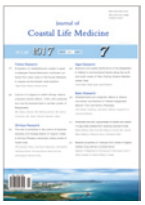
Journal of Coastal Life Medicine

journal homepage: www.jclmm.com



Original article https://doi.org/10.12980/jclm.5.2017J7-52

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

Tijjani Rufa'i Buhari[1,2*], Ahmad Ismail[1]

[1]*Department of Biology, Faculty of Science, University Putra Malaysia, Serdang 43300, Selangor, Malaysia*

[2]*Department of Biological Science, Northwest University, Kano P.M.B.3220, Kano, Nigeria*

ARTICLE INFO

Article history:
Received 17 Mar 2017
Received in revised form 12 Jun 2017
Accepted 29 Jun 2017
Available online 13 Jul 2017

Keywords:
Metallothionein
Heavy metals pollution
Periophthalmodon schlosseri
Bio monitoring
West coast of Peninsular Malaysia

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 d^{10} electron configuration metals, including essential (Zn and Cu) and non-essential (Cd and Hg) trace elements[8]. They are inducible 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], antioxidant 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

*Corresponding author: Tijjani Rufa'i Buhari, Department of Biology, Faculty of Science, University Putra Malaysia, Serdang 43300, Selangor, Malaysia.
Tel: +2348094079355
Fax: 0644025560
E-mail: trbuhari@yahoo.com; trbuhari@nwu.edu.ng

Foundation Project: Supported by Ministry of Higher Education Malaysia under Fundamental Research Grant Scheme (Vot: 5523641).
The journal implements double-blind peer review practiced by specially invited international editorial board members.

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 45OT 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 were then dissected out from the samples and immediately frozen in liquid nitrogen and later stored at –80 °C until further processing for MT and metal analyses. The authors declare that this experiment followed the ethical

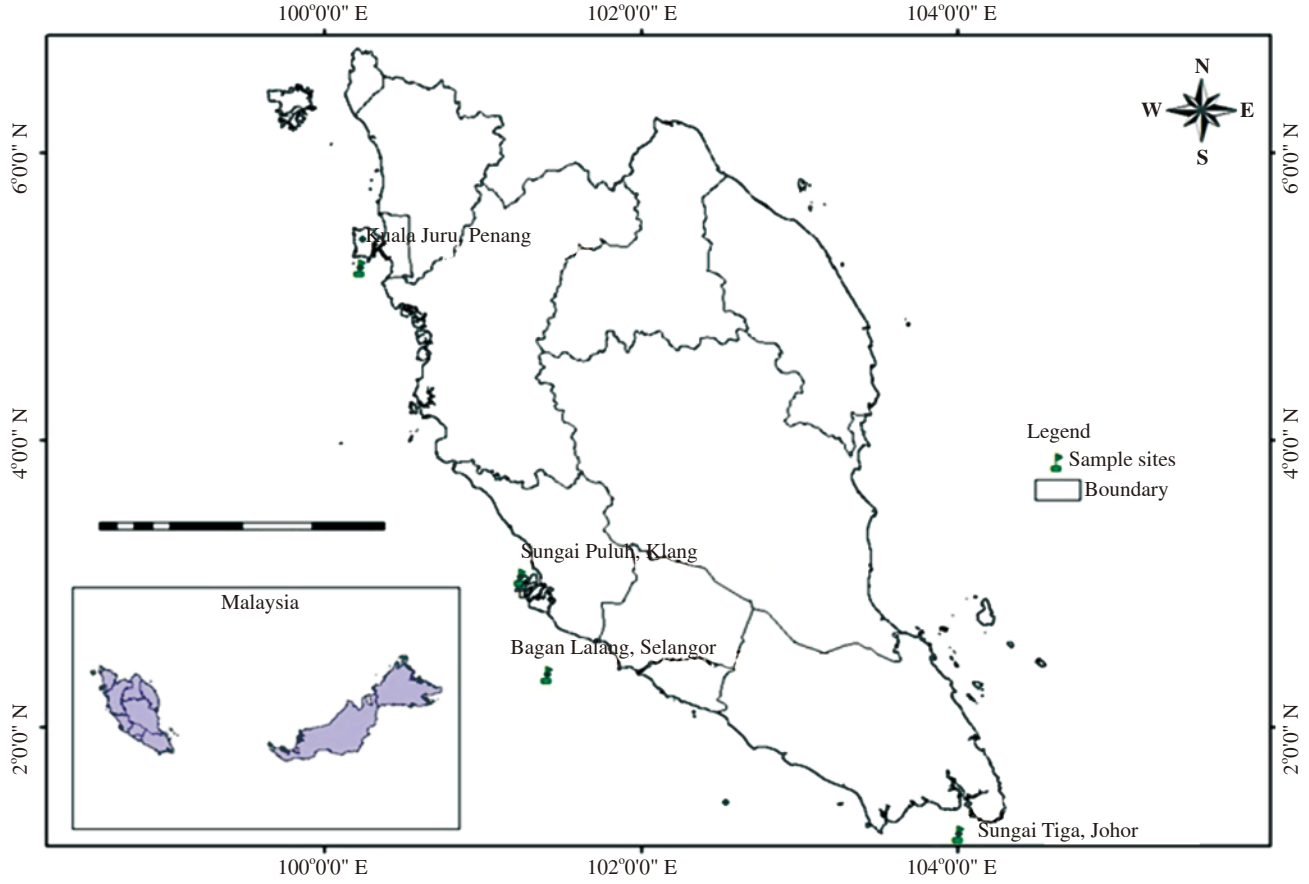


Figure 1. Map of west coast of Peninsular Malaysia showing the sampling sites.

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 40 ˚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% HNO_3 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 1 000 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 AAnalyst 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 AAnalyst 800 (n = 3).

Heavy metal	CRM	Certified value (μg/g)	Measured value (μg/g)	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 mmol/L leupeptin, 0.5 mmol/L phenylmethylsulfonyl fluoride and 0.01% β-mercaptoethanol and centrifuged for 20 min at 30 000 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 6 000 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 6 000 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 6 000 r/min at –4 ˚C. The supernatant was discarded and dried the pellet with N_2 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 5.5'-dithio-bis-2-nitrobenzoic acid adjusted pH 8 with 0.2 mol/L Na-phosphate (NaH_2PO_4) were added at room temperature. After centrifugation at 3 000 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 while the lowest value was found in muscle as 4.99 μg/g wet weight. The order of MT content was in the decreasing order of liver > gills > muscle.

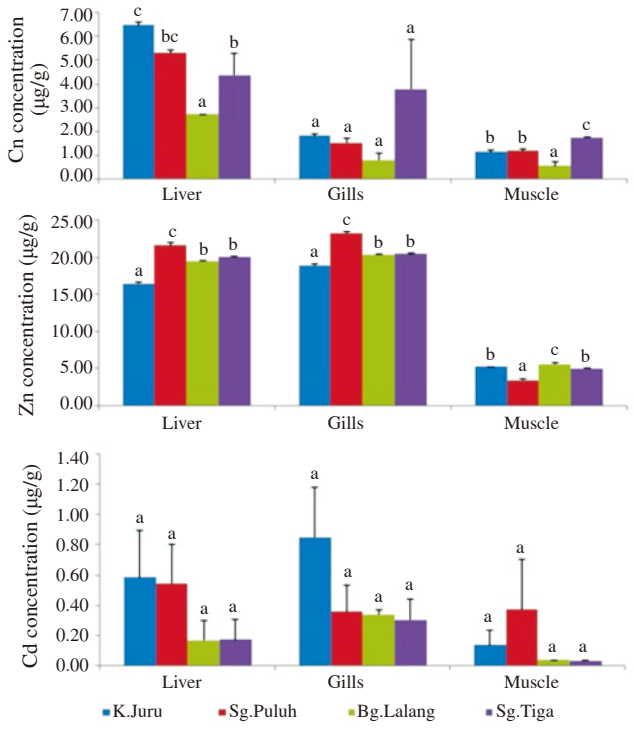


Figure 2. Metal concentrations (μg/g w/w) in liver, gills and muscle of *P. schlosseri* ($n = 4$).
Data are expressed as mean ± SE. Different alphabets indicate a significant difference between ($P < 0.05$) the sampling sites.

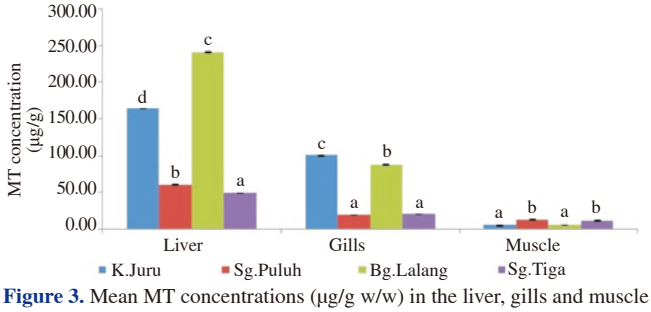


Figure 3. Mean MT concentrations (μg/g w/w) in the liver, gills and muscle of *P. schlosseri* ($n = 4$).
Data are expressed as mean ± SE. Different alphabets indicate a significant difference ($P < 0.05$) between the sampling sites.

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.

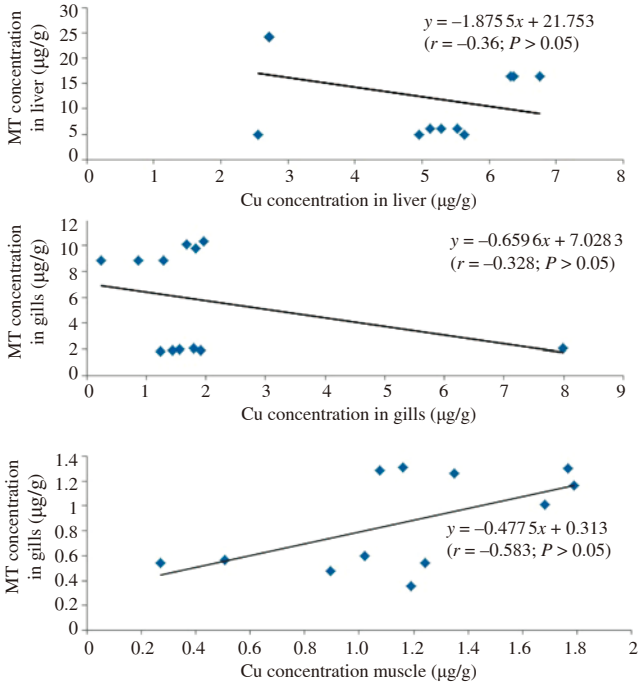


Figure 4. Correlations between MT concentrations in tissues and Cu concentrations in the liver, gills and muscle of *P. schlosseri*.
A significant difference was observed at ($P < 0.05$) between MT and Cu concentrations in the tissues.

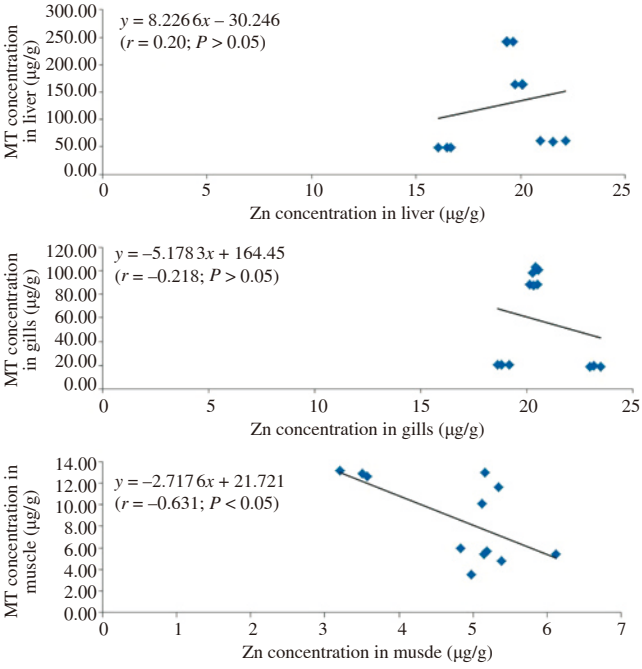


Figure 5. Correlations between MT concentrations in tissues and Zn concentrations in the liver, gills and muscle of *P. schlosseri*.
A significant difference was observed at ($P < 0.05$) between MT and Zn concentrations in the tissues.

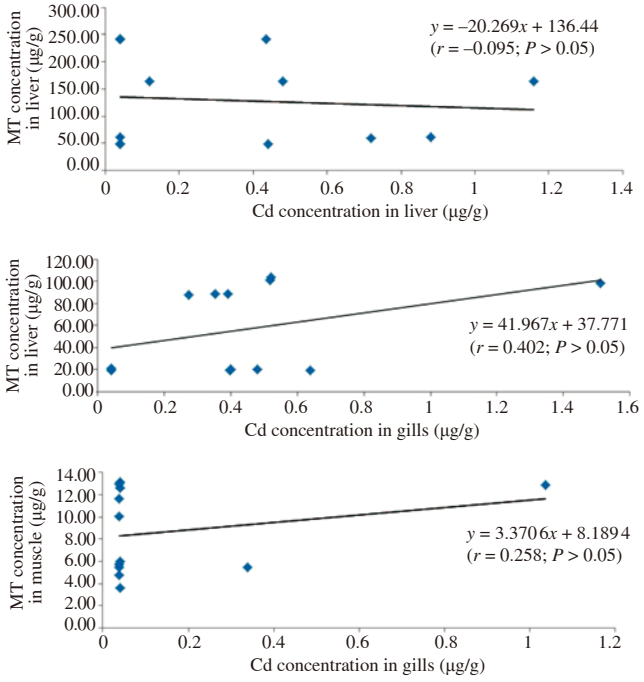


Figure 6. Correlations between MT concentrations in tissues and Cd concentrations in the liver, gills and muscle of *P. schlosseri*.
A non-significant difference was observed at ($P > 0.05$) between MT and Cd concentrations in the tissues.

4. Discussion

4.1. Metals concentrations in the tissues of mudskipper P. schlosseri

Metals concentrations in the examined tissues were highest at sampling sites with high anthropogenic activities. The lowest concentrations of the three examined metals were recorded in muscle. The highest concentration of metals in liver and gills is probably due to their physiological roles in fish metabolism. Gills are the primary site of metal uptake from water[31], especially if metals are bound to particulate matters[32,33], while the liver as metabolically active tissue is the accumulation place of metals[34]. Result of this study is in agreement with many studies conducted in different areas which show many species of fish having tendency to accumulate heavy metals in high values in their target organs, such as liver and gill[35,36]. Ambedkar and Muniyan[37], Shahat *et al.*[38], Lemus *et al.*[39] and Ekpo *et al.*[40] have reported high metals accumulation in fish liver tissues and least in muscles tissues.Metals accumulation in the tissues of Gudgeon fish (*Gobio gobio*) was reported by Van Campenhout *et al.*[41]; gill and kidney accumulated most of the Cd and Zn, whereas liver preferentially accumulated Cu. The gills of aquatic organisms constitute a key interface for the uptake of dissolved metals ions from water. Fish gills are highly sensitive to metal exposure, since the absorption takes place primarily through this organ[42-44], high metals content in gills could be attributed to the direct contact of this tissue with water and also as one of the major route of dissolved metals uptake in fish. The concentration of metals in the gill reflects the level of the metals in the waters where the fish live, whereas the concentration in liver represents storage of metals[45,46].

The high accumulation of Cu more than Zn in the liver could be explained by the findings of Roch *et al.*[47] that showed Cu has greater affinity for protein and is able to displace Zn. Furthermore, liver is usually not an important storage site for Zn[48]. The gills are in contact with the external medium and are considered to be central in the uptake of dissolved substances from the water being the prime target for the toxic action of waterborne metals such as Cd, Cu and Zn[49]. The constant contact of gills with water and resuspended sediment particle might explain the high metal concentration in this organ. Gills are also involved in Zn regulation, either reducing influx or increasing efflux rates, reaching a steady state[50] and probably site of transient metal accumulation from where the absorbed metals are distributed throughout the whole body and accumulated in specific organs[51]. Cd concentration in gills was higher than in liver, which is in agreement with reported values of Cd levels in gills[52] that Cd level in gills was higher than or comparable with that in the liver of *Cyprinus carpio* and some other fish species. This could be due to the natural environmental waterborne exposure to the metal. Cd ions (Cd^{2+}) that are in direct contact with gills could bind in a non-specific manner to the mucopolysaccharides (constituents of mucoproteins, glycoproteins) present on the outside of the gills[53].

Zn and Cd concentrations in gills probably reflected uptake of these metals from water since gills generally accumulate higher metals concentrations during waterborne exposure. High concentrations of heavy metals in liver and gill tissues are attributed to the affinity or strong coordination of MT with metals. These proteins are synthesized in the liver and gill tissues when fishes are exposed to heavy metals and help the fish to detoxify metals[54], while the low concentrations of metals in muscle are due to the fact that, muscle is not an active tissue in heavy metals accumulation[55-57].

The lower rate of bioaccumulation of metals in muscle may be 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.

4.2. 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[63,64]. It has been reported by Kovarova *et al*.[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 thionein ligands has been reported in fish gill[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[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[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*.[59] has showed a 2 fold decrease in MT mRNA level with the increasing metal concentration. Study by Sevcikova *et al*.[71], on the effect of metals on MT content in fish from Skalka and Želivka 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*.[72].

Nevertheless, literatures present many contraindications and inconsistencies in MT induction. Many researchers had 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[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*.[76] and Wall *et al*.[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[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[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[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[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[83]. At high pollution stress however, protein synthesis can be suppressed indicating disturbance of normal metabolic processes[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 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[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[87-89]. Copper may become toxic and can be bound to MT for detoxification[90]. Long and Wang[91] have reported an increase of MT in all tissues and muscle of marine fish *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[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[85] in these tissues. Biochemical mechanisms within organisms tend to regulate essential trace elements at constant concentrations[93]. It is well established that MT has a role in Zn homeostasis and Zn is a constituent element of MT[94]. Zn toxicity effects will occur during exposure to elevated concentrations[95], although Zn toxicity is rather uncommon in fish[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[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[97]. Similar results from different field studies have reported no significant correlations between fish tissues and heavy metals concentrations. Kavarova *et al*.[98] have reported no significant correlations between Cd liver content and MT while Mieiro *et al*.[22] 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[59].

Furthermore, the non-significant correlation observed between MT and metals concentrations in the tissues of *P. schlosseri* suggest the possible involvement of other MT-binding metals[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.

Acknowledgments

The authors wish to express their appreciation to the Ministry of Higher Education Malaysia for funding this study under Fundamental Research Grant Scheme (Vot: 5523641). The authors are grateful to Umar Musa Umar of Geography Department Northwest University, Kano, for producing the map of the sampling sites.

References

[1] Knapen D, Reynders H, Bervoets L, Verheyen E, Blust R. Metallothionein gene and protein expression as a biomarker for metal pollution in natural gudgeon populations. *Aquat Toxicol* 2007; **82**(3): 163-72.

[2] Stauber JL, Davies CM. Use and limitations of microbial bioassays for assessing copper bioavailability in the aquatic environment. *Environ Rev* 2000; **8**(4): 255-301.

[3] Wells PG, Depledge MH, Butler JN, Manock JJ, Knap AH. Rapid toxicity assessment and biomonitoring of marine contaminants-exploiting the potential of rapid biomarker assays and microscale toxicity tests. *Mar Poll Bull* 2001; **42**(10): 799-804.

[4] Jebali J, Banni M, Guerbej H, Almeida EA, Bannaoui A, Boussetta H. Effects of malathion and cadmium on acetylcholinesterase activity and metallothionein levels in the fish *Seriola dumerilli*. *Fish Physiol Biochem* 2006; **32**(1): 93.

[5] Gauthier L, Tardy E, Mouchet F, Marty J. Biomonitoring of the genotoxic potential (micronucleus assay) and detoxifying activity (EROD induction) in the River Dadou (France), using the amphibian *Xenopus laevis*. *Sci Total Environ* 2004; **323**(1-3): 47-61.

[6] Lopes PA, Pinheiro T, Santos MC, da Luz Mathias M, Collares-Pereira MJ, Viegas-Crespo AM. Response of antioxidant enzymes in freshwater fish populations (Leuciscus alburnoides complex) to inorganic pollutants exposure. *Sci Total Environ* 2001; **280**(1-3): 153-63.

[7] Samecka-Cymerman A, Kempers AJ. Biomonitoring of water pollution with *Elodea canadensis*. A case study of three small polish rivers with different levels of pollution. *Water Air Soil Poll* 2003; **145**(1): 139-53.

[8] Isani G, Carpene E. Metallothioneins, unconventional proteins from unconventional animals. A long journey from nematodes to mammals. *Biomolecules* 2014; **4**(2): 435-57.

[9] Farrell AP, Wood CM, Brauner CJ. *Homeostasis and toxicology of non-essential metals*. London: Academic Press; 2012, p. 505.

[10] Roesijadi G. Metallothioneins in metal regulation and toxicity in aquatic animals. *Aquat Toxicol* 1992; **22**(2): 81-113.

[11] Roesijadi G, Robinson W. Metal regulation in aquatic animals: mechanisms of uptake, accumulation, and release. In: Mullins DC, Ostrander GK, editors. *Aquatic toxicology-molecular, biochemical and cellular perspectives*. Florida: Lewis Publishers; 1994, p. 387-419.

[12] Sanders B. Stress proteins. Potential as multitiered biomarkers. In: Shugart L, McCarthy J, editors. *Environmental biomarkers*. Chelsea: Lewis Publishers; 1990, p. 165-91.

[13] Wang C, Zhang F, Cao W, Wang J. The identification of metallothionein in rare minnow (*Gobiocypris rarus*) and its expression following heavy metal exposure. *Environ Toxicol Pharmacol* 2014; **37**(3): 1283-91.

[14] Atif F, Kaur M, Yousuf S, Raisuddin S. *In vitro* free radical scavenging activity of hepatic metallothionein induced in an Indian freshwater fish, *Channa punctata* Bloch. *Chem Biol Interact* 2006; **162**(2): 172-80.

[15] Sato M, Kondoh M. Recent studies on metallothionein. Protection against toxicity of heavy metals and oxygen free radicals. *Tohoku J Exp Med* 2002; **196**(1): 9-22.

[16] Wright J, George S, Martinez-Lara E, Carpene E, Kindt M. Levels of cellular glutathione and metallothionein affect the toxicity of oxidative stressors in an established carp cell line. *Mar Environ Res* 2000; **50**(1-5): 503-8.

[17] Shariati F, Shariati S. Review on methods for determination of metallothioneins in aquatic organisms. *Biol Trace Elem Res* 2011; **141**(1-3): 340-66.

[18] Ceratto N, Dondero F, van de Loo J-W, Burlando B, Viarengo A. Cloning and sequencing of a novel metallothionein gene in *Mytilus galloprovincialis* Lam. *Comp Biochem Physiol C* 2002; **131**(3): 217-22.

[19] George S, Gubbins M, MacIntosh A, Reynolds W, Sabine V, Scott A, et al. A comparison of pollutant biomarker responses with transcriptional responses in European flounders (*Platichthys flesus*) subjected to estuarine pollution. *Mar Environ Res* 2004; **58**(2): 571-5.

[20] Tom M, Chen N, Segev M, Herut B, Rinkevich B. Quantifying fish metallothionein transcript by real time PCR for its utilization as an environmental biomarker. *Mar Poll Bull* 2004; **48**(7): 705-10.

[21] Aldo Viarengo, Burlando B, Dondero F, Marro A, Fabbri R. Metallothionein as a tool in biomonitoring programmes. *Biomarkers* 1999; **4**(6): 455-66.

[22] Mieiro CL, Bervoets L, Joosen S, Blust R, Duarte AC, Pereira ME, et al. Metallothioneins failed to reflect mercury external levels of exposure and bioaccumulation in marine fish - Considerations on tissue and species specific responses. *Chemosphere* 2011; **85**(1): 114-21.

[23] Williams CR, Gallagher EP. Effects of cadmium on olfactory mediated behaviors and molecular biomarkers in coho salmon (*Oncorhynchus kisutch*). *Aquat Toxicol* 2013; **140**: 295-302.

[24] Montaser M, Mahfouz ME, El-Shazly SAM, Abdel-Rahman GH, Bakry S. Toxicity of heavy metals on fish at Jeddah coast KSA: metallothionein expression as a biomarker and histopathological study on liver and gills. *World J Fish Mar Sci* 2010; **2**(3): 174-85.

[25] Isani G, Monari M, Andreani G, Fabbri M, Carpene E. Effect of copper exposure on the antioxidant enzymes in bivalve mollusc *Scapharca inaequivalvis*. *Vet Res Commun* 2003; **27**: 691-3.

[26] Kim JH, Wang SY, Kim IC, Ki JS, Raisuddin S, Lee JS, et al. Cloning of a river pufferfish (*Takifugu obscurus*) metallothionein cDNA and study of its induction profile in cadmium-exposed fish. *Chemosphere* 2008; **71**(7): 1251-9.

[27] Ismail A. Heavy metal concentrations in sediments off Bintulu, Malaysia. *Mar Poll Bull* 1993; **27**(12): 706-7.

[28] Ismail A, Yap CK. Concentrations of Cd, Cu and Zn in sediments collected from urban lakes at Kelana Jaya, Peninsular Malaysia. *Wetlands* 2004; **2**(4): 248-58.

[29] Viarengo A, Ponzano E, Dondero F, Fabbri R. A simple spectrophotometric method for metallothionein evaluation in marine organisms. An application to Mediterranean and Antarctic molluscs. *Mar Environ Res* 1997; **44**(1): 69-84.

[30] Linde AR, Garcia-Vazquez E. A simple assay to quantify metallothionein helps to learn about bio indicators and environmental health. *Biochem*

Mol Biol Educ 2006; **34**: 360-3.

[31] Dogru MI, Orun I, Dogru A, Kandemir S, Altas L, Erdogan K, et al. Evaluation of metal accumulation, oxidative stress, biochemical and hematological parameters in *Sander lucioperca* L., 1758 from Bafra (Samsun) fish lakes. *Fresen Environ Bull* 2011; **20**(10): 2731-6.

[32] Kargin F. Metal concentrations in tissues of the freshwater fish *Capoeta barroisi* from the Seyhan River (Turkey). *Bull Environ Contam Toxicol* 1998; **60**(5): 822-8.

[33] Klavins M, Potapovics O, Rodinov V. Heavy metals in fish from lakes in Latvia. Concentrations and trends of changes. *Bull Environ Contam Toxicol* 2009; **82**(1): 96-100.

[34] Yilmaz F, Ozdemir N, Demirak A, Tuna AL. Heavy metal levels in two fish species *Leuciscus cephalus* and *Lepomis gibbosus*. *Food Chem* 2007; **100**(2): 830-5.

[35] Canpolat Ö, Calta M. Heavy metals in some tissues and organs of *Capoeta capoeta umbla* (Heckel, 1843) fish species in relation to body size, age, sex and seasons. *Fresen Environ Bull* 2003; **12**(9): 961-6.

[36] Fernandes C, Fontainhas-Fernandes A, Peixoto F, Maria AS. Bioaccumulation of heavy metals in Liza saliens from the Esmoriz-Paramos coastal lagoon, Portugal. *Ecotoxicol Environ Saf* 2007; **66**(3): 426-31.

[37] Ambedkar G, Muniyan M. Analysis of heavy metals in water, sediments and selected freshwater fish collected from Gadilam River, Tamilnadu, India. *Int J Toxicol Appl Pharmacol* 2012; **2**(2): 25-30.

[38] Shahat M, Ameer OSO, Abdallah AT, Abdelsater N, Moustafa MA. The distribution of certain heavy metals between intestinal parasites and their fish hosts in the River Nile at Assuit Province, Egypt. *Egypt J Hosp Med* 2011; **43**: 241-57.

[39] Lemus M, Blanco I, Hernández M, León A, Centeno L, Chung K, et al. Relation between metallothionein, RNA/DNA and heavy metals in juveniles of *Colossoma macropomun* (Cuvier, 1818) in natural conditions. In: E3S Web of Conferences 1, 12001 (2013): EDP Sciences. [Online] Available from: https://www.dawn.com/news/418705/overfishing-pollution-continue-to-threaten-marineresources [Accessed on 15th May, 2017]

[40] Ekpo FE, Agu NN, Udoakpan UI. Influence of heavy metals concentration in three common fish, sediment, and water collected within quarry environment, Akamkpa LG Area, Cross River State, Nigeria. *J Toxicol Sci* 2013; **3**: 1-11.

[41] Van Campenhout K, Bervoets L, Blust R. Metallothionein concentrations in natural populations of gudgeon (*Gobio gobio*): relationship with metal concentrations in tissues and environment. *Environ Toxicol Chem* 2003; **22**(7): 1548-55.

[42] Brunelli E, Mauceri A, Maisano M, Bernabo I, Giannetto A, De Domenico E, et al. Ultrastructural and immunohistochemical investigation on the gills of the teleost, *Thalassoma pavo* L. exposed to cadmium. *Acta Histochem* 2011; **113**: 201-13.

[43] Fasulo S, Marino S, Mauceri A, Maisano M, Giannetto A, D'Agata A, et al. A multibiomarker approach in *Coris julis* living in a natural environment. *Ecotoxicol Environ Saf* 2010; **73**: 1565-73.

[44] Carvalho CS, Bernusso VA, Araújo HSS, Espíndola ELG, Fernandes MN. Biomarker responses as indicator of contaminant effects in *Oreochromis niloticus*. *Chemosphere* 2012; **89**: 60-9.

[45] Rao LM, Padmaja G. Bioaccumulation of heavy metals in *M. cyprinoids* from the harbor waters of Visakhapatnam. *Bull Pure Appl Sci* 2000; **19**: 77-85.

[46] Romeo M, Siau Y, Sidoumou Zn, Gnassia-Barelli M. Heavy metal distribution in different fish species from the Mauritania coast. *Sci Total Environ* 1999; **232**(3): 169-75.

[47] Roch M, Nordin RN, Austin A, McKean CJP, Deniseger J, Kathman RD, et al. The effects of heavy metal contamination on the aquatic biota of Buttle Lake and the Campbell River drainage (Canada). *Arch Environ Contam Toxicol* 1985; **14**(3): 347-62.

[48] Hogstrand C, Haux C. Naturally high levels of zinc and metallothionein in liver of several species of the squirrelfish family from Queensland, Australia. *Mar Biol* 1996; **125**(1): 23-31.

[49] Soto M, Marigómez I, Cancio I. *Biological aspects of metal accumulation and storage.* Basque Country: University of the Basque Country Bilbo; 2008.

[50] Kraemer LD, Campbell PGC, Hare L. Dynamics of Cd, Cu and Zn accumulation in organs and sub-cellular fractions in field transplanted juvenile yellow perch (*Perca flavescens*). *Environ Poll* 2005; **138**(2): 324-37.

[51] Dallinger R, Prosi F, Segner H, Back H. Contaminated food and uptake of heavy metals by fish: a review and a proposal for further research. *Oecologia* 1987; **73**(1): 91-8.

[52] Canli M, Ay Ã, Kalay M. Levels of heavy metals (Cd, Pb, Cu, Cr and Ni) in tissue of *Cyprinus carpio*, *Barbus capito* and *Chondrostoma regium* from the Seyhan River, Turkey. *Turk J Zool* 1998; **22**(2): 149-58.

[53] Isani G, Andreani G, Cocchioni F, Fedeli D, Carpene E, Falcioni G. Cadmium accumulation and biochemical responses in *Sparus aurata* following sub-lethal Cd exposure. *Ecotoxicol Environ Saf* 2009; **72**(1): 224-30.

[54] Negi RK, Maurya A. Heavy metal concentrations in tissues of major carp and exotic carp from Bhagwanpur fish pond, India. *J Fish Aquat Sci* 2015; **10**(6): 543-52.

[55] Okunola OJ, Alhassan Y, Yebpella GG, Uzairu A, Tsafe AI, Abechi ES, et al. Risk assessment of using coated mobile recharge cards in Nigeria. *J Environ Chem Ecotoxicol* 2011; **3**(4): 80-5.

[56] Jalal KCA, Mohd AY, Habab M, Kamaruzzaman BY. Bioaccumulation of selected metals in fresh water Haruan fish (*Channa striatus*) collected from Pahang river basin, Malaysia. *Orient J Chem* 2013; **29**(4): 1553-8.

[57] Abdel-Tawwab M, El-Sayed GO, Shady SH. Growth, biochemical variables, and zinc bioaccumulation in Nile tilapia, *Oreochromis niloticus* (L.) as affected by water-born zinc toxicity and exposure period. *Int Aquat Res* 2016; **8**(3): 197-206.

[58] Jithesh M, Radhakrishnan MV. Seasonal variation in accumulation of metals in selected tissues of the Ribbon fish, *Trichiurus lepturus* collected from Chaliyar River, Kerala, India. *J Entomol Zool Stud* 2017; **5**(1): 51-6.

[59] Tiwari M, Nagpure NS, Saksena DN, Lakra WS. Metallothionein mRNA expression in freshwater teleost, *Channa punctata* (Bloch) under the influence of heavy metal, cadmium - a dose kinetic study. *Int Aquat Res* 2011; **3**: 21-9.

[60] Sinaie M, Bastami KD, Ghorbanpour M, Najafzadeh H, Shekari M, Haghparast S. Metallothionein biosynthesis as a detoxification mechanism in mercury exposure in fish, spotted scat (*Scatophagus argus*). *Fish Physiol Biochem* **36**(4): 1235-42.

[61] Hogstrand C, Haux C. Binding and detoxification of heavy metals in lower vertebrates with reference to metallothionein. *Comp Biochem Physiol C* 1991; **100**(1-2): 137-41.

[62] Paulino MG, Benze TP, Sadauskas-Henrique H, Sakuragui MM, Fernandes JB, Fernandes MN. The impact of organochlorines and metals on wild fish living in a tropical hydroelectric reservoir: bioaccumulation and histopathological biomarkers. *Sci Total Environ* 2014; **497**: 293-306.

[63] Wu SM, Chen CC, Lee YC, Leu HT, Lin NS. Cortisol and copper induce metallothionein expression in three tissues of tilapia (*Oreochromis mossambicus*) in organ culture. *Zool Stud* 2006; **45**(3): 363-70.

[64] Ghedira J, Jebali J, Bouraoui Z, Banni M, Guerbej H, Boussetta H. Metallothionein and metal levels in liver, gills and kidney of *Sparus aurata* exposed to sublethal doses of cadmium and copper. *Fish Physiol Biochem*; 2010; **36**(1): 101-7.

[65] Kovarova J, Kizek R, Adam V, Harustiakova D, Celechovska O, Svobodova Z. Effect of cadmium chloride on metallothionein levels in carp. *Sensors* 2009; **9**(6): 4789-803.

[66] Lange A, Ausseil O, Segner H. Alterations of tissue glutathione levels and metallothionein mRNA in rainbow trout during single and combined exposure to cadmium and zinc. *Comp Biochem Physiol C* 2002; **131**(3): 231-43.

[67] De Boeck G, Ngo TTH, Van Campenhout K, Blust R. Differential metallothionein induction patterns in three freshwater fish during sublethal copper exposure. *Aquat Toxicol* 2003; **65**(4): 413-24.

[68] Burkhardt-Holm P, Bernet D, Hogstrand C. Increase of metallothionein-immunopositive chloride cells in the gills of brown trout and rainbow trout after exposure to sewage treatment plant effluents. *Histochem J* 1999; **31**(6): 339-46.

[69] Dang ZC, Flik G, Ducouret B, Hogstrand C, Bonga SEW, Lock RAC. Effects of copper on cortisol receptor and metallothionein expression in gills of *Oncorhynchus mykiss*. *Aquat Toxicol* 2000; **51**(1): 45-54.

[70] Olsvik PA, Gundersen P, Andersen RA, Zachariassen KE. Metal accumulation and metallothionein in two populations of brown trout, *Salmo trutta*, exposed to different natural water environments during a run-off episode. *Aquat Toxicol* 2000; **50**(4): 301-16.

[71] Sevcikova M, Modra H, Kruzikova K, Zitka O, Hynek D, Adam V, et al. Effect of metals on metallothionein content in fish from Skalka and Želivka reservoirs. *Int J Electrochem Sci* 2013; **8**: 1650-63.

[72] Fonseca VF, França S, Serafim A, Company R, Lopes B, Bebianno MJ, et al. Multi-biomarker responses to estuarine habitat contamination in three fish species: *Dicentrarchus labrax*, *Solea senegalensis* and *Pomatoschistus microps*. *Aquat Toxicol* 2011; **102**(3): 216-27.

[73] Berthet B, Mouneyrac C, Amiard JC, Amiard-Triquet C, Berthelot Y, Rainbow PS, et al. Response to metals of the polychaete annelid *Hediste diversicolor*, a key species in estuarine and coastal sediments. *Arch Environ Contam Toxicol* 2003, **45**: 468-78.

[74] Geffard A, Amiard-Triquet C, Amiard JC, Mouneyrac C. Temporal variations of metallothionein and metal concentrations in the digestive gland of oysters (*Crassostrea gigas*) from a clean and a metal-rich site. *Biomarkers* 2001; **6**(2): 91-107.

[75] Pedersen SN, Lundebye AK. Metallothionein and stress protein levels in shore crabs (*Carcinus maenas*) along a trace metal gradient in the Fal estuary (UK). *Mar Environ Res* 1996; **42**(1): 241-6.

[76] Rotchell JM, Clarke KR, Newton LC, Bird DJ. Hepatic metallothionein as a biomarker for metal contamination. Age effects and seasonal variation in European flounders (*Pleuronectes flesus*) from the Severn Estuary and Bristol Channel. *Mar Environ Res* 2001; **52**(2): 151-71.

[77] Wall KL, Jessen-Eller K, Crivello JF. Assessment of various biomarkers in winter flounder from coastal Massachusetts, USA. *Environ Toxicol Chem* 1998; **17**(12): 2504-11.

[78] McGeer JC, Szebedinszky C, McDonald DG, Wood CM. Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout 2: tissue specific metal accumulation. *Aquat Toxicol* 2000; **50**(3): 245-56.

[79] Walker CJ, Gelsleichter J, Adams DH, Manire CA. Evaluation of the use of metallothionein as a biomarker for detecting physiological responses to mercury exposure in the bonnethead, *Sphyrna tiburo*. *Fish Physiol Biochem*2014; **40**(5): 1361-71.

[80] Wu SM, Weng CF, Hwang JC, Huang CJ, Hwang PP. Metallothionein induction in early larval stages of tilapia (*Oreochromis mossambicus*). *Physiol Biochem Zool* 2000; **73**(5): 531-7.

[81] George SG, Hodgson PA, Tytler P, Todd K. Inducibility of metallothionein mRNA expression and cadmium tolerance in larvae of a marine teleost, the turbot (*Scophthalmus maximus*). *Toxicol Sci* 1996; **33**(1): 91-9.

[82] Chatterjee A, Maiti IB. Purification and immunological characterization of catfish (*Heteropneustes fossilis*) metallothionein. *Mol Cell Biochem* 1987; **78**(1): 55-64.

[83] Lannig G, Flores JF, Sokolova IM. Temperature-dependent stress response in oysters, *Crassostrea virginica*: pollution reduces temperature tolerance in oysters. *Aquat Toxicol* 2006; **79**(3): 278-87.

[84] Pottinger TG, Carrick TR, Yeomans WE. The three-spined stickleback as an environmental sentinel: effects of stressors on whole-body physiological indices. *J Fish Biol* 2002; **61**(1): 207-29.

[85] Filipovi V, Raspor B. Metallothionein and metal levels in cytosol of liver, kidney and brain in relation to growth parameters of *Mullus surmuletus* and *Liza aurata* from the Eastern Adriatic Sea. *Water Res* 2003; **37**(13): 3253-62.

[86] Roméo M, Cosson RP, Gnassia-Barelli M, Risso C, Stien X, Lafaurie M. Metallothionein determination in the liver of the sea bass *Dicentrarchus labrax* treated with copper and B (a) P. *Mar Environ Res* 1997; **44**(3): 275-84.

[87] Aucoin J, Blanchard R, Billiot C, Partridge C, Schultz D, Mandhare K, et al. Trace metals in fish and sediments from Lake Boeuf, Southeastern Louisiana. *Microchem J* 1999; **62**(2): 299-307.

[88] Finney LA, O'Halloran TV. Transition metal speciation in the cell: insights from the chemistry of metal ion receptors. *Science* 2003; **300**(5621): 931-6.

[89] Kennish MJ. *Practical handbook of estuarine and marine pollution*. Boca Raton: CRC Press; 1996.

[90] Peña MMO, Lee J, Thiele DJ. A delicate balance: homeostatic control of copper uptake and distribution. *J Nutr* 1999; **129**(7): 1251-60.

[91] Long A, Wang WX. Metallothionein induction and bioaccumulation kinetics of Cd and Ag in the marine fish *Terapon jarbua* challenged with dietary or waterborne Ag and Cu. *Mar Ecol Prog Ser* 2005; **291**: 215-26.

[92] Sorensen EMB. *Metal poisoning in fish*. Boca Raton: CRC Press; 1991.

[93] Skinner C, Turoczy NJ, Jones PL, Barnett D, Hodges R. Heavy metal concentrations in wild and cultured Blacklip abalone (*Haliotis rubra* Leach) from southern Australian waters. *Food Chem* 2004; **85**(3): 351-6.

[94] Hamza-Chaffai A, Cosson RP, Amiard-Triquet C, El Abed A. Physico-chemical forms of storage of metals (Cd, Cu and Zn) and metallothionein-like proteins in gills and liver of marine fish from the Tunisian coast: ecotoxicological consequences. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 1995; **111**(2): 329-41.

[95] Eisler R. *Zinc hazards to fish, wildlife, and invertebrates: a synoptic review.* U.S. Fish and Wildlife Service Biological Report 10. Washington, DC: US Department of Interior; 1993.

[96] Klaasen CD. *Casarett and Doull's toxicology – The basic science of poisons.* McGraw Hill: Medical Publishing Division; 2001.

[97] de Lafontaine Y, Gagné F, Blaise C, Costan G, Gagnon P, Chan HM. Biomarkers in zebra mussels (*Dreissena polymorpha*) for the assessment and monitoring of water quality of the St Lawrence River (Canada). *Aquat Toxicol* 2000; **50**(1): 51-71.

[98] Kovarova J, Celechovska O, Kizek R, Adam V, Harustiakova D, Svobodova Z. Effect of metals, with special attention of Cd, content of the Svitava and Svratka rivers on levels of thiol compounds in fish liver and their use as biochemical markers. *Neuro Endocrinol Lett* 2009; **30**(1): 169-76.