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REVIEW ARTICLE

Metallothionein - A potential biomarker to assess the metal contamination in marine fishes - A review

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Abstract: In this review, the fish Metallothionein (MT) is broadly discussed in relation to their utilization as a biomarker to assess the heavy metal contamination in marine ecosystem. Heavy metals are the main marine pollutants whose bioaccumulation in fishes results in deleterious effects on physiology, biochemistry and behavior. To assess the health status of fishes, an early determination of metal levels along with a biomarker like MT will help us to understand the bioavailability and toxicity of the metals. MTs constitute a family of low-molecular-weight, cysteine-rich proteins functioning in the regulation of essential metals and detoxification of both essential and nonessential metals. Since 1980, MT has been the useful tool for toxicity assessment of metals before sub lethal and lethal damage to organisms because of quick induction of MTs as metals enter the tissue. Various studies conducted on the induction, regulation and estimation of the MT by the metals showed that many variables such as sexual maturity, age, tissue type, metal type, route of exposure, changes in the environmental conditions of the living habitat of the organisms under study and presence of exogenous and endogenous substances do create fluctuations in the level of MT. Keeping in view of the above factors, this review attempts to understand the effectiveness of this biomarker in assessing the health status of the fishes.

Keywords: Biomarker, Heavy Metal, Marine Environment, Metallothionein, Marine Pollution

Introduction

Heavy metals are relatively dense metals or metalloids whose density ranges from 3.5 to 7 g cm^{-3}. Irrespective of their atomic mass or density, any metal which is toxic can be termed as heavy metal [1]. Heavy metals constitute the transition metals, some metalloids, lanthanides and actinides [2]. The family of heavy metals mainly include Lead (Pb), Cadmium (Cd), Mercury (Hg), Arsenic (As), Chromium (Cr), Copper (Cu), Selenium (Se), Nickel (Ni), Silver (Ag) and Zinc (Zn). Other less common metallic contaminants include Aluminium (Al), Cesium (Cs), Cobalt (Co), Manganese (Mn), Molybdenum (Mb), Strontium (Sr) and Uranium (U) [3]. Some heavy metals are biologically important such as Zinc, Iron etc. but some such as Mercury, Lead etc. are extremely toxic even in small doses [2]. Heavy metals are indestructible and have high tissue biomagnification and bioaccumulation ability which makes them the major anthropogenic contaminant in all coastal and marine environments [4]. Heavy metals in the environment directly and indirectly affect the living organisms which need the natural resources present in the environment for their sustenance. Heavy metal pollution of the natural resources leads to molecular, physiological, biochemical and behavioral changes in the organisms [5]. As per Utpal Singha, "Metals are the chemical toxicants that can change the environmental homogeneity by their prolonged persistence and complex interactions. Bioaccumulation of any metal above its threshold level invariably results in stress often results in irreversible physiological conditions" [6]. The primitive phase of heavy metal monitoring in marine environment consisted of quantification of metals in sea water and sediments. Such quantification gave information on levels of heavy metals but did not provide any information on effects of heavy metals on biota. But these traditional methods suffered one main drawback of being less sensitive towards low concentration of heavy metals. Furthermore, the complete analysis of deleterious effects of heavy metals in biological systems is onerous because these effects take a long time to manifest. Till the effects become evident, the destruction of the biological systems might have gone to the point where deleterious effects of heavy metals cannot be reversed [6,7]. Therefore, it is very difficult to determine the extent of heavy metal contamination in the environment just by quantitative methods. This has motivated the researchers to focus their attention towards effect monitoring along with quantitative estimation of heavy metals. New methods were developed which could detect early warning signals reflecting the adverse effects of heavy metals. These efforts gave rise to a new terminology called biomarker [8]. Biomarkers are an effective tool to study and measure those changes and forecast the immediate consequences happening due the contamination of the environment [9].

Biomarkers

Biomarkers can be defined as "the measurement of the body fluids and cells or tissues that indicate in biochemical or cellular terms, the presence of contaminants or the magnitude of the host response" [10]. Biomarkers are broadly classified as Exposure biomarker and Effect biomarker. Exposure biomarkers are indicative of exposure to

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a specific agent. Some of them are Acetylcholinesterase (AChE) activity, Antioxidant enzymes and Metallothionein. Effect biomarkers comprise of the biological changes in organisms caused by contaminants. Some of them are DNA damage and Lipid peroxides. Among them Metallothionein is considered as an important biomarker due to it falling under exposure biomarker and also it being a toxicity specific biomarker [11]. Fishes are mainly employed in marine contamination analysis via MT analysis.

Preference of Fishes in Marine Heavy Metal Pollution Assessment

To study the extent of the effects of heavy metal pollution in the marine ecosystem, fishes are used for the measurement of biomarkers. Fishes from an integral part of the food chain for the general population living in the coastal areas due to their ubiquitous nature [12,13]. Intake of fishes affected by the pollution can cause terminal and non terminal diseases in the population due to which monitoring of fishes for biomarkers is mandatory for assessing the extent of heavy metal pollution in the marine system. Fish biomarkers have increasingly become the worldwide recognized tool for the assessment of the impact of pollution in the marine environment and some are already incorporated in the environmental monitoring programs [14,15,16]. In fishes relationship between metal exposure and metallothionein induction is easier to demonstrate because heavy metal detoxification mainly depends on metal binding to metallothioneins, as there is less or no interference from biomineralization processes [17]. Organisms such as fishes which live in the coastal areas get affected by multiple pollution sources from urban, industrial and agricultural activities and get exposed to multiple varieties of contaminants these are the reason why they serve as the best bioindicator of marine pollution. Fishes selected from contaminated region have to be categorized based on age, sex, site of collection and morphometric parameters such as length, weight etc for accurate quantification of heavy metals [18].

Metallothionein (MTs)

Metallothionein (MT) was discovered in 1957 by Margoshes and Vallee [19]. They are ubiquitous and inducible proteins characterized by low molecular mass (MW 6-8kDa), whose isoelectrical point is 8.3. The molecule of MT is made up of 61 amino acids [20]. MT is devoid of aromatic amino acids with high cysteine content (20-30%) [21]. They are very stable and bind to metals via metal thiolate bonds [22]. It is found to be present in the tissues of all vertebrates and many invertebrates. Their main function is to regulate the metabolically important metals such as copper (Cu) and zinc (Zn) [23]. Since they bind the I b and II b metals, they also play an important role in binding to Ag, Pb and Hg in the suitable physiological conditions [10]. MTs are also involved in many other body processes such as metal ion homeostasis (Zn, Cu) and detoxification of metals such as Cd, Hg, Pt, Ag, scavenging of reactive oxygen species [24], cell proliferation, apoptosis, protection against ionizing radiation and chemo-resistance [25]. MTs are composed of two main globular subunits or domains each comprising 10 amino acids residue: β-Domain which binding 4 divalent or 6 monovalent metal ions and α-Domain binding 4 bivalent or 6 monovalent metal ions. The primary structure of fish MT displays the marked variation in comparison with mammalian MT. Some of the variations in fish MTs are (a) Displacement of the carboxy terminal cysteine. (b) At the juxtaposition with cysteine lysine residues present. The fish MT is more thermal sensitive than mammalian MT. Furthermore, the fish MT has more metal exchange capability then mammalian MT. The mechanism of metal detoxification by MTs occurs via metal initiated transcription activation of MTs genes resulting induction of synthesis of MTs and subsequent binding of free metals [26]. The relationship between the concentration of metals in the marine environment and the concentration of MTs in tissues of fishes has led to their use for monitoring the biological effects of metal exposure in marine environments [10,27].

Metallothionein (MTs) as a Biomarker

Since 1980s, Metallothionein has been used as a biomarker for the analysis of biological effects of heavy metals in aquatic organisms [28]. Some of the biomarkers such as Glutathione (GSH), Glutathione S-transferases (GSTs), Superoxide dismutase (SOD), Catalase (CAT), Malondialdehyde (MDA) etc can be used as biomarkers to assess the heavy metal contamination only after the sublethal and lethal damage to the tissues in the organisms. These damages cannot be reversed by remedial actions. MT can be a useful substitute for the toxicity assessment of metals before organisms experience sublethal and lethal damage because MT will be induced as soon as the metals enter the tissue [29] [30]. MT is already added to the "core biomarker" in European intercalibration exercises for improvement of their quantitative determination of heavy metals in marine environment [31].

Numerous studies such as,

a) Studies on Cadmium induced Metallothionein expression in a variety of fish species including the Turbot (*Scophthalmus maximus*) [32], Carp (*Cyprinus carpio)* [33], Goldfish (*Carassius auratus)* [34], Sole (*Solea solea*) [35], Zebra fish (*Danio rerio)* [36], Rainbow trout (*Oncorhynchus mykiss)* [37] and Tilapia (*Oreochromis mossambicus*) [38], showed increase in the concentration of MTs with respect to increase in the concentration of Cadmium.

b) Further studies concluded that MT transcript proteins can be induced in fish by other bivalent metals, including Zinc, Copper, Lead, Iron, Cobalt and Mercury [39,40].
c) Studies on regulation of MT gene expression provided evidence that there is an induction in MT gene expression with respect to increasing intercellular levels of heavy metals which indicates that increase in metallothionein level is the direct response to the extent of heavy metal contamination in the cells [41].
d) Kling and Olsson characterized the four metallothionein genes and demonstrated that four metallothionein genes share common promoter regions and carry several metal responsive elements (MREs), which effectively bind metal transcription factors (MTF) to enhance the large synthesis of MTs in target organs [42].

All these above studies highlight that there is well established relationship between the tissue concentration of heavy metals and metallothionein which supports the utilization of MT as a biomarker.

Factors Affecting the Metallothionein Estimations

There are many fluctuations while using MTs as a biomarker because MT induction, function and concentration in tissues vary depending on different factors. Many researchers have worked to determine the different factors which results in MT diversity. When teleosts (*Teleostei*) are exposed to different metals, some endogenous and exogenous factors such as reproductive steroids, stress hormones, seasonal changes, temperature, salinity and reproductive and dietary status modify MT levels teleosts [22,43,44,45] and display marked variations in the inductive response among different species [46]. Stipulating the standard MT levels in fishes is difficult because of the variations in the level of MTs in the many interspecies of fishes and differential responses to different environmental contamination levels. Some fishes like Brown trout (*Salmo trutta)* are an exception as the MTs level does not depend on either age or sex [47]. Determination of hepatic MT levels is an affirmed and appropriate biomarker for evaluating the biological consequences of metal contamination. Several studies indicate that the strength of relationships between metals and MT synthesis implies an induced response primarily from metal exposure. Including a measurement of hepatic MT as part of a suite of sub-lethal effects is likely to enhance environmental quality assessment [48]. In order to give the genetic basis for sex, exogenous chemicals and temperature mediated variations of MTs induction the following research was carried out by Kathleen A., *et al.,* in 2001. They characterized the MT messenger RNA (mRNA) expression in male and female non-spawning and spawning killifish (*Fundulus heteroclitus*) following an 8-day exposure to specific sublethal stressors, which included temperature perturbation (26°C or 10°C) and 6 ppb of waterborne cadmium chloride ($CdCl_2$). As a result the liver, gill, and intestine MT mRNA expression was significantly ($P < 0.05$) increased in non-spawning killifish exposed to 26°C compared with those exposed to 19°C (control). They observed a significant ($P < 0.05$) increase in gill MT mRNA induction in non-spawning killifish exposed to 6 ppb of waterborne $CdCl_2$ compared with controls. The results of their study demonstrated significant MT mRNA induction in non-spawning killifish following short-term exposure to physiological and chemical stressors [49]. Some of the factors which affect the MT levels are:

1.1 Age

Age is an important factor which affects the tissue concentration of MTs along with its metal sequestering ability. In order to determine the effect of age on MT concentrations, Wu *et al.,* studied the effect of Cd on different larval stages of Tilepia (*Oreochromis mossambicus)* and found that 3rd day older larva died when exposed to Cd. But no mortality has been observed in newly hatched larva. That is because in 1st day of hatching, the larva had MTs in peak and declined rapidly. After 3 days it had reduced to its half of initial concentration. So they demonstrated that different sensitivity to Cd might be associated with the ability to synthesize the additional MT upon Cd exposure in different stages of larval age or development and also due to inability of MT gene expression [50]. Sole *et al.,* used larvae of benthic fish Senegal sole (*Solea senegalensis)* and measured the levels of MT by pulse polarography for 28 days after hatching. They found that MT is at high concentration during endogenous phase i.e. 0 to 2 days post hatch (dph) and low concentration was noted in 3rd dph, when the egg –yolk sac was fully reabsorbed. Study state value was recorded thereafter that is from 6 to 28 dph. This clearly demonstrates the age dependent MT variations in fishes [51].

1.2 Sex

It has been found that during the sexual maturity of female fishes the MT concentration fluctuates to maximum extents. In Rainbow Trout (*Salmo trutta*) at the onset of sexual maturity, enormous quantity of vitellogenin is produced in the liver. When MT levels were determined during an annual reproduction cycle, it was observed that MT levels began to increase at the onset of vitellogenesis and the level peaked when spawning. Elevated MT levels were also found in male fish at the time of spawning. The actual MT m RNA levels during spawning was found to be 2 folds in

male fishes and about 7 folds in female fishes. Thus it can be said that the hepatic MT levels are down-regulated during the period of sexual maturation in female fish [52].

1.3 Tissue type

MTs induction varies among tissues. In some tissues, MTs induction is at the peak whereas in other tissues it will be minimum. Tissues found in kidney, liver and muscles which are directly involved in metal uptake, storage, detoxification and excretion, have greater levels of MT gene transcription [28]. MTs in fishes are usually analyzed in the liver. But various studies on different fishes showed that muscles, kidneys, gills, skin and brain can be used for analysis. Still the Liver measurement remains the most used since it reflects early exposure to contaminants because the liver is the main detoxification organ of the body [53,54,55]. According to the Mediterranean Action Plan, digestive glands have been the preferred organs for MT analysis in fishes [31]. Richard P. C. in 1994 experimentally demonstrated the MT induction in the liver by metals and gave the following order of potency for MT induction in liver: mercury > silver > cadmium > zinc. Pedersen *et al.,* reported a clear induction of MT in the gills of the Crab (*Carcinus manenas)* related to the presence of copper in the field [56]; whereas Schlenk and Brouwer demonstrated that copper induced MT synthesis in the hepatopancreas of the blue crab (*Callinectes sapidus)* both in the field and in laboratory. Intraperitoneal injection of cadmium, copper and zinc has shown an accountable induction of metallothionein gene in gills. Even a small dose of Cd induces the MT gene transcription but MT level was undetectable [57,58].

1.4 Metal type

Even though all metals can induce the MTs but some metals are potent inducer of MTs. Cu, and Zn have been found to be the most potent inducer of metallothionein in vertebrates followed other metals sharing stoichiometric characteristics with copper or zinc such as Cd and Hg while Cu and Ag are often found to be poor inducers. This shows that Zn can be better investigated and quantified by MT analysis. MT induction by Cd and Hg is highly variable and it depends on conditions of exposure [44]. Li Zhang, Wen-Xiong Wang in their study found that MT concentration elevated in fishes by Zinc exposure, but its concentration increased significantly only in higher exposure of Zn [59]. Additionally, Ni ions have a very high affinity for cysteine [60]. Aruna Chatterjee and Indu B. Maiti worked on catfish (*Heteropneustes fossilis)* and demonstrated that induction of metallothionein by cadmium is dose dependent. Single acute dose of Cd results in low production of MT. But chronic doses produced more metallothionein. Zn and Cu induced metallothionein to a lower extent compared to Cd. They concluded that MT induction is dependent on both metal type and dose of each metal [61].

1.5 Route of exposure, experimental conditions and habitat

The potency of metal to induce the MT may depend upon fish species, tissue physiological condition and experimental conditions. For instance, Chowdhury M. J. *et al.,* in 2005 exposed Rainbow trout (*Oncorhynchus mykiss*) to a sublethal concentration of waterborne Cd (0 or 3 µg/L) or dietary Cd (0 or 500 mg/kg dry wt) for 30 days to induce acclimation. When tissue metallothionein (MT) levels were examined after exposure, they observed highest MT in the kidney flowed by gills and liver in the waterborne exposure group. In the dietary exposure group MT was high in kidney followed by the order kidney >> cecae and posterior intestine > liver and stomach > mid-intestine > gills. Reflecting a variation and specificity depending on the route of exposure, tissue and metal type [62]. Cosson in 1994 while studying the induction of MTs in gill tissues of the Carp found the following order of potency for MT induction: Hg>Cd>Ag>Zn [63]. Experimental errors such as improper handling of fish, prolonged freezing of fish and habitat variation such as anoxic environment, nutrition deficiency and presence of vitamins and herbicides in habitat independently induced MT but lower than that caused by metals [64].

1.6 Seasonal variations

Studies on winter Flounder (*Pseudopleuronectes americanus*) have shown that there is an increased zinc binding to metallothionein during summer feeding season. They found the marked increase in metallothionein levels during summer [65]. Olsson in 1993, estimated the MT concentration in the kidneys and liver of Rainbow Trout (*Oncorhynchus mykiss)* in different degrees of water temperature with different time durations with Cd exposure. They found that MT induction was completely inconstant during various water temperatures. High induction of MT in Liver and kidney was found at 6°C for 4-month duration. A model involving three-factor analysis of variance (ANOVA) was used to examine variation in MT and metal concentrations with respect to season, year and site; with age-class included as a covariate in the analysis. Hepatic concentrations of MT and Cd (and to some degree, Cu but not Zn) increased significantly with age. The model explained 38, 25, 17 and 26% of the variation in MT, Cu, Zn and Cd respectively with significant changes due to season and to a lesser extent over the year. Site was only a significant factor for Cd [66]. Correlation between the individual concentration of MT and each metal alone or in combination was poor. The study

emphasizes the importance of seasonal variation and other factors in biomonitoring programmes and highlights the limitations of using MT as a biomarker for metal contamination in flounders [67].

1.7 Exogenous and endogenous substances

Many of the endo or exogenous substances affect the MT induction. It has been demonstrated that MT synthesis may be reduced in the presence of high levels of organic contaminants due to an increased demand for cysteine residues for glutathione (GSH) synthesis. Estradiol and estrogeic polychlorinated biphenyl (PCBs) appeared to inhibit calcium mediated MT induction in Arctic Char (*Salvelinus alpines)* [68]. Even estrogen is a potent inducer of MTs. Study on rainbow trout demonstrates the inducibility of metallothionein by cortisol treatment of primary hepatocytes. A 90% elevation above control levels was achieved within 8 days of treatment. Zinc treatment was performed to evaluate the system in which 100 μM zinc in the culture medium resulted in a 350% increase of the metallothionein levels [69]. Su-Mei Wu studied the effects of exogenous cortisol and progesterone on metallothionein expression and tolerance to waterborne cadmium in tilapia (*Oreochromis mossambicus*). They reared adult and larval tilapia with artificial feed containing different concentrations of cortisol or progesterone and then tilapia was exposure to Cd. They found that glucocorticoids can induce the expression of metallothionein (MT) which consequently enhances the tolerance to metal toxicity in tilapia [70].

Analysis of MTs

From the physiological point of view it is very difficult to classify the MTs. This has lead to the development of numerous methods for MT quantification. Since MT has no known catalytic function, measurement of their concentration is purely based upon quantitative assay of the protein itself [71]. A number of methods have been employed such as Electroanalytical technique, UV-Vis Spectrophotometry, Metal Saturation Assay and Immunological assay such as Enzyme Linked Immuno Sorbent Assay (ELISA) and Radioimmuno Assay (RIA). The basis for Spectrophotometric assay is the ability of absorption of radiation in combination with mercaptons [72]. Principles of analytical methods for MT estimation are based on the criteria of:

- Detection of bound metal ions.
- Detection of free –SH groups.
- Protein mobility in electric fields.
- Interactions with different other types of sorbets

Some of the major methods for analyzing the MTs are:

2.1 Separation procedure

Detection methods are always coupled with separation procedure. Some important separation procedures are chromatography, electrophoresis etc.

2.1.1 Chromatographic method

MT has got small molecular mass which makes them suitable candidate for chromatographic quantification. If metal content is low in marine environment as detected by Atomic Absorption Spectroscopy then ion exchange or gel chromatography is mainly employed. MT is size specific; therefore, gel chromatography is the best technique for estimation. The column packing agent or gel for separation of MT should possess the pore size of 10-100 nm. Silicates or Organic polymers have the pore size between this range, which is why they are proven to be the best packing agents for this process. The mobile phase is always either water or buffer because they are neutral with respect to interaction with MT and prevent metal dissociation from MT.

Furthermore, ion exchange chromatography has also been proven to be a good detection technique. In ion exchange chromatography, as MT has got high affinity for stationary phase, a high ionic strength buffer is utilized to elute out the MT from stationary phase. High ionic strength buffer alters the three dimensional structure of MT [73]. To overcome this co-polymeric styrenedivinyl benzene is used as column packing material.

If metal content is very high in the marine environment, then MT quantification is done by fluorescence detection using High Performance Liquid Chromatography (HPLC). Supelcosil LC-18 (0.46 cm x 25 cm, 5μm particle) is used as a column, fluorescence light detector is used as a detector with excitation wavelength 382nm, emission wavelength is 470nm and sample injection volume is 20 μL [74,75,76]. Mass Spectrometry is most often used to identify MTs from fractions obtained from chromatography techniques [77].

2.1.2 Electrophoresis

Electrophoresis has become best alternative to chromatographic techniques as it can provide high resolution, high efficiency and rapid analysis from small sample size of MT. MT can be separated and detected by both native and denatured sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For proper separation of MT the gel should have 15-17% acrylamide in it. Furthermore, gradient gel is also considered as proper gel for MTs separation. Native electrophoresis uses Tris-glycine as buffer. Coomassie Blue and Silve- staining are used for quantification of MT-1 and MT-2 isoforms of MT [78]. Coomassie Blue and Silver- staining are time

consuming process because MT does not contain aromatic amino acids so, Fluorescent staining is preferred. Small molecular mass of MT makes it susceptible for re-oxidation during electrophoresis run, which results in migration of protein of interest at molecular masses higher than expected and formation of thin smear, smudgy bands. As a preventive measure Carboxymethylation of MT by iodoacetic acids is done prior to the electrophoresis [79]. Different isoforms of MTs have different Isoelectric point so Capillary Electrophoresis is mainly employed.

2.2 Direct detection of MTs

Many methods have been proposed to determine and quantify the MTs without using separation techniques.

2.2.1 Electrochemical methods

Electrochemical methods are highly sensitive to low concentration of MT. It can detect 10^{-7} to 10^{-10}M. This method is based on electroactivity of sulfhydryl (–SH) moieties of MTs which tend to oxidized or catalysed to evolve hydrogen from electrolytes. Some of the electrochemical methods are voltammetry, chronopotentiometry and polarography [80]. On the hanging mercury droplet MT is accumulated, supporting substances are washed off after which MT modified electrode is inserted into the measuring vessel. In order to increase the sensitivity Adsorption transfer stripping technique (AdTS) is utilized[81]. This method needs high purity preparation due to interference caused by different compounds in the sample. Apart from the above voltammetric methods, differential pulse voltammety with a modification called after its founder "Brdicka reaction" is the widely used electrochemical method for detection of MTs since Olafson optimized it on fish tissues [82].

2.2.2 Immunochemical methods:

This method exploits the antigen-antibody reaction to detect the MT levels in the sample. High sensitivity of this method makes it the second most important method of MTs estimation. Some of the most recurrently used immunochemical methods are enzyme linked immunosorbent assay (ELISA) with enzymatically –labeled antibodies, radioimmuno assay (RIA) using isotopically –labeled antibodies and Western blotting. ELISA can be used for direct detection of MT with little modification in sample preparation procedure. Sona Krizkova, *et al.,* in 2009 compared differential pulse voltammetry Brdickas reaction with ELISA and result were satisfactory [83]. ELISA can detect up to Nanogram/milliliter (ng/ML) of MT in the sample. Electrophoresis prior to the immunoassay enhances the sensitivity of ELISA. Enzymatic degradation of MT, Cross reactions of polyclonal antibodies and interference of metals creates alteration in the ELISA results. So ELISA is mainly used for the detection of MTs rather than quantification. Blotting techniques are employed in MT quantification which uses immobilized antibodies on membrane such as nitrocellulose or polyvinylidene fluorides. MT1, one of the main isoforms of MT is better detected by Dot blot and Western blot methods. Reducing agents such as mercaptoetanol or Tween 20 are used in order to inhibit MT oxidization, which might interfere with estimation [84,85,86,87].

2.2.3 Mass Spectroscopy (MS)

MT analysis by MS uses soft ionization technique to prevent MT distraction and MT-metal dissociation. Main techniques used are electrospray ionization (ESI) and matrix- assisted laser desorption –ionization (MALDI). If MT is separated via capillary electrophoresis (CP) or liquid chromatography (LC) then ESI is used as detection system. ESI can be used for the masses more than 500 Daltons. [88] As ESI is a soft ionizing technique it preserves the MT metal complex and is widely used in the detection of MT isoforms [89]. F. Benavente *et al.,* used ESI-time-of-flight (TOF) analyzer for MT detection in *Mytilus edulis* and the results were satisfactory [90]. If the size of the sample is large then MALDI-TOF is utilized as it is more tolerant than ESI. MALDI-TOF is utilized in studying interaction between metals and MTs. ESI-MS and MALDI-TOF are the good detection techniques for MTs provided the samples should be devoid of contaminants [91].

2.3 Indirect detection of MTs

2.3.1 Spectrophotometric method

Spectrophotometric techniques are widely used as they can be coupled with all most all the separation techniques, specific for MT isoforms and analyses the impure samples on large scale [92]. The process of sample preparation for MT estimation by spectrophotometer carried out in the following way - Tissue is homogenized and the homogenate is subjected to acidic ethanol or chloroform fractionation in order to get the partially purified metalloprotein fraction. This fraction is subjected to spectrophotometric estimations [28]. The following precautions have to be taken during sample preparation:

a) MT Should not get complete precipitated during fractionization.
b) Avoid the oxidation of free sulfhydryl groups (SH) of MT.
c) Avoid the contamination by hydrophilic low molecular weight thiols.
d) Inhibit the enzymatic degradation of MTs.

Further MT is denatured by low pH and high ionic strength, Quantification is done by Ellman's reagent. Ellman's reagent (5,5'-dithiobis- (2-nitrobenzoic acid) or DTNB) is a chemical used to

quantify the number or concentration of thiol groups in a sample. Sensitive method can quantify even in nanoMolar (nmol) of MT in the biological sample. It is one of the simple, repeatable, reliable and cost effective methods to estimate the MT in biological samples [28].

2.3.2 Saturation assay

Saturation assay was the first method used for quantification with lower limits of detection. The principle behind the saturation assay is selective affinity of MTs towards metals.MT does not blind all metals in same affinity some metals are high affinity metals for MTs. Affinity of MTs for heavy metals are like this (Hg>Ag> Cu>Cd>Zn)[93]. Mercury has high affinity towards MT. It results in displacement of other metals by Hg during MTs estimation which may interfere in MTs estimation with respect to other metals. Removal of unbound or nonspecifically bound metals is achieved by addition of Egg white solutions [94]. In addition to mercury even cadmium and silver are also considered as good metals for MT estimation. If Ag is used then Calcium level in the test solution should be minimum because presence of Ca may lead to precipitation of Ag [95].

Experimental problems with MTs

(a) MT oxidation and MT polymorphisms are the main concepts which create problems in the MT analysis. When MTs are fully saturated with metals the remaining free sulphur group makes MT susceptible for oxidation under aerobic condition. To avoid the formation of their bonds with oxygen anaerobic handling of sample is mandatory and even the addition of reducing agents such as 2-Mercaptoethanol to crack the disulfide bonds is also effective to inhibit the oxidation of MTs [85].

(b) Increase in MT level is observed during spawning period of fish and this is associated with MT induction by reproductive steroids and also increased Zn that occur in female fishes during sexual maturity. This may interfere with the metal analysis in fishes using MTs [96].

(c) Rothchell J.M. *et al.,* 2001 observed the increase in the MTs as the temperature decreases but he was unable to demonstrate the mechanism behind it. Cd has a higher affinity than Zn for most MTs and is likely that Cd might have displaced Zn from MT binding site. Potentially this could cause cellular effect even in tissues where all the Cd is immobilized by Metallothionein [97, 98].

(d) Maximum induction of MTs in tilapia occurred nearly 2 mg/kg of $CdCl_2$ and MT did not increase proportionally with dose greater than 2 mg/kg. Administration of higher doses of Cd led to excess cellular Cd, which in turn decreased the MT levels in fish tissue indicating that MTs analysis is not good for higher doses of contaminants [99]. Some authors have suggested that the gills do not constitute a good organ for quantification [100] because MT induction is dependent on the cell type and occurs primarily in the chloride cells [101, 53].

Future prospects

MT is already employed as a biomarker for the analysis of heavy metal contamination in the marine environment but as this field is still at its infancy there is much to be discovered and understood. Some of them are:

(a) Exact mechanism of action of MT in fish need to be elucidated.

(b) Response of MT towards other pollutants such as PCB's and PAH's if any needs to be researched.

(c) Research should also be carried out on characterization of MT in fishes along with comparative analysis of MT in fishes and MT in other organisms.

(d) Inorganic Hg has been shown to be a strong inducer of MT in various invitro studies such as fish cell culture, but invivo influence has not been properly evaluated mainly because in the tissue Hg will be in its organic-mercury complex. Therefore, at present the invivo interactions of Hg and MTs is not clear so MTs are not recommended as a biomarker for Hg contamination analysis (OSPAR commission). MTs can be the good biomarker if proper studies carried out in order to understand the invivo interaction between Hg and MT.

(e) Main drawback of using MT as a biomarker is that MT is not standard in all tissue and its value fluctuates based on hormone level, environmental condition, dietary status, developmental stages of organisms and etc. If standard methods are developed which can overcome this interference, then MT can be a good biomarker. Different methodology used by researchers for the MT analysis creates difficulty in comparison and standardization of results. MT can be a reliable biomarker if analytical methodology is standardized or new innovative methods are discovered which can overcome all current drawbacks.

Conclusions

Presence of heavy metals in the environment is menacing for both present and future generations. Metals are resistant to biodegradation and have high affinity for bioaccumulation or biomagnification in fatty tissues of marine organisms resulting in grievous injury to the heath of organisms and the ecosystem. Prior determination of heavy metals and analysis of its contamination levels in environmental components are obligatory for healthy ecosystem. Many efforts have been put forward towards the detection of heavy metals using ecosystem

components such as organisms, sediments, soil etc. which are called as bioindicators. Traditional quantification of heavy metals in ecosystem components failed due to lack of information on biological effect of metals which results in use of biological response as parameters for determination of heavy metals called biomarkers. Some of the biological responses are oxidative stress, cytological damage, reproductive impairments, inhibition of AchE (Acetylcholinesterase), stress proteins inductions, immunological impairments etc. Among them stress proteins such as MTs are well known as an efficacious biomarker. Metallothionein induction and regulation have been the main subjects for various studies since its discovery. It results in generation of immense amount of data on induction and regulation of MTs. Metallothionein regulation is quite complex due to interference of several factors such as period of sexual maturity, age, habitat and environmental conditions. Due to the quick response of MTs towards metal contamination and proportionate increase of tissue concentration of MTs with respect to the tissue concentration of metals it promises to be the potent biomarker for heavy metal contamination.

Various studies conducted on the regulation of the MT by the metals in the environment provide a solid base for the monitoring of pollutants in the environment. Although many other variables such as sexual maturity and the changes in the environmental conditions of the living habitat of the organisms under study do create fluctuations in the levels of MT which tend to hamper the study of MT but still it is the foremost biomarker among all for monitoring the extent and level of pollution in the environment. George and Olsson in 1994 suggested that the use of fish MT be accepted in monitoring. However, to obtain an appropriate interpretation of the data resulting from MT analysis it is necessary to use fish species for which the basal MT level are widely known. Aquatic animals are considered as the main bioindictor for the estimation of heavy metal pollution but variations in the habitat of aquatic animals such as water pH, salinity, temperature, turbidity, dissolved oxygen content etc. create problems in effectively measuring the level of MTs. MT can a promising biomarker only if precautions are taken towards the selection of fish type, organ and method of analysis as well as the environmental factors.

References

1. Singh R, Gautam N, Mishra A and Gupta R, Heavy metals and living system: An overview. Indian Journal of Pharmacology, 43 (3), 2011, 246-253

2. Singh J, Upadhayay SK, Pathak RK and Gupta V. Accumulation of Heavy Metal in Soil and Paddy Crop (*Oryza sativa*), Irrigated with Water of Ramgarh Lake, Gorakhpur, UP, India. Environmental Toxicology and Chemistry, 93, 2011, 462-473. .

3. Mcintyre T, Phytoremediation of heavy metals from soils. Advances in Biochemical Engineering / Biotechnology, 78, 2003, 97–123.

4. Ruilian Y, Xing Y, Yuanhui Z, Gongren H and Xianglin T, Heavy metal pollution in intertidal sediments from Quanzhou Bay, China. Journal of Environmental Sciences, 20, 2008, 664–669.

5. Guanghua L, Xiaofan Y, Zhihua L, Haizhou Z and Chao W. Contamination by metals and pharmaceutical in northern Taihu Lake (China) and its relation to integrated biomarker response. Ecotoxicology, 2012, 1002-1004

6. Utpal Singha Roy, Chattopadhyay B, Datta S and Mukhopadhyay SK, 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). Environmental Research, Engineering and Management, 4 (58), 2011, 10-17.

7. Bayne BL, Brown DA, Burns K, Dixon DR, Ivanovici A, Livingstone DR, Lowe DM, Moore MN, Stebbing ARD and Widdows J, The effects of stress and pollution on marine animals in Environmental Toxicology and Risk Assessment 4 (New York, USA: Praegez Publshers, 1985).

8. Bucheli TD and Fent K, Induction of cytochrome P450 as a biomarker for environmental contamination in aquatic ecosystems. Critical Reviews in Environmental Science and Technology, 25 (3), 1995, 201-268.

9. Beliaeff B and Burgeot T, Integrated Biomarker Response: A useful tool for ecological risk assessment. Environmental Toxicology and Chemistry, 21, 2002, 1316.

10. Livingstone DR, Biotechnology and pollution monitoring: use of molecular biomarker in the aquatic environment. Journal of Chemical Technology and Biotechnology, 57, 1993, 195–211.

11. Monserrat JM, Geracitano LA and Bianchini A. Current and future prospective using Biomarker to assess pollution in aquatic ecosystem. Comments on toxicology, 9, 2003, 255-269.

12. Suter GWH, Ecological Risk Assessment (Boca Raten, FL, USA: Lewis Publishers, 1993).

13. Beyer J, Fish biomarker in marine pollution monitoring: evaluation and validation in laboratory and field studies, University of Bergen, Norway, 1996.

14. Cajaraville MP, Bebianno MJ, Blasco J, Porte C, Sarasquete C and Viarengo A, The Use of Biomarker to Assess the Impact of Pollution in the Coastal Environment of the Iberian Peninsula: A Practical Approach, Science of the Total Environment, 247, 2000, 295-311.

15. Cajaraville MP, Bebianno MJ, Blasco J, Porte C, Sarasquete C and Viarengo A, The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. Science of the Total Environment, 247, 2000, 201–212

16. Viarengo A, Lowe D, Bolognesi C, Fabbri E & Koehler A, 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, 146 C, 2007, 281–300.

17. Mason AZ and Jenkins KD, Metal detoxification in aquatic organisms. In: Metal speciation and bioavailability in aquatic systems (London, UK: John Wiley & Sons Ltd, 1995)

18. Depledge MH and Fossi MC, The Role of Biomarker in the Environmental Assesment. Ecotoxicology, 3, 1994, 161-172.

19. Margoshes M and Vallee BL, A cadmium protein from equine kidney cortex. Journal of American Chemical Society, 79, 1957, 4813-4814.

20. Decataldo A, Di Leo A, Giandomenico S and Cardellicchio N. Association of metals (Mercury, Cadmium and Zinc) with metallothionein like proteins in storage organs of standard dolphins from the Mediterranean Sea (Southern Italy). Journal of Environmental Monitoring, 6, 2004, 361-367.

21. Alhaman J, Romero-Ruiz A, Jebali J and Lopez-Barea J, Total metallothionein quantification by reversed-phase high performance liquid chromatography coupled to fluorescence detection monobromobimane derivation. Environmental Research Journal, 5, 2011, 1-17.

22. Kaegi JH and Schaffer A, Biochemistry of metallothionein. Biochemistry, 27, 1988, 8509-8515.

23. Roesijadi G, Metallothioneins in metal regulation and toxicity in aquatic animal. Aquatic Toxicology, 22, 1992, 81-114.

24. Doki Y and Monden M, Can metallothionein be a useful molecular marker for selecting hepatocellular carcinoma patients for platinum based chemotherapy, Journal of Gastroenterology, 39, 2004, 1228-1229.

25. Prusa R, Blastik O, Potesil D, Trnkova L, Zehnalek J, Adam V, Petrlova J and Jelen F, Analytic method for determination of metallothionenis as tumor biomarker. Clinical Chemistry 51, 2005, A 56-A 56.

26. Hogstrand C and Haux C, Binding and detoxification of heavy metals in lower vertebrates with reference to metallothionein. Comparative Biochemistry and Physiology, 100C, 1991, 137-141.

27. Hylland K, Haux C and Hogstrand C, Hepatic metallothionein and heavy metals in dad *limanda limanda* from the German Bight. Marine Ecology Progress Series, 91,1992, 89-96.

28. Viarengo A, Ponzano E, Dondero F and Fabbri R, A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic molluscs. Marine Environmental Research, 44, 1997, 69–84

29. Pavicic J, Skreblin M, Raspor B and Branica M. Metal pollution assessment of marine environment by determination of metal – binding proteins in *Mytillus sp.* Marine Chemistry, 22, 1987, 235-248.

30. Paek SM, Soohee C and In-Sook L, Level of heavy metals in the Onsan Bay in Korea and involvement of metals binding proteins in the accumulation of Cadmium in *Littorina brevicula.* The Korean Journal of Ecology, 22, 1999, 95-100.

31. UNEP/RAMOGE, Manual on the Biomarkers Recommended for MED POL Biomonitoring Programme, UNEP, Athens, 1999, 92.

32. George SG, Hodgson PA, Tytler P and Todd K, Inducability of Metallothionein mRNA expression and Cadmium tolerance in larva of teleosts the turbot (*Scophthalmus maximus).*

Fundamental Application of Toxicology, 33, 1996, 91-99.

33. Smet HD, Wachter BD, Lobinski R and Blust R, Dynamics of (Cd, Zn)- metallothioneins in gills, liver and kidney of common carp (*Cyprinus carpio)* during cadmium exposure. Aquatic Toxicology, 52, 2001, 269–281.

34. Choi CY, An KW, Nelson ER and Habibi HR, Cadmium affects the expression of metallothionein (MT) and glutathione peroxidase (GPX) mRNA in goldfish (*Carassius auratus)*. Comparative Biochemistry and Physiology, 145C, 2007, 595–600.

35. Rovira MS, Fernández-Díaz C, Canavate JP and Blasco J, Effects on metallothionein levels and other stress defenses in Senegal sole larvae exposed to cadmium. Bulletin of Environmental Contamination and Toxicology, 74, 2005, 597–603.

36. Chen WY, John JAC, Lin CH and Chang CY, Expression pattern of metallothionein, MTF-1 nuclear translocation, and its DNA-binding activity in zebrafish (*Danio rerio*) induced by zinc and cadmium. Environmental Toxicology and Chemistry, 26, 2007, 110–117.

37. Lange A, Ausseil O and Segner H, Alterations of tissue glutathione levels and metallothionein mRNA in rainbow trout during single and combined exposure to cadmium and zinc. Comparative Biochemistry and Physiology, 131, 2002, 231–243.

38. Wu SM, Weng CF, Yu MJ, Lin CC, Chen ST, Hwang JC and Hwang PP, Cadmium-inducible metallothionein in tilapia (*Oreochromis mossambicus*). Bulletin of Environmental Contamination and Toxicology, 62, 1999, 758–768.

39. Carginale V, Scudiero R, Capasso C, Capasso A, Kille A, Di Prisco G and Parisi E. Cadmium-induced differential accumulation of metallothionein isoforms in the Antarctic icefish, which exhibits no basal metallothionein protein but high endogenous mRNA levels. Biochemical Journal 332, 1998, 475–481.

40. Riggioa M, Filosaa S, Parisib E and Scudieroa R, Changes in zinc, copper and metallothionein contents during oocyte growth and early development of the teleost *Danio rerio* (zebrafish). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 135 (2), 2003, 191–196

41. Thiele DJ, Metal Regulated Transscription in Eukaryotes. Nucleic Acid Research, 210, 1992, 1183-1188.

42. Kling P and Olsson PE, Metal regulation of the rainbow trout metallothionein-A gene. In: proceedings of the Seventh International Symposium on Responses of Marine Organisms to Pollutants. 1993

43. Olsson PE, Kling P, Erkell LJ and Kille P, Structural and functional analysis of the rainbow trout (*Oncorhynchus mykiss)* metallothionein-A gene. European Journal of Biochemistry, 230, 1995, 344–349.

44. Olsson PE, Metallothionein in fish: induction and use in environmental monitoring, In: Toxicological aquatic pollution: physiological, molecular and cellular approaches (Cambridge, UK: Cambridge University Press, 1996).

45. Hylland K, Nissen-Lie T, Christensen PG and Sandvik M, Natural Modulation of hepatic Metallothionein andcytochrome P4501A in flounder, *Platichthys flesus*. Marine Environmental Research, 46, 1998, 1-5.

46. Cinier CD, Petit-Ramel M, Faure R and Bortolato M, Cadmium accumulation and metallothionein biosynthesis in *Cyprinus carpio* tissues. Bulletin of Environmental Contamination and Toxicology, 61, 1998, 793–799

47. Linde AR, Sanchez-Galan S, Klein D, Garcia-Vazquez E and Summer KH, Metallothionein and heavy metals in Brown trout (*salmo trutta*) and European eel (*Anguilla anguilla*): a comparative study. Ecotoxicology Environmental Safety, 44, 1999, 168-173.

48. Langston WJ, Chesman BS, Burt GR, Pope ND and McEvoy J, Metallothionein in liver of eels (*Anguilla Anguilla)* from the Thames Estuary: an indicator of environmental quality. Marine Environmental Research, 53, 2002, 263–293.

49. Kathleen A, Van Cleef Toedt, Lisa AE, Kaplan E and Crivello JF, Killifish metallothionein messenger RNA expression following temperature perturbation and cadmium exposure. Cell Stress Chaperones, 6 (4), 2001, 351–359.

50. Wu SM, Lin HC and Yang WL, The effects of maternal Cd on the metallothionein expression in tilapia (*Oreochromis mossambicus*) embryos and larvae. Aquatic Toxicology, 87, 2008, 296–302.

51. Sole M, Potrykus C, Fernandez-Diaz and Blasco J, Variations on the stress defense and metallothionein levels in the Senegal sole, *Solea senegalensis,* during early larval stages. Fish Physiology and Biochemistry, 30, 2004, 57-66.

52. Olsson PE and Haux C, Rainbow trout metallothioneins. Inorganica Chimica Acta, 107, 1985, 67-71

53. Dang ZC, Berntssen HMG, Lundebye KA, Flik G, Bonga SEW and Lock RAC, Metallothionein and Cortisol Receptor Expression in Gills of Atlantic Salmon, *Salmo Salar* Exposed to Dietery Cadmium. Aquatic Toxicology, 53, 2001, 91-101.

54. Langstone WJ, Chesman BS, Burt GR, Pope MD and Mc Evoy J, Metallothionein in Liver of Eels *Anguilla Anguilla* from the Thames Estuary: An Indicator of Environmental Quality. Marine Environmental Research, 53, 2002, 263-293.

55. Pathiratne A, Chandrasekera LWHU and Pathiratne KAS, Use of Biomarkers in Nile Tilapia (*Oreochromis nilticus)* to Assess the Impact of Pollution in Bolgoda Lake, An Urban Waterbody in Sri Lanka. Environmental Monitoring and Assessment, 15, 2009, 361-374.

56. Pedersen SN, Lundebye AK and Depledge MH, Filed application of metallothionein and stress protein biomarker vin the shore crab (*Carcinus maenas)* exposed to trace metal. Aquatic Toxicology, 37, 1997, 183-200.

57. Richard P. Cosson, Heavy metal intracellular balance and relationship with metallothionein induction in the liver of carp after contamination by silver, cadmium and mercury following or not pretreatment by zinc. Biometals, 7 (1), 1994, 9-19.

58. Zafarullah M, Olsson PE and Gedamu L, Endogenous and heavy metal ion induced metallothionein gene expression in salmonid tissues and cell lines. Gene 83 (1), 1989, 85–93.

59. Li Zhang and Wen-Xiong Wang, Effects of Zn pre-exposure on Cd and Zn bioaccumulation and metallothionein levels in two species of marine fish. Aquatic Toxicology, 73 (4), 2005, 353–369.

60. Costa M, Zhuang Z, Huang X, Cosentino S, Klein CB, and Salnikow K, Molecular mechanisms of nickel carcinogenesis. Science of the Total Environment, 148, 1994, 191–199.

61. Chatterjee A and Maiti BI, Induction and turnover of catfish (*Heteropneustes fossilis*) metallothionein. Molecular and Cellular Biochemistry, 108 (1), 1991, 29-38.

62. Chowdhury M, Baldisserotto B and Wood CM, Tissue-Specific Cadmium and Metallothionein Levels in Rainbow Trout Chronically Acclimated to Waterborne or Dietary Cadmium. Archives of Environmental Contamination and Toxicology 48 (3), 2005, 381-390.

63. Cosson RP, Heavy metal intracellular balance and relation with metallothionein induction in the gills of crap after contamination by Ag, Cd and Hg followed by pretreatment with Zn or not. Biological Trace Elemement Research, 46, 1994, 229-245.

64. Kagi JHR, Evolution, structure and chemical activity of class I metallothioneins: an overview (Basel, Switzerland: Birkhäuser Verlag, 1993)

65. Shears MA and Fletcher GL, Hepatic metallothionein in the winter flounder (*Pseudopleuronectes americanus*), Canadian Journal of Zoology, 1985, 1602-1606.

66. Olsson PE, Åke Larsson and Haux C, Influence of seasonal changes in water temperature on cadmium inducibility of hepatic and renal metallothionein in rainbow trout. Marine Environmental Research, 42 (1-4), 1996, 41–44.

67. Rotchell JM, Clarke KR, Newton LC and Bird DJ, Hepatic MT's as a Biomarker for metal contamination: age effect and seasonal variation in European flounder (*Pleuronectes flesus*) from the Severn Estuary and Bristol Channel. Marine Environmental Research, 52, 2001, 151-171.

68. Gerpe M, Kling P, Berg AH and Olsson PE, Arctic char (*Salvelinus alpinus*) metallothionein: cDNA sequence, expression and tissue-specific inhibition of cadmium mediated metallothionein induction by 17β-estradiol, 4-OH-PCB 30 and PCB 104. Environmental Toxicology and Chemistry, 19, 2000, 638–645.

69. Hyllner SJ, Andersson T, Haux C and Olsson PE, Cortisol induction of metallothionein in primary culture of rainbow trout hepatocytes. Journal of Cellular Physiology 139 (1), 2005, 24–28.

70. Su-Mei Wu, Yi-Ying Chou and Am-Ni Deng, Effects of Exogenous Cortisol and Progesterone on Metallothionein Expression and Tolerance to Waterborne Cadmium in Tilapia (*Oreochromis mossambicus*). Zoological Studies 41 (1), 2002, 111-118.

71. Huggett RJ, Biomarkers: Biochemical, Physilogical and Histological marker of Anthropogenic Stress (Chelsea, MI: Lewis Publishers, 1992).

72. Dobrio M, Adela R, Rodrigues, Gey Bordin, Maria J, Bebeanno, Mare. A. Ley, Ivan Sestakova, Milan Vossat and Monica Nordberg, Recent Development in Quantification method of MTs. Journal of Inorganic Biochemistry 88, 2002, 23-134.

73. Lobinski R, Chassaigne H and Szpunar J, Analysis for metallothioneins using coupled techniques. Talanta 46 (2), 1998, 271-89.

74. Alhama J, Romero-Ruiz A and Lopez–Barea J, Metallothionein quantification in clam by reversed- phase high–performance liquid chromatography coupled to fluorescence detection after monobromobimane derivatization. Journal of chromatography A 1107, 2002, 52-58.

75. Jebali J, Banni M, Gerbej H, Boussetta H, Lopez-Barea J and Alhama J, Metallothionein induction by Cu, Cd and Hg in *Dicentrarchus labrax* liver: Assessment by RP-HPLC with florescence detection and spectrophotometry. Marine Envrionmental Research 65, 2008, 358-363.

76. Romerio-Ruiz A, Alhama J, Blasco J, Luis Gomez-Ariza J and Lopez-Barea J, New Metallothionein assay in *Scrobicularia plana*: Heating effect and correlation with other biomarkers. Environmental Pollution 156, 2008, 1340-1347.

77. Apostolova M, Bontchev PR, Nachev C and Sirakova I, Apometallothionein in rat liver. Journal of Chromatography 620 (2), 1993, 191-197.

78. McCormick CC and Lin LY, Quantification and identification of metallothioneins by gel electrophoresis and silver staining. Methods in Enzymology, 205, 1991, 71.

79. Conklin DR, Cowan KS and Aschner M, Detection of metallothionein (MT) proteins with radiolabeled [c-14] iodoacetamide. Toxicology methods 6 (3), 1996, 149-155.

80. Orihuela R, Domenech J, Bofill R, You C, Mackay EA, Kagi JHR, Capdevila M and Atrian S, The metal-binding features of the recombinant mussel (*Mytilus edulis)* MT-10-IV metallothionein. Journal of Biological Inorganic Chemistry 13, 2008, 801-812.

81. Adam V, Krizkova S, Zitka O, Trnkova L, Petrlova J, Beklova M and Kizek R, A determination of apo-metallothionein using adsorptive transfer stripping technique in connection with differential pulse voltammetry. Electroanalysis (NY) 19, 2007, 339-347.

82. Sestakova I and Navratil T, Voltammetric methods in metallothionein research. Bioinorganic Chemistry and Applications 3, 2005, 43-53.

83. Krizkova S, Fabrik I, Adam V, Kukacka J, Prusa R, Chavis GJ, Trnkova L, Strnadel J, Horak V and Kizek R, Comparison of metallothionein detection by using Brdicka reaction and enzyme-Linked immunosorbent assay employing chicken yolk antibodies. Journal of Sensors 8, 2009, 3106.

84. Butcher H, Kennette W, Collins O, Demoor J and Koropatnick J, A sensitive time-resolved fluorescent immunoassay for metallothionein protein. Journal of Immunological Methods 272, 2003, 247-256.

85. Dabrio M, Adela R, Rodriguez, Bordin G, Bebeanno MJ, Ley MA, Sestakova I, Vosak M and Nordberg M, Recent development in quantification method of MTs. Journal of Inorganic Biochemistry 88, 2002, 123-134.

86. Chan HM, Pringle GA and Cherian MG, Heterogeneity of antibodies to metallothionein isomers and development of a simple enzyme-linked immunosorbent assay. Journal of Biochemical and Molecular Toxicology 7 (4), 1992, 219-227.

87. Benavente F, Andon B, Gimenez E, Barbosa J and Sanz-Nebot V, Modeling the migration behavior of rabbit liver apothioneins in capillary electrophoresis. Electrophoresis 29 (13), 2008, 2790.

88. Rosenberg E, The potential of organic (electrospray and atmospheric pressure chemical ionisation) mass spectrometric techniques coupled to liquid-phase separation for speciation analysis. Journal of Chromatography A 1000, 2003, 841-889.

89. Kaltashov IA, Zhang MX, Eyles SJ and Abzalimov RR, Investigation of structure,

dynamics and function of metalloproteins with electrospray ionization mass spectrometry. Analytical and Bioanalytical Chemistry, 386 (3), 2006, 472-481.

90. Orihuela R, Domenech J, Bofill R, You C, Mackay EA, Kagi JHR, Capdevila M and Atrian S, The metal-binding features of the recombinant mussel *Mytilus edulis* MT-10-IV metallothionein. Journal of Biological Inorganic Chemistry 13, 2008, 801-812.

91. Karotki AD and Vasak M, Reaction of human metallothionein-3 with cisplatin and transplatin. Journal of Biological Inorganic Chemistry 14, 2009, 1129-38.

92. Szpunar J, Advances in analytical methodology for bioinorganic speciation analysis: metallomics, metalloproteomics and heteroatom-tagged proteomics and metabolomics. Analyst 130, 2005, 442-465.

93. Klaverkamp JF, Wautier K and Baron CL, A modified mercury saturation assay for measuring metallothionein. Aquatic Toxicology 50, 2000, 13-25.

94. Huang ZY, Shen JC, Zhuang ZX, Wang XR and Lee FSC, Metallothionein as a biomarker for mercury in tissues of rat fed orally with cinnabar. Applied Organometallic Chemistry 18, 2004, 255-261.

95. Bienengraber M, Forderkunz S, Klein D and Summer KH, Determination of Cu-Containing Metallothionein: Comparison of Ag Saturation Assay, Thiomolybdate Assay and Enzyme-Linked Immunosorbent Assay. Analytical Biochemistry 228, 1995, 69-73.

96. Olsson PE, Kling P, Erkell LJ and Kille P, Structural and functional analysis of the rainbow trout (*Oncorhynchus mykiss*) metallothionein-A gene. European Journal of Biochemistry 230, 1995, 344–349.

97. Rotchell JM, Clarke KR, Newton LC and Bird DJ, Hepatic MT's as a Biomarker for metal contamination: age effect and seasonal variation in European F European flounder (*Pleuronectes flesus*) from the Severn Estuary and Bristol Channel. Marine Environmental Research 52, 2001, 151-171.

98. Hollis L, Hogstrand C and Wood CM, Tissue specific Cadmium accumulation, MT induction and tissue Zn and Cu levels during chronic sub lethal Cadmium exposure in juvenile Rainbow Trout. Archives of Environmental Contamination and Toxicology 41, 2001, 468-474.

99. Ueng YF, Lai CF, Merg LM, Hug YY and Uerg TH, Effect of Cadmium and Environmental pollution on MTs and Cytochrome P450 in Tilapia. Bulletin of Environmental Contamination and Toxicology 57, 1996, 125-131.

100. Olsvik PA, Gundersen P, Andersen RA and Zachariassen KE, Metal accumulation and metallothionein in two populations of brown trout, *Salmo trutta*, exposed to different natural water environments during a runoff episode. Aquatic Toxicology 50, 2000, 301-316.

101. Burkhardt-Holm P, Bernet D and Hogstrand C, Increase of metallothionein-immunopositive chloride cells in the gills of brown trout and rainbow trout after exposure to sewage treatment plant effluent. Histochemical Journal 31, 1999, 339-346.

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