulate stress and also able to reduce metal toxicity in a cell (Coyle *et al*., 2002). Mortality rate and metal toxicity will increase with the absence or disruption of metallothionein synthesis since metallothionein is able to regulate xenobiotics that are harmful to the internal environment. Thus, the increase of metallothionein expression will protect the cell from

stress and lethal effect. Apart from that, over expression of metallothionein can also induce transcription, replication and new protein synthesis inside the cell (Dondero *et al*., 2005).

5.2 Micronucleus and nuclear abnormalities

Micronucleus is a small part of chromosomes found in the cytoplasm detached from the main nucleus of the cell. Micronucleus can be formed from a whole lagging chromosome or just a part of it. It has been known that during anaphase of cell division, the spindle fiber is damaged, thus, unable to attach to the centromere for proper segregation to form new cells. This will cause the micronuclei to be left in the cytoplasmic fluid instead of being a part of the main nucleus (Çavaş, 2008). Since a part of the chromosome is separated from the nucleus, the cell is known to have abnormalities in the structure of the chromosome. In contrast, cells that have lost a whole chromosome are considered as aneuploid.

Micronucleus test with the observation of other nuclear abnormalities is an effective test to determine the genotoxicity and cytotoxicity of an organism. Furthermore, the test is fairly simple, easy to handle, reliable and inexpensive (Rodriguez *et al*., 2003). The result of micronucleus test observed can be an effect of chromosome breakage, chromosome loss, chromosome rearrangement, cell division inhibition, necrosis and apoptosis (Fenech, 2000).

Apart from the observed micronuclei, the nuclei that undergo alterations will also tend to look different from the normal nucleus or known as nuclear abnormalities. The main reason for the presence of the binucleated cell is related to interference of

cytokinesis during cell divison of the mother cell into daughter cells (Rodilla, 1993). Lobed and blebbed nuclei might have been formed through the replication of cells that have mutated chromatids. These chromatids lacked telomere, which resulted in the sister chromatids to attach to each other and undergo replication process via breakage-fusion-bridge cycle (Fenech & Crott, 2002). On the other hand, nuclear alterations of aneuploids contributed to a cell with a notched nucleus when being observed under the microscope (Ventura *et al*., 2008). Many parts of the fish can be used to perform micronucleus test such as erythrocyte, gill and liver. Erythrocytes are usually being used in genotoxicity studies as it is easier to obtain and handle. Moreover, erythrocytes have proven to be a good indicator in genotoxicity tests.

Many studies have been conducted using this method. However the studies vary in terms of time of exposure before conducting the micronucleus test. The treatment might be too short for sufficient induction of micronuclei and also after a certain prolonged time of treatment, micronuclei will decrease in number. According to Yadav & Trivedi (2009), the frequency of micronuclei increases with the time of treatment to copper and after 96 hours of treatment, the frequency started to decrease gradually depending on the dose of the treatment. Several chromosomal abnormalities were found on *Hoplias malabaricus* after being treated with lead for a certain period of time. The chromosomal abnormalities seemed to decrease during the end of the exposure period rather than the middle part of the treatment. This could be due to the fact that most of the repair mechanism occured and were activated during the earlier stages of exposure to the metal (Cestari *et al*., 2004). In another study, the increase of micronuclei number can only be seen on a neotropical freshwater fish after 24 to 96 hours of exposure to lead (Monteiro *et al*., 2011). It has been suggested that the usage of liver and gill as test samples are more sensitive to prolonged exposure to the heavy metals, whereas

erythrocytes are sufficient enough for a shorter time of treatment (Çavaş *et al*., 2005). Clastogenic effect was observed on liver of the mouse fetus once it was exposed to lead (Nayak *et al*., 1989). Mouse that has been exposed to lead has shown an increase in total micronuclei observed compared to the control sample (Jagetia & Aruna, 1998) which is similar to what was observed in this experiment.

Results obtained from this study shows that micronuclei of samples exposed with lead are only significant at the highest dose. This was also reported from studies conducted on *Carassius auratus* using lead acetate as the toxicant (Çavaş, 2008). Lead was also observed to be able to induce significant increase in blood of samples which was 18-fold higher than the control samples (Minozzo *et al*., 2004). According to the result obtained from this study, copper did not have as much effect as lead on the sample. This shows that the concentration of copper used for this study was at a non-toxic level as the sample was more resistant to copper or basal level of copper is already high and well regulated in the cell. Production of reactive oxygen species such as hydroxyl radicals which resulted in oxidative stress has been one of the reasons why heavy metals were able to induce the synthesis of micronucleus (Bonacker *et al*., 2005). The reactive oxygen species will cause damage to the DNA by affecting their bases which resulted in breaks in the DNA strand (Ahmad *et al*., 2006).

5.3 Banding Pattern of RAPD

The RAPD is an easy, simple, reliable and an inexpensive experimental method. An added advantage of using RAPD method for genotoxicity screening is that it can also detect temporary DNA damage in a cell (Atienzar *et al*., 2006). RAPD are most commonly used for phylogenetic studies between species or organisms. Apart from

phylogenetic studies, RAPD can also be used to detect mutations or DNA alteration between the same species and organism. One can expect to observe different banding patterns between untreated sample and treated samples because of the effect of chemicals or other xenobiotics that has affected the integrity of the DNA. If alteration of DNA did not take place, the banding pattern between treated and untreated samples will be the same. In this study, it is as expected that there would be a slight difference between the samples in the banding pattern which are either missing bands or additional bands. In a study conducted on *Oreochromis niloticus* that were exposed to different concentration of ammonia, additional and missing bands can be observed on the RAPD PCR products (Abumourad *et al*., 2012). Based on another study, results obtained shows that there were more loss of bands compared to the addition of new bands when bean seedlings were exposed to a toxic chemical and most missing bands were those with a higher molecular weight (Cenkci *et al*., 2009).

A study conducted on loach with exposure to a particular chemical has shown 78% differences in the banding pattern of treated sample compared to the control sample (Nan *et al*., 2013). The observed differences included lost bands, extra bands and changes of the intensities of the band, which resulted from oxidative DNA damage and also DNA modification. Although differences in band intensities could be considered as indicator of alteration or damage of DNA, the present study however, focuses on the addition and absence of bands. Inaccurate loading of initial DNA concentration might have affected the intensities of the PCR products. Other reasons that may have caused the differences of the banding pattern are DNA-protein cross-links, chromosomal rearrangement and DNA strand breaks (Atienzar *et al*., 2000). Primer binding sites will change with chromosomal rearrangements and DNA damages

that occur on these sites, thus resulting in different banding patterns of RAPD PCR products (Aydin *et al*., 2012).

Nevertheless, if there is no difference in the banding pattern of the PCR products, it does not necessarily mean that there was no DNA damage or alteration present in the sample. It could be that on that particular run, the primer has amplified regions which are not affected by any DNA damage (Cambier *et al*., 2010). However, the differences in banding pattern could be observed later with a repeat run of RAPD using the same primer. A slight change in the binding site of an oligo primer might create different bands than the control sample (Atienzar, 2002). For this present study, scoring data were generated from the variation of banding patterns to calculate Jaccard distance of the exposed samples to the control sample. Although most studies using RAPD were analysed based on the observed variability of the banding patterns, additional steps of scoring and determining the distance (Jaccard) was made for this study in order to have a quantitative data to support the qualitative data obtained through agarose gel viewing. Through the qualitative data, how much the DNA changes or differences of the exposed sample from the control sample can be estimated and tabulated. UPGMA tree was also generated from the scoring data to support the qualitative data.

Lead at 1.5mg/L has the furthest distance from the control sample when compared to the other treated samples. This shows that the sample has a higher DNA damage compare to the other treated samples. Because the samples are from the same species, the further the Jaccard distance of a sample shows that particular sample has undergone a more extensive DNA damage. On the contrary, sample exposed to 0.5mg/L of copper has the least effect of DNA damage as shown by the closest distance to the

control sample. The tree that was constructed is in concordance with data of Jaccard distance which reflects on a dose dependent relationship with the metal concentrations and genomic DNA alterations. Other similar studies were done by scoring banding pattern to determine the changes of total bands in control, polymorphic and varied bands on samples of *Evernia prunastri* (Duman *et al*., 2011). Cenkci *et al*., (2010) used similar method in evaluation of genotoxicity of herbicides in bean seedlings.

Although the method would not be sufficient solely on its own, complementary method that is coupled with RAPD for further study of genotoxicity will produce a good and reliable data. Which in this case, real time PCR and micronucleus test are being strongly supported by RAPD data which was similar to the study conducted by Cambier *et al*., (2010). The study they conducted shows that the genotoxicity effect of cadmium on exposed fish can be observed in the banding pattern of RAPD. Aydin *et al*., (2012), also recommends using additional biomarker that complements the RAPD results to further strengthen the data collected during experiment. They have found out that banding patterns of RAPD are concurrent with their observation on the rate of germination in cucumbers that were treated with copper and zinc. The data from Jaccard distance of the exposed sample for this study are similar to the results obtained for gene expression and micronucleus test with lead having the most impact on the test conducted compared to copper.

CHAPTER 6

CONCLUSION

The metallothionein expression levels were induced with the different concentration of metals. The concentration that has the largest effect to the lowest is as follows:

Pb 1.5mg/L* > Cu 1.5mg/L* > Pb 1.0 mg/L* > Cu 1.0mg/L > Pb 0.5mg/L > Cu 0.5mg/L > control

The values that were significantly different from the control samples were only those of 1.0mg/L of lead, 1.5mg/L of both lead and copper. The highest fold induction of all samples was lead with concentration of 1.5mg/L with a 7.61-fold increase followed by the same concentration of copper with 5.05-fold higher than the control sample. Thus lead was able to have a greater impact in inducing higher fold induction. In other words, the production of metallothionein was induced significantly higher with exposure of lead than copper at the same concentration.

The difference in micronucleus was observed significantly only for samples that were exposed to lead with the concentration of 1.0 and 1.5 mg/L. Copper at any concentration was not able to produce a significant number of observed micronucleus. Although lead has significant values, the frequency of micronucleus were less when compared with the frequency of nuclear abnormalities that were much more visible on the slides. Concentrations that were able to significantly induce nuclear abnormalities

were 0.5, 1.0 and 1.5mg/L of lead and also 1.5mg/L of copper. Thus, lead showed more impact than copper based on the micronucleus and nuclear abnormalities present.

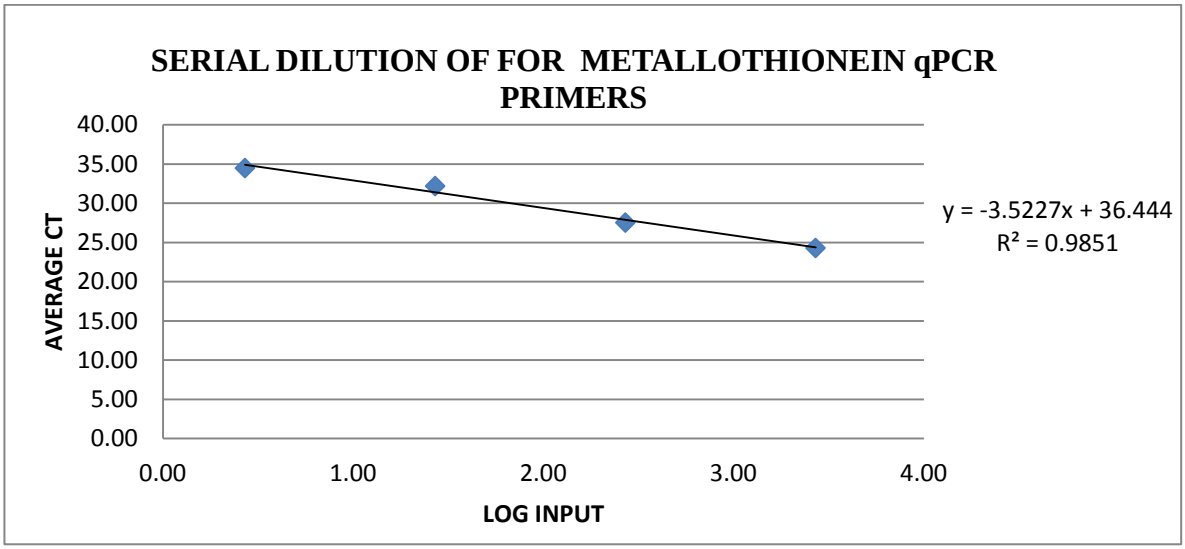
RAPD was conducted to calculate the Jaccard distance of the exposed samples to the control sample. The arrangement of the distance from furthest to the closest sample to control is as follows:

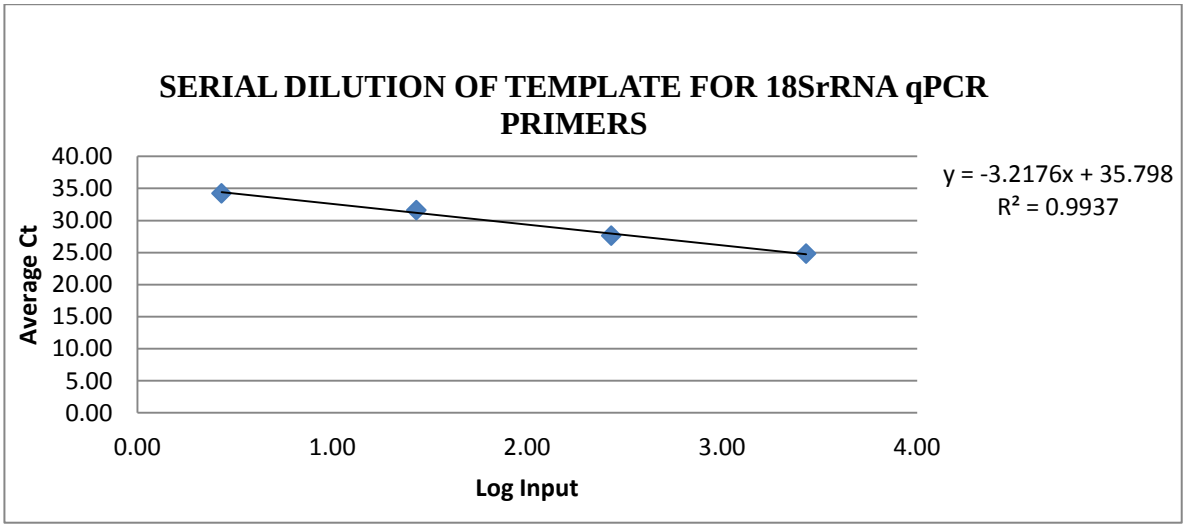
Pb 1.5mg/L > Cu 1.5mg/L > Pb 1.0mg/L > Pb 0.5mg/L > Cu 1.0mg/L > Cu 0.5mg/L

The results were similar to the order of samples in gene expression of metallothionein. However lead at 0.5mg/L has a higher Jaccard distance which is 0.200 compared to copper at 1.0mg/L which is 0.171. 0.5mg/L lead was the furthest from control sample with 0.297, followed by copper with 0.229 at the same concentration. The closest distance was sample of copper exposure at 0.5mg/L which was 0.029.

In comparing overall results, it can be seen that the other test results relatively supports the findings of the realtime PCR for metallothionein gene expression. It also shows that lead gives a higher impact compared to copper on metallothionein gene expression level, Jaccard distance, frequency of micronucleus and nuclear abnormalities. However, copper at the highest concentration can also give rise to a significant level of impact on the test results. Apart from that, metallothionein can be said to be a good potential biomarker for further toxicological studies.

APPENDIX I


SERIAL DILUTION OF FOR METALLOTHIONEIN qPCR PRIMERS
AVERAGE CT
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
0.00
1.00
2.00
3.00
4.00
LOG INPUT
y = -3.5227x + 36.444
R² = 0.9851


SERIAL DILUTION OF TEMPLATE FOR 18SrRNA qPCR PRIMERS
Average Ct
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
0.00
1.00
2.00
3.00
4.00
Log Input
y = -3.2176x + 35.798
R² = 0.9937

APPENDIX II

Statistical analysis for metallothionein gene expression.

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	101.149	6	16.858	28.202	.000
Within Groups	8.369	14	.598		
Total	109.517	20			

Post Hoc Test

Metallothionein gene expression for all samples

Tukey HSD

		Subset for alpha = .05			
Conc	N	1	2	3	4
CTL	3	1.0033			
Cu 0.5	3	1.5867	1.5867		
Pb 0.5	3	1.8767	1.8767		
Cu 1.0	3	2.0267	2.0267		
Pb 1.0	3		3.4167	3.4167	
Cu 1.5	3			5.0533	
Pb 1.5	3				7.6367
Sig.		.673	.122	.200	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Multiple Comparisons (Tukey HSD)

(I) Conc	(J) Conc	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
CTL	Cu 0.5	-.58333	.63127	.962	-2.7389	1.5722
	Cu 1.0	-1.02333	.63127	.673	-3.1789	1.1322
	Cu 1.5	-4.05000(*)	.63127	.000	-6.2055	-1.8945
	Pb 0.5	-.87333	.63127	.802	-3.0289	1.2822
	Pb 1.0	-2.41333(*)	.63127	.024	-4.5689	-.2578
	Pb 1.5	-6.63333(*)	.63127	.000	-8.7889	-4.4778
Cu 0.5	CTL	.58333	.63127	.962	-1.5722	2.7389
	Cu 1.0	-.44000	.63127	.991	-2.5955	1.7155
	Cu 1.5	-3.46667(*)	.63127	.001	-5.6222	-1.3111
	Pb 0.5	-.29000	.63127	.999	-2.4455	1.8655
	Pb 1.0	-1.83000	.63127	.122	-3.9855	.3255
	Pb 1.5	-6.05000(*)	.63127	.000	-8.2055	-3.8945
Cu 1.0	CTL	1.02333	.63127	.673	-1.1322	3.1789
	Cu 0.5	.44000	.63127	.991	-1.7155	2.5955
	Cu 1.5	-3.02667(*)	.63127	.004	-5.1822	-.8711
	Pb 0.5	.15000	.63127	1.000	-2.0055	2.3055
	Pb 1.0	-1.39000	.63127	.353	-3.5455	.7655
	Pb 1.5	-5.61000(*)	.63127	.000	-7.7655	-3.4545
Cu 1.5	CTL	4.05000(*)	.63127	.000	1.8945	6.2055
	Cu 0.5	3.46667(*)	.63127	.001	1.3111	5.6222
	Cu 1.0	3.02667(*)	.63127	.004	.8711	5.1822
	Pb 0.5	3.17667(*)	.63127	.003	1.0211	5.3322
	Pb 1.0	1.63667	.63127	.200	-.5189	3.7922
	Pb 1.5	-2.58333(*)	.63127	.015	-4.7389	-.4278
Pb 0.5	CTL	.87333	.63127	.802	-1.2822	3.0289
	Cu 0.5	.29000	.63127	.999	-1.8655	2.4455
	Cu 1.0	-.15000	.63127	1.000	-2.3055	2.0055
	Cu 1.5	-3.17667(*)	.63127	.003	-5.3322	-1.0211
	Pb 1.0	-1.54000	.63127	.252	-3.6955	.6155
	Pb 1.5	-5.76000(*)	.63127	.000	-7.9155	-3.6045
Pb 1.0	CTL	2.41333(*)	.63127	.024	.2578	4.5689
	Cu 0.5	1.83000	.63127	.122	-.3255	3.9855
	Cu 1.0	1.39000	.63127	.353	-.7655	3.5455
	Cu 1.5	-1.63667	.63127	.200	-3.7922	.5189
	Pb 0.5	1.54000	.63127	.252	-.6155	3.6955
	Pb 1.5	-4.22000(*)	.63127	.000	-6.3755	-2.0645
Pb 1.5	CTL	6.63333(*)	.63127	.000	4.4778	8.7889
	Cu 0.5	6.05000(*)	.63127	.000	3.8945	8.2055
	Cu 1.0	5.61000(*)	.63127	.000	3.4545	7.7655
	Cu 1.5	2.58333(*)	.63127	.015	.4278	4.7389
	Pb 0.5	5.76000(*)	.63127	.000	3.6045	7.9155
	Pb 1.0	4.22000(*)	.63127	.000	2.0645	6.3755

* The mean difference is significant at 0.05 level.

APPENDIX III

Statistical analysis for total of micronucleus and nuclear abnormalities.

		Sum of Squares	df	Mean Square	F	Sig.
NUCLEAR ABNORMALITIES	Between Groups	.034	6	.006	44.440	.000
	Within Groups	.002	14	.000		
	Total	.036	20			
MICRONUCLEI	Between Groups	.000	6	.000	12.901	.000
	Within Groups	.000	14	.000		
	Total	.000	20			

NUCLEAR ABNORMALITIES

Tukey HSD

		Subset for alpha = .05			
CONCENTRATION	N	1	2	3	4
Pb Ctl	3	.01122200			
Pb 0.5	3	.01944442	.01944442		
Pb 1.0	3	.02899999	.02899999		
Pb 1.5	3		.04811111	.04811111	
Cu 0.5	3			.07199999	
Cu 1.0	3				.10544444
Cu 1.5	3				.12288877
Sig.		.492	.084	.198	.512

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 3.000.

MICRONUCLEI

Tukey HSD

CONCENTRATION	N	Subset for alpha = .05		
		1	2	3
Pb Ctl	3	.00000000		
Pb 0.5	3	.00000000		
Pb 1.0	3	.00011111		
Pb 1.5	3	.00022220		
Cu 0.5	3	.00133333	.00133333	
Cu 1.0	3		.00188890	.00188890
Cu 1.5	3			.00311113
Sig.		.142	.894	.206

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

Statistical analysis for all nuclear abnormalities.

		Sum of Squares	df	Mean Square	F	Sig.
NOTCHED	Between Groups	.002	6	.000	36.438	.000
	Within Groups	.000	14	.000		
	Total	.002	20			
LOBED	Between Groups	.011	6	.002	39.900	.000
	Within Groups	.001	14	.000		
	Total	.012	20			
BINULCEI	Between Groups	.000	6	.000	20.756	.000
	Within Groups	.000	14	.000		
	Total	.000	20			
BLEBBED	Between Groups	.001	6	.000	17.911	.000
	Within Groups	.000	14	.000		
	Total	.001	20			

NOTCHED

Tukey HSD

CONC.	N	Subset for alpha = .05				
		1	2	3	4	5
CTL	3	.00322233				
Pb 0.5	3	.00666667	.00666667			
Pb 1.0	3	.01044200	.01044200	.01044200		
Pb 1.5	3		.01344433	.01344433	.01344433	
Cu 0.5	3			.01466677	.01466677	
Cu 1.0	3				.01877777	
Cu 1.5	3					.03188867
Sig.		.062	.088	.496	.255	1.000

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 3.000.

LOBED

Tukey HSD

		Subset for alpha = .05			
CONCENTRATION	N	1	2	3	4
CTL	3	.00611133			
Pb 0.5	3	.01033333	.01033333		
Pb 1.0	3	.01366667	.01366667		
Pb 1.5	3		.02644433	.02644433	
Cu 0.5	3			.04166667	
Cu 1.0	3				.06411133
Cu 1.5	3				.06533300
Sig.		.817	.126	.163	1.000

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 3.000.

BINUCLEI

Tukey HSD

CONCENTRATION	N	Subset for alpha = .05		
		1	2	3
CTL	3	.00000000		
Pb 0.5	3	.00000000		
Pb 1.0	3	.00011100		
Pb 1.5	3	.00011100		
Cu 0.5	3	.00133333	.00133333	
Cu 1.0	3		.00244433	.00244433
Cu 1.5	3			.00333333
Sig.		.081	.192	.405

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

BLEBBED

Tukey HSD

CONCENTRATION	N	Subset for alpha = .05		
		1	2	3
CTL	3	.00188867		
Pb 0.5	3	.00244467		
Pb 1.0	3	.00477800	.00477800	
Pb 1.5	3	.00811100	.00811100	
Cu 0.5	3		.01433333	.01433333
Cu 1.0	3			.02011133
Cu 1.5	3			.02233333
Sig.		.347	.051	.133

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.

APPENDIX IV

LABEL	BAND (kb)	CTL	Pb 0.5	Pb 1.0	Pb 1.5	Cu 0.5	Cu 1.0	Cu 1.5
F	2.2	1	1	1	1	1	1	1
D	1.75	1	1	1	1	1	1	1
G	1.7	0	0	1	1	0	0	0
I	1.5	1	1	1	1	1	1	1
J	1.4	1	1	1	1	1	1	1
R1	1.35	1	1	1	0	1	1	1
E	1.3	1	1	1	1	1	1	1
R2	1.25	1	1	1	1	1	1	1
A	1.2	1	1	1	1	1	1	1
K	1.1	1	1	1	1	1	1	1
Q1	0.95	1	0	0	0	0	0	0
R3	0.91	1	1	1	1	1	1	1
L	0.9	1	1	1	1	1	1	1
O	0.89	1	1	1	1	1	1	1
Q2	0.85	1	1	1	1	1	1	1
R4	0.83	1	1	1	1	1	1	1
B	0.8	1	1	1	1	1	1	1
P	0.76	1	1	1	1	1	1	1
C	0.75	0	0	0	1	0	0	0
M	0.72	1	1	1	1	1	1	1
R5	0.71	1	1	1	0	1	1	1
Q3	0.7	1	0	0	0	1	0	0
R6	0.69	1	1	1	1	1	1	1
Q4	0.68	1	0	0	0	1	0	0
N	0.65	1	1	1	1	1	1	1
R7	0.63	1	1	1	1	1	1	0
H	0.6	1	1	1	1	1	1	1
Q5	0.59	1	0	0	0	1	1	0
R8	0.53	1	1	1	1	1	1	1
Q6	0.51	1	1	1	1	1	1	1
R9	0.47	1	1	1	1	1	1	1
Q7	0.43	1	0	0	0	1	0	0
R10	0.4	1	1	1	1	1	1	1
Q8	0.38	1	0	0	0	1	0	0
Q9	0.32	1	0	0	0	1	0	0
R11	0.31	1	1	1	1	1	1	1
R12	0.3	1	1	1	1	1	1	1
TOTAL BANDS		35	28	29	28	34	29	27
GEN. DISTANCE (D) WITH CTL		-	0.200	0.222	0.297	0.029	0.171	0.229

REFERENCES

Abumourad, I. M. K., Hanna, M. I. & Girgis, S. M. (2012). Genotoxicity assessment of ammonia in cultured Orechromis niloticus using RAPD assay. *Journal of Genetic Engineering and Biotechnology, 20,* 209-212.

Ahmad, I., Maria, V. L., Oliveira, M., Pacheco, M. & Santos, M. A. (2006). Oxidative stress and genotoxic effects in gill and kidney of Anguilla Anguilla L. exposed to chromium with or without pre-exposure to β-naphthoflavone. *Mutation Research, 608,* 16-28.

Ahmed, M. M. M., Ali, B. A., & El-Zaeem, S. Y. (2004). Application of RAPD markers in fish: Part I – some genera (*Tilapia, Sarotherodon and Oreochromis*) and species (*Oreochromis aureus* and *Oreochromis niloticus*) of tilapia. *International Journal of Biotechnology, 6(1),* 86-93.

Alloway, B. J. (1995). *Heavy metals in soil*. Blackie Academic & Professional.

Almeida, J. A., Novelli, E. L. B., Silva, M. D. P., & Júnior, R. A. (2001). Environmental cadmium exposure and metabolic responses of the Nile tilapia. *Oreochromis niloticus Environmental Pollution, 114(2),* 169-175.

Alonso, M. L., Prieto F., Miranda, M. & Castillo, C. (2005). The role of metallothionein and zinc in hepatic copper accumulation in cattle. *The Veterinary Journal, 169,* 262-267.

Andrews, G. K. (1990). Regulation of metallothionein gene expression. *Programme Food Nutritional Science, 14(2-3),* 193-258.

Atienzar, F. A., Cordi, B., Donkin, M. E., Evenden, A. J., Jha, A. N & Depledge, M. H. (2000). Comparison of ultraviolet-induced genotoxicity detected by random amplified polymorphic DNA with chlorophyll fluorescence and growth in a marine macroalgae, *Palmariapalmata*. *Aquatic Toxicology, 50(1-2),* 1-12.

Atienzar, F. A., Venier, P., Jha, A. N. & Depledge, M. H. (2002). Evaluation of the random amplified polymorphic DNA (RAPD) assay for the detection of DNA damage and mutations. *Mutation Research 521,* 151-163.

Atienzar, F. A. & Jha, A. N. (2006). The random amplified polymorphic DNA (RAPD) assay and related techniques applied to genotoxicity and carcinogenesis studies: A critical review. *Mutation Research, 613,* 76-102.

Atli, G., Alptekin, O., Tükel, S., & Canli, M. (2006). Response of catalase activity to Ag^{+}, Cd^{2+}, Cr^{6+}, Cu^{2+} and Zn^{2+} in five tissues of freshwater fish *Oreochromis niloticus. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 143(2),* 218-224.

Atli, G. & Canli, M. (2003). Natural occurrence of metallothionein-like proteins in the liver of fish *Oreochromis niloticus* and effects of cadmium, lead, copper, zinc and iron exposures on their profiles. *Environmental Contamination Toxicology, 70* (619-627).

Atli, G. & Canli, M. (2007). Enzymatic responses to metal exposures in a freshwater fish *Oreochromis niloticus. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 15(2),* 282-287.

Atli, G. & Canli, M. (2008). Responses of metallothionein and reduced glutathione in a freshwater fish *Oreochromic niloticus* following metal exposures. *Environmental Toxicology and Pharmacology, 25,* 33-38.

Aydin, S. S., Gökçe, E., Büyük, İ. & Aras, S. (2012). Characterization of stress induced by copper and zinc on cucumber (*Cucumis sativus* L.) seedlings by means of molecular and population parameters. *Mutation Research, 746,* 49-55.

Baykan, U., Atli, G., & Canli, M. (2007). The effects of temperature and metal exposures on the profiles of metallothionein-like proteins in *Oreochromis niloticus. Enviromental Toxicology and Pharmacology, 23*, 33-38.

Baysoy, E., Atli. G., Gürler, C. Ö., Dogan, Z., Eroglu, A., Kocalar, K. & Canli, M. (2012). The effects of increased freshwater salinity in the biodisponibility of metals (Cr, Pb) and effects on antioxidant systems of *Oreochromis niloticus. Ecotoxicology and Environmental Safety, 84,* 249-253.

Benjamin, D., & Thatheyus, A. J. (2012). Acute toxicity of nickel and cadmium to the Cichlid fish, *Oreochromis mossambicus. Research in Zoology 2(5),* 1922.

Bonacker, D., Stoiber, T., Böhm, K. J., Prots, I., Wang, M., Under, E., … Degen, G. H. (2005). Genotoxicity of inorganic lead salts and disturbance of microtubule function. *Environmental and Molecular Mutagenesis, 45(4),* 346-353.

Cambier, S., Gonzalez, P., Durrieu, G. & Bourdineaud, J. P. (2010). Cadmium-induced genotoxicity in zebrafish at environmentally relevant doses. *Ecotoxicology and Environmental Safety, 73,* 312-319.

Çavaş, T. (2008). In vivo genotoxicity of mercury chloride and lead acetate: Micronucleus test on acridine orange stained fish cells. *Food and Chemical Technology, 46,* 352-358.

Çavaş, T., Garanko, N. N. & Arkhipchuk, V. V. (2005). Induction of micronuclei and binuclei in blood, gill and liver cells of fishes subchronically exposed to cadmium chloride and copper sulphate. *Food and Chemical Technology, 43*, 569-571.

Cenkci, S., Yildiz, M., Ciğerci, İ. H., Konuk, M. & Bozdağ, A. (2009). Toxic chemicals-induces genotoxicity detected by random amplified polymorphic DNA (RAPD) in bean (*Phaseolus vulgaris* L.) seedlings. *Chemosphere, 76,* 900-906.

Cenkci, S., Yildiz, M., Ciğerci, İ. H, Bozdağ, A., Terzi, H. & Terzi W., S., A. (2010). Evaluation of 2,4-D and Dicamba genotoxicity in bean seedlings using comet and RAPD assays. *Ecotoxicology and Environmental Safety, 73*, 1558-1564.

Cestari, M. M., Lemos, P. M. M., Ribeiro, C. A. O., Costa, J. R. M. A., Pelletier, E., Ferraro M. V. M., … Fenocchio, A. S. (2004). Genetic damage induced by thropic doses of lead in the neotropical fich *Hoplias malabaricus* (Characiformes, Erythrinidae) as revealed by the comet assay and chrosomal aberrations. *Genetic and Molecular Biology, 27(2)*, 270-274.

Chan, W. W. L., & Chan, K. M. (2008). Cloning and characterization of a tilapia *(Oreochromis aureus)* metallothionein gene promoter in Hepa-T1 cells following the administration of various heavy metal ions. *Aquatic Toxicology*, 86, 59-75.

Cheung, A. P. L., Lam, T. H. J., & Chan, K. M. (2004). Regulation of tilapia metallothionein gene expression by heavy metal ions. *Marine Environmental Research, 58(2-5)*, 389-394.

Cheung, A. P. L., Lam, T. H. J, & Chan, K. M. (2005). Tilapia metallothionein genes: PCR-cloning and gene expression studies. *Biochimica et Biophysica Acta 1731*, 191-201.

Çoğun, H. Y., Yüzereroğlu, T. A., & Kargin, F. (2003). Accumulation of copper and cadmium in small and large Nile tilapia *Orecochromic niloticus. Bulletin of Environment Contamination Toxicology, 71*, 1265-1271.

Çoğun, H. Y., & Kargin, F. (2004). Effects of pH on the mortality and accumulation of copper in tissues of *Oreochromis niloticus. Chemosphere, 55*, 277-282.

Coyle, P., Philcox, J. C., Carey, L. C. & Rofe, A. M. (2002). Metallothionein: The multipurpose protein. *CMLS Cellular and Molecular Life Sciences, 59*, 627-647.

Dang, Z. C., Flik, G., Ducouret. B., Hogstrand, C., Bonga, S. E. W., & Lock, A. C. (2000). Effects of copper on cortisol receptor and metallothionein expression in gills of *Oncorhynchus mykiss. Aquatic Toxicology, 51*, 45-54.

De Boeck, G., Huong Ngo, T. T., Campenhout K. V. & Blust, R. (2003). Differential metallothionein induction patterns in three freshwater fish during sublethal copper exposure. *Aquatic Toxicology, 65*, 413-424.

Dondero, F., Piacentini, L, Banni, M., Rebelo, M, Burlando, B. & Viarengo, A. (2005). Quantitative PCR analysis of two molluscan metallothionein genes unveils differential expression and regulation. *Gene, 345,* 259-270.

Duman, D. C., Atakol, O. & Aras, S. (2011). Assessment of air pollution genotoxicity by RAPD in *Evernia prunastri* L. Ach. From around iron-steel factory in Karabük, Turkey. *Journal of Environmental Sciences, 23(7)*, 1171-1178.

Ergene, S., Çavaş, T., Çelik, A., Köleli, N., & Aymak, C. (2007). Evaluation of river water genotoxicity using the piscine micronucleus test. *Environmental and Molecular Mutagenesis, 48(6),* 421–429.

Fenech, M. (2000). The in vitro micronucleus technique. *Mutation Research, 455,* 81-95.

Fenech, M. & Crott, J. W. (2002). Micronuclei, nucleoplasmic bridges and nuclear buds induced in folic acid deficient human lymphocytes – evidence for breakage-fusion-bridge cycles in the cytokinesis-block micronucleus assay. *Mutation Research, 504,* 131-136.

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

Frieauff, W., Pőtter-Locher, F., Cordier, A., & Suter, W. (1998). Automatic analysis of the in vitro micronucleus test on V79 cells. *Mutation Research 413,* 57-68.

Ge, H., Liu, y., Jiang, M., Zhang, J., Han, H. & Chen, H. (2013). Analysis of genetic diversity and structure of eggplant populations (*Solanum melongena* L.) in China using simple sequence repeat markers. *Scientia Horticulturae, 162,* 71-75.

Hayat, S., Javed, M., & Razzaq, S. (2007). Growth performance of metal stressed major carps viz. *Catla catla*, *Labeo rohita* and *Cirrhina mrigala* reared under semi-intensive culture system. *Pakistan Veterinary Journal, 27(1),* 8-12.

Hoshina, M. M., Angelis, D. D. F. D., & Marin-Morales, M. A. (2008). Induction of micronucleus and nuclear alterations in fish (*Oreochromis niloticus*) by a petroleum refinery effluent. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 656(1-2),* 44-48.

Hou, Y. M., Kim, R & Kin, S. H. (1988). Expression of the mouse metallothionein-I gene in Escherichia coli: Increased tolerance to heavy metals. *Biochimica et Biophysica Acta (BBA) – Gene Structure and Expression, 951(1),* 230-234.

Jagetia, G. C. & Aruna, R. (1998). Effect of various concentrations of lead on the induction of micronuclei in mouse bone marrow. *Mutation Research, 415,* 131-137.

Jiraungkoorskul, W., Sahaphong, S., Kosai, P., & Kim, M. H. (2007). Micronucleus test: The effect of ascorbic acid on cadmium exposure in fish (*Puntius altus). Research Journal of Environment Toxicology, 1(1),* 27-36.

Jones, C. J. , Edwards, K. J. , Castaglione, S. , Winfield, M. O. , Sala, F. & Van, C. D. W. (1997). Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. *Molecular Breeding, 3(5),* 381-390.

Li, J. S., Li, J. L., & Wu, T. T. (2007). The effects of copper, iron and zinc on digestive enzyme activity in the hybrid tilapia *Oreochromis niloticus (L.)* x *Oreochromis aureus (Steindachner). Journal of Fish Biology, 71,* 1788-1798.

Lima, P. L., Benassi, J. C., Pedrosa, R. C., Magro, J. D., Oliveira T. B., & Filho, D. W. (2006). Time-course variations of DNA damage and biomarkers of oxidative stress in tilapia (Oreochromis niloticus) exposed to effluents from a swine industry. *Archives of Environmental Contamination And Toxicology, 50,* 23–30.

Livak, K. J. & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. *Methods, 25,* 402-408.

Low, K. H., Zain., S. M., & Abas, M. R. (2011). Evaluation of metal concentrations in red tilapia (*Oreochromis* sp.) from three sampling sites in Jelebu, Malaysia using principle component analysis. *Food Analytical Methods, 4,* 276-285.

M'kandawire, E., Syakalima, M., Muzandu, K., Pandey, G., Simuunza, M., Nakayama, S. M. M., … Ishizuka, M. (2012). The nucleotide sequence of metallothioneins (MT) in liver of the Kafue lechwe (Kobus leche kafuensis) and their potential as biomarkers of heavy metal pollution of the Kafue River. *Gene, 506,* 310-316.

Martins, C. I. M., Eding, E. H., & Verreth, J. A. J. (2011). The effect of circulating aquaculture systems on the concentrations of heavy metals in culture water and tissues of Nile tilapia *Oreochromis niloticus. Food Chemistry, 126,* 1001-1005.

Matsumoto, S. T., Mantovani, M. S., Malaguttii, M. I. A., Dias, A. L. Fonseca, I. C., & Marin-Morales, M. A. (2006). Genotoxicity and mutagenicity of water contaminated with tannery effluents, as evaluated by the micronucleus test and comet assay using the fish *Oreochromis niloticus* and chromosome aberrations in onion root-tips. *Genetics and Molecular Biology, 29(1),* 148-158.

Minissi, S. & Lombi, E. (1997). Heavy metal content and mutagenic activity, evaluated by *Vicia faba* micronucleus test, of Tiber river sediments. *Mutation Research, 393,* 17-21.

Minozzo, R., Deimling, L. I., Gigante, L. P. & Santos-Mello, R. (2004). Micronuclei in peripheral blood lymphocytes of workers exposed to lead. *Mutation Research, 565,* 53-60.

Monteiro, V., Cavalcante, D. G. S. M., Viléla, M. B. F. A., Sofia, S. H. & Martinez, C. B. R. (2011). *In vivo* and *in vitro* exposures for the evaluation of the genotoxic effects of lead on the Neotropical freshwater fish *Prochilodus lineatus*. *Aquatic Toxicology, 104,* 291-298.

Nan, P., Xia, X. H., Du, Q. Y., Chen, J. J., Wu, X. H. & Chang, Z. J. (2013). Genotoxic effects of 8-hydroxylquinoline in loach (*Misgurnus anguilicaudatus*) assessed by the micronucleus test, comet assay and RAPD analysis. *Environmental Toxicology and Pharmacology, 35,* 434-443.

Nayak, B. N., Ray M., Persaud T. V. & Nigli M. (1989). Maternal and fetral chromosomal aberrations in mice following prenatal exposure to subembryotoxic dose of lead nitrate. *Acta Anatomica, 135(2), 185*-188.

Olurin, K. B., & Aderibigbe, O. A. (2006). Length-weight relationship and condition factor of pond reared juvenile *Oreochromis niloticus*. *World Journal of Zoology, 1(2),* 82-86.

Pena-Mendoza, B., Gómez-Màrquez, J. L., Salgado-Ugarte, I. H., and Ramírez-Noguera, D. (2005). Reproductive biology of *Oreochromis niloticus* (Perciformes: Cichlidae) at Emiliano Zapata Dam, Morelos, Mexico. *Revista de Biologia Tropical, 53*, 515-522.

Popma, T., and Masser, M. (1999). Tilapia Life History and Biology. *Southern Regional Aquaculture Center Publication 283.*

Rishi, K.K., & Grewal, S. (1995). Chromosome aberration test for the insecticide, dichlorvos, on fish chromosomes. *Mutation Research/Genetic Toxicology, 344(1-2),* 1-4.

Rodilla, V. (1993). Origin and evolution of binucleated cells and binucleated cells with micronuclei in cisplatin-treated CHO cultures. *Mutation Research/Genetic Toxicology, 300(3-4),* 281-291.

Rodriguez, V. M., Jimenez-Capdeville, M. E. & Giordano, M. (2003). The effects of arsenic exposure on the nervous system. *Toxicology Letters, 145,* 1-18.

Roesijadi, G. (1996). Metallothionein and its role in toxic metal regulation. *Comparative Biochemical and Physiology, 11(2),* 117-123.

Saeed, S. M., & Shaker, I. M. (2008). Assessment of heavy metals pollution in water and sediments and their effect on *Oreochromis niloticus* in the northern Delat Lakes, Egpyt. *International Symposium on Tilapia in Aquaculture, 8,* 475-489.

Schlenk, D., & Rice, C. D. (1998). Effect of zinc and cadmium treatment on hydrogen peroxide-induced mortality and expression of glutathione and metallothionein in a teleost hepatoma cell line. *Aquatic Toxicology, 43(2-3),* 121-129.

Shariati, F., & Shariati, S. (2011). Review on methods on determination of metallothionein in aquatic organisms. *Biological Trace Element Research 141*, 340-366.

Shlomo, A. B., Pichurin, O., Barshop, N. J., Wawrowsky, K. A., Taylor, J., Culler, M. D., … Melmed, S. (2007). Selective regulation of somatostatin receptor subtype signaling: Evidence for constitutive receptor activation. *Molecular Endocrinology, 21,(10),* 2565-2578.

Terradas, M., Martín, M., Tusell, L. & Genescà, A. (2010). Genetic activities in micronuclei: Is the DNA entrapped in micronuclei lost for the cell? *Mutation Research, 705,* 60-67.

Thirumoorthy, N., Kumar, K. T. M., Sundar, A. S., Panayappan, L., & Chatterjee, M. (2007). Metallothionein: An overview. *World Journal of Gastroenterology, 13(7),* 993-996.

Tom, M., Chen, N., Segev, M., Herut, B., & Rinkevich, B. (2004). Quantifying fish metallothionein transcript by real time PCR for its utilization as an environmental biomarker. *Marine Pollution Bulletin, 48(7-8),* 705-710.

Ventura, B. C., Angelis, D. F. & Marin-Morales, M. A. (2008). Mutagenis and genotoxic effects of the Atrazine herbicide in *Oreochromis niloticus* (Perciformes, Cichlidae) detected by the micronuclei test and the comet assay. *Pesticide Biochemistry and Physiology, 90,* 42-51.

Wegwu, M. O., Ibeh, G. O., & Abber, B. W. (2010). Evaluation of toxicity of varied concentrations of mercury and copper on *Oreochromis niloticus. Scientia Africana, 9(1),* 86-92.

Wu, S. M., Chen, C. C., Lee, Y. C. Leu, H. T. & Lin, N. S. (2006). Cortisol and copper induce metallothionein expression in three tissues of tilapia (*Oreochromis mossambicus*) in organ culture. *Zoological Studies, 45(3),* 363-370.

Wu, S. M., Lin, H. C. & Yang, W. L. (2008). The effects of maternal Cd on the metallothionein expression in tilapia (*Oreochromis mossambicus*) embryos and larvae. *Aquatic Toxicology, 87,* 296-302.

Yadav, K. K. & Trivedi, S. P. (2009). Sublethal exposure of heavy metals induces micronuclei in fish, *Channa punctata*. *Chemosphere, 77,* 1495-1500.