Metallothionein as a Biomarker to Assess the Effects of Pollution on Indian Major Carp Species from Wastewater-Fed Fishponds of East Calcutta Wetlands (a Ramsar Site)

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Metals are one of the major chemical toxicants that can perturb environmental homogeneity by their prolonged persistence and complex interactions. Bioaccumulation of any metal above its threshold level invariably results in stress often leading to irreversible physiological conditions. The present investigation was carried out to study the potential stress that fish species are facing in wastewater-fed (contaminated) fishponds in East Calcutta Wetlands (ECWs), manifested in total protein and metallothionein (MT) concentrations. Indian major carps (IMCs) – rohu (*Labeo rohita*), katla (*Catla catla*) and mrigel (*Cirrhinus mrigala*) were used as suitable fish models. Total protein concentration of different fish tissues were found to be always lower in wastewater-fed fishponds when compared to fish tissues from freshwater-fed (uncontaminated) fishponds, while an indirect enzyme-linked immunosorbent assay (ELISA) revealed a higher expression of MT in all the fish tissues collected from wastewater-fed fishponds. Major significances drawn from the present study were that fish species cultivated in east Calcutta wetland, with its ecosystem being under a potential threat of contamination and stress induced by composite effluents, could lead to adverse physiological conditions. Moreover, these findings could be important in terms of designing biomarkers for an early environmental warning system and also for monitoring fish health.

Key words: *metallothionein, heavy metals, East Calcutta Wetlands, biomonitoring, Indian major carps.*

1. Introduction

Urbanization and industrialization have persistently contaminated most of the natural environment, and consequently major cities around the globe are under a budding threat of pollution. Wetland ecosystems are the most productive and diverse ecosystems with multifarious functions but are fragile in nature and indiscriminate anthropogenic activities have resulted in the conversion of wetlands worldwide. Wetland ecosystems that receive composite effluents tinted with heavy metals are of prime environmental concern especially for the biotic components that interact with the contaminated aquatic environment (Abdelmeguid et al. 2002; Wong 2003; Raychaudhuri et al. 2008). In the eastern fringe of city Calcutta (recently renamed as Kolkata) there lies East Calcutta Wetland, a Ramsar site (No. 1208) in West Bengal, India (lat 22°33′ - 22°40′N, long 88°25′ - 88°35′ E), the biggest urban wetland ecosystem covering around 12,771 ha area. This wetland receives untreated industrial run-off from almost 6000 large- and small-scale industrial establishments (including tanneries) along with Calcutta metropolitan's (12 million inhabitants) domestic sewage throughout the year. Nearly 50,000

m^3 of contaminated composite effluent traverses through a web of canals into the wetlands each day that has been effectively utilized by local fisherman for better yield in pisciculture in an informal indigenous method (Ghosh 2004). Spread over 3,832.27 ha (over 30% of the total wetland area) are 286 large wastewater-fed fish ponds (locally known as *Bheris*), producing nearly 10,915 metric tons of fish annually (Chattopadhyay et al. 2002). Thus cultivated fish species along with other biota thriving in and around ECW ecosystems are under potential threat of elemental contamination. Bioaccumulation of hazardous metals in different fish tissues cultivated in contaminated areas around the world is on record (Jana and Sahana 1988; Canli and Erdem 1994; Kalay and Canli 2000; Rauf et al. 2009) and previous findings of our research lab have shown a higher accumulation pattern of trace metals in tissues of cichlids (Chatterjee et al. 2006) and IMCs (Aich et al. 2011) from wastewater-fed fishponds of the East Calcutta Wetlands.

Stress caused by both bio-active and non-bio-active metals induces a variety of complex changes in fish physiology and the possible consequent physiological alterations are also on record (Sorensen 1991; Heath 1995; Cicik and Engin 2005; Farombi et al. 2007). Since metals are non-biodegradable in nature, the only possibility for biota to avoid metal poisoning is by reducing the metal uptake rate, chelating the metal with some ligand or by increasing the rate of excretion. Metallothionein (MT) is a ubiquitous, low molecular weight, cysteine-rich (>30%) protein that avidly binds various transition elements, especially group IB and IIB metals (Waalkes et al. 1984; Hamer 1986; Olsson et al. 1990). Although the precise functions of MT is not clear, the protein is generally regarded to be involved in intracellular homeostasis of Cu and Zn, enzyme activation, the acute phase response to infections, free radical scavenging and detoxification of metals (Karin 1985; Hamer 1986; Bremner and Beattie 1990; Roesijadi 1996). Since metallothionein is induced by metals it has been proposed as an excellent environmental biomarker (Linde-Arias et al. 2008).

The present investigation was carried out to assess the effects of pollution loads on different fish tissues of IMCs from wastewater-fed fishponds of ECWs manifested in total protein and metallothionein expression and to compare it to that of fish species collected from unpolluted control sites. Indian major carps (IMCs) – rohu (*Labeo rohita*), katla (*Catla catla*) and mrigel (*Cirrhinus mrigala*) were taken as test species because these are the most popularly cultured fish species in this part of the world due to their huge market demand. Also, the differential utilization of physical and trophic state by these species has been found to play an important role in differential metal accumulation from ECWs (Aich et al. 2011). Thus during the present investigation it has been also observed whether the differential accumulation pattern of trace metals could result in differential total protein and MT synthesis and expression in different fish tissues.
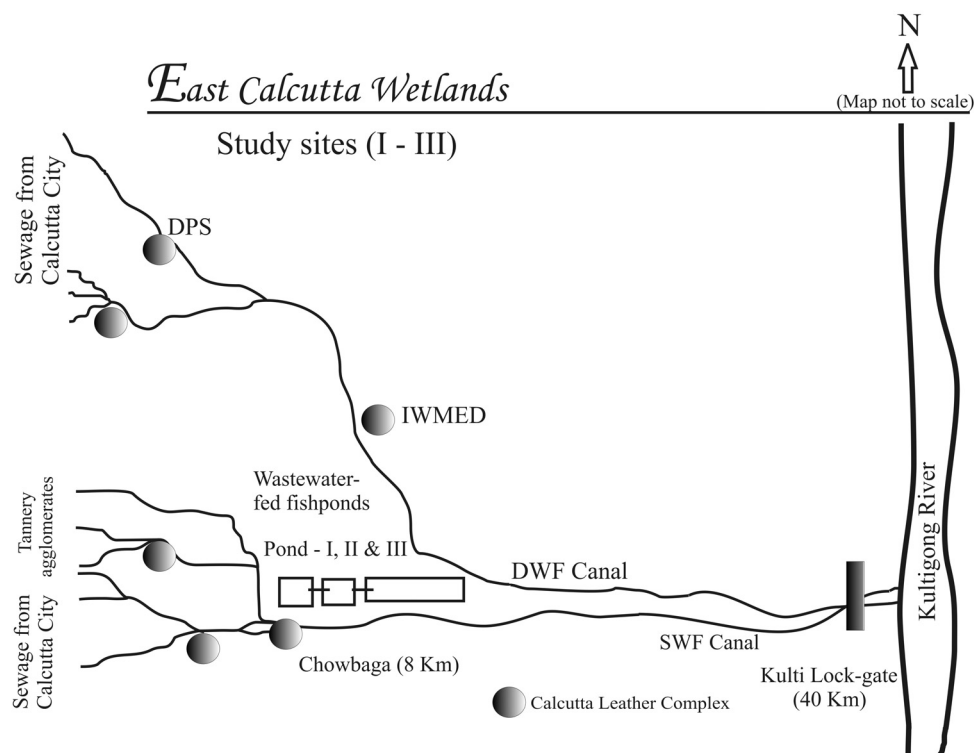

Fig. 1.   *Study sites showing major wastewater carrying canals in the East Calcutta Wetlands and the contaminated study sites (three wastewater-fed fishponds) for collection of abiotic and biotic samples*

2. Materials and methods

2.1. Study area

Samples were collected from three wastewater-fed fishponds located in and around the Chowbaga region, nearly 8 km downstream from the source of the Storm Weather Flow (SWF) canal in the ECW ecosystem (Fig. 1). Preference was given to that site as it was the merging site for municipal sewage with industrial wastewater making it altogether a composite effluent. This almost untreated raw wastewater has been effectively utilized by local fishermen in an informal, indigenous pattern for pisciculture.

2.2. Sample collection

Eighteen mature IMCs (six each of rohu, katla and mrigel) were collected from three wastewater-fed fishponds (2 individuals of 3 species from each pond). Yearlings of fish specimen of both sexes in equal proportion weighing 80 ± 20 g were collected and were anesthetized with MS222 immediately and later dissected for livers, gills, muscles and brains. Control specimens (six each of rohu, katla and mrigel) for comparison, weighing 75 ± 20 g, were similarly collected from three natural-water-fed fishponds, free from any effluent contamination, located at Ashoknagar, West Bengal, India (about 60 km west of the contaminated sampling sites). The total sampling was replicated for four times for avoiding possible sampling errors.

2.3. Measurement of metallothionein

The anesthetized fish specimens collected both from contaminated and uncontaminated sites were dissected for livers, gills, muscles and brains. Soluble tissue extracts were prepared by homogenizing the tissue with 35% w/v of homogenization buffer (50mm Tris-HCL, pH 7.4; containing .1 mM PMSF, 0.5mM DTT and 150mM NaCl) in a Teflon homogenizer at 1000 – 1200 rpm. The homogenates were centrifuged at 12,000 g for 40 minutes at 4° C. Supernatants were collected carefully and after suitable dilution with PBS (pH 7.4) (final concentration 20µg/mL) 100 µL of tissue homogenate were coated on the wall of a 96-well PVC micro titer plate, sealed and kept at 4° C overnight.

Standard ELISA protocol (Derango and Page 1996) was applied to measure the metallothionein content. Primary antibody against metallothionein (Ab 36882) and HRP tagged secondary antibody (Ab 6721) were purchased from Abcam, UK. TMB (SRL) was used as the substrate against HRP along with 5% H_2O_2. An automatic micro titer plate ELISA reader (LabLife-elite 96s Microplate ELISA reader, Wipro, India) was used to measure the absorbance at 650 nm. A second reading was also noted at 450 nm after addition of 2.0 M H_2SO_4 which stops the reaction. A standard curve was generated each time the assay was performed by metallothionein standard (Sigma M 7641, USA). Back ground, non-specific binding was assessed through negative control wells that received all the components except tissue homogenate. Hornitzky and Searson (1986) were followed to obtain the ELISA values which were plotted in the regression curve obtained from a standard curve to get the high precision metallothionein value.

2.4. Measurement of total protein content

The protein content of various tissue fractions was calculated after Lowry et al. (1951) using bovine serum albumin as standard and absorbance was recorded by using a UV-Vis spectrophotometer (Perkin Elmer Lambda 25).

2.5. Reagents used

All gravimetric analysis, reagents and standard preparation were performed using Mettler AE 240 monopan electronic balance and all the reagents used were of analytical grade and obtained from MERCK, Germany, if otherwise not mentioned.

2.6. Statistical analysis

Statistical analysis for significant difference in total protein and MT expression among different fish species from respective controls for the same time period have been indicated by asterisks (*) ($p < 0.05$, ANOVA with Tukey's comparisons) was performed using statistical software SPSS release 10.0; 1997). Graphical plots were made by using SciDAVis release 0.2.0; 2010.

3. Results and discussion

The metal concentrations in wastewater-fed fishponds of ECWs are sufficiently indicative of distinct adverse physiological responses of the fish thriving therein. Proteins are the most important macromolecules that control crucial functions in essentially all biological processes. In fish species protein acts as the major macronutrient that controls energy gain or loss (Diana 1982) and an increase in pollutants, including heavy metals, often results in decreased total protein concentration in fish tissues (Goel et al. 1988; Almeida et al. 2001). Total protein concentration (mg g^{-1} of wet tissue; mean ± SD) of different fish tissues collected from both contaminated and uncontaminated fishponds have been presented in Figs 2 – 5. The pattern for total protein expression for different fish tissues collected from both contaminated and uncontaminated sites for all the species is: liver > gill ≥ muscle > brain. It is

interesting to note that total protein concentration is always found to be invariably higher in uncontaminated fish tissues.

It can be inferred from the study of total protein that IMCs thriving at ECWs are facing potential stress from metals. At this point it is noteworthy to mention that the ECW areas are well-known for good fish yield through wastewater pisciculture and the present work has been initiated with a basic question how the fish yield is so good even though the ambient water is contaminated with such a high concentrations of elements.

Here the study of stress protein metallothionein comes handy. The tissue specific expression of metallothionein is given in Figs 6 – 9. The pattern for MT expression for different fish tissues collected from wastewater-fed fishponds is: liver>muscle>gill>brain and for freshwater-fed fish pond it is: liver>gill>muscle>brain. The tissue specific metallothionein expression has almost complemented the tissue specific metal concentration for IMCs previously reported from this area (Aich et al. 2011).
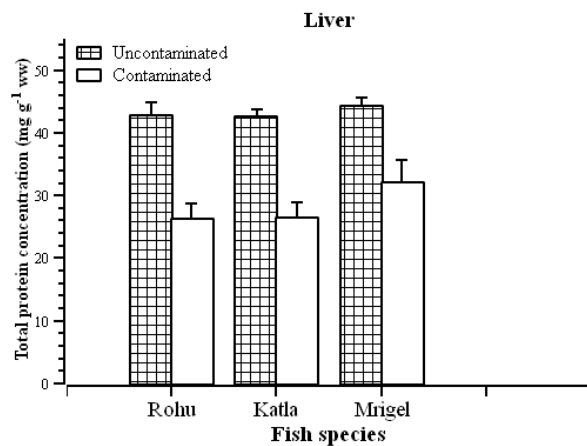

Fig. 2.   Total protein content (mg g⁻¹ of wet tissue; mean ± SD) in liver of IMCs collected from both uncontaminated and contaminated (wastewater-fed) fishponds
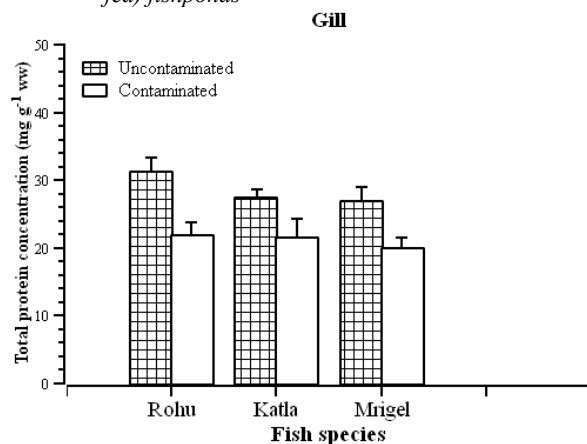

Fig. 3.   Total protein content (mg g⁻¹ of wet tissue; mean ± SD) in gill of IMCs collected from both uncontaminated and contaminated (wastewater-fed) fishponds

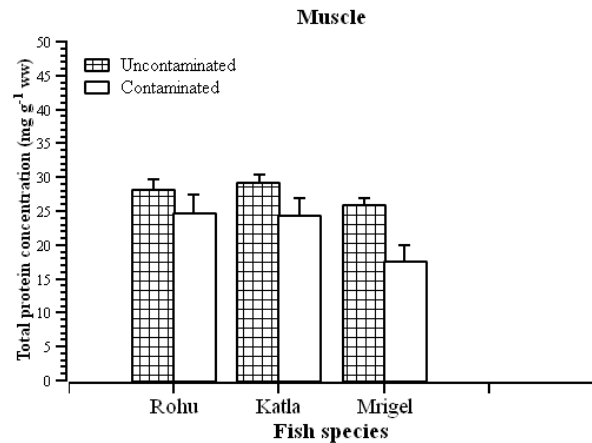*Fig. 4.   Total protein content (mg g⁻¹ of wet tissue; mean ± SD) in muscle of IMCs collected from both uncontaminated and contaminated (wastewater-fed) fishponds*
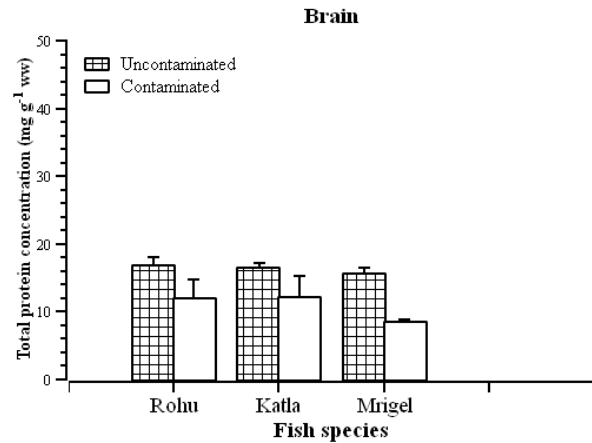

Fig. 5.   Total protein content (mg g⁻¹ of wet tissue; mean ± SD) in brain of IMCs collected from both uncontaminated and contaminated (wastewater-fed) fishponds

These findings are similar to those obtained by Stewart et al. (1996) where metallothionein was found to be positively correlated with Cd, Zn and Cu in kidney and liver of adult lesser-black backed gulls, *Larus fuscus*. There is increasing evidence that an elevated level of metallothionein in fish indicates that the fish has been exposed to metals like Cu, Zn, Cd or Hg (Roch et al. 1982; Olsson and Haux 1986; Sulaiman et al. 1991; Hylland et al. 1992). In teleosts at least four metallothionein genes have been characterized that share common promoter regions and carry several metal responsive elements (MREs), which effectively bind metal transcription factors (MTF) (Kling and Olsson 1993). Previous findings have also confirmed that pre-exposure of an organism or cultured cells to metals can enhance the tolerance to subsequent metal induced toxicities by inducing metallothionein (Irato et al. 1996; Dethloff et al. 1999; Wu et al. 2000; Wu and Hwang 2003; Zhang et al. 2006). This *de novo* synthesis of metallothionein, which is dose and time dependent, results in increased

chelation of heavy metals rendering the heavy metals less toxic (Wicklund-Glynn and Olsson 1991).

Though the binding affinity for metals to thionein is the highest for Hg followed by Cu, Cd and Zn, the inducing ability of these metals for metallothionein is quite different. In most studied vertebrates Zn has been found to be the most potent inducer of metallothionein followed by Cd and Hg, while Cu is often found to be a poor inducer (Olsson 1996). The present findings indicate that higher bioavailability of some metals like Zn and Cu induced higher metallothionein expression in the fishes collected from contaminated fishponds, which effectively chelated both bioactive and non-bioactive metals, reflected by higher metallothionein concentrations in the concerned organs.
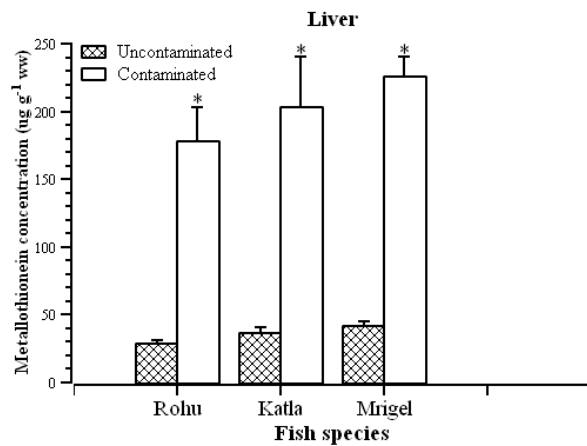
These findings conform to the previous studies, where metallothionein levels have been found to increase with environmental exposure to heavy metal mixtures, mainly containing Cu and Zn (Roch et al. 1982; Hogstrand et al. 1991). Moreover, it has been argued that the toxicity of heavy metals such as Cd and Hg will only occur when the binding capacity of metallothionein is exceeded and the excess heavy metal is free to bind some other proteins in the cell.

Fig. 8. *Metallothionein content (µg g^{-1} of wet tissue; mean ± SD) in muscle of IMCs collected from both uncontaminated and contaminated (wastewater-fed) fishponds. Significant difference in MT expression among different fish species from respective controls for the same time period has been indicated by asterisks (*) (p < 0.05, ANOVA with Tukey's comparisons)*
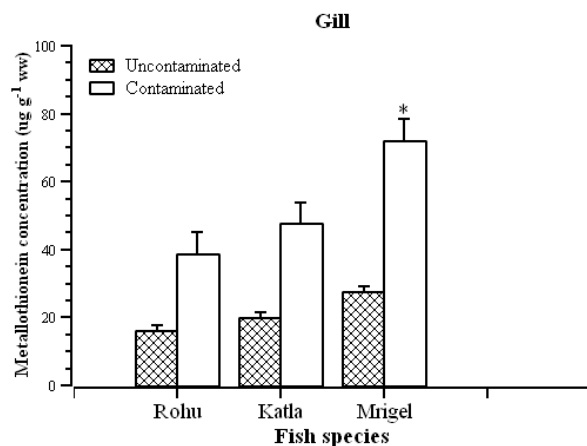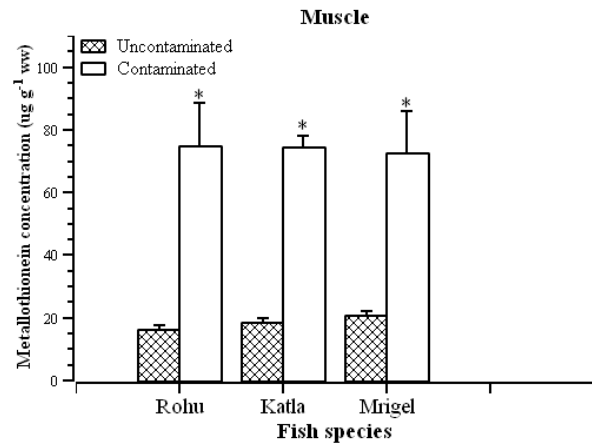

Fig. 6. *Metallothionein content (µg g^{-1} of wet tissue; mean ± SD) in liver of IMCs collected from both uncontaminated and contaminated (wastewater-fed) fishponds. Significant difference in MT expression among different fish species from respective controls for the same time period has been indicated by asterisks (*) (p < 0.05, ANOVA with Tukey's comparisons)*

Fig. 9. *Metallothionein content (µg g^{-1} of wet tissue; mean ± SD) in brain of IMCs collected from both uncontaminated and contaminated (wastewater-fed) fishponds*

Fig. 7. *Metallothionein content (µg g^{-1} of wet tissue; mean ± SD) in gill of IMCs collected from both uncontaminated and contaminated (wastewater-fed) fishponds. Significant difference in MT expression among different fish species from respective controls for the same time period has been indicated by asterisks (*) (p < 0.05, ANOVA with Tukey's comparisons)*
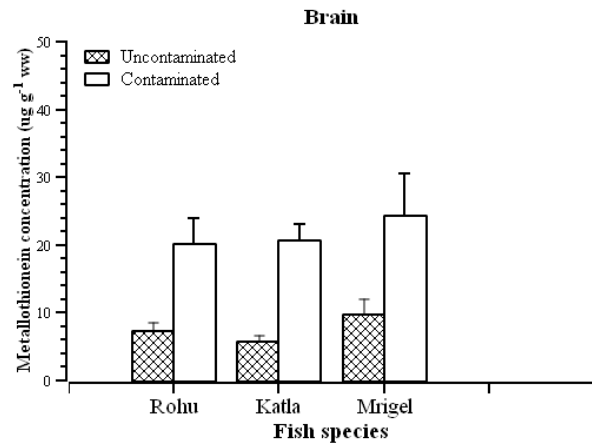
There it seems to be a threshold level for heavy metal toxicity. However, it has been argued that the kinetics of metal uptake and metallothionein synthesis are both to be taken into account, whereby the pathological effects would appear when the rate of metal uptake exceeds the rate of metallothionein synthesis (McCarter et al. 1982). Thus it can be inferred that IMCs thriving at ECWs evolved in a manner to express higher metallothionein to arrest excess elements in the system that would otherwise interfere with the physiological processes. Liver was

the site for the highest metallothionein synthesis and thereby it arrested the highest portions of elements which entered the fish biosystem to maintain the proper metal homeostasis and to lessen the stress, otherwise intolerable.

4. Conclusions

Since its discovery metallothionein has been subjected to various studies and an increasing amount of information on the induction and regulation of this protein by metals in the environment provides a suitable system for monitoring purposes. Though metallothionein regulation is fairly complex, considering certain factors such as the period of sexual maturation and ambient temperature fluctuation, it promises to be a potent indicator for environmental monitoring.

So far as IMCs of ECWs are concerned, there remains a potential stress due to exposure to adverse ambient conditions loaded with metals and that was reflected in the lower total protein concentration in all the wastewater-fed fish tissues. Despite this stressful ambient environment, fish yield in wastewater-fed fish ponds in ECW was 2.85 metric ton per ha of water body per annum and this could be attributed to the fact that fish species thriving at ECWs are still able to effectively trap the metals by metallothionein, or, in another sense, the stress due to higher ambient metal concentration is still within the threshold limit.

Acknowledgements

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