Evaluation of metallothionein content in giant mudskipper *Periophthalmodon schlosseri* collected from west coast of Peninsular Malaysia to assess environmental metal pollution

Tijjani Rufa'i Buhari1,2*, Ahmad Ismail1

1Department of Biology, Faculty of Science, University Putra Malaysia, Serdang 43300, Selangor, Malaysia
2Department of Biological Science, Northwest University, Kano P.M.B.3220, Kano, Nigeria

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**ABSTRACT**

**Objective:** To evaluate the concentrations of metallothionein (MT) in giant mudskippers *Periophthalmodon schlosseri* (*P. schlosseri*) and to elucidate their potential use in bio monitoring of coastal environment.

**Methods:** The giant mudskippers were collected from four sampling sites on the west coast of Peninsular Malaysia and analyzed for heavy metals – Cu, Zn and Cd, and MT concentrations in liver, gills and muscle by using an air-acetylene flame atomic absorption spectrophotometer, Perkin Elmer Analyst 800 and UV-Visible Recording Spectrophotometer Shimadzu UV-160 A Model, respectively.

**Results:** One-way analysis of variance (ANOVA) with Duncan multiple comparisons of the measured metals and MT concentrations in the liver, gills and muscle of *P. schlosseri* were statistically significant (*P* < 0.05) between the sampling sites. Non-significant difference (*P* > 0.05) was observed between Cu concentrations in the gills and Cd concentration in the liver, gills, and muscle at all the four sampling sites. Zn concentration in the tissues was significantly different (*P* < 0.05) among the sampling sites. Statistically significant positive correlation (*P* < 0.05; *r* = 0.583) and negative correlation (*P* < 0.01; *r* = -0.737) were observed between MT concentration in muscle with Cu and Zn concentrations in muscle, respectively.

**Conclusions:** The highest MT concentrations were found at sampling sites that mostly showed low metals concentrations in the tissues. The use of MT induction in *P. schlosseri* as a valuable biochemical tool for assessment of metals bioavailability and environmental impact needs further research to explore its feasibility as a biomarker of metal exposure.

1. **Introduction**

Apart from measuring metal concentrations in water and sediment, a series of methods and biomarkers have been proposed and extensively tested[1], to evaluate, assess and monitor pollution in the environment. The use of toxicity tests and/or biomarkers have been suggested as useful tools to link biological responses with contaminants in the environment[2,3], especially those from sediments. The examination of the dynamics of metal accumulation as well as the quantification of changes in the sub-cellular partitioning of metals over time could afford insight into potential mechanisms of toxicity and detoxification at the cellular level[4]. The potential utility of biomarkers for monitoring both environmental quality and the health of organisms inhabiting polluted ecosystems has received increasing attention during recent years[5-7]. Nowadays, biomarker, such as metallothioneins (MTs), has been commonly employed in studies of ecotoxicology and environmental monitoring on heavy metals and was proven to be an invaluable tool for monitoring the effects of metal influxes into the environment. MTs are ubiquitous low molecular weight cysteine-rich proteins characterized by high affinity for divalent cations[8] and their constitutive thiol (-SH) group possesses high capacity to bind divalent cations[9]. They are promising biomarkers for metal-specific stress in fish[10-12] and their synthesis is one of the best known biochemical responses to metal exposure. MTs are involved in the regulation of the essential metals copper and zinc and in the detoxification of non-essential metals[13] and excessive activity[14,15] and radical scavenging[16] and helps in metal ion homeostasis in a cell[17]. The exposure of aquatic organisms to excess essential and non-essential metals induces MT expression.
in different species and tissues. MT gene expression has often been proposed as a sensitive and efficient biomarker for evaluating the cumulative biological effects of metal exposure[18-20]. MT protein concentrations have been demonstrated to increase significantly as a result of metal exposure in tissues of a wide range of aquatic species, both in laboratory studies and in field surveys[21]. MT determinations in experimental aquatic organisms exposed to heavy metal and organisms in natural condition have demonstrated its expression, which supports its use as a biomarker, however in natural environments, is not always possible to establish associations between this protein and levels of metals in tissues[22]. MT mRNA levels were highly sensitive indicator of laboratory cadmium exposures in coho salmon[23]. Montaser et al.[24] denoted to an increase in MT-gene expression in the level of mRNA synthesis due to metal pollution in Naso hexacanthus fish at Jeddah Coast. MTs have been isolated and characterized in several aquatic organisms including fish[25]. Fish are commonly used as indicators of environmental chemical pollution, and MT induction is considered a useful biomarker of exposure to trace metals[8]. Study of MTs in fish is an interesting and ever-expanding area of research as fish live in challenging environments and MT induction is recognized as one of the robust adaptive and stress responses to such challenges[26]. Although MT has been found in a wide range of animal species, to our knowledge, no available information is on MT induction in Periophthalmodon species from the west coast of Peninsular Malaysia or elsewhere. The objectives of the present study were to determine the concentrations of Cu, Zn and Cd, and MT content in liver, gills and muscle of Periophthalmodon schlosseri (P. schlosseri) and to evaluate the use of MT as a biomarker of environmental metal pollution in the coastal environment.

2. Materials and methods

2.1. Description of sampling sites

Samplings of P. schlosseri were conducted in the west coast of Peninsular Malaysia (Figure 1), in September 2010 at Sungai Tiga (Sg. Tiga), Johor (01°25.841’ N, 104°00.281’ E) and Sungai Puluh (Sg. Puluh), Klang (03°04.786’ N, 101°23.903’ E) and in March and June 2010 at BaganLalang (Bg. Lalang), Selangor (02°36.669’ N, 101°41.100’ E) and Kuala Juru (K. Juru), Penang (05°19.683’ N, 100°22.949’ E) respectively. The coordinates of the sampling sites were recorded with Global Positioning System (GPS) (Garmin OREGON 450T 850 MB water proof GPS).

2.2. Sample collection

Fish were collected from the four sampling sites using trap net and brought to Ecotoxicology Laboratory, Department of Biological Sciences, Universiti Putra Malaysia in a plastic aquarium containing some sediment and water. Stomach and intestines of the fish were emptied and dissected immediately or put in labelled plastic bags and kept in deep freeze at –20°C until further analysis. In the laboratory, the fish samples were removed from the refrigerator and plastic bags, rinsed with double distilled water then thawed at room temperature. Their length and weight were recorded to the nearest centimeter and gram respectively before dissection. The tissues of P. schlosseri including liver, gills and muscle of Periophthalmodon schlosseri (P. schlosseri) and to evaluate the use of MT as a biomarker of environmental metal pollution in the coastal environment.

Figure 1. Map of west coast of Peninsular Malaysia showing the sampling sites.
guidance for animal research.

2.3. Metal analysis

About 4 P. schlosseri from each sampling sites with body length (mm) and weight (g) that ranged from (192.50 ± 4.01) mm to (271.00 ± 2.40) mm and (68.02 ± 2.33) g to (227.30 ± 14.87) g, respectively, were dissected on clean plastic material using stainless steel kits and glass equipment. The dissected parts were pooled into three different parts, namely, livers, gills and muscle. These tissues were chosen because they play a role in metals uptake, bioaccumulation, formation of metal-complexes, storage and detoxification processes. Sample of each wet weight part was weighed separately (0.5–1.0 g) in triplicate and placed in digestion tubes. To each digestion tube 10 mL concentrated nitric acid (AnalaR grade, BDH 69%) was added and placed in a hot block digester unit at –4 °C for 1 h. The temperature was then increased to 140 °C for at least 3 h[27]. The digested samples were diluted to 40 mL with double distilled water. The samples were then filtered (diameter: 110 mm) through filter papers into pill box and the filtrate was stored until metal determination.

The procedures of quality assurance and quality control (QA and QC) were employed to ensure the validity of the analytical data[28]. To avoid contamination, all reagents were handled carefully; all plastics and glassware used were washed with detergent, Deacon 90, rinsed with double-distilled water and soaked in 10% HNO₃ for at least 24 h, then rinsed with double-distilled water and allowed to dry at room temperature. The QA and QC were controlled by procedural blanks, sample replicates and dogfish liver DOLT–3 from National Research Council Canada (NRCC). During the period of atomic absorption spectrophotometer (AAS) metal analysis, a quality control sample was routinely included every 5–10 samples. Procedural blanks and quality control samples made from standard solutions for Cu, Zn and Cd were analyzed after every 5–10 samples to ensure the sensitivity and recovery of the instrument used. All metal concentrations in the tissues are expressed in μg/g on a wet weight basis. Multilevel calibration standards were analyzed to generate calibration curves against which sample concentrations were calculated. Standard solutions were prepared from 1000 mg/L stock solutions of each metal (BDH Spectrosol®). The quality of the method was checked with a Dogfish liver DOLT–3 from NRCC. These were checked to accuracy of the digestion method with the certified values supplied by the NRCC. The results of similarly digested samples analyzed for Cu, Zn and Cd, by the flame AAS Perkin Elmer AAAnalyst 800 showed acceptable recoveries of the metals. About 95.5%–105.6% for dogfish liver of recoveries of these metals had been observed in Table 1. The percentage recoveries (n = 3) for each metal, for the certified and measured concentration ranged from 95.5%, 96.7% and 105.6% for Cu, Zn and Cd, respectively.

### Table 1

Comparison of analytical result of DOLT-3 with certified concentrations using AAS Perkin Elmer AAAnalyst 800 (n = 3).

| Heavy metal | CRM       | Certified value | Measured value | Recovery (%) |
|-------------|-----------|-----------------|----------------|--------------|
| Cu          | DOLT-3    | 31.2 ± 1.0      | 29.8 ± 2.7     | 95.5         |
| Zn          | DOLT-3    | 86.6 ± 2.4      | 83.8 ± 3.5     | 96.7         |
| Cd          | DOLT-3    | 19.4 ± 0.6      | 20.5 ± 0.4     | 105.6        |

2.4. MT assay

MT content was analyzed in livers, gills and muscle by the assay of Viarengo et al.[29]. Pooled tissue of 4 P. schlosseri (1.0 g) was homogenized in 3 mL of 20 mmol/L Tris-HCl buffer (pH 8.6) containing 0.5 mol/L sucrose, 0.006 mol/L leupeptin, 0.5 mmol/L phenylmethylsulfonyl fluoride and 0.01% β-mercaptoethanol and centrifuged for 20 min at 30000 r/min at 4 °C. The supernatant (1 mL) of the sample was purified with 1.05 mL of cold ethanol (–20 °C) and 80 μL chloroform and centrifuged for 10 min at 6000 r/min at –4 °C. To the supernatant 40 μL 37% concentrated HCl and 6 mL of cold ethanol were added and allowed the protein to denature at 1 h at –20 °C. The mixture was centrifuged for 10 min at 6000 r/min at –4 °C and the pellet was saved. The supernatant was discarded and 1 mL of previously described homogenizing buffer solution, 6 mL cold ethanol and 80 μL chloroform were added before centrifuging and then centrifuged for 10 min at 6000 r/min at –4 °C. The supernatant was discarded and dried the pellet with N₂ gas. The pellet was re-suspended in 150 μL 0.25 mol/L NaCl and 150 μL 1 mol/L HCl with 4 mmol/L ethylene diamine tetraacetic acid. To assess MT content of a sample, 4.2 mL of a solution containing 2 mol/L NaCl and 0.43 mmol/L L 5,5'-dithio-bis-2-nitrobenzoic acid adjusted pH 8 with 0.2 mol/L Na-phosphate (NaH₂PO₄) were added at room temperature. After centrifugation at 3000 r/min for 5 min, supernatant absorbance was measured at 412 nm in a UV-Visible Recording Spectrophotometer Shimadzu UV-160 A Model. The MT concentration was estimated using GSH as a reference standard[29]. GSH contains one cysteine per molecule; thus, it is a standard for quantifying cysteine in protein analyses. The amount of MT in the samples was estimated using the GSH standard, assuming that 1 mol of MT contains 20 mol of cysteine[30].

2.5. Statistical analysis

All statistical analyses of data were carried out using SPSS statistical package programs version 17 and graphs were plotted with Microsoft Excel 2007. Data were tested for the basic assumptions of normality and homogeneity of variance in exploratory data analysis in SPSS 17. One-way analysis of variance (ANOVA) was calculated, and post host comparison was made using Duncan’s multiple range test at 0.05 confidence level. Correlations in Cu, Zn and Cd concentrations in liver, gills and muscle with Cu–MT, Zn–MT and Cd–MT concentrations respectively were determined for all samples examined using Pearson’s correlation coefficients (r); P < 0.05 was established as the limit of a statistically significant correlation.

3. Results

The concentration of heavy metals (Zn, Cu and Cd) in the tissues of P. schlosseri were presented in Figure 2. Metals concentration in the examined tissues of P. schlosseri ranged from 0.56 to 6.48, 3.43 to 23.22 and 0.04 to 0.85 μg/g wet weight for Cu, Zn and Cd respectively. One-way ANOVA with Duncan’s multiple comparison analysis shows that most of the measured metals in the liver, gills and muscle of P. schlosseri were statistically significant (P < 0.05) between the sampling sites. The concentration of Cu was recorded highest in liver as 6.48 μg/g wet weight and no significant different (P > 0.05) was observed between Cu concentrations in gills at all the four sampling sites. Zn and Cd concentrations were highest in gills...
as 23.22 and 0.85 μg/g wet weight, respectively. Zn concentration in the tissues was significantly different \((P < 0.05)\) between the sampling sites. Non-significant difference \((P > 0.05)\) was observed between Cd concentration in the liver, gills and muscle and between the sampling sites. The lowest concentrations of the three examined metals were recorded in muscle.

The MT level in tissues of P. schlosseri was presented in Figure 3, where statistical significant difference was observed among the sampling sites. Liver recorded the highest MT value as 241.59 μg/g wet weight. The order of MT content was in the decreasing order of sampling sites. Liver recorded the highest MT value as 241.59 μg/g wet weight. The order of MT content was in the decreasing order of liver > gills > muscle.

**Figure 2.** Metal concentrations (μg/g w/w) in liver, gills and muscle of P. schlosseri \((n = 4)\).

Data are expressed as mean ± SE. Different alphabets indicate a significant difference between \((P < 0.05)\) the sampling sites.

**Figure 3.** Mean MT concentrations (μg/g w/w) in the liver, gills and muscle of P. schlosseri \((n = 4)\).

Data are expressed as mean ± SE. Different alphabets indicate a significant difference \((P < 0.05)\) between the sampling sites.

The relationships between MT and metals concentrations in the liver, gills and muscle of P. schlosseri were shown in Figures 4–6 for Cu, Zn and Cd, respectively. Non-significant \((P > 0.05)\) correlations were observed between MT concentrations in liver and gills with Cu concentrations in the same tissues. A significant \((P < 0.05)\) positive correlation was found between MT concentration in muscle \((r = 0.583)\) with Cu concentration in muscle. Correlations between MT concentrations in tissues and Zn concentration in the liver, gills and muscle of P. schlosseri were presented in Figure 5. A non-significant \((P > 0.05)\) correlation was observed between MT and Zn concentrations in liver, and a non-significant \((P > 0.05)\) negative correlation was observed between MT and Zn concentrations in gills \((r = -0.218)\). Zn concentration in muscle showed a significant \((P < 0.05)\) negative correlation with MT concentration in muscle \((r = -0.631)\). A non-significant correlation \((P > 0.05)\) was observed between MT and Cd concentrations in liver, gills and muscle.

**Figure 4.** Correlations between MT concentrations in tissues and Cu concentrations in the liver, gills and muscle of P. schlosseri.

A significant difference was observed at \((P < 0.05)\) between MT and Cu concentrations in the tissues.

**Figure 5.** Correlations between MT concentrations in tissues and Zn concentrations in the liver, gills and muscle of P. schlosseri.

A significant difference was observed at \((P < 0.05)\) between MT and Zn concentrations in the tissues.
metals are bound to particulate matters [32,33], while the liver as metabolically active tissue is the accumulation place of metals [34]. Cu. The gills of aquatic organisms constitute a key interface for most of the Cd and Zn, whereas liver preferentially accumulated al. represents storage of metals in the waters where the fish live, whereas the concentration in liver reflects the level of the metals in the gill. The highest concentration of metals in liver and gills is probably due to the direct contact of this tissue with water and also attributed to the lack of direct contact of muscle with water medium and for not being an active site for detoxification, therefore transport of metals from other tissues to muscle does not seem to arise[58]. The metals concentrations in the tissues of P. schlosseri were in the order of Zn > Cu > Cd.

4. MT levels in the tissues of mudskipper P. schlosseri

Metal regulation in teleost fish occurs mainly via MT induction, although the inductive response in different species and tissues varies significantly[9]. Currently, no evidence exists for the presence of metal binding proteins with a higher affinity for Cd than MT in teleost[59]. The results of MT induction in the tissues of giant mudskipper P. schlosseri shows a significant difference between the sampling sites. The highest MT was recorded in liver while the lowest MT was found in muscle. The pattern of MT content in the tissues was in the decreasing order of: liver > gills > muscle. This result confirms earlier studies. The highest concentration of MT seen in the liver, is in accordance with the findings of some authors such as Tom et al.[22] and Sinaie et al.[60] who reported highest concentrations of MT in liver while lowest average MT was reported in muscle of Colossoma macropomum by Lemus et al.[39]. High levels of copper in the liver was ascribed to copper binding to MT, which serves as a detoxification mechanism[61]. Liver is a highly specialized metabolic organ which can induce MT biosynthesis much higher than the gills. Paulino et al.[62] has reported higher concentrations of contaminants in the liver of Astyanax fasciatus and Pimelodus maculatus when compared to the gills. Many researches have shown that liver has higher MT content than gills; the result of the present study is in agreement with the
results observed by other workers and reported in the literature\cite{63,64}. It has been reported by Kovarova et al.\cite{65} that MT total values were elevated in fish liver in correlation to physiologically occurred levels. The low MT content in gills could be attributed to low binding capacity of gills to metals, particularly Cu which induced MT production as compared to liver. Synthesis of metal-binding thionien ligands has been reported in fish gill\cite{66,67}, but the amount is likely low because this synthesis occurs primarily in chloride cells of the gills and much less in the other cell types\cite{68,69} which comprise a minority (< 10%) of the branchial epithelial surface area.

In the present study the values of MT in gills were found lower compared to liver but higher than muscle. MT concentration in aquatic animals has been associated with increased levels of metals in the aquatic environment and with the length of exposure time\cite{70}. But in the present study, the highest MT contents do not correspond with highest heavy metals concentrations in tissues i.e. highest MT contents were found at sampling sites that mostly showed low metals concentrations in tissues. A similar finding by Tiwari et al.\cite{59} has showed a 2 fold decrease in MT mRNA level with the increasing metal concentration. Study by Sevcikova et al.\cite{71}, on the effect of metals on MT content in fish from Skalka and Zelivka reservoirs, indicated that MT did not seem to be induced by high metal contamination. Low MT levels was also observed in polluted sites characterized by metals contamination, petroleum/crude hydrocarbon inputs and combustion PAH sources as reported by Fonseca et al.\cite{72}.

Nevertheless, literatures present many contraindications and inconsistencies in MT induction. Many researchers have reported that some species do not show increased MT concentrations, at least in some organs at sites where metals are present and bio available at high concentrations\cite{41,73-75}. However, despite the high levels of Cu detected at Sg. Puluh and K. Juru, the MT concentrations at these sites were low as compared to Bg. Lalang. This might be due to the continued exposure of fish to high levels of heavy metals in the sediment at these sites and has selected for resistant strains of mudskipper in which the expression of MT was attenuated. This finding is similar to the results reported by Rotchell et al.\cite{76} and Wall et al.\cite{77} that once maximum induction has occurred, MT levels will no longer reflect the degree of metal exposure. Because over time tissues metal concentrations increase and then stabilize\cite{78} and ultimately, the internal physiology of the animal either returns to the pre-exposure condition or new equilibrium is established. Intensity of MTs synthesis is, thus, tissue-specific, concentration and time-dependent\cite{65}. Generally, the MT expression level is dose-dependent on heavy metals. However, the response of MT to metals is not positively correlated when the amount of metals overdoses\cite{79}. In addition, many studies have illustrated that MT mRNA and the MT protein are highly correlated with heavy metal levels at low doses, but the expression is reduced at high doses\cite{80,82}; during exposure to low pollution protein synthesis is known to increase due to induction of proteins involved in the protection of the cell against harmful conditions, such as stress proteins, MTs, antioxidant enzymes and biotransformation enzymes, which is expected to reflect in elevated transcriptional activity and thus higher RNA: DNA ratios\cite{83}. At high pollution stress however, protein synthesis can be suppressed indicating disturbance of normal metabolic processes\cite{84}. Therefore increase or decrease in protein synthesis and thus RNA: DNA ratios can be expected as a result of pollutant exposure depending on the stress level.

Metals uptake depends not only on bioavailability, but on ecological needs and metabolic activity of species. Therefore, the low copper accumulation and higher MT concentration in the liver of fish from Bg. Lalang might suggest an imbalance between copper uptake and detoxification/excretion rate.

4.3. Relationships between MT levels and heavy metals concentrations in the tissues of \textit{P. schlosseri}

The significant positive correlation observed between MT in muscle and Cu concentration in muscle suggest Cu and MT concentrations increases simultaneously in muscle tissue. Several authors have interpreted the positive correlations between metal and MT content in fish tissue as the metal sequestration by MT and the poor correlation as metals exceeding the binding capacity of MT or the involvement of non-MT proteins\cite{76,85,86}.

Copper is a redox-active transition metal, an essential requirement as a cofactor for biochemical activity and a potential to catalyze toxic reactions when accumulated in excess of cellular requirement\cite{87-89}. Copper may become toxic and can be bound to MT for detoxification\cite{90}. Long and Wang\cite{91} have reported an increase of MT in all tissues and muscle of marine fish \textit{Terapon jarbua} with increasing waterborne or dietary Cu concentration. They showed that the positive correlation between MT and Cu concentration in muscle implied that the accumulated Cu was sequestered by MT. Cu accumulation is stimulated in muscle when the storage limits of liver are reached. Sorensen\cite{92} has reported that fish tend to store excess copper in the liver and regulate accumulation in the muscle tissue.

The negative correlations between Zn and MT concentrations in gills and muscle might suggest metabolic regulation\cite{85} in these tissues. Biochemical mechanisms within organisms tend to regulate essential trace elements at constant concentrations\cite{93}. It is well established that MT has a role in Zn homeostasis and Zn is a constituent element of MT\cite{94}. Zn toxicity effects will occur during exposure to elevated concentrations\cite{95}, although Zn toxicity is rather uncommon in fish\cite{48}, the free Zn$^{2+}$ is indeed, extremely toxic to the cell. Therefore, Zn has the ability to induce the synthesis of MT, which is a factor in regulating the metabolism of Zn, including absorption and storage\cite{96}.

Cd was the only metal that did not show significant correlation with MT level in any examined tissue, which could be attributed to low level of Cd bioaccumulation which may alter the correlations coefficients\cite{97}. Similar results from different field studies have reported no significant correlations between fish tissues and heavy metals concentrations. Kavarova et al.\cite{98} have reported no significant correlations between Cd liver content and MT while Mieiro et al.\cite{122} observed no significant correlations between total mercury content and MT levels in different fish tissues from a mercury-contaminated area. The non-significant correlations observed between Cd and MT concentrations might further be explained by one of the functions of MT in the homeostatic regulation of zinc and copper, and the concentration of these essential elements in organs will decrease the ability of cadmium to be bound to this protein, however, proteins with a lower affinity for the metal may also bind Cd because of their relative high concentrations in the cell compared to MT\cite{59}.

Furthermore, the non-significant correlation observed between MT and metals concentrations in the tissues of \textit{P. schlosseri} suggest the possible involvement of other MT-binding metals\cite{76}. Among the metals analyzed, only Zn showed a strong and constant negative
relationship with MT levels in both gills and muscle, whereas Cu and Cd did not show this trend. Therefore, there is a need for further research on MT induction in P. schlosseri to explore its use as a valuable biomarker of environmental metal exposure.

Conflict of interest statement

We declare that we have no conflict of interest.

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