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Metallothionein-Like Protein Levels in Java Medaka Fish (*Oryzias javanicus*) Exposed to Different Concentrations of Cadmium

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Abstract

In this study, induction of metallothionein (MT) and levels of cadmium (Cd) in the Java medaka fish (*Oryzias javanicus*) are studied after long time (60 days) exposure of juvenile fish to different concentrations of Cd. Statistically significant differences in Cd and MT content in the gills, caudal muscles and livers of the fish groups exposed to this metal are found between the control group and other groups exposed to different concentrations of metal ($p < 0.05$). Correlation between Cd content and MT in all body sections of Java medaka fish are statistically significant and the correlation is positive; the Cd and MT concentrations in all studied tissues indicate that an increase in Cd levels is followed with an increase in MT levels also ($p < 0.01$). Long term effects and MT protein results indicate that this fish is a suitable bioindicator for the monitoring of particular metals, such as Cd, and for ecotoxicology studies in the estuary and coastal areas.

Keywords: Metallothionein (MT), cadmium (Cd), Java medaka fish, ecotoxicology

Introduction

Metallothionein (MT) is a family of cysteine-rich, low molecular weight (MW ranging from 500 to 14000 Da) proteins. They are localized to the membrane of the Golgi apparatus. MT has the capacity to bind both physiological (such as zinc (Zn), copper (Cu), selenium (Se)) and non-essential (such as cadmium (Cd), mercury (Hg), silver (Ag), arsenic (As)) heavy metals through the thiol group of its cysteine residues, which represents nearly 30 % of its amino acidic residues [1].

MT was discovered in 1957 by Vallee and Margoshe from purification of a Cd-binding protein from horse (equine) renal cortex [2]. MT's function is not clear, but experimental data suggest MT may provide protection against metal toxicity, be involved in the regulation of homeostasis of essential metals (Zn, Cd and Cu), and provide protection against oxidative stress [3]. Their production is dependent on the availability of dietary minerals, such as Zn, Cu, Cd and Se, and the amino acids histidine and cysteine. With careful experimental design, mainly through choice of species and tissue analyzed, MT concentration can be an unfailing biomarker that the cell is responding to raised exposure to the trace metals [4].

MT as a biomarker of exposure is attractive because it is easily quantified by using a highly sensitive, simple, low-cost spectrophotometric technique [5], and it can provide sub-lethal diagnosis of contamination and assessment of sub-lethal exposure to metals ions [6]. Some studies have been carried out on fish to examine MT's possible role in intracellular homeostasis of essential elements and the detoxification on non-essential elements, but study on fish MT is still in its infancy [7]. MT is present in all tissues, but gills and the liver are the most usually surveyed organs for MT induction in fish, due to their role in metal uptake and bioaccumulation/detoxification respectively [8].

There are several methods to determine MTs such as spectrophotometric methods, electrochemical methods, chromatography, saturation-based methods, immunological methods, electrophoresis, and RT-

PCR [9]. In the current study, a spectrophotomety was applied to quantify the MT concentrations in different tissues of Java medaka fish (*Oryzias Javanicus*), because this method is simple, repeatable and is a low cost detection of minimal concentration of MTs in biological samples, as described by Viarengo *et al*. [5]. The main aim of this study is to assess the effectiveness of using metallothionein (MT) as a biomarker for metal stress in Java medaka fish (*Oryzias avanicus*) exposed to different concentrations of Cd.

Material and method

Animal treatment

Specimens of juvenile Java medaka (*Oryzias javanicus*) of 0.109 - 0.117 gr (1.4 - 1.7 cm), collected from the Linggi Estuary of the Malaysian Peninsular, were purchased from the Medaka laboratory in the Biology Department of the Faculty of Science at UPM. Two sets of glass aquariums, every set with 6 aquariums (5 different concentrations of metal and one control with no concentration of metal), were prepared. Every aquarium was filled with natural Linggi Estuary water (**Table 1**), as the main habitat water of the fish, with manual water changing occurring once every week (10 litres per one aquarium). There were 30 juveniles fish inside every aquarium.

After a one week acclimation period, doses of 100, 200, 300, 400, 500 µg/L and 0 (control) were added into the aquariums. The chosen concentration was significantly below LC50 amounts of those metals: 6 - 7 mg/L Cd from juvenile to adult Java Medaka fish (the authors' previous and unpublished study). Temperature was maintained between 29 - 31 °C and dissolved oxygen was at a saturation level of at least 90 % at all times (**Table 1**).

The fish were fed with artemia nauplii 4 times every day, and the light cycle was 12 h light; 12 h dark over all of the 60 day experiment period. Mortality of fish in the 60 day experiment period averaged less than 10 percent in all aquariums. After 60 days of exposure, fish were taken from the aquariums and 3 parts of the fish were removed for study, gill, caudal muscle and liver, and washed briefly an in ice-cold homogenizing buffer. Each organ was divided in 2 parts. Two sets of samples were obtained. The first set was used for metallothienein analysis and the second set of tissues was used for Cd amounts analysis. All samples were flash frozen into liquid nitrogen and stored at −80 °C until the start of analysis. Cd content in samples was determined in gill, caudal muscle and liver by atomic absorption spectrophotometry (AAS) with Perkin Elmer Model Analyst 880 using the acid digestion method.

Table 1 Mean amount of physicochemical parameters in aquariums.

Parameter	Salinity (ppt)	pH	Temperature (°C)	Conductivity (ms)	O2 (mg/lit)
Mean	19.4 - 19.9	7.6 - 7.9	29.3 - 31.3	13.84 - 13.99	6.8 - 7.1

Metallothionein determination

MT content was analyzed in frozen/ground tissue (gill, caudal muscle and liver) by the assay of Viarengo *et al*. [5] and modification of the method described by Ghedira *et al*. [10]. Pooled tissue of every aquarium fish (1 g) was homogenized 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 % *b*-mercaptoethanol, and spun 20 min at 30,000 g. To 1 ml supernatant, 1.05 ml of cold (–20 °C) absolute methanol and 80 µl chloroform were added, and the sample was spun at 6,000 g for 10 min at 4 °C to the supernatant.

The MT containing pellet was washed with 2 ml 87 % ethanol, 1 % chloroform and 12 % homogenizing buffer, and spun and dried under a N_2 gas. The pellet was resuspended in 150 µl 0.25 M NaCl, and the metals released from MT with 150 µl of 1 N HCl plus 4 mM EDTA. To assess MT content

of a sample, 4.2 ml of 2 M NaCl with 0.43 mM, 5-dithio-bis-2-nitrobenzoic acid adjusted to pH 8 with 0.2 M Na-phosphate was added at room temperature. After centrifugation at 3,000 g for 5 min, supernatant absorbance was measured, at 412 nm in a UV-Visible Recording Spectrophotometer Shimadzu UV-160 A- Model, and the results were compared to glutathione (GSH) calibration cure as a reference standard [5].

Statistical analysis

All analyses were carried out in 5 replicates. Results are expressed as the means ±SE. The data were analysed by one-way analysis of variance (ANOVA) using SPSS software. The means obtained from each set were compared using Duncan's multiple range tests at the 0.05 confidence level by Pearson correlation analysis.

Results

Cd and MT content in muscle, liver and gill of fish groups to different concentration of Cd were compared. Cd content and MT levels in muscle differed significantly among fish groups exposed to different concentrations of Cd for 60 days (**Figures 1**, **2**). Statistically significant differences in Cd content in fish groups exposed to this metal were found between the control group and groups exposed to different concentrations of metal.

There was significant difference between the control group and groups exposed to 50, 100, 200, 400 and 500 µg/L Cd, then between the group exposed to 50 µg/g and groups exposed to 100, 200, 400 and 500 µg/L of Cd ($p < 0.05$). The lowest Cd content was in the control group (1.23±0.01 µg/g) and the highest was in the group exposed to 500 µg/L (3.70±0.06 µg/g) (**Figure 1**). MT in muscles of fish groups exposed to Cd were generally the highest in the group exposed to 400µg/L Cd (39.71±0.16 µg/g) followed by the group exposed to 500 µg/L (39.55±0.3 µg/g) and the group exposed to 200 µg/L (39.0.3±0.08) (**Figure 2**). Significant differences among groups were found between the control group and groups exposed to 50, 100, 200, 400 and 500µg/L Cd, then between group exposed to 50 µg/L and groups exposed to 100, 200, 400 and 500µg/L Cd ($p < 0.05$).

Amounts of Cd and MT were varied significantly among fish groups exposed to different concentrations of Cd for 60 days (**Figures 3**, **4**). For all concentration groups exposed to Cd, the lowest Cd content was in the control group (4.59±0.06 µg/g) and the highest Cd was in the group exposed to 500 µg/L (13.09±0.15 µg/g) (**Figure 4**). Significant differences among groups exposed to Cd were found between the control group and over all 5 concentrations of Cd, then between groups exposed to 50 and 100 µg/L with the other 3 groups (200, 400 and 500 µg/L) ($p < 0.05$).

MT in liver of fish groups exposed to Cd were generally the highest in the group exposed to 500 µg/L Cd (349.71±9.35 µg/g) and the lowest was in the control group (126.91±5.42µg/g) (**Figure 3**). Statistically significant differences among groups were found between the control group and groups exposed to 50, 100, 200, 400 and 500 µg/L Cd, then between the group exposed to 50 µg/L with groups exposed to 100, 200, 400 and 500 µg/L Cd, and also the group exposed to 100 µg/L with groups exposed to 200 400 and 500 µg/L Cd ($p < 0.05$).

Cd and MT content in gill differed significantly among fish groups exposed to different concentration of Cd for 60 days (**Figures 5**, **6**). Statistically significant differences in Cd content in gill of fish groups exposed to this metal were found between the control group and groups exposed to different concentrations of metal. There was significant difference between the control group and groups exposed to 50, 100, 200, 400 and 500 µg/L Cd, then between the group exposed to 50µg/L and groups exposed to 100, 200, 400 and 500 µg/L Cd ($p < 0.05$). The lowest Cd content was in the gill of the control group (2.27±0.07 µg/g) and the highest was in the group exposed to 500 µg/L (4.91±0.42 µg/g) followed by the group exposed to 200 µg/L (4.86±0.23 µg/g) (**Figures 5**, **6**).

MT in gills of fish groups exposed to Cd were generally the highest in the group exposed to 500µg/L Cd (65.65±05.23 µg/g), followed by the group exposed to 400 µg/L (64.33±6.12 µg/g) and the group exposed to 200 µg/L (64.11±3.21) (**Figures 5**, **6**). Significant differences among groups were found between the control group and groups exposed to 50, 100, 200, 400 and 500 µg/g Cd, then between the

group exposed to 50 µg/L and groups exposed to 100, 200, 400 and 500 µg/L Cd ($p < 0.05$).There were no any significant differences between the groups exposed to 200 and 400 µg/L ($p > 0.05$).

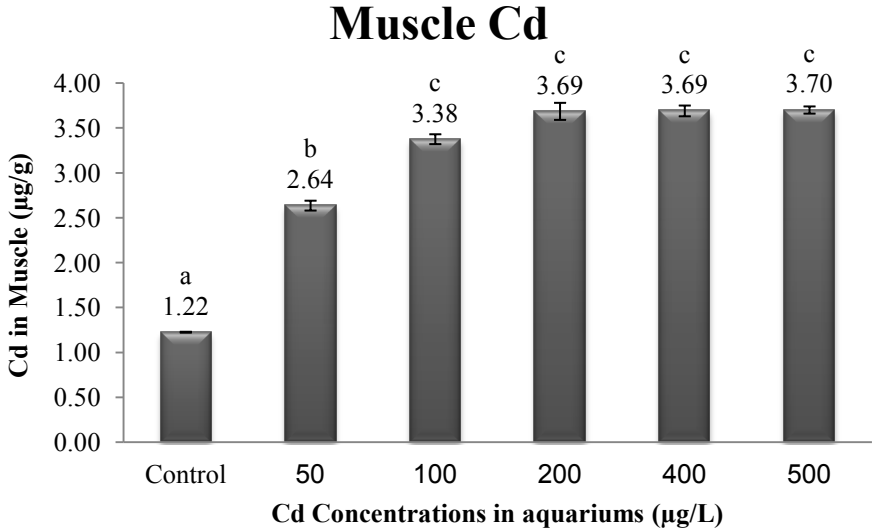

Figure 1 Cd in fish muscle according to exposure to Cd in different concentrations (µg/L) with standard errors after 60 days. Different superscript letters indicate significant differences ($p < 0.05$).

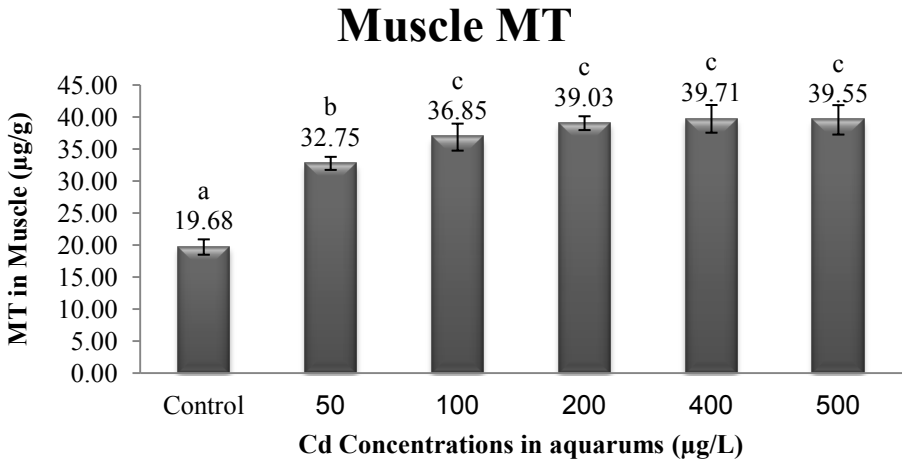

Figure 2 MT in fish muscle according to exposure to Cd in different concentrations (µg/L) with standard errors after 60 days. Different superscript letters indicate significant differences ($p < 0.05$).

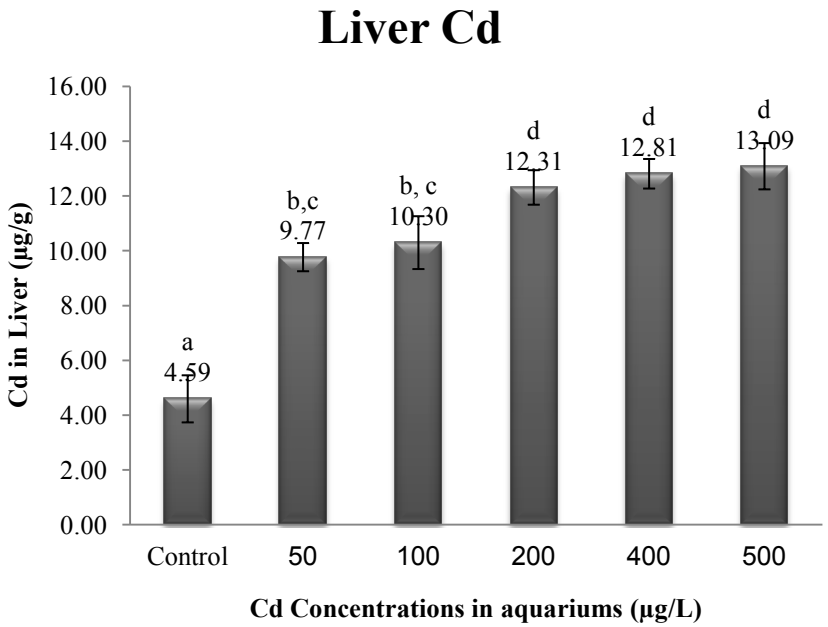

Figure 3 Cd in fish liver according to exposure to Cd in different concentrations (µg/L) with standard errors after 60 days. Different superscript letters indicate significant differences ($p < 0.05$).

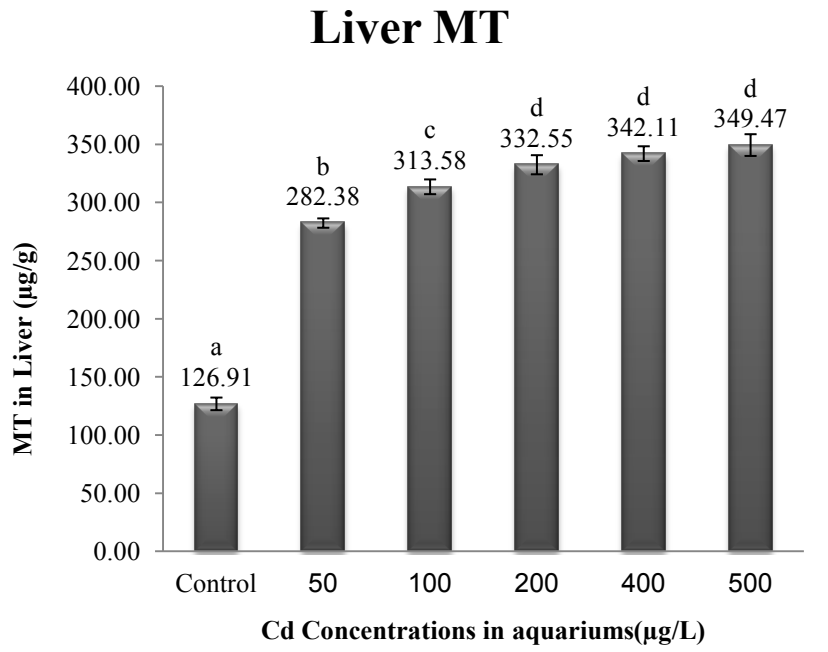

Figure 4 MT in fish liver according to exposure to Cd in different concentrations (µg/L) with standard errors after 60 days. Different superscript letters indicate significant differences ($p < 0.05$).

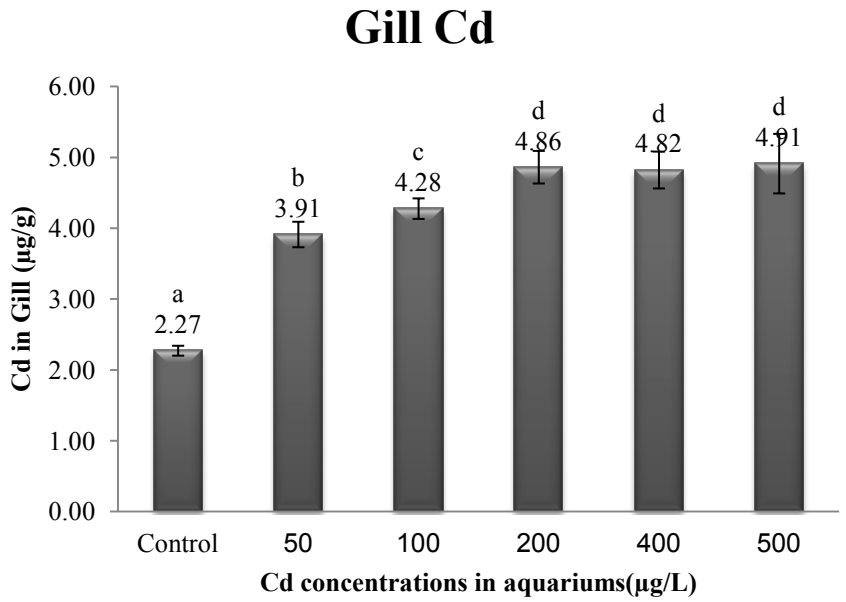

Figure 5 Cd in fish gill according to exposure to Cd in different concentrations (µg/L) with standard errors after 60 days. Different superscript letters indicate significant differences ($p < 0.05$).

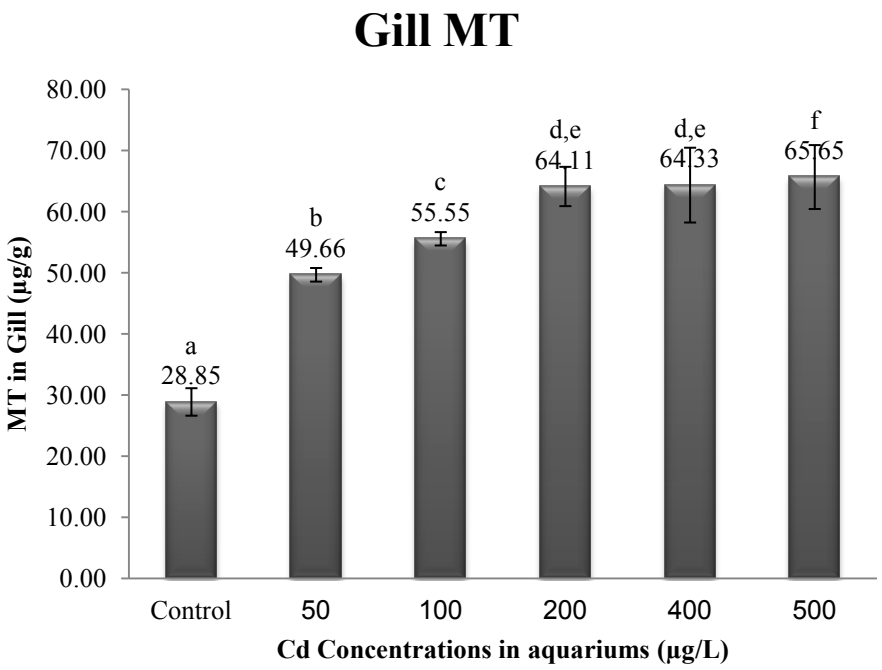

Figure 6 MT in fish gill according to exposure to Cd in different concentrations (µg/L) with standard errors after 60 days. Different superscript letters indicate significant differences ($p < 0.05$).

Correlation between Cd and MTs levels

Correlation between Cd content and MT in all analysed tissues of Java medaka fish (muscle, gill and liver) was statistically significant. The correlation was positive; increasing the Cd content in body sections, the MT increased also ($p < 0.01$) (**Table 2**).

Table 2 Results of Pearson's Correlation Analysis, relationships between Cd concentration in different tissue of *Java medaka* and MTs levels.

	Liver-MT	Gill-MT	Muscle-MT	Liver- Cd	Gill-Cd	Muscle-Cd
Liver-MT	*1*					
Gill-MT	*.983***	*1*				
Muscle-MT	*.996***	*.989***	*1*			
Liver- Cd	*.982***	*.994***	*.982***	*1*		
Gill-Cd	*.987***	*.995***	*.990***	*.987***	*1*	
Muscle-Cd	*.984***	*.989***	*.994***	*.971***	*.989***	*1*

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

Discussion

Results showed a gradual enhancement of Cd content in muscle with concentration of Cd in water. Muscle is a kind of tissue which is extrinsic to the target organs for Cd accumulation and for MT production [8]. In this study, MT levels had positive correlation with concentration of Cd in different body sections of fish. In liver and gill, the situation was more sensible because of accumulation of this metal and because of enhancement of MT synthesis in these tissues. The highest content of Cd in liver (13.09±0.85µg/g) was found in the group exposed to 500 µg/g Cd. It has been reported that the first target organ for Cd after exposure of Cd salt in water is the liver [11].

Exon *et al*. [12] reported that, under lower concentrations of Cd, higher concentrations of Cd were found in kidneys than in livers. Exposure to higher concentrations of Cd resulted in similar accumulation in liver and kidney tissue during the first several hours or days of the exposure. After longer time of exposure, the ratio changed, where higher Cd levels were found in kidneys [12]. As mentioned above, Cd may bind to MT, displacing Zn. Cd presence inside a cell also may induce synthesis of new MT, and bind to it. Cd-MT complexes formed in such a way may be transported to the kidneys. Metal binding capacity depends on initial MT level, and on intensity of further MT synthesis. The most intense MT synthesis was usually observed in liver. Hepatic MT concentrations in fish showed marked elevations in proportion to the metal concentrations lower than 10 mg/L. Intensity of MT synthesis is, thus, tissue-specific, concentration and time-dependent [13]. The ability of fish species to synthesize MT is also different among them. A difference between red-blooded and white-blooded Antarctic teleost was demonstrated.

There, the highest MT contents were found in liver (acute study of 60 min of exposure to high Cd concentration), and hepatic concentration of Cd, Cu and Zn and MT showed positive correlation in red-blooded teleosts (*Trematomus bernacchii*) but not in white-blooded teleosts (*Chionodraco hamatus*) [13]. Sex-differences in the synthesis of MT were demonstrated too. The levels of hepatic MT in the male dab, *Limanda limanda*, were better correlated with Cd exposure in the mixture with Cu than in females [14]. The age of fish is other thing that needs to be correlated. It is not surprising that the hepatic and kidney Cd levels increase with the age of fish, due to the gathering of various metals. Similar behaviour was shown in MT application. In accordance, Filipović Marijić and Raspor (2006) reported an age-related increase in Cd and MT levels in kidney and Cd in liver of Mullus barbatus. This is reliable with the

findings that both Cd and MT concentration in fish body sections increase under conditions of chronic exposure to this ion [15].

Symptoms of Cu and Cd effects show that, in low concentrations, fish had unusual swimming behaviour, and in high concentrations they had increased activity and irritability, imbalance, skin colour, formation of mucus on skin, vertical swimming, slipped eye, formation of blood spots around eye and under stomach, gill hyperaemia, and curvature of the spine at the end of the 60 day experiment. In this study, MT in liver section showed high levels (above 349 μg/g) in fish exposed to 500 μg/g of Cd and low levels (below 135 μg/g) in the control group of fishes exposed to Cd. Similar trends could be seen in MT in gill, although the stability of MT content in fish groups exposed to higher Cd concentration was noticeable. This finding could confirm the meaning that synthesis of MT and binding capacity of these proteins is restricted [16]. Furthermore, Dallinger *et al.* [16] observed that MT isolated from livers and kidneys contained mainly Cd, and thus displaced Zn and Cu. Moreover, they observed that MT induction usually needs some time to develop and die down after a certain time of experience [9].

It is obvious that MT can detoxify certain concentrations of Cd only. Cd ions, which cannot be bound by MT, interact with high molecular mass proteins. This phenomenon may result in toxic effects. Evidently, the Cd portion not bound in MT results in Cd toxicity [17]. Very similar results were presented in another study [6], in which it was shown that most of the Cd (60 %) was located in the heat stable cytosolic component, probably bound by MT, protecting the liver from Cd toxicity. In another study [18], the authors paid their attention to Cd distribution in yellow perch (*Perca flavescens*) liver. Perches were placed under several natural long-term exposure of water-born Cd. They showed that fish hepatic cellular components, fractionated by differential centrifugation, sequestered Cd in constant ratios. Cd was bound by both Cd-sensitive and resistant cellular components. Bio-accumulated amounts were in correlation with the exposure intensity (**Table 3**).

It was demonstrated that induction of MT synthesis by Cd depends on metal uptake by the fish. Metal uptake in fish involves gills, intestine and skin, but the virtual amount of these ways varies, depending somewhat on the chemical and physical features of water and sediments [19]. 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 [18] and other permeable body surfaces. Metals bound to solid particles are ingested and detached from their carrier particles in the digestive system and absorbed through the gut epithelium [20]. The experimental method for Cd disclosure as compared to the pharmacological injection method showed that absorption of Cd by liver was much more competent (17 - 18 %) than through the physiological intake method (0.32 - 0.44 %) in the sentinel fish (*Lithognathus mormyrus*) [21].

It is shown that MT has an important role in Cd detoxification in fish. Level of Cd was closely related to the increase of MT level. Correlation between Cd and Zn content and MT concentration in liver, gill and muscle tissue were positive, and this fact was statistically significant in most of the groups exposed to Cd. The same values were reported in the study of fish, where Cd was administered to the water for 29 days in 4 concentrations (0, 0.8, 4 and 20 μM). It was reported that Cd accumulation in tissue followed the order: kidney > liver > gills. Concentrations of Cd binding metallothioneins (Cd-MTs) were in the following order: liver > kidney > gills [22]. The results of the present study show that Cd accumulation in medaka fish tissue were in the following order: liver > gill > muscle for Cd, and concentration of MT binding with Cd were in the following order: liver > gill > muscle, for fish groups exposed to Cd concentrations. However, in another study [23] the authors showed that accumulation capacity of every single organ depends on other metals in water. Common carps coexposed to Cd, mercury and lead had the amounts of Cd in this order: kidney > gills > liver > muscle, and MT were in order: gills > kidney > liver > muscle. The reasoning of these results should be investigated in a further study.

Table 3 Different levels of MT in different aquatic organism.

Organism	Organel	Determined range	Reference
Liza aurata	Liver Kidney Brain	2.32±0.35 mg/g 1.56±0.21 mg/g 1.34±0.24 mg/g	[26]
Mullus surmuletus	Liver Kidney Brain	2.11±1.13 mg/g 3.41±0.89 mg/g 1.36±0.15 mg/g	[26]
Anguilla Anguilla	Liver	280 - 2580 µg/g	[27]
Brown trout	Liver	110 µg/g	[28]
European eel	Kidney Liver	13 µg/g 100 - 180 µg/g	[25]
Eel Anguilla Anguilla	Liver	350 - 3500 µg/g	[29]
Common carp	Liver	15 nmol SH/mg	[30]
Sparus auratus	Liver, gill	50 - 450 µg/g	[10]
Seriola dumerilli	Different parts	200 - 1800 ng/g	[16]
Salmo salar	Different parts	120 - 168 µg/g	[22]
Sparus auratus	Different orgalels	22.04 - 399.53 µg/g	[10]
zebra mussel	Different parts	69.33 - 346.329 µg/g	[31]
Oryzias Javanicus (Exposed to Cd)	Different parts	32.75 - 349.42 µg/g	This study

Conclusions

The results show that Cd accumulation in medaka fish tissue were in the following order: liver > gill > muscle for both Cd, and concentration of MT binding with Cd were in the following order: liver > gill > muscle, for fish groups exposed to Cd. In this study, the results of other authors [24], who concluded that the concentration of cellular stress proteins (including MT) is a good indicator of water pollution, have been confirmed. However, it was reported that MT level was a good bioindicator of heavy metals pollution in *Salmo trutta*, but not in *Anguilla anguilla* [25]. Therefore, it could be concluded that not every fish species is suitable for biomonitoring. It has been demonstrated herein that the Java medaka fish (*Oryzias javanicus*) could be considered as a specimen of fish suitable for this purpose. This study of MT as biomarker suggested that there was a connection between some heavy metal contamination and MT production in Java medaka fish (*Oryzias javanicus*). Also, the higher heavy metal concentrations and MT levels approved the role of MT in metal homeostasis and detoxification. Therefore, MT content can be used as an effective biomarker for metal stress through *Oryzias javanicus*.

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