Bioaccumulation of Zinc in Java Medaka Fish *(Oryzias javanicus)* and Identifying of Metallothionein-Like Protein

Khodadoust, Daryoush, Ahmad, Ismail

Department of Biology, Faculty of Science, University Putra Malaysia, UPM, 43400, Serdang, Selangor Malaysia, aismail@science.upm.edu.my

Abstract: Metallothioneins (MTs) have been broadly measured for their probable use as exact bioindicator to be a sign of the existence of heavy metal contamination, since their induction has been observed to be noticeably elevated after heavy metal exposure in a large amount organism considered. In this study with the results from spectrophotometric method induction of Metallothionein (MT) and levels of Zinc of the Java Medaka fish *(Oryzias javanicus)* were studied after long time (60 days) exposure of juvenile fishes to different concentrations of zinc. Statistically significant differences in Zn and MT's content in different organs of fish groups exposed to this metal was found between control group and other groups with diverse concentrations of metal ($p<0.05$). Correlation between Zn content and MTs in all body sections of Java Medaka fish *(Oryzias javanicus)* were statistically significant and the correlation was positive; increasing the Zn content in body sections, the MTs levels increased also ($p<0.01$). Also the Metallothioneins (MTs) content in the tissues of Java Medaka fish *(Oryzias javanicus)* showed a significant difference between the different tissues of this fish. The order of MT content was in the decreasing order of: visceral organs > gill > muscle. Long term effect and MTs protein results indicate that this fish *(Oryzias javanicus)* is more useful and accurate to monitor particular metals and ecotoxicology studies in the estuary and coastal areas.

[Daryoush Kh, Ismail A**. Bioaccumulation of Zinc in Java Medaka Fish *(Oryzias javanicus)* and Identifying of Metallothionein-Like Protein**. *Life Sci J* 2018;15(3):42-53]. ISSN: 1097-8135 (Print) / ISSN: 2372-613X (Online). http://www.lifesciencesite.com. 6. doi:10.7537/marslsj150318.06.

Keywords: Bioaccumulation, Java medaka, Zinc, Metallothionein, Bioindicator

1. Introduction

The measurement of heavy metals concentration in living organisms has been verified to be a worthwhile chase but fails to explain the impacts of metals on health condition or as an early warning signal on living creature. The approach taken by biological monitoring provides a holistic view of a potential or on-going environmental impact by not only providing information on organism health, but also serving as an early warning system to environmental risks (Bigot *et al*., 2009). The effects of pollutants in living organism can be detected even at sub lethal concentrations or complex chemical mixtures, at molecular/biochemical levels. Biochemical responses are typically the first line of defense in the cell against pollutants. Changes at biochemical levels may induce structural or functional change at a higher level, such as hormonal regulation, immune system, and metabolism in an organism. These changes may finally impair the growth, reproduction and survival ability of the organism (Adams *et al*., 1990; Filipovi *et al*., 2003). In other hand the coastal portion of seawater and brackish water of estuaries are often contaminated by many kinds of pollutants and human pathogens because of human activities (Chua, 1992; Paez-Osuna and Tron-Mayen, 1996).). Zinc (Zn) is an ubiquitous and nutritionally essential metal playing a role as cofactor in more than 200 metalloenzymes and a functional component of transcription factor proteins contributing to gene expression and regulation (Klaassen, 2001). Furthermore, zinc also plays important roles in nervous and immune systems, in the optimal metabolism of vitamin A, and in normal calcification of bone. However, although excessive exposure of animals to zinc is relatively uncommon, it does occur around the world, especially in coastal areas, and many studies have monitored the concentration of zinc in the field (Paez-Osuna and Ruiz- Fernandez, 1995; Pederson et al., 1997; Mantelatto et al., 1999; Turoczy et al., 2001), as well as having investigated the acute and chronic toxicity of zinc to aquatic organisms (Vanegas et al., 1997; Zyadah and Abdel-Baky, 2000). The LC50-96 h for Zn were determined at 9.75 (9.65 - 9.85) mg/l for juveniles and 14.32 (13.94 - 14.70) mg/l for adults of Java Medaka fish (*Oryzias Javanicus*) (Daryoush & Ismail., 2012).

The use of toxicity tests and/or biomarkers has been suggested as useful tools to link biological responses with contaminants in the environment (Stauber & Davies, 2000; Wells *et al*., 2001).

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 (Gauthier *et al*., 2004).

Nowadays, biomarkers, such as glutathione (GSH), 7-ethoxyresorufin O-deethylase (EROD) and metallothionein (MT), have been commonly employed in studies of ecotoxicology and environmental monitoring (on persistent organic pollution and heavy metals, respectively) in supplement with traditional quantifications of pollutants (Schreiber *et al*., 2006;). Metallothionein has been applied in both laboratory and field studies (Hansen *et al*., 2006) as a biomarker of metal exposure.

Metallothioneins (MTs) are low molecular mass (6-8000 daltons), cysteine-rich (20-30%), metal binding proteins (Huang *et al*., 2007) involved in the binding and regulation of essential metals such as Cu and Zn, and the detoxification of non-essential metals such as Cd and Hg (Viarengo *et al*., 2007). MT plays an important role in the detoxification of heavy metals due to its strong binding ability to bind metals; for example a studies by (Leung *et al*., 2005) showed that everyone mole of MT binds 7 moles of cadmium or zinc. MT induction represents an early response to heavy metals presence (Chesman *et al*., 2007; Van Campenhout *et al*., 2008). Several works, including both laboratory and field studies have established a dose dependent relationship between Metallothionein (MT) and trace metal exposure. Since their discovery in 1957, the existence of MTs was firmly established in a large number of animals including fish, in which they occur in visceral organs, kidney, gill and muscle (De Boeck *et al*., 2003; Scudiero *et al*., 2005).). Fish Metallothionein (MT) have attracted special attention in the past 10–15 years, and have been studied in detail mainly under experimental conditions (Smirnov *et al*., 2005).

In fish, the efforts to establish a role for MT have been focused on the potential function of MT in reducing the toxicity of heavy metals (Park *et al*., 2001), because metal toxicity is often related to binding of the metal to non-thionein ligands with a physiological important role that can be inactivated by metal binding (Mason and Jenkins, 1995).

Several studies in fish have demonstrated relationships between exposure to metals and MT expression (Ghedira *et al*., 2010; Migliarini *et al*., 2005; Urena *et al*., 2007). In general, MT binds to heavy metals and reduces their toxicity. The use of biomarkers in biomonitoring can give “early warning” signals for assessing the e□ects of contaminants on organisms (Li *et al*., 2012) and to detect and identify possible pollutants, so that unacceptable and irreversible effects at higher levels of biological organisation can be avoided (Shulkin *et al.,* 2003), rather than a mere quantification of their environmental levels. This makes MT a useful biomarker in monitoring heavy metal pollution.

Hence, the objectives of our present study were first to identify the MTs or MT-like proteins of Java Medaka fish *(Oryzias javanicus)* as a foundation, then to investigate the induction of MTs and metal accumulation patterns within the tissues of Java Medaka fish *(Oryzias javanicus)* under long-term exposure to Zn, and finally to further explore and discuss the practicality of using MTs as biomarkers for monitoring heavy metal pollution.

2. Material and Methods

Animal treatment

Specimens of juvenile Java medaka (*Oryzias javanicus*) of 0.109-0.117gr (1.4-1.7 cm) were prepared at the medaka laboratory (collected from Linggi River estuary of Peninsular Malaysia) in the Biology Department of Faculty Science of UPM. One set of glass aquariums, each set with six aquariums (five different concentrations of metal and one control with no concentration of metal) were prepared. Every aquarium was filled with natural Linggi estuary filtered water as the main habitat water of the fish with manual water being changed weekly (10 litres per aquarium). There were 30 juvenile fish inside each aquarium, with mean length of fish being 1.4 ± 0.7 cm and mean weight of 0.109 ± 0.06 gr.

After one week acclimation period, every aquarium was added the desired concentration of Zinc as $znso_4$. The doses were 200, 400, 600, 800, 1000 µg/l and 0 (control). The chosen concentration was significantly below LC50 amounts of Zn 9-13.5 mg/litter from juvenile to adult Java Medaka fish from our previous study (Daryoush & Ismail, 2012). Temperature was maintained between 29 and 31 ºC and dissolved oxygen was at least 90% saturation at all times and Zn amount of aquarium waters (Linggi estuary water) were between 0.02-0.08 mg/Lit (Table 1).

Table 1: Mean amount of physicochemical parameters in aquariums

Parameter	Salinity (ppt)	pH	Temperature (°C)	Conductivity (ms)	O_2 (mg/lit)	Zn (mg/Lit)
Mean	19.4 – 19.9	7.6 – 7.9	29.3 –313	13.84-13.99	6.8-7.1	0.02-0.08

The fish were fed with artemia nauplii four times daily and the light cycle was 12 hours light; 12 hours dark during all 60 days of the experiment period. Mortality of fishes in the 60 days experiment period averaged less than 10% in the all aquariums.

After 60 days of exposure, fish were cached from aquariums and three parts of fish were separated as gill, caudal muscle, and visceral organs and washed briefly in ice-cold homogenizing buffer and each organ was divided into two parts. Two sets of samples were obtained. The first set was used for Metallothienein (MT) analysis and the second set of tissues was used for cadmium and zinc analysis. All samples were flash frozen in liquid nitrogen and stored at - 80ºC until start of analysis.

Determination of zinc concentrations in the samples. The filtrates obtained from biological samples were determined for Zinc by using an air-acetylene flame atomic absorption spectrophotometer (AAS) Perkin Elmer Analyst 800. Standard solutions were prepared from 1000 mg/L stock solution of each metal (BDH Spectroso L) and the wavelength was 213.9, nm for Zn. The data were presented in µg/g dry weight. Multiple-level calibration standards were analyzed to generate calibration curves against which sample concentrations were calculated.

Determination of Metallothioneins (MTs) induction. Metallothionein (MT) content was analyzed in visceral organs, gills and muscle by the assay of Viarengo *et al*. (1997). Pooled tissue of 3-6 Java Medaka fish *(Oryzias javanicus)* (1.0g) was homogenised in 3 ml of 20 mM Tris-HCl buffer (pH 8.6) containing 0.5 M sucrose, 0.006 mM leupeptin, 0.5 mM Phenylmethylsulfonyl fluoride (PMSF) and 0.01% β- mercaptoethanol and centrifuged for 20 minutes at 30,000 g 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 minutes at 6000 g 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 hour at -20°C. The mixture was centrifuged for 10 minutes at 6000 g at -4°C and the pellet was saved.

The supernatant was discarded and 1 mL of previously described homogenizing buffer solution was added, 6 mL cold ethanol and 80 µL chloroform and centrifuged for 10 minutes at 6000 g at -4°C. The supernatant was discarded and dried the pellet with N_2 gas. The pellet was resuspended in 150 µL 0.25 M Nacl and 150 µL 1 N HCl with 4 mM EDTA. To assess MT content of a sample, 4.2 ml of a solution containing 2 M Nacl and 0.43 Mm 5.5'-dithio-bis-2-nitrobenzoic acid (DTNB) adjusted pH 8 with 0.2 M Na-phosphate (NaH_2PO_4) were added at room temperature. After centrifugation at 3000 g for 5 minutes, supernatant absorbance was measured at 412nm in aUV-Visible Recording Spectrophotometer Shimadzu UV-160 A Model. The MT concentration was estimated using GSH as a reference standard (Viarengo *et al*., 1997) and modification of the method described by Ghedira *et al* (2010). Scheme of MT assay is presented in Figure 1.

GSH contains one cysteine per molecule; thus, it is a standard for quantifying cysteine in protein analyses. The amount of metallothionein in the samples was estimated using the GSH standard, assuming that 1 mol of MT contains 20 mol of cysteine (Linde and Garcia-Vazquez, 2006).

Statistical analyses. Statistical analysis was performed with one-way analysis of variance (ANOVA) to determine the treatment and time effects on MT induction and metal accumulation after exposure to zinc. Duncan's multiple-range test was used to evaluate the mean difference among individual groups at a 0.05 significance level. Results are reported as the mean and the standard errors (SE).

Result And Discussion

Comparison of Zn and MT levels in Java medaka fish tissues exposed to different concentration of Zn. Zinc and it's depending Metallothionein content in muscle, visceral organs and gill in fish groups to different concentration of zinc were compared.

Muscle: Zinc content and MTs levels in muscle differed significantly among fish groups exposed to different concentrations of Zn for 60 days (Figures 2 and 3). Statistically significant differences in zinc content in fish groups exposed to that metal were found between control group and groups exposed to different concentrations of metal.

Significant differences in zinc content in fish groups exposed to zinc were found between the groups exposed to control group and groups exposed to 300, 400 and 500µg/l zinc and between group exposed to 100 µg/l and groups exposed to 300, 400 and 500 µg/l zinc and between group 200 µg/l and the groups exposed to 300, 400 and 500 µg/l zinc ($p<0.05$).

About MTs level in fish exposed to different zinc concentrations in the 60 days, the highest content was found in the group exposed to 500 µg/l zinc (53.69±4.36 µg/g) followed by group exposed to 400 µg/l (53.22±1.45 µg/g) and the lowest content was found in the control group (26.08±0.15 µg/g) followed by group exposed to 100 µg/l zinc (27.16±0.17 µg/g) (Figure 3). Significant differences among groups were found between control group and groups exposed to 400 and 500µg/l zinc, then between group exposed to 100 µg/l and groups exposed to 400 and 500µg/l zinc and groups 200 and 300 µg/l with groups 400 and 500µg/l ($p<0.05$).

Visceral organs. Zinc and MTs in visceral organs differed significantly among fish groups exposed to different concentrations of zinc for 60 days (Figures 4 and 5).

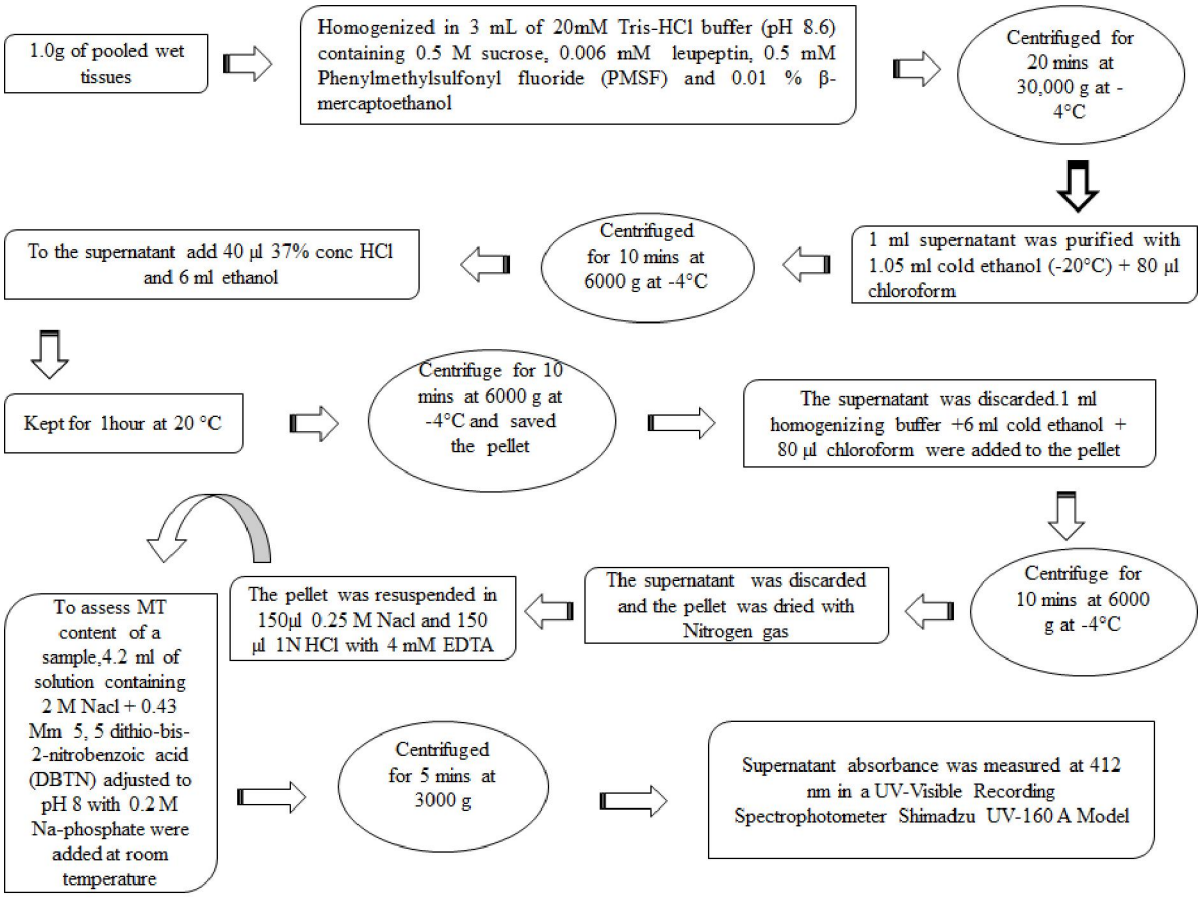

Figure 1: Scheme of Metallothionein assay (Viarengo et al. 1997)

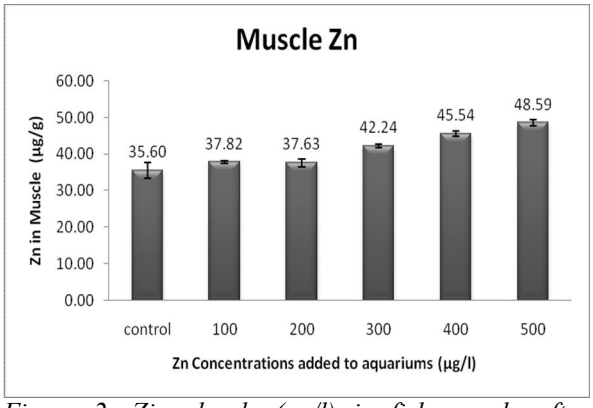

Figure 2: Zinc levels (µg/l) in fish muscle after exposure to differentconcentrations of Zn with standard errors after 60 days.

Significant differences in zinc content in visceral organs of fish groups exposed to zinc were found between the group exposed to control group and groups exposed to 200, 300, 400 and 500µg/l zinc and between group exposed to 100 µg/l and groups exposed to 300, 400 and 500 µg/l zinc and between group exposed to 200 µg/l with the groups exposed to 300, 400 and 500 µg/l zinc ($p<0.05$).

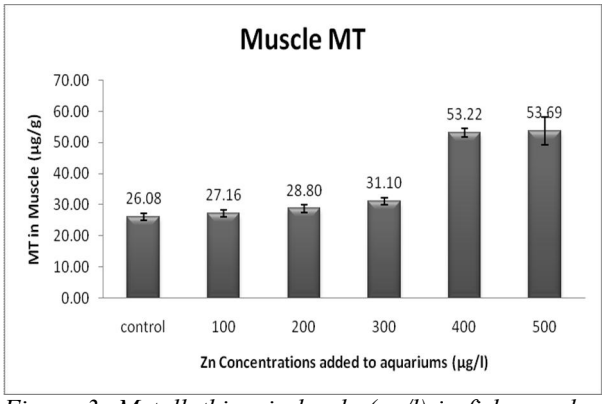

Figure 3: Metallothionein levels (µg/l) in fish muscle after exposure to different concentrations of Zn, with standard errors after 60 days.

About MTs level in fish groups exposed to different zinc concentrations in the 60-day experiment period, the highest content was found in the group exposed to 300 µg/l zinc (229.99±2.49 µg/g) followed

by group exposed to 500 µg/l (227.80±4.18 µg/g) and the lowest content was found in the control group (176.60±4.27 µg/g) followed by group exposed to 100 µg/l zinc (176.92±5.47 µg/g) (Figure 5). Significant differences among groups were found between control group and all other groups exposed to different zinc concentrations, then between group exposed to 100 µg/l and all other groups exposed to zinc and at last between group 200 µg/l and with groups exposed to 300, 400 and 500µg/l ($p<0.05$).

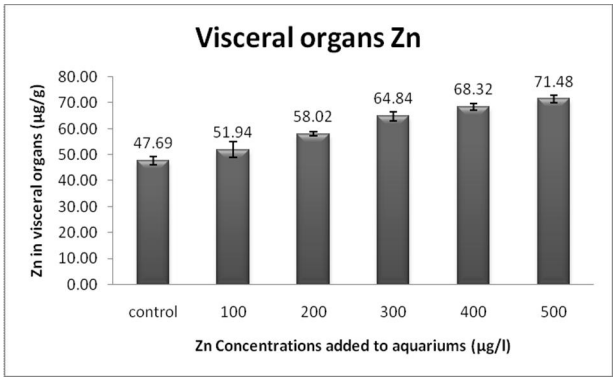

Figure 4: Zinc levels (µg/l) in fish visceral organs after exposure to different concentrations of Zn, with standard errors after 60 days

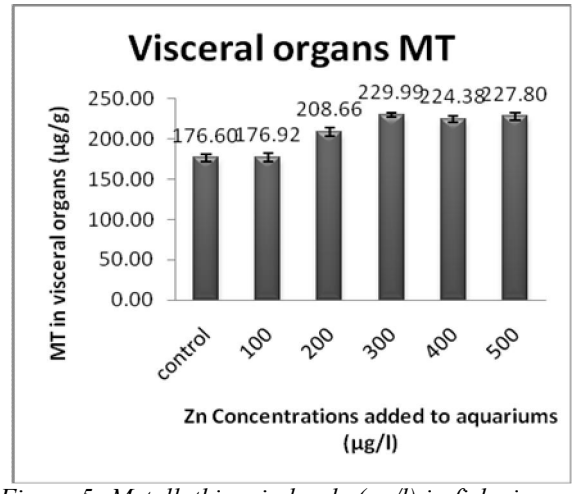

Figure 5: Metallothionein levels (µg/l) in fish visceral organs after exposure to different concentrations of Zn, with standard errors after 60 days.

Gill

Zinc and Metallothionein content in gill differed significantly among fish groups exposed to different concentrations of Zn for 60 days (Figures 6 and 7).

Statistically significant differences in zinc content in gills of fish groups exposed to that metal were found between control group and groups exposed to different concentrations of metal.

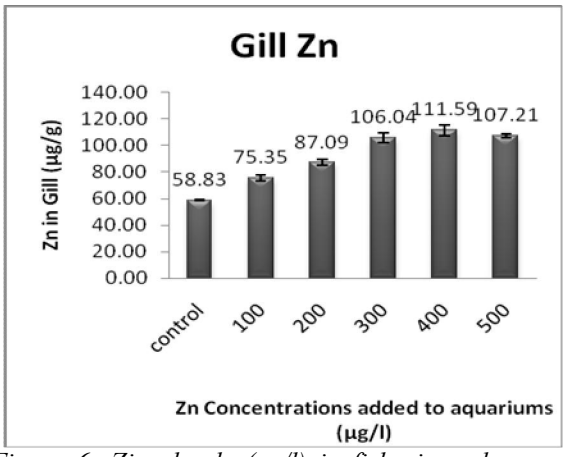

Figure 6: Zinc levels (µg/l) in fish visceral organs after exposure to different concentrations of Zn, with standard errors after 60 days.

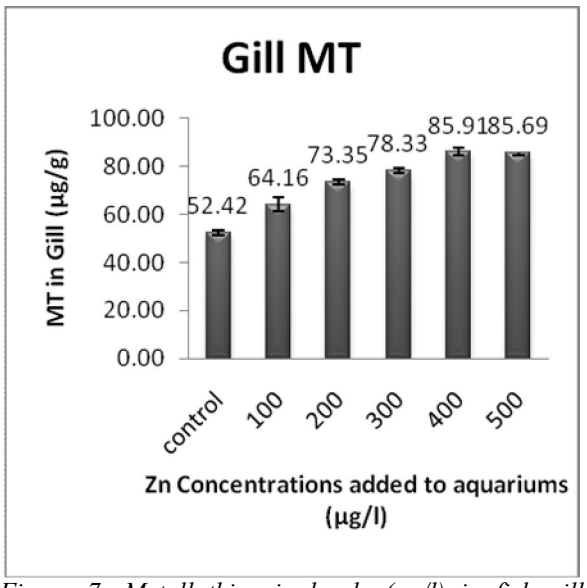

Figure 7: Metallothionein levels (µg/l) in fish gill after exposure to different concentrations of Zn, with standard errors after 60 days.

Significant differences in zinc content in gills of fish groups exposed to zinc were found between the group exposed to control group and groups exposed to200, 300, 400 and 500µg/l zinc, and between group exposed to 100 µg/l and groups exposed to200, 300, 400 and 500 µg/l zinc and between group 200 µg/l and the groups exposed to 300, 400 and 500 µg/l zinc ($p<0.05$). No significant difference between groups exposed to 300, 400 and 500 µg/l zinc was found ($p>0.05$).

As for the MTs level in gills of fish groups exposed to different concentrations of zinc in the 60-day experiment period, the highest content was found in the group exposed to 400 µg/l zinc (85.91±1.58 µg/g) followed by group exposed to 500 µg/l

(85.69±1.19 µg/g) and the lowest content was found in the control group (52.42±0.42 µg/g) (Figure 7). Significant differences among groups were found between control group and groups exposed to all other concentrations of zinc, then between group exposed to 100 µg/g and groups exposed to 200, 300, 400 and 500µg/l zinc and between group 200 µg/l with groups 300,400 and 500µg/l also ($p<0.05$). No significant difference between groups exposed 400 and 500 µg/l was found ($p>0.05$).

Correlation between cadmium and zinc content with MTs levels.

Correlation between cadmium content and MTs levels in all three body sections of java medaka fish (Muscle, Gill and Visceral organs) was statistically significant. The correlation was positive; increasing the cadmium content in body sections also increased ($p<0.01$) (Table 2).

Muscle is a kind of tissue which is extrinsic to the target organs for Zinc accumulation and for Metallothionein (MT) production (Jezierska and Witeska 2001). In our study, MTs levels had positive correlation with concentration of Zn in different body sections of fish. In Visceral organs and gill the situation was more pronounced because of accumulation of this metal and because of enhancing of Metallothionein (MT) synthesis in these tissues. The highest content of Zinc in visceral organs (111.59±0.85µg/g) was found in the group exposed to 500µg/g Zn. It has been reported that the first target organ for zinc after exposure of zinc salt in water is the visceral organs, especially the liver (Glynn and Olsson 1991; Jezierska and Witeska 2001).

Table 2: Results of Pearson's Correlation Analysis, relationships between Zn concentration in different tissue of O.javanicus and MTs levels

	Visceral organs MT	Gill MT	Muscle MT	Visceral organs ZN	Gill ZN	Muscle Zn
Visceral organs MT	1					
gill MT	.914**	1				
Muscle MT	.663*	.798**	1			
Visceral organs ZN	.914**	.957**	.838**	1		
Gill ZN	.938**	.969**	.743**	.930**	1	
Muscle Zn	.800**	.869**	.909**	.892**	.849**	1

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

The ability of fish species to synthesize Metallothioneins (MTs) is also different among them. A difference between red-blooded and white-blooded Antarctic teleost has been demonstrated. There, the highest MTs contents are found in the visceral organs (acute study of 60 minutes of exposure to high Cd concentration), and hepatic concentration of Cd, Cu and Zn and Metallothioneins (MTs) shows positive correlation in red-blooded teleosts (*Trematomus bernacchii*) but not in white-blooded teleosts (*Chionodraco hamatus*) (Santovito, Irato et al. 2000).

Sex-differences in the synthesis of Metallothioneins (MTs) have been demonstrated too. The levels of hepatic MTs in the male dab, *Limanda limanda*, are better correlated with cadmium exposure in the mixture with Cu than in females (Hylland, Haux et al. 2002). The age of fish is another factor that needs to be correlated. It is not amazing that the hepatic and kidney cadmium levels increase with the age of fish, due to gathering of various metals. Similar behaviour has been shown in Metallothioneins (MTs) application. This is based on findings that both Cd and MT concentration in fish body sections increase under conditions of chronic exposure to this ion (Brown, Bay et al. 1990; Chaterjee and Maiti 1991).

Symptoms of Zn effects show that in low concentrations, fish had unusual swimming activity and in high concentrations they showed increased activity and irritability, imbalance, changed skin colour, formation of mucus on skin, vertical swimming and slipped eye, formation of blood spots around eye and under stomach, gill hyperaemia, and curvature of the spine at the end of 60 days experiment especially in the two last higher concentrations (Figure 8).

In our study, MTs in visceral organs section showed high levels (above85.91 µg/g) in fish exposed to 400µg/g of zinc and low levels (below 52.42µg/g) in the control group of fish exposed to zinc. A similar trend could be seen in MTs in gill, although the

stability of MTs content in fish groups exposed to higher Zn concentration was noticeable. This finding could confirm the notion that synthesis of Metallothioneins (MTs) and binding capacity of these proteins is restricted (Dallinger, Egg et al. 1997). Furthermore, Dallinger *et al* (1997) observed that MTs isolated from visceral organss and kidneys contained cadmium mainly and thus displaced zinc and copper. Moreover, they observed that Metallothioneins (MTs) induction usually needs some time to develop and settle in after a certain time of experience (Bigot, Doyen et al. 2009).

It is obvious that MTs can detoxify certain concentration of zinc only. Zinc ions, which cannot be bound by MTs, interact with high molecular mass proteins. This phenomenon may result in toxic effects. Very similar results were presented in another study (Chan 2005) in which it was shown that most of the cadmium (60%) was located in the heat stable cytosolic component, probably bound by Metallothioneins (MTs), protecting the visceral organs from cadmium toxicity. In this study (Yudkovski, Rogowska-Wrzesinska et al. 2008), the authors paid their attention to cadmium distribution in yellow perch (*Perca flavescens*) visceral organs. Perches were under several natural long-term exposures to water-borne cadmium. They showed that fish hepatic cellular components, fractionated by differential centrifugation, sequestered cadmium in constant ratios. Cadmium was bound by both cadmium-sensitive and resistant cellular components. Bio-accumulated amounts were in correlation with the exposure intensity.

It was demonstrated that induction of MTs synthesis by Zn depends on the way of metal uptake by the fish. The way for metal uptake in fish appears to be via gills, intestine and skin, but the virtual amount of these ways varies, depending somewhat on the chemical and physical features of water and sediments (Couillard 1997).

In the environment, metals are presented as free ions or as complexes with suspended particles and sediments. Transition metal ions dissolved in the ambient water are adsorbed through the gills (Yudkovski, Rogowska-Wrzesinska et al. 2008) and other permeable body surfaces. Metals bound to solid particles are ingested detached from their carrier particles in the digestive system and absorbed through the gut epithelium (De Smet and Blust 2001).

It has been shown that absorption of Zinc by visceral organs is much more competent (17-18%) than through physiological intake (0.32-0.44%) in the sentinel fish (*Lithognathus mormyrus*) (Alvarado, Quesada et al. 2006).

We have shown that MTs have an important role in zinc detoxification in fish. The level of zinc was closely related to the increase of MTs level. Correlation between zinc content and Metallothioneins (MTs) concentration in tissues of visceral organs, gill and muscle was positive and this fact was statistically significant in most of the groups exposed to Zn. In the similar study, Zn accumulations in the tissues were reported in the following order: kidney > visceral organs > gills and concentration of Zn binding Metallothioneins (MTs) ((Zn)-MTs) was in the following order: visceral organs > kidney > gills (Berntssen, Aspholm et al. 2001).

Figure8: Noticeable changes in the body of O. javanicus fish after expose to Zinc concentrations.

Table 3. Different levels of Metallothionein in different aquatic organism.

Organism	Organel	Determined range	Reference
Liza aurata	Visceral organs Kidney Brain	2.32±0.35 mg/g 1.56±0.21 mg/g 1.34±0.24 mg/g	(Filipovi and Raspor 2003)
Mullus surmuletus	Visceral organs Kidney Brain	2.11±1.13 mg/g 3.41±0.89 mg/g 1.36±0.15 mg/g	(Filipovi and Raspor 2003)
Anguilla anguilla	Visceral organs	280–2580 µg/g	(Langston, Chesman et al. 2002)
Brown Trout	Visceral organs	110 µg/g	(Linde, Sánchez-Galán et al. 1998)
European eel	Kidney Visceral organs	13 µg/g 100-180 µg/g	(Linde, Sanches-Gallan et al. 2001)
Eel Anguilla anguilla	Visceral organs	350-3500 µg/g	(Noel-Lambot, Gerday et al. 1978)
Common carp	Visceral organs	15 nmol SH/mg	(Gorbi, Baldini et al. 2005)
Sparus auratus	Visceral organs, gill	50-450 µg/g	(Ghedira, Jebali et al. 2010)
Seriola dumerilli	Different parts	200-1800 ng/g	(Dallinger, Egg et al. 1997)
Salmo salar	Different parts	120-168 µg/g	(Berntssen, Aspholm et al. 2001)
Sparus auratus	Different orgalels	22.04-399.53 µg/g	(Ghedira, Jebali et al. 2010)
zebra mussel	Different parts	69.33-346.329 µg/g	(Frank, Singer et al. 2008)
Oryzias javanicus	Gill	52.42-85.69 µg/g	This study
Oryzias javanicus	Visceral organs	176.60-227.80 µg/g	This study
Oryzias javanicus	Caudal muscle	26.08-53.69 µg/g	This study

And our results show Zn accumulation in the medaka fish tissues was in the following order: visceral organs > gill > muscle, and concentration of MTs binding with Zn it was in the following order: visceral organs > gill > muscle; for fish groups exposed to Zn in different concentrations. However, in another study(Huang, Zhang et al. 2007) the authors showed that accumulation capacity of every single organ depends on other metals in water. Common carps coexposed to cadmium, mercury and lead had the amounts of cadmium in this order: kidney > gills > visceral organs > muscle and Metallothioneins (MTs) were in the order of: gills > kidney > visceral organs > muscle. Detailed explanation of the reason for this would require further study.

Conclusion: Our results showed that Zn accumulation in the medaka fish tissues was in the following order: visceral organs > gill > muscle for Zn, and concentration of MTs binding with Zn was in the following gill > visceral organs > muscle for fish groups exposed to different Zn concentrations. Range of MT in the gill, caudal muscle and visceral organs was: were 26.68-53.69 µg/g, 52.42-85.69 µg/g and 176.60-227.80 µg/g caudal muscle, gill and visceral organs respectively in fish exposed to different Zn concentrations. The results show that with increasing level of both zn concentrations, level of MT also increased. Values were elevated in fish visceral organs in correlation to physiologically occurred levels. The low MT content in gills and caudal muscle could be attributed to low binding capacity of this organs to metals. Synthesis of metal-binding thionein ligands has been reported in fish gill (De Boeck *et al*., 2003; Lange *et al*., 2002) 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 (Chao Dang et al., 2000) which comprise a minority (<10%) of the branchial epithelial surface area. Some investigators have suggested that the gill does not constitute a good organ for MT quantification (Hamza-Chaffai *et al*., 1997). In the present study the values of MT in gills were found lower compared to visceral organs but higher than muscle. In addition, many studies have illustrate 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 (Wu *et al*., 2000) when the heavy metal treatment dose or time exceeds the fish's resistance, and similar effects are not specifically limited to heavy metals as seen in in vivo studies. Over time tissues metal concentrations increase and then stabilize (McGeer *et al*., 2000) and ultimately, the internal physiology of the animal either returns to the pre-exposure condition or new equilibrium is established. Metals uptake depends not only on bioavailability, but on ecological needs and metabolic activity of species.

The results of our study are confirmed by the findings of other authors (Fabrik, Ruferova et al. 2008) who concluded that the concentration of cellular stress proteins (including Metallothioneins

(MTs)) is a good indicator of water pollution. However, it has been reported that MT level is a good bioindicator of heavy metals pollution in *Salmo trutta*, but not in *Anguilla anguilla* (Linde, Sanches-Gallan et al. 2001). Therefore, it could be concluded that not every fish species is suitable for biomonitoring. We have demonstrated in our study that the Java Medaka fish (*Oryzias Javanicus*) could be considered as a specimen of fish suitable for this purpose. This study of MTs as biomarker suggests that there is connection between some heavy metals contamination and MTs production in Java Medaka fish (*Oryzias Javanicus*). Also, the higher heavy metals concentrations and MTs levels have confirmed the role of MTs in metals homeostasis and detoxification. Therefore, MTs content can be as an effective biomarker for metal stress in *Oryzias. Javanicus (Table 3)*.

Recommendations for future research

1. Biological factors such as age, sex, reproductive status as well as hormone levels may influence MT induction, therefore increasing response variability should be considered into account when MT is to be used as a biomarker.

2. The presence of different variety of MT inductors unrelated to metals contaminants in the environment, requires more detailed studies to establish a relationship and patterns between these environmental contaminants and MT induction in Java Medaka fish in the west coast of Peninsular Malaysia.

References

1. Bigot, A., Doyen, P., Vasseur, P., & Rodius, F. (2009). Metallothionein coding sequence identification and seasonal mRNA expression of detoxification genes in the bivalve Corbicula fluminea. *Ecotoxicology and Environmental Safety, 72*(2), 382-387.
2. Adams, S., Shugart, L., Southworth, G., & Hinton, D. (1990). Application of bioindicators in assessing the health of fish populations experiencing contaminant stress. In J. F. McCarthy & L. R. Shugart. *Biomarkers of Environmental Contamination* (pp.333 – 353). Chelsea, Michigan: Lewis Publishers.
3. Filipovi, V., & Raspor, B. (2003). Metallothionein and metal levels in cytosol of visceral organs, kidney and brain in relation to growth parameters of Mullus surmuletus and Liza aurata from the Eastern Adriatic Sea. *Water Research, 37*(13), 3253-3262.
4. Chua, T. E., 1992. Coastal aquaculture development and the environment: the role of coastal area management. Mar. Pollut. Bull. 25, 98–103.
5. Paez-Osuna, F., Ruiz-Fernandez, C., 1995. Trace metals in the Mexican shrimp Penaeus vannamei from estuarine and marine environments. Environ. Pollut. 87, 243– 247.
6. Klaassen, C. D., 2001. Toxicology, Sixth edition The McGraw-Hill Companies, New York.
7. Pederson, S. N., Lundebye, A.-K., Depledge, M. H., 1997. Field application of metallothionein and stress protein biomarkers in the shore crab Carcinus maenas exposed to trace metals. Aquat. Toxicol. 37, 183– 200.
8. Mantelatto, F. L. M., Avelar, W. E. P., Silva, D. M. L., Tomazelli, A. C., Lopez, J. L. C., Shuhama, T., 1999. Heavy metals in the shrimp Xiphopenaeus kroyeri from Ubatuba Bay Saint Paulo, Brazil. *Bull. Environ. Contam.* Toxicol. 62, 152–159.
9. Turoczy, N. J., Mitchell, B. D., Levings, A. H., Rajendram, V. S., 2001. Cadmium, copper,
10. Vanegas, C., Espina, S., Botello, A. V., Villanueva, S., 1997. Acute toxicity and synergism of cadmium and zinc in white shrimp Penaeus setiferus juveniles. Bull. Environ. Contam. Toxicol. 58, 87– 92.
11. Zyadah, M. A., Abdel-Baky, T. E., 2000. Toxicity and bioaccumulation of copper, zinc, and cadmium in some aquatic organisms. *Bull. Environ. Contam. Toxicol*.
12. Daryoush, K. and A. ismail (2012). "Acute toxicity test of Zn, on Java medaka (Oryzias javanicus) fish as an indicator of estuary pollution." Scientific Research and Essays **7**(39): 3302-3306.
13. Stauber, J., & Davies, C. (2000). Use and limitations of microbial bioassays for assessing copper bioavailability in the aquatic environment. *Environmental Reviews*. 8: 255-301.
14. Gauthier, L., Tardy, E., Mouchet, F., & Marty, J. (2004). Biomonitoring of the genotoxic potential (micronucleus assay) and detoxifying activity (EROD induction) in the River Dadou (France), using the amphibian *Xenopus laevis*. *Science of the Total Environment.* 323: 47-61.
15. Schreiber, E. A., Otter, R. R., & van den Hurk, P. (2006). A biomarker approach to measure biological effects of contaminant exposure in largemouth bass from Lake Conestee, South Carolina, USA.
16. Hansen, B., Romma, S., Garmo, Q., Olsvik, P., & Andersen, R. (2006). Antioxidative stress proteins and their gene expression in brown trout (*Salmo trutta*) from three rivers with different heavy metal levels. *Comparative Biochemistry and Physiology.*

17. Huang, Z. Y., Q. Zhang, et al. (2007). "Bioaccumulation of metals and induction of metallothioneins in selected tissues of common carp (*Cyprinus carpio*)." *Appl. Organometal. Chem* **21**: 101-107.
18. Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E., & Koehler, A. (2007). The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*. 146: 281-300.
19. Leung, K. M. Y., R. E. Dewhurst, H. Halldorsson and J. Svavarsson (2005). "metallothioneins and trace metals in the dogwhelk *Nucella lapillus* (L.) collected from icelanic coasts." Mar. Pollut. Bull. 46,:: 729-733.
20. Chesman, B., O'hara, S., Burt, G., & Langston, W. (2007). Hepatic metallothionein and total oxyradical scavenging capacity in Atlantic cod *Gadus morhua* caged in open sea contamination gradients. *Aquatic Toxicology*. 84: 310-320.
21. Van Campenhout, K., Goenaga Infante, H., Goemans, G., Belpaire, C., Adams, F., Blust, R., *et al*. (2008). A field survey of metal binding to metallothionein and other cytosolic ligands in liver of eels using an on-line isotope dilution method in combination with size exclusion (SE) high pressure liquid chromatography (HPLC) coupled to Inductively Coupled Plasma time-of-flight Mass Spectrometry (ICP-TOFMS). *Science of the Total Environment*. 394: 379-389.
22. De Boeck, G., Ngo, T. T. H., Van Campenhout, K., & Blust, R. (2003). Differential metallothionein induction patterns in three freshwater fish during sublethal copper exposure. *Aquatic Toxicology*. 65: 413-424.
23. Scudiero, R., Temussi, P. A., & Parisi, E. (2005). Fish and mammalian metallothioneins: a comparative study. *Gene*. 345: 21-26.
24. Smirnov, L., Sukhovskaya, I., & Nemova, N. (2005). Effects of environmental factors on low-molecular-weight peptides of fishes: a review. *Russian Journal of Ecology*. 36: 41-47.
25. Park, J. D., Liu, Y., & Klaassen, C. D. (2001). Protective effect of metallothionein against the toxicity of cadmium and other metals. *Toxicology*. 163: 93-100.
26. Ghedira, J., Jebali, J., Bouraouni, z., Banni, M., Guerbej, H., & Boussetta, H. (2010). Metallothionein and metal levels in visceral organs, gills and kidney of Sparus aurata exposed to sublethal doses of cadmium and copper. *Fish Phyiol Biotchem, 36*, 101-107.
27. Migliarini, B., Campisi, A., Maradonna, F., Truzzi, C., Annibaldi, A., Scarponi, G., *et al*. (2005). Effects of cadmium exposure on testis apoptosis in the marine teleost Gobius niger. *General and Comparative Endocrinology*. 142: 241-247.
28. Urena, R., Peri, S., Del Ramo, J., & Torreblanca, A. (2007). Metal and metallothionein content in tissues from wild and farmed Anguilla anguilla at commercial size. Environment International. 33: 532-539.
29. Li, W.-H., Y. Tian, et al. (2012). "Source and risk assessment of PCBs in sediments of Fenhe reservoir and watershed, China." Journal of Environmental Monitoring.10.1039/C2EM10983B.
30. Shulkin, V., & Presley, B. (2003). Metal concentrations in mussel *Crenomytilus grayanus* and oyster *Crassostrea gigas* in relation to contamination of ambient sediments. *Environment International*. 29: 493-502.
31. Viarengo, A., Ponzano, E., Dondero, F., & Fabbri, R. (1997). A simple spectrophotometric method for metallothionein evaluation in marine organism: an application to Mediterranean and Antarctic molluscs. *Mar Environ Res, 44*, 69-84.
32. Linde, A. R., Sanches-Gallan, S., Valles-Mota, P., & Garcia-Vasquez, E. (2001). Metallothioneins (MTs) as bioindicator of freshwater metal pollution: European eel and brown trout. *Ecotoxicol. Environ. Safe., 49*, 60-63.
33. Jezierska, B., & Witeska, M. (2001). Metal toxicity to fish. *university of Podlasie: Siedlce, poland.*, 51-68.
34. Glynn, A. W., & Olsson, P. E. (1991). Cadmium turnover in minnows (*Phoxinus phoxinus*) preexposed to waterborn cadmium. *Environ. Toxicol. Chem., 10*, 338-394.
35. Santovito, G., Irato, P., Piccinni, E., & Albergoni, V. (2000). Relationship between metallothionein and metal contents in red-blooded and white-blooded Antarctic teleosts. *Polar. Biol, 23*(383-391).
36. Hylland, K., Haux, C., & Hogstrand, C. (2002). Hepathic metallothionein and heavy metals in dab (*Limanda limanda*) from the German bright. *Ecol. Progr., 91*(89-96).
37. Brown, D. A., Bay, S. M., & Hershelm, G. P. (1990). Exposure of scorpionfish (*Scorpaena guttata*) to cadmium: effects of acute and chronic exposure on the cytosolic distribution of cadmium, copper and zinc. *Aquat. Toxicol, 16*, 295-310.
38. Chaterjee, A., & Maiti, I. B. (1991). Induction and turnover of catfish (*Heteropneustes fossilis*) metalothionein. *Moll. Cell. Biochem, 108*, 29-38.

39. Dallinger, R., Egg, M., Köck, G., & Hoffer, R. (1997). The role of metallothionein in cadmium accumulation of Arctic char (*Salvelinus alpinus*) from high Alpine lakes. *Aquat. Toxicol., 38*, 48-66.
40. Yudkovski, Y., Rogowska-Wrzesinska, A., Yankelevich, I., Shefer, E., Herut, B., & Tom, M. (2008). Quantitative immunochemical evaluation of fish metallothionein upon exposure to cadmium. *Mar. Environ. Res., 65*(427-436).
41. Couillard, Y. (1997). Technical evaluation of metallothionein as a biomarker for the mining industry.. *Ottawa, Ontario. Natural Resources Canada. CANMET. AETE Project 2.2.1, 191 p.*
42. De Smet, H., & Blust, R. (2001). Stress responses and changes in protein metabolism in carp (*Cyprinus carpio*) during cadmium exposure.. *Ecotox. Environ. Safe, 48*, 255-262.
43. Alvarado, N. E., Quesada, I., Hylland, K., Marigomez, I., & Soto, M. (2006). Quantitative changes in metallothionein expression in targed cell types in the gills of turbot (*Scopthalmus maximus*) exposed to Cd, Cu, Zn and after a depuration treatment. *Aquatic. Toxicol., 7*, 64-77.
44. Berntssen, M. H. G., Aspholm, O. O., Hylland, K., Bonga, S. E. W., & Lundebye, A. K. T. (2001). Tissue metallothionein, apoptosis and cell proliferation responses in Atlantic salmon (*Salmo salar* L.) parrfed elevated dietary cadmium. *Comp. Bioch. Physiol., 128*, 299-310.
45. Langston, W. J., Chesman, B. S., Burt, G. R., Pope, N. D., & McEvoy, J. (2002). Metallothionein in visceral organs of eels Anguilla anguilla from the Thames Estuary: an indicator of environmental quality? *Mar Environ Res, 53*(3), 263-293.
46. Linde, A. R., Sánchez-Galán, S., Izquierdo, J. I., Arribas, P., Marañón, E., & García-Vázquez, E. (1998). Brown Trout as Biomonitor of Heavy Metal Pollution: Effect of Age on the Reliability of the Assessment. *Ecotoxicology and Environmental Safety, 40*(1-2), 120-125.
47. Gorbi, S., Baldini, C., & Regoli, F. (2005). Seasonal variability of Metallothioneins (MTs), cytochrome P450, bilemetabolites and oxyradicalmetabolismin the European eel Anguilla anguilla L. (Anguillidae) and striped mullet Mugil cephalus L. (Mugilidae). *Arch Environ Contam Toxicol, 49*(1), 62-70.
48. Frank, S. N., Singer, C., & Sures, B. (2008). Metallothionein (MT) response after chronic palladium exposure in the zebra mussel, Dreissena polymorpha. *Environmental Research, 108*(3), 309-314.
49. Chao Dang, Z., Flik, G., Ducouret, B., & Hogstrand, C. (2000). Effects of copper on cortisol receptor and metallothionein expression in gills of *Oncorhynchus mykiss*. *Aquatic Toxicology*. 51: 45-54.
50. Hamza-Chaffai, A., Cosson, R., Amiard-Triquet, C., & El Abed, A. (1995). 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. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*. 111: 329-341.
51. Wu, S. M., Weng, C. F., Hwang, J. C., Huang, C. J., & Hwang, P. P. (2000). Metallothionein induction in early larval stages of tilapia *Oreochromis mossambicus*). *Physiological and Biochemical Zoology*. 73: 531-537.
52. McGeer, J. C., Szebedinszky, C., Gordon McDonald, D., & Wood, C. M. (2000). Effects of chronic sublethal exposure to waterborne Cu, Cd or Zn in rainbow trout 2: tissue specific metal accumulation. *Aquatic Toxicology*. 50: 245-256.
53. Fabrik, I., Ruferova, Z., Hilscherova, K., Adam, V., Trnkova, L., & Kizek, R. A. (2008). Determination of Metallothionein in Larvae of Freshwater Midges (*Chironomus riparius*) Using Brdicka Reaction. *Sensors 8*, 4081-4094.
54. Noel-Lambot, F., Gerday, C., & Disteche, A. (1978). Distribution of Cd, Zn and Cu in the visceral organs and gills of the eel Anguilla anguilla with special reference to Metallothioneins (MTs). *Comp Biochem Physiol 61*(1), 177-187.
55. Alhama, J., Romero-Ruiz, A., & Lpez-Barea, J. (2006). Metallothionein quantification in clam by reversed-phase high-performance liquid chromatography coupled to fluorescence detection after monobromobimane derivatization. *Journal of Chromatography A, 1107*, 52-58.
56. Amiard, J. C., amiard-Triquet, C., Barka, S., pellerin, J., & Rainbow, P. S. (2006). Metallothioneins (MTs) in aquatic invertebrates: their role in metal detoxification and their use as biomarkers.. *Aquatic Toxicology, 76*, 160-202.
57. Campbell, P. G. C., Giguere, A., Bonneris, E., & Hare, L. (2005). Cadmium-handling strategies in two chronically exposed indigenous freshwater organisms-the yellow perch (*Perca flavescens*) and the floater mollusc (*Pyganodon grandis*). *Aquat. Toxicol., 72*, 83-97.
58. Chan, K. M. (2005). Metallothionein: Potential biomarker for monitoring heavy metal pollution in fish around Hong Kong. *Marine Pollution Bulletin, 31*(4-12), 411-415.

59. Exon, J. H., Lamberton, J. G., & Koller, L. D. (1997). Effect of chronic oral exposure and withdrawal on cadmium residues in organs of mice. *Bull. Environ. Contam. Toxicol, 18*, 74-76.
60. Ghedira, J., Jebali, J., Bouraoui, Z., Banni, M., Guerbej, H., & Boussetta, H. (2010). Metallothionein and metal levels in liver, gills and kidney of *Sparus aurata* exposed to sublethal doses of cadmium and copper. *Fish Physiology and Biochemistry*. 36: 101-107.
61. Huang, Z. Y., Zhang, Q., Chen, J., Zhuang, Z. X., & Wang, X. R. (2007). Bioaccumulation of metals and induction of Metallothioneins (MTs) in selected tissues of common carp (*Cyprinus carpio*). *Appl.Organometal. Chem, 21*, 101-107.
62. Kille, P., J. Kay, M. Leaver, & George., S. (1992). Induction of piscine metallothionein as a primary response to heavy metal pollutants: Applicability of new sensitive molecular probes. *Aquat. Toxicol, 22*, 279-286.
63. Lange, A., Ausseil, O., & Segner, H. (2002). Alterations of tissue glutathione levels and metallothionein mRNA in rainbow trout during single and combined exposure to cadmium and zinc* 1. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*. 131: 231-243.
64. Margoshes, M., & Vallee, B. L. (1957). A cadmium protein from equine kidney cortex. *Journal of American Chemical Society, 79*(4813-4819).
65. Paez-Osuna, F., Tron-Mayen, L., 1996. Concentration and distribution of heavy metals in tissues of wild and farmed shrimp Penaeus vannamei from the northwest coast of Mexico. Environ. *Int.* 22, 443– 450.
66. Romero-Ruiz, A., Alhama, J., Blasco, J., Gómez-Ariza, J. L., & López-Barea, J. (2008). New metallothionein assay in Scrobicularia plana: Heating effect and correlation with other biomarkers. *Environmental Pollution, 156*(3), 1340-1347.
67. Sigel, A., Sigel, H., & Sigel, R. K. O. (2009). *Metallothioneins (MTs) and Related Chelators*. Metal Ions in Life Sciences. *Cambridge: RSC Publishing*.
68. mercury, and zinc concentrations in tissues of the king crab Pseudocarcinus gigas from southeast Australian waters. *Environ. Int*. 27, 327– 334.
69. Wells, P. G., Depledge, M. H., Butler, J. N., & Manock, J. (2001). Rapid toxicity assessment and biomonitoring of marine contaminants--exploiting the potential of rapid biomarker assays and microscale toxicity tests. *Marine Pollution Bulletin*. 42: 799-804.

3/25/2018