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Time kinetic study of metallothionein mRNA expression due to cadmium exposure in freshwater murrel, *Channa punctata* (Bloch)

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Abstract: *Metallothionein (MT) is a low molecular weight, cysteine-rich protein whose cysteinyl residues are involved in the metal coordination. MT induction and the associated increase in binding of metals by MT have been used as prima facie evidence for functions in metal detoxification, sequestration and storage. A quantitative real-time reverse-transcriptase polymerase chain reaction (q-RT-PCR) study was carried out for 14 days using freshwater murrel, Channa punctata (Bloch). After exposure to 3.74 mg L^{-1} waterborne cadmium, the MT gene was over expressed in liver during the first 8 hours of contamination but repressed from day 1 to 14. In contaminated kidney, this gene was repressed the first day and up-regulated on day 14. In contaminated gills, the MT gene expression remained at the basal level at all the time points. The study indicates that MT mRNA expression was found to be tissue specific with respect to duration of the metal exposure.*

Key Words: *Cadmium, Channa punctata, Metallothionein, Time kinetic study, Tissue specific.*

Introduction

Living organisms respond at the cellular level to a variety of stimuli as diverse as heat, heavy metals, anoxia, amino acid analogues, infection, and inflammatory agents by synthesizing different groups of highly conserved polypeptides (Hamer, 1986; Morimoto *et al.*, 1990). Out of these a group of induced proteins is represented by low-molecular-mass, cysteine-rich, heavy-metal-binding proteins known as metallothioneins (MTs). They lack aromatic amino acids, have a selective capacity to bind heavy-metal-ions *via* mercaptide linkages, and are ubiquitous in distribution (Olsson, 1993; Roesijadi, 1994; Bae *et al.*, 2005).

Metallothioneins are induced by a variety of agents, including heavy metals, endotoxins, UV radiation, cytokines, etc.- the most potent being heavy metals, and are encoded by a multigene family (Hamer, 1986; Gedamu and Zafarullah, 1993). MT gene transcription is induced by heavy metals through metal response elements (MRE) that are present in multiple copies in the proximal promoters of MT genes. MREs were shown to mediate transcriptional response of MT to zinc (Zn), cadmium (Cd), oxidative stress and to hypoxia (Murphy *et al.*, 1999; Andrews, 2000; Bourdineaud *et al.*, 2006).

They are capable of donating copper (Cu) and Zn to appropriate receptor molecules such as metalloenzymes (Brouwer and Brouwer-Hoexum, 1991) and transcriptional factors (Zeng *et al.*, 1991a, b), thus regulating metal-dependent activities through highly specific molecular interactions. With both essential and nonessential metals, binding to MT limits metal availability at inappropriate sites and is thereby believed to confer protection against toxicity. For proteins previously compromised by binding a toxic metal such as Cd, a rescue function, whereby ZnMT serves as a receptor of Cd and, in the case of Zn metalloproteins, donor of Zn has been proposed as a mechanism for restoring functional properties of these structures (Huang, 1993).

In fish, the expression and role of MT mRNA have mostly been studied in organs that play a central role in metal uptake and accumulation i.e., the liver, kidney and gills. It has also been shown that significant differences can appear in the expression and induction of MT among different fish species (De Boeck *et al.*, 2003) and among tissues within the same fish species (Olsson, 1993; Campenhout *et al.*, 2004). 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 (Viarengo *et al.*, 1999; Dragun *et al.*, 2009).

Cellular levels of specific transcripts or proteins are often measured in relation to a variety of biological events, using a selection of qualitative and quantitative methodologies. Evaluating biological effects of xenobiotics discharged into natural environments is no exception. It may be reasonably assumed that the expression of a broad selection of genes is affected by a variety of environmental stimuli. Therefore, changes of expression levels, measured in a native sentinel species may serve as biomarkers for the effect of present and future environmental perturbations. MT mRNA expression has often been proposed as a sensitive and efficient biomarker for evaluating the cumulative biological effects of metal exposure (Ceratto *et al.*, 2002; George *et al.*, 2004; Tom *et al.*, 2004).

Numerous findings are available that relate to the mechanism of regulation of metal-induced MT gene transcription in several fish species. Studies using Indian freshwater fishes as sentinels in aquatic environments are scanty. Thus, with this goal the present toxicogenomic study has been carried out using an Indian freshwater murrel, *Channa punctata* (Bloch), a fish species widely distributed in Indian freshwater system, as a model fish. The study deciphers the interaction of metallothionein with compromised target molecule and provides information on the duration dependent mRNA expression pattern as well as toxicological significance of metallothionein gene transcription under the influence of heavy metal, cadmium.

Materials and Methods

Experimental animals: The fish species selected for the present study was freshwater snakehead murrel, *Channa punctata* (Bloch, Family Channidae and Order Perciformes). Healthy specimens were procured from local sources. The specimens had an average wet weight and length of 21.34 ± 2.79 g and 12.05 ± 0.56 cm (mean ± S.E.), respectively. The specimens were given prophylactic treatment by bathing them in 0.05% (w/v) potassium permanganate ($KMnO_4$) solution for 2 min to avoid any dermal infections. The fishes were then acclimatized for 15 days under laboratory conditions prior to heavy metal exposure. The fishes were fed, *ad libitum,* with boiled chicken eggs, goat liver and poultry waste material. No mortality was occurred during the acclimation period.

Test chemical: For the present study, analytical-grade cadmium chloride ($CdCl_2.H_2O$) (98%), manufactured by Himedia Lab. Ltd., Mumbai, India was used as the test compound (as a source of heavy metal, cadmium).

Determination of sub lethal concentration: The acute toxicity bioassay to determine the 96 h median lethal concentration ($^{96}LC_{50}$) of cadmium was conducted in the semi-static system with the change of test water on every 24 h, following standard methods (APHA *et al.*, 2005). The 96 h LC_{50} value of Cd was determined as 14.95 mg L^{-1} by using SPSS computer statistical software (SPSS 16.0.2, 2008). Based on the LC_{50} value, the single sub lethal concentration of cadmium (1/4th of 96 h LC_{50} = ~3.74 mg L^{-1}) was determined for time kinetic study of MT mRNA expression, since the study aimed to evaluate the duration dependent MT mRNA expression.

In vivo exposure experiment: The fish specimens were exposed to the aforementioned

Table 1: Primer sequences used in RT-PCR for the amplification of Metallothionein and Beta actin mRNA

Primer	Sequence (5'->3')	Length (bp)	TM (°C)	GC %	Reference (GenBank Accession No.)
MT- F' Forward	CTGCAACTGCGGAGGA	16	57.92	62.50	FJ869867
MT-2 R' Reverse	GGTGTCGCATGTCTTTCCTT	20	60.12	50.00	
β-actin F' Forward	GTGCCCATCTACGAGGGTTA	20	59.96	55.00	GQ219743
β-actin R' Reverse	AAGGAAGGAAGGCTGGAAGA	20	60.32	50.00	

FJ869867 C. punctata MT, 559 bp DNA; GQ219743 C. orientalis Beta-actin 314 bp, mRNA

Table 2 . Metal concentration in the test water at various time points

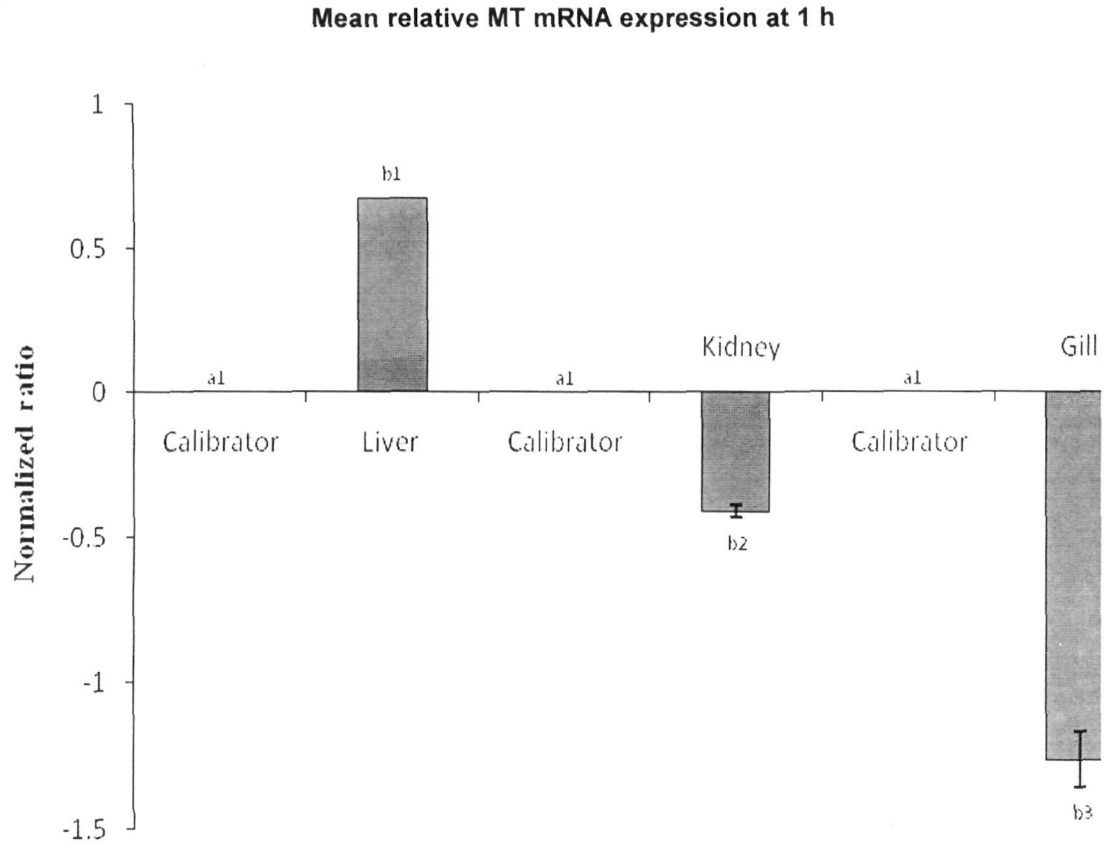
Time Point	CD Concentration (mg L^{-1})		Decrease in Concentration (%)
	Dissolved	Measured (Mean)	
o h	3.74	3.48	6.95
1 h		3.45	7.75
2 h		3.41	8.82
4 h		3.38	9.63
8 h		3.21	14.17
24 h		2.91	22.19
72 h		2.81	24.87
14 d		2.91	22.19

FJ869867 *C. punctata* MT, 559 bp DNA; GQ219743 *C. orientalis* Beta-actin 314 bp, mRNA

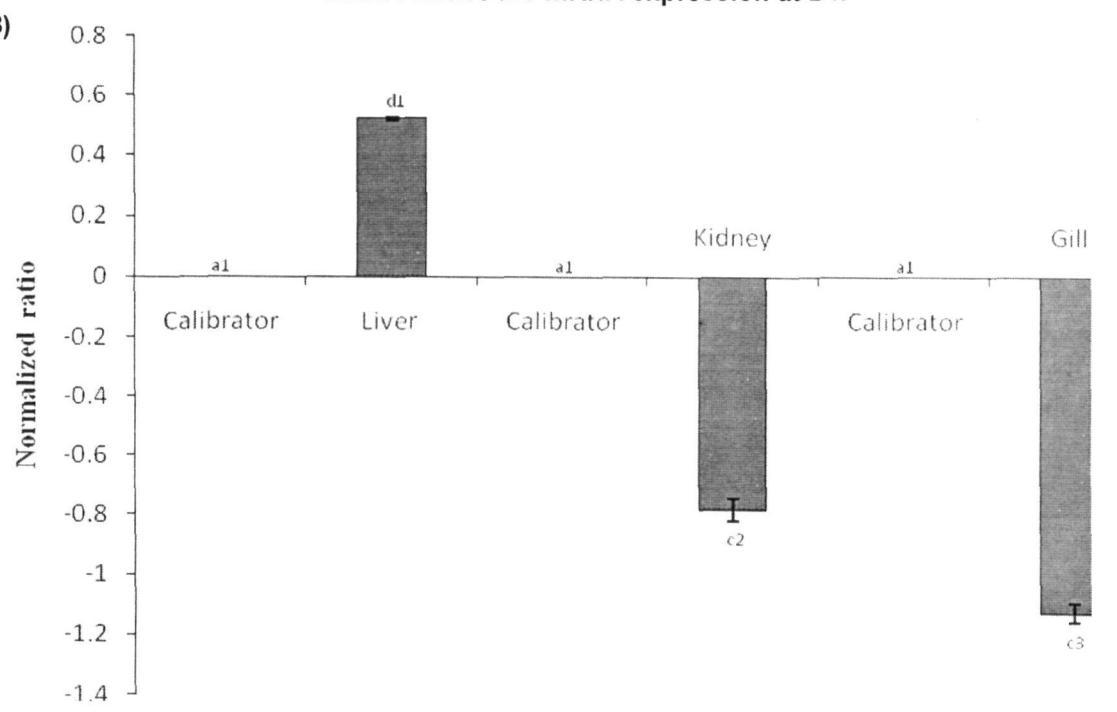
test concentration of the waterborne Cd in the semi-static system. The exposure was continued up to 14 days and tissue sampling was done at eight time points- t_1= 0 h, t_2= 1 h, t_3= 2 h, t_4= 4 h, t_5= 8 h, t_6= 24 h, t_7= 72 h, and t_8= 14 d at the rate of five fish per duration (n=5). The fish maintained in tap water without the test chemical (control group) were considered as calibrator (t_1= 0 h) for the time-course study. During experiment, the test solution was changed after every 24 h to maintain the appropriate concentration of the metal in the test aquaria. The physico-chemical properties of test water were determined according to the standard procedures (APHA *et al.*, 2005).

At each sampling time point, the liver, kidney, and gill tissues were taken from each individual. The tissue sample were placed in sterile tubes and immediately stored in RNA*later* RNA Stabilization Reagent (Qiagen GmbH, Germany) following the manufacturer's instructions, for

(1A)

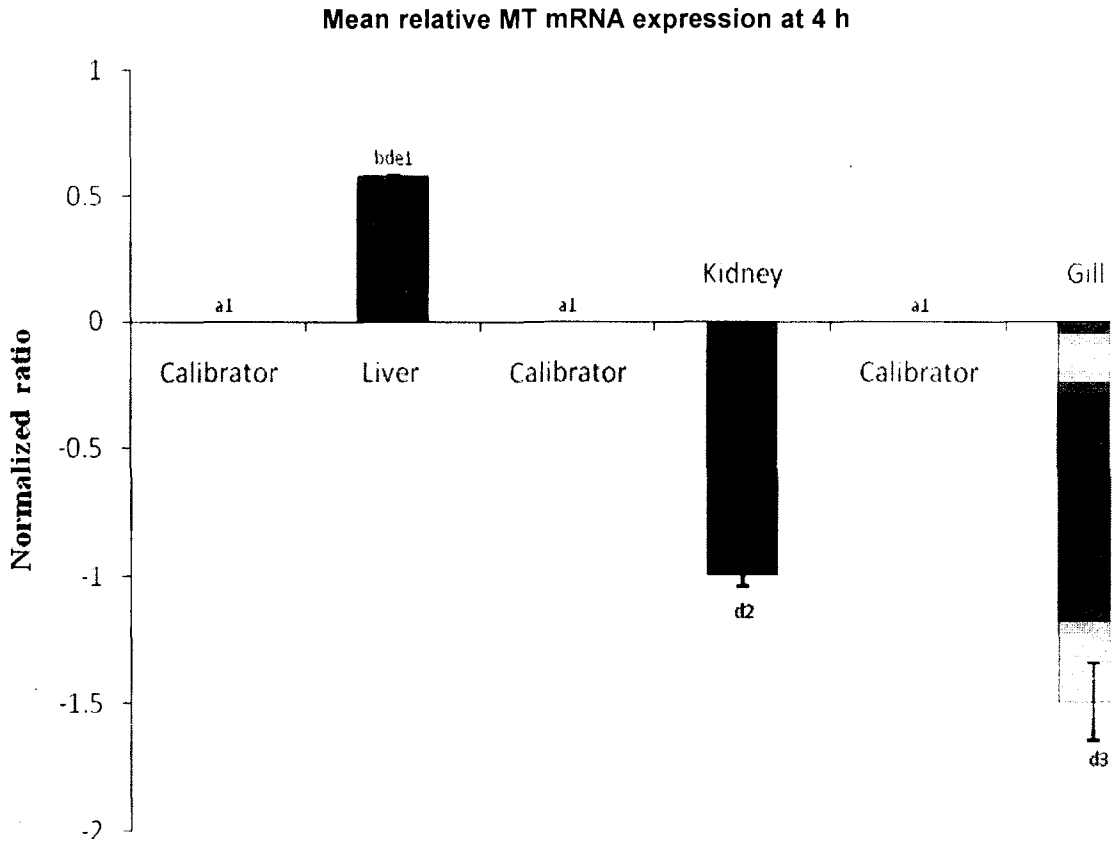

Mean relative MT mRNA expression at 1 h
Normalized ratio
1
0.5
0
-0.5
-1
-1.5
b1
a1
a1
a1
b2
b3
Calibrator
Liver
Calibrator
Kidney
Calibrator
Gill

(1B)

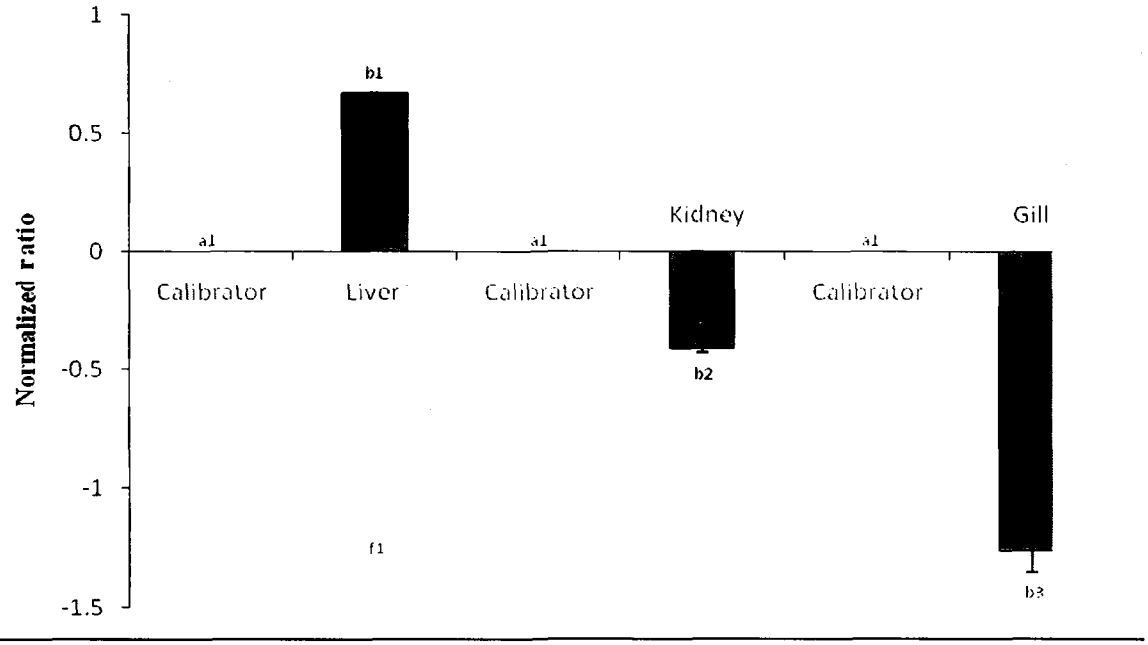

Mean relative MT mRNA expression at 2 h
Normalized ratio
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
-1.4
d1
a1
a1
a1
c2
c3
Calibrator
Liver
Calibrator
Kidney
Calibrator
Gill

Time kinetic study of metallothionein mRNA expression due to cadmium exposure

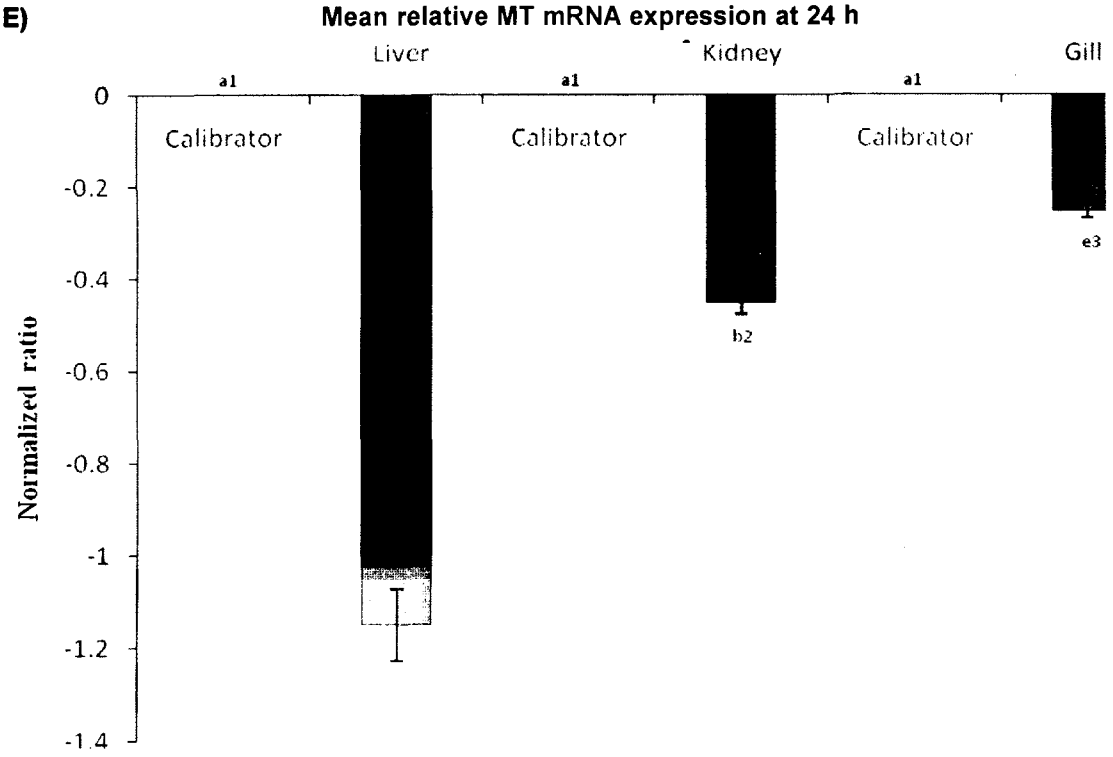
(1 C)



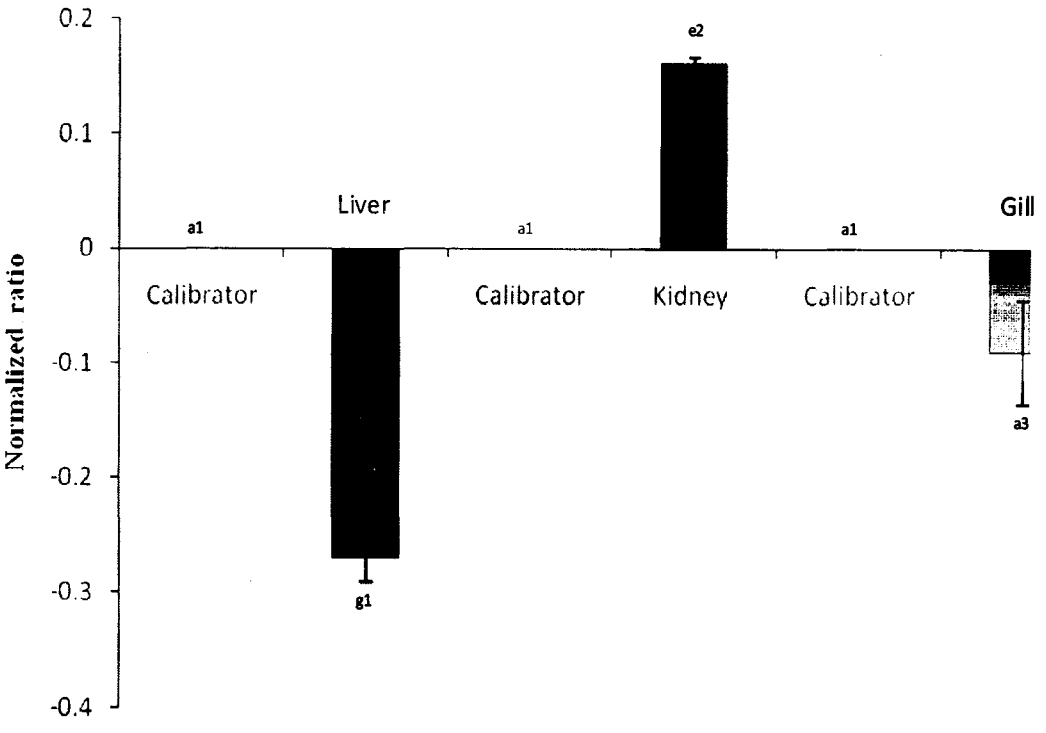
(1 D)



(1 E)


Mean relative MT mRNA expression at 24 h
Liver
Kidney
Gill
a1
a1
a1
0
-0.2
-0.4
-0.6
-0.8
-1
-1.2
-1.4
Calibrator
Calibrator
Calibrator
e3
b2
Normalized ratio

(1 F)


Mean relative MT mRNA expression at 72 h
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
e2
Liver
Gill
a1
a1
a1
Calibrator
Calibrator
Kidney
Calibrator
g1
a3
Normalized ratio

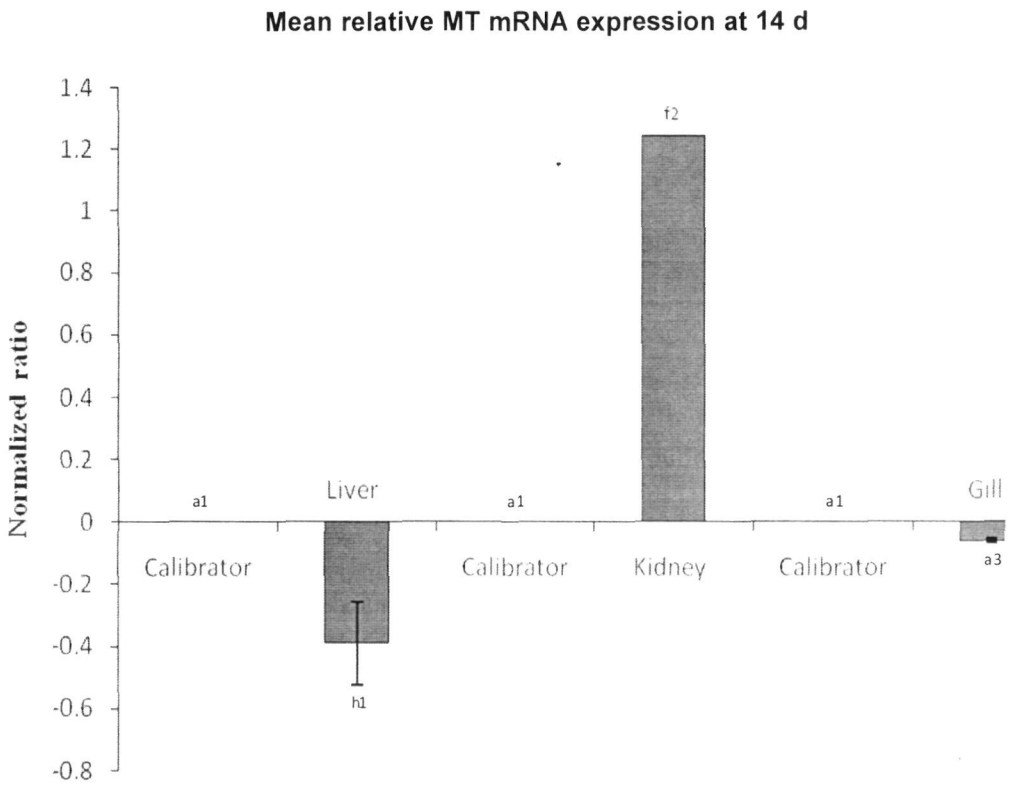


Fig.1. Mean relative [log 2^ (-ΔΔ"C_T)] metallothionein mRNA expression (± S. D.) in liver, kidney, and gill tissues of *C. punctata* (n = 5) after exposure to Cd for 1 h, **A**; 2 h, **B**; 4 h, **C**; 8 h, **D**; 24 h, **E**; 72 h, **F**; 14 d, **G**. Calibrator refers to the relative expression (ΔC_T) in control sample (t_1= 0 h).
Different alphabet superscript letters denote significant difference ($P < 0.01$) in mRNA expression between time points within tissues. Different numeric superscript values denote significant difference in mRNA expression ($P < 0.01$) between tissues within time point

further RNA extraction.

Primers for the real time amplification of MT cDNA: The quantitative real-time reverse-transcriptase polymerase chain reaction (q-RT-PCR) primers (MT-2 F', forward; MT-2 R', reverse) for the real-time PCR amplifications of MT were designed on the basis of the sequence information of *C. punctata* MT gene (GenBank Accession No. **FJ869867**).

Beta-actin (â-actin) was used as an internal control (housekeeping gene) to normalize mRNA levels in the real-time PCR amplification study. The sequence information of â-actin mRNA in *C. orientalis* (GenBank Accession No. **GQ219743**) was used for the designing of primers (â-actin F', forward; â -actin R', reverse) (Table 1).

Sequence information was submitted to Operon (Operon Biotechnologies GmbH, Germany) for primer production. Melt curve and primer efficiency analyses were conducted for each primer for quality control purposes. PCR product generated by each primer was electrophoresed on an agarose gel to ensure that amplicons of the correct size were being obtained.

Total RNA extraction: Total RNA was isolated from each sample using acid guanidinium isothiocyanate-phenol-chloroform extraction

following the TRI Reagent extraction protocol (Molecular Research Center, Inc. Cincinnati, OH) (Chomczynski and Sacchi, 1987). For quality control purposes, samples were quantified spectrophotometrically at 260 and 280 nm (PowerWave XS Microplate Spectrophotometer, Bio Tek Instruments, Inc., USA). Only samples with absorbance260 to absorbance280 ratios (A_{260}/A_{280}) greater than 1.7 were used in subsequent analyses.

RNA integrity was checked by denaturating formaldehyde agarose (FA) gel electrophoresis with MOPS (3[N-morpholino] propanesulfonic acid, 200 mM; sodium acetate, 20 mM; ethylenediaminetetraacetic acid, 10 mM) buffer (Sambrook and Russell, 2001). Visual inspection of the gels showed no signs of RNA degradation in any of the samples.

Expression of MT mRNA: q-RT-PCR amplifications were carried out using the QuantiTect SYBR Green RT-PCR kit according to the manufacturer's instructions (Qiagen). Reaction mixture included 25 µl of kit MasterMix (contains nucleotides, buffer, *Taq* enzyme, $MgCl_2$, and SYBR Green I dye), 1 µl of 50 µM gene specific primer and 50 ng total RNA. The mixture was placed in a LightCycler real-time PCR (LightCycler 480 System, Roche Diagnostics GmbH, Roche Applied Science, Mannheim, Germany) and incubated at 50 °C (30 min), 95 °C (15 min), 94 °C (15 s), 56 °C (30 s) and 72 °C (30 s) to reverse transcription, PCR initial activation, denature the cDNA, anneal the primers and extend the product, respectively. Reactions were carried out for a total of 45 cycles. All amplifications were performed in triplicates.

Fluorescence was plotted against cycle number, generating a curve for each sample showing increasing fluorescence with cycle number using computer software (provided with LightCycler RT-PCR System). Depending on the amount of gene-specific cDNA in each sample, it took a particular number of cycles to reach the threshold level of fluorescence (C_T value). For the q-RT-PCR data analysis a method of comparison of C_T values, the $2^{\wedge} (- \Delta \Delta "C_T)$ method, was used (Livak and Schmittgen, 2001).

Cadmium analysis: At all time points, cadmium level was analyzed using an atomic absorption spectrophotometer (AAnalyst 300 Spectrometer, Perkin Elmer, USA). Three replicate samples were analyzed to obtain an average cadmium concentration at each sampling. The sample digestion and analysis was done following the standard methods (APHA *et al.*, 2005).

Data analysis: Statistical analyses of MT mRNA expression data were performed using SPSS computer software. To achieve homogeneity of variance, all gene expression data [$2^{\wedge} (-\Delta \Delta "C_T)$ values] were log transformed. Comparisons between the effects of exposure duration on mean relative tissue specific mRNA expression was done using multiple comparison technique, Tukey's HSD post hoc test (á = 0.01).

Results and Discussion

Fish MTs attracted special attention in the past 10-15 years because of increasing pollution of the aquatic environment with technogenic xenobiotics (in particular, heavy metals). The transcript level of MTs is usually determined by various PCR based methods. In this study, we examined tissue specific time kinetics of *C. punctata* MT mRNA expression in adaptation to environmental factor, with emphasis on the Cd in water using q-RT-PCR.

During the experiment temperature of the test water varied from 19.3 to 22.5 °C, and pH values ranged from 7.14 to 7.95. The dissolved oxygen concentration was normal, varying from 6.72 to 8.13 mg L^{-1}, during the experiment. The conductivity of the test water ranged from 239-303 µS cm^{-1}. The total hardness and total alkalinity of the test water varied from 169 to 198, and 242 to 278 mg L^{-1} as $CaCO_3$, respectively during experimental period.

The measured concentration of cadmium in the test water using AAS at each time point (t_1= 0 h,

t_2= 1 h, t_3= 2 h, t_4= 4 h, t_5= 8 h, t_6= 24 h, t_7= 72 h, and t_8= 14 d) is shown in Table 2. The measured concentration of waterborne Cd was found to be slightly lesser in comparison to that of the dissolved at all time points, and showed decrease in level from 6.95 to 24.87% in the 0-14 d time course.

Decrease in metal concentration in the test water was found to be directly proportional to duration. The decrease in the concentration after metal analysis may be attributed to the precipitation of the metal in test water as well as the hardness and relative higher pH of test solution causing lesser solubility of Cd (Playle *et al.*, 1993; Adhikari, 2003).

The varied MT mRNA expression pattern was observed according to the tissues viz. liver, kidney, and gill in the time course study for 14 days after waterborne exposure to 3.74 mg L^{-1} cadmium in *C. punctata* (Fig. 1A-G). In the time kinetic study the MT transcript level was found in the following order: liver > kidney > gill, in the early exposure phase (up to 8 h) due to waterborne Cd uptake (Fig. 1A-D). As the exposure increases with time, the transcript level was found in the order: kidney > gill > liver, in the late exposure phase (post 8 h exposure) (Fig. 1F, G). Liver shows biphasic reaction as the up regulation in MT transcript level was observed up to 8 h only, whereas kidney and gill have the same below basal level, and seems to be the major site for metal uptake in an initial defence function in post 8 h exposure no significant transcript level was observed. It appears that liver Cd-MT complex are either degraded post 8 h of Cd exposure or are transported elsewhere to different organs.

Twenty four hours after cadmium exposure MT mRNA is observed below the basal level in liver, kidney and gill of *C. punctata*. From 72 h kidney shows elevated MT mRNA expression, before this at all time points it was below basal level. Since MT gene up-regulation was not seen in kidney at any time point of our study except on day 3 and 14, we presume Cd-MT complex may have migrated to kidney by day 3rd, which is obsered by elevated expression of mRNA.

The weakest MT mRNA expression was found in gill tissues, where at all the time points it was below basal level. Although induction of MT in gill and kidney has been reported in fishes, liver seems to be more actively involved in the formation and storage of MT as compared to other organs (Marr *et al.*, 1996). It may be co-related to the fact that in comparison to the liver and kidney relatively little Cd accumulated in the gills. The similar transcript level order was also found by several other authors and may be attributed to the lower metal-binding capacity of the gills as a consequence of the low gill MT mRNA present (Cattani *et al.*, 1996; De Smet and Blust, 2001; Lange *et al.*, 2002).

The initial up regulation in liver MT mRNA level attests liver as a potent organ for Cd detoxification in the early exposure phase in *C. punctata*. It is in accordance with previous studies revealing that Cd treatment causes induction of MT-like protein (MTLP) in liver (De Smet *et al.*, 2001; Atif *et al.*, 2005, 2006). MT induction has been described to play a regulatory role, which is mainly aimed at detoxification (Roesijadi *et al.*, 2009). Liver has been shown as the prime induction site for MT mRNA in fish exposed to paper mill effluent (Ahmad *et al.*, 2000), and copper (Parvez *et al.*, 2003). The differences in the level of MT mRNA in the different tissues of the test fish can primarily be ascribed to the differences in the physiological role of each tissue (Karuppasamy, 2004). Regulatory ability and functions are also other factors that could influence the expression pattern in the different tissues (Murugan *et al.*, 2008).

Liver MT mRNA was found to be significantly heightened with 0.67, 0.37, 0.52, and 0.58 (logarithm values) or around 5-, 2-, 3-, and 4-fold induction level, after 1, 2, 4, and 8 h cadmium exposure, respectively ($P < 0.01$). It diminishes significantly after a 1 h exposure ($P < 0.01$). Post 2 h Cd exposure liver showed significant up regulation of MT transcript level. Non significant up regulation in transcript level

was found on 8 h in comparison to that of 4 h post Cd exposure.

Post 24 h Cd exposure elevation in MT mRNA level was observed in kidney and transcript level was found linearly related with the duration of exposure in this tissue and it was found to be significantly up regulated in time dependent manner with 0.16 and 1.24 (logarithm values) or around 2-, and 18-fold induction level post day 3 and 14 cadmium exposure, respectively ($P < 0.01$).

On day 14 the MT mRNA level was found to be heightened with > 7 fold expression relative to that of on day 3 (Fig. 1F,G). After Cd absorption by the gills, the metal is probably transferred to the liver where it induces the synthesis of MT, which in turn is delivered to the blood and finally accumulated in the kidney, as previously demonstrated (Webb, 1987). Kidney responds to Cd by both reabsorbing circulatory Cd-MT mRNA complex that has been released from the liver and gut, and filtered into the renal tubules, as well as by synthesizing renal MT for Cd storage (Zalups and Ahmad, 2003). It is likely that murrel also respond in a similar way, a relatively greater amount of Cd-MT complex released from extrarenal tissues reabsorbed in the kidney and more renal MT was synthesized (Chowdhury *et al.*, 2005). After complete saturation of the total Cd binding sites in hepatic MT, the new incoming metal is subsequently taken up by the kidney through blood circulation. Inter-tissue differences in the MT mRNA expression level reflect capability of the MT present to bind Cd.

Piscine MT mRNA level seems to be variable in a tissue specific manner according to species. De Smet *et al.* (2001) reported first induction of MT in kidney in spite of gill and liver after waterborne exposure of Cd in common carp. Whereas in rainbow trout kept for four months in water with a Cd concentration of 200 ìg L^{-1}, the increased MT level being recorded in the liver after one month, in the kidney after three months, and in the gill after four months (Olsson *et al.*, 1989). In gilthead sea bream exposed to waterborne Cu, the highest MT mRNA concentrations were found in the liver followed by gills and kidney, indicating that the liver is the first organ where MT induction takes place (Isani *et al.*, 2003).

The metal sequestering function of MT protein that has been shown to be involved in essential metal homeostasis and in the detoxification of heavy metals is performed by the formation of a metal-metallothionein complex (Roesijadi, 1996). Metals induce MT by displacing zinc from intracellular binding sites, makes additional Zn available for interacting with the inhibitor (Palmiter, 1994). Increased metal influx result in induction of thionein, mediated by displaced Zn, and increased metal flux to MTs. Metals-induced MT synthesis occurs through the presence of metal regulatory elements (MREs) present in the promoter regions of MT genes. It is believed that metals-induced MT synthesis is initiated by the interaction of a metal with a positively-acting metal transcription factor (MTF). These transcription factors then bind with the MREs, initiating transcription, and the production of metallothionein-specific mRNA. MTs have also been shown to have antioxidative regulatory elements (AREs) and can be induced under oxidative stress conditions (Roesijadi, 1996).

An understanding of the relationships between MT mRNA expression and exposure to exogenous stresses will undoubtedly contribute to improved strategies for the use of MT in assessing aquatic organismal health leading to biomarker approach for the environmental bio-monitoring. For this to be achieved there needs to be a better understanding of MT function and the dynamics of MT mRNA induction.

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