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Review Article

A critical review on integrating multiple fish biomarkers as indicator of heavy metals contamination in aquatic ecosystem

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Abstract: An immense amount of data is available on biomarkers related to different eco-toxicants. But data on contaminant-specific biomarkers in fishes is sparse. Traditionally, detection and quantification of heavy metals in sediment, water, and biota gave us valuable information on the quantity and the type of heavy metal present in the ecosystem. This information can be utilized to select a heavy metal specific biomarker. For an instance, if Cadmium (Cd), Zinc (Zn) and Cupper (Cu) are at high concentration, then Metallothionein (MT) can be a good candidate biomarker. Along with this, Superoxide dismutase (SOD) is a very potent indicator of Iron (Fe) and Mercury (Hg) contamination and also Catalase (CAT) is specific for Cadmium (Cd) and Zinc (Zn) exposure. For these kinds of selection of biomarker, the researchers should know heavy metals type specific biomarker. This review is the small effort towards cumulating the heavy metals type specific biomarker. This demonstrates the exposure and effects of heavy metals in fishes by integrating the heavy metal quantification and biomarker selection.

Keywords: Biomarker, Heavy metals, Metallothioneins, Oxidative stress, Genotoxicity.

Introduction

Humanity's interest in the technological innovation is universal and enduring. From prehistoric period, humans have a natural instinct to explore the unknown, discover new worlds and push the boundaries of scientific and technological knowhow. But the human's greed to exploit Mother Nature in the name of technological and scientific progression has aggravated the heavy metal pollution in the environment due to an imbalance in biogeochemical cycles. Heavy metals are those whose density ranges from 3.5 g/cm3 to 7 g/cm3. Heavy metals are naturally occurring elements of all ecosystems. They can exist in elemental form and in a variety of other chemical compounds. The volatile heavy metals can be widely transported on a very large scale in the ecosystem (Nriagu, 1995). Heavy metals include the transition metals, some metalloids, lanthanides, and actinides.

Some heavy metals such as Zinc (Zn), Iron (Fe) etc. are biologically important. Many metalloenzymes like Carboxypeptidase, Aldolase, and metal activated enzymes like ATPase are dependent on them. But metals like Mercury (Hg), Lead (Pb) etc. are extremely toxic even in small doses. Heavy metals are the most pestilential pollutants owing to their indestructible nature and high tissue biomagnification and bioaccumulation ability. These properties of heavy metals make them to accumulate in aquatic organisms and persist in water and sediments (Luoma and Rainbow, 2008). Fishes are one of the main nutritional components consumed by humans. Furthermore, fishes are at the apex of the aquatic food chain, so they are good bioindicators for metal contamination. Fishes from the contaminated site have been proven to be better bioindicators (Livingstone, 2003).

"Bioaccumulation of any metal above its threshold level results in irreversible physiological conditions" (Utpal Singha Roy *et al.,* 2011). The traditional methods for analyzing heavy metals in aquatic ecosystem includes; quantification of heavy metals in water, sediments, or a member of the indigenous biota. The analysis of water and sediments is expensive and laborious; demanding multiple sampling due to many endogenous as well as exogenous factors affecting the sample. Moreover, these traditional methods lack the information on biological implications of metals on biota (David J.H. Phillips, 1977). These complications have led to the scenario for investigating the early warning signals like biomarkers, to analyze the heavy metals contamination in the organism.

Heavy metal toxicity in fishes

Metal ions exceeding the threshold level cause elevated toxicity and have a detrimental impact on systemic circulation and organ system. Metals mediate the gene activation of stress proteins and

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also induce oxidative stress. Heavy metals inhibit the functions of structural proteins, enzymes, and nucleic acids by forming metal complexes. Additionally, they also cause morphological alterations, chromosomal aberrations, and bring about impairment in the immune system (Coen *et al.,* 2001). The heavy metal toxicity in aquatic organisms primarily depends on metal solubility, water pH, and ecosystem complexity. In fishes, gills, digestive tract and body surface are predominantly involved in metal uptake (Tao *et al.,* 2001).

Heavy metals induce toxicity by the following major mechanisms:

a) Redox-active metals contain an unpaired electron in their d-orbital and are capable of generating free radical by redox cycling mechanisms. Some of them are Iron (Fe), Copper (Cu), Chromium (Cr) and Vanadium (V).

b) Metals without redox potential, impair the antioxidant defenses by structural and functional alteration of thiol-containing antioxidants and enzymes. Metals like Mercury (Mg), Nickel (Ni), Lead (Pb), and Cadmium (Cd) belong to this class (Stohs and Bagchi, 1995).

c) Ferrous iron can undergo Fenton reaction in which hydrogen peroxide oxidizes ferrous iron (II) to ferric iron (III), a hydroxyl radical, and a hydroxyl anion (Valko *et al.,* 2005). The superoxide radical can reduce iron to its ferrous form. Copper, Cobalt, Chromium, Titanium, Vanadium and their complexes can also get involved in the Fenton reaction and Haber-Weiss reaction (Lushchak, 2011). Many of these metals are in oxidized form but to undergo Fenton reaction, metals should be in a reduced state. Iron can attain hypervalency state as Ferryl ion (Fe^{+4}) which is more toxic.

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

$$O_2^{\cdot -} + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$$

d) Heavy metals also activate the redox-sensitive transcription factors such as Activator protein 1(AP-1), p53, and Nuclear factor-κB (NF-κB). These transcription factors regulate the expression of DNA repair genes and induce apoptosis, cell division and cell differentiation (Valko *et al.,* 2005).

e) Heavy metals like Silver (Ag^{2+}), Lead (Pb^{2+}), and Mercury (Hg^{2+}) noncompetitively inhibit enzymes by binding with cysteinyl- sulfhydryl group (Valko *et al.,* 2005).

Fishes as bioindicators

Fishes are the largest, ubiquitous and diverse group of organisms. They occur in different trophic levels including the apex predators. Fishes are the valuable food source for humans due to their high mineral and protein content with low-fat residue and optimum ratio of unsaturated fatty acids. They are available in different species, sizes, and ages which allow easy comparative analysis. Because of their well-developed osmoregulatory, endocrine, nervous, and immune systems, they are preferred in toxicological research (Song *et al.,* 2012). According to Rayment & Barry (2000), fishes are a valuable bioindicator because fish sample preparation and chemical analysis is relatively simple, more rapid and less expensive in comparison to water and sediment analysis. The metabolically active tissues, which possess high bioaccumulation ability such as gills, liver, kidney and muscles, are usually employed for metal estimations (Heier *et al.,* 2009). The outer surface of the gills contains negatively charged sites which act as ligands for positively charged metal ions to dock (Playle *et al.,* 2011). Elevated quantity of heavy metals in gills reflects the acute exposure to heavy metal contaminated water. On the contrary, heavy metal concentration higher than the permissible limit in the liver and kidney represents chronic exposure of heavy metals. The metabolically active organs of fishes express various metal-binding proteins such as metallothioneins and tend to accumulate metal in specified organs (Atli & Canli, 2003). But knowledge on fish age, sex, species, breeding season, habitat and morphometric measurements such as length, width and weight etc. are mandatory to achieve accuracy in analysis.

Biomarkers

The primitive quantitative methods of heavy metal analysis in water and sediments are proven to be incapable of evaluating the implications of heavy metals on organisms. Further investigations showed that a correlation must be established among external levels of exposure, internal levels of tissue contamination and early adverse effects with extent and severity of tissue damage by heavy metals. At this juncture, different researchers decided to use various sub-organismal alterations caused by heavy metals such as Biochemical, Hematological, Immunological, Genotoxic etc., as diagnostic and predictive markers of heavy metal contamination in organisms and named them as a Biomarkers (Livingstone, 1993). The molecular, biochemical and physiological biomarkers can be used as early warning signals of heavy metal contamination in organisms. Biomarkers at the molecular level are the first to respond to heavy metal contamination, followed by responses at the biochemical and physiological and finally at morphological/histological levels (Lam & Gray, 2003).

Biomarkers can be classified into three types

a) Exposure biomarkers: These constitute a qualitative and quantitative analysis of extrinsic

substances or its metabolites or those products which are the result of interaction between extrinsic substances and target cells or molecules.

b) Effect biomarkers: This group of biomarkers comprises of those biochemical, physiological, or behavioural (or other) alterations in the organisms brought about by heavy metals.

c) Susceptibility biomarkers: Each organism reacts differently with different pollutants. This inherited or adapted ability of an organism to react with heavy metals is termed as susceptibility biomarker (Millward *et al.,* 2000).

Biomarkers are even classified into specific and nonspecific biomarkers. Metallothionein is defined as a specific biomarker as it indicates the presence of heavy metals. Some biomarkers which react in the same way to many toxicants are called nonspecific biomarkers.

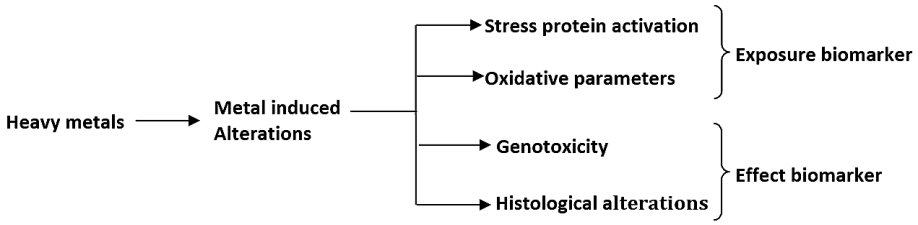


Fig. (1). Depicting the classification of biomarker

Some of the fish biomarkers for heavy metals are:

Stress proteins

The sub-lethal exposure to heavy metals invokes several cellular defense mechanisms in fishes. The gene expression of heavy metal- binding proteins in the affected cells is the main defense against heavy metals. The stress caused by heavy metals induces the synthesis of many stress proteins which minimize the detrimental effects of heavy metals. These stress proteins such as Heat Shock Proteins (HSP) and Metallothionein (MT) act as molecular chaperones (Bauman *et al.,* 1993). Heat shock proteins are the conserved proteins which are involved in folding, assembly and translocation of proteins. They also take part in the prevention of protein aggregation and miss-folding. Heavy metals damage the three-dimensional conformation of proteins by the destruction of intramolecular noncovalent interactions leading to misfolding or denaturation of proteins. But as soon as cells get exposed to heavy metals, the accelerated induction of stress protein genes and subsequent synthesis of these proteins provide the protection. These proteins have been regarded as a suitable biomarker as they are specific and are a quick inductive signal of metal contamination before the lethal effect of heavy metals (Currie *et al.,* 2000). In terms of stress biomarkers the Metallothioneins (MT) is the potent biomarker for heavy metals.

Metallothioneins (MT)

MTs are ubiquitous, heat-stable, soluble and inducible metalloproteins. Their molecular weight is 2.0-7.0 kDa. MT is generally composed of 61 amino acids but as an exception, cloudy catshark (*Scyliorhinus torazame*), which is a cartilaginous fish, contains 68 amino acids (Cho *et al.,* 2005). One of the remarkable features of the MT structure is the recurrence of Cys–X–Cys tripeptide sequences, where X stands for an amino acid residue other than cysteine. They have high cysteine content (20-30%), but they lack aromatic amino acids (Results in virtual lack of absorbance at 280nm). MT binds to metal by metal thiolate bond or mercaptid linkage and this is mainly due to cysteinyl thiolate sulfur atoms of the cysteine, which gives rise to metal-tetrathiolate cluster (Kaegi *et al.,* 1988). MT can bind to the extremely high concentration of metals (6-12g at/mol). Metallothionein's apoprotein, thionein is induced by exposure to Cadmium (Cd), Copper (Cu), Mercury (Hg), and Zinc (Zn). So it mediates the homeostasis of the metabolically important metals such as Zinc (Zn) and Copper (Cu). MTs also bind to Ib and IIb metals and detoxifies the toxic metals such as Cadmium (Cd), Mercury (Hg), Platinum (Pt) and Silver (Ag) (Doki *et al.,* 2004). Four MT isoforms have been discovered and named as MT-1, MT-2, MT-3, and MT-4. Out of them, only one or two MT isoforms have been detected in fishes (Bargelloni *et al.,* 1999).

MT genes have metal response elements (MRE) in the proximal promoter region. Mainly metal-responsive transcription factor 1 (MTF1), a zinc finger transcription factor belongs to the Cys2His2 family, binds to this MRE resulting in the induction of MT synthesis as soon as metal enters the tissue Fig.2. (Saydam *et al.,* 2002). As metals initiate the MT synthesis and also there is a well-established correlation between MT induction and intracellular heavy metals, it can be used as a potential biomarker for heavy metal contamination (Van Der Oost *et al.,* 2003). Schlenk *et al.,* (1997) exposed catfish (*Ictalurus punctatus*) to low-level Arsenic and found the dose-dependent increase in MT

expression. There was a marked time-dependent increase in hepatic MT expression as a result of acute Cu exposure to pre-spawning juvenile catfish.

Stipulating the standard MT levels in fishes is the tough task, as MT levels in fishes can fluctuate due to many exogenous and endogenous parameters. Rotchell *et al.,* (2001) studied age-dependent variations in hepatic MT levels of European Founders (*Pleuronectes fesus*) and found the peak induction at a younger age of fish. MT concentration is at its peak once fishes reach to 3 years of age. These variations were not in correlation with metal concentration. Along with age, reproductive steroids, stress hormones, seasonal changes, temperature, salinity and reproductive and dietary status modify the MT levels in fishes (Olsson, 1996). Many researchers also found that at the onset of vitellogenesis, the concentration of MT's increased irrespective of heavy metals in female fishes. On the other hand, mild variations in MT levels were seen in spermatogenesis of male fishes. Liver, as well as kidneys, are proven to the best organs for MT analysis, because of the fact that they have high concentrations of metallothioneins. But various studies showed that muscles, gills, skin, and brain can serve as samples for better analysis. Zinc and Copper are the potent inducers of metallothioneins followed by Cadmium and Mercury as they have stoichiometric similarities with Zn and Cu. Ag is often found to be a poor inducer of MT. MT induction by Cadmium is highly variable (Zhang *et al.,* 2005). Route of exposure of heavy metals does have an impact on the expression of tissue dependent MTs. Many researchers have found that waterborne exposure induces MT in kidneys followed by gills and liver. And in the dietary exposure following order of MT expression was observed, kidney >> cecae and posterior intestine > liver and stomach > midintestine> gills (Chowdhury *et al.,* 2005). MT level in fishes also depends on seasonal conditions, for example, Olsson *et al.,* 1996 found maximum MT induction during autumn and winter and less induction during summer. Hence, they concluded that MT level fluctuates with temperature. Furthermore, some endo or exogenous substances have an impact on MT levels. Estradiol and Estrogenic Polychlorinated Biphenyl (PCBs) inhibits the calcium-mediated MT synthesis. Estrogen, Cortisol, and Progesterone are proven to be an inducer of MT synthesis. Also, improper handling of fish like prolonged freezing, anoxic conditions, malnutrition and presence of herbicides adversely affects the MT level resulting in the improper quantification of MT in fishes. MT's have high cysteine content which make them susceptible to oxidation resulting in improper estimations. Furthermore, MT's have a saturation point. Once MTs get saturated with metal, excess metals spill over into cellular components and cause deleterious effects (Dabrio *et al.,* 2002)

Advantages

a) Stress proteins are very specific for heavy metal induction. Stress protein gene mainly MT gene activates as soon as metal enters the fish and MT can be estimated in tissue before the deleterious effect of heavy metals on fish. So it is rightly called the early warning signal and a potent biomarker for heavy metals.
b) Stress protein like MT is very specific for metal contamination such as zinc and copper.
c) In comparison to other biomarkers, there is a huge progression in MT estimation methodologies.
d) Some of the properties of MT such as having many –SH groups, mobility under the influence of electricity, thiolate bond formation and dynamic interaction with sorbents make MT easy for isolation as well as estimation.
e) Immense research has been undertaken to establish a correlation between MT level and tissue concentrations of various heavy metals and the significant positive correlations were observed by many researchers.
f) There is a huge amount of data on MT which makes researchers to easily get the information on it so one can explore new horizons in this field utilizing the current knowledge.

Disadvantages

a) Many stress proteins like MT have fluctuations in their levels due to many factors like temperature, salinity etc. which may affect the estimations.
b) MT structural analysis and isoform detection is yet to be studied
c) MT induction and expression is limited to a particular dose of heavy metals. If the heavy metal concentration goes above the limit, the MT estimation is not applicable. For example, highest induction of MT was seen in tilapia upon exposure of 2mg/kg of $CdCl_2$. But a further increase in $CdCl_2$ did not show any significant increase in MT level (Ueng *et al.,* 1996).
d) Many researchers use different methods for isolation and analysis of MT due to which no one can compare the results.

Various studies show that MT is a good biomarker for heavy metals in fishes even though many factors hamper the estimation. So, a proper study on fish species, sex, age, morphometric measurements such as length, width, weight of fish and season, water pH, temperature, salinity etc. is much appreciated before considering MT as a biomarker. The result will be more promising if one correlates the MT levels with these factors.

Finally, the stipulation of a standard method for MT analysis is much to be desired.

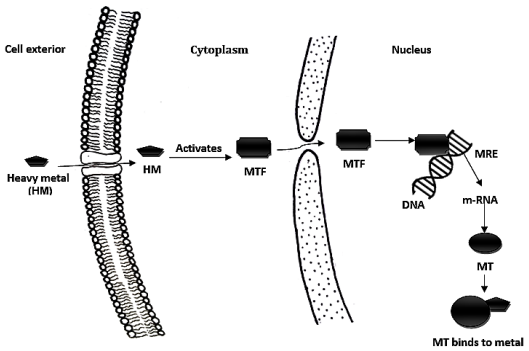


Fig. (2): Heavy metal induced MT gene activation and MT biosynthesis, HM-Heavy metal, MTF-Metal transcription factor, MRE- Metal-responsive element, MT- Metallothionein.

Oxidative stress parameters

Metals cause oxidative stress by initiating the production of Reactive Oxygen Species (ROS) or by reducing the concentration of antioxidants. The oxidative stress is the result of an imbalance between the synthesis of ROS and antioxidant defenses in fishes (Nishida, 2011). The lone pair of electron in the valence shell makes them highly reactive. Some of the ROS are superoxide anion radicals (O^{2-}), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^+). They are extremely reactive and react with important macromolecules, leading to the inactivation of enzymes, lipid peroxidation and DNA damage (Winston and Di Giulio, 1991). Antioxidants provide electrons to ROS and turn them nonreactive. The antioxidant system in fishes includes the enzymes and low molecular weight antioxidants. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-s-transferase (GST) are the main enzymatic antioxidants. Reduced glutathione (GSH) and oxidized glutathione disulfide (GSSG) constitute non-enzymatic antioxidants. Some of the byproducts of lipid peroxidation such as MDA serve as an effective indicator of oxidative stress. Yildirim *et al.,* (2011) collected C. trutta from both the heavy metal contaminated site and uncontaminated site in Munzur River. They found the significant increase in CAT activity and MDA level and decrease GSH level, which exhibits the heavy metal contamination. SOD mediates the dismutation of the O^{2-} (superoxide radical) to H_2O and H_2O_2 and H_2O_2 is detoxified by CAT. Due to early inhibitory function SOD–CAT system, this system acts as a primary line of defense as well as a prime biomarker for oxidative stress (Pandey., *et al.,* 2003).

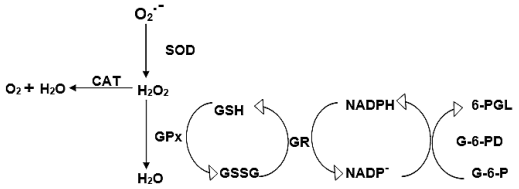


Fig. (3): Schematic representation of Antioxidants functional network. SOD = Superoxide dismutase, CAT = Catalase, GPx = Glutathione peroxidase, GR = Glutathione reductase, GSH=Glutathione, G-6-P = Glucose -6- phosphate, G-6-PD = Glucose-6-phospshate dehydraogenase, 6-PGL = 6-Phosphogluconolactone.

Superoxide dismutase (SOD)

SOD is the metalloenzyme mainly involved in the conversion of superoxide radical (O^{2-}) to hydrogen peroxide (H_2O_2), which itself is a Reactive Oxygen Species(ROS). H_2O_2 is neutralized by CAT. SOD is a very potent indicator of Iron and Mercury contamination. SOD is the kinetic perfection enzyme (Stegeman *et al.,* 1992). Li *et al.,* (2008) used embryonic and adult medaka (*Oryzias latipes*) exposed to nano-iron. They observed the dose-dependent inhibition of SOD activity in embryos. They also noticed that the cerebral and hepatic SOD was initially reduced, but subsequently increased with the duration of exposure in adult fishes. The increased erythrocyte SOD was observed in cichlid fish collected from iron contaminated river (Ruas *et al.,* 2008). SOD-CAT induction can be the potent biomarker for mercury exposure. Asagba *et al.,* (2008) showed an increase in SOD activity in the liver of C. gariepinus on exposure to cadmium for 21 days.

$$O_2^{\cdot-} + O_2^{\cdot-} \xrightarrow{SOD} H_2O_2 + O_2$$

Catalase (CAT)

CAT is a hematin-containing enzyme that is involved in the conversion of hydrogen peroxide (H_2O_2) to molecular oxygen (O_2) and water. It is a well-developed marker for cadmium and zinc exposure. Cadmium (Cd) directly binds to CAT and inhibits its activity. The significant decrease in CAT activity was observed in the kidney of the sea bass (*Dicentrarchus labrax*) upon exposure to cadmium (Cd) (Romeo *et al.,* 2000). CAT is substrate specific. CAT presented a negative correlation upon cadmium exposure as CAT is localized in peroxisomes and is involved in fatty acid metabolism. So changes in CAT activity are difficult to interpret. Vaglio and Landriscina (1999) found a significant decline in the CAT activity in Gilt-head bream (*Sparus aurata*) on days dependent exposure of $CdCl_2$. In support of this, Elia *et al.,* (2007) demonstrated the dose-dependent negative correlation between the CAT activity and cadmium in Black bullhead (*Ameiurus melas*). Many researchers have noticed that cadmium at higher concentration inhibit the activity of CAT. For

example, in killifish (*Fundulus heteroclitus*) cadmium concentration higher than 1 mg/L inhibits CAT activity (Pruel and Engelhardt 1980). CAT also shows a negative correlation with zinc as zinc-induced H_2O_2 inhibits CAT activity (Maria *et al.,* 2009).

$$H_2O_2 \xrightarrow{\text{CATALSE}} O_2 + H_2O$$

Glutathione enzyme system

Glutathione peroxidase (GPOX), Glutathione reductase (GRED), Glutathione-s-transferase (GST) and Glutathione reductase (GR)

GPOX is the tetrameric, selenium-dependent enzyme which reduces the peroxides to the corresponding alcohols using GSH as a cofactor. Glutathione peroxidase (GPx) is a selenoenzyme. It catalyzes the reduction of H_2O_2 to water and also converts lipid hydroperoxides (LOOH) to the corresponding stable alcohols (LOH) with associated oxidation of reduced GSH to its oxidized GSSG. The oxidized GSSG will be reduced by Glutathione reductase (GR) at the expense of NADPH. Diana *et al.,* 2009 exposed matrinxa (*Brycon amazonicus*) to $HgCl_2$ and observed the absence in the induction of GPx activity in the gills, heart and white muscle. Glutathione reductase (GRED) is involved in the homeostasis of GSH/GSSG in oxidative stress condition (Winston and Di Giulio, 1991). It converts the oxidized glutathione (GSSG) to reduced glutathione (GSH) with concomitant oxidation of NADPH to $NADP^+$. Lead (Pb) interacts with glutathione reductase (GR) via formation of complex with selenium and also reduces GPx activity (Ercal *et al.,* 2001). Many researchers have shown that Hg induces GST activity. Exposure of *S. senegalensis* showed that GR has negative correlation with lead (Pb). Lead binds to the disulfide bond in the active site of GR and results in its inhibition. This inhibition prevents the reduction of GSSG (Winston and Di Giulio, 1991). The exposure of *Cyprinus carpio* to 10–100mM Zn revealed that GST is negatively correlated to zinc Franco *et al.,* (2008).

Glutathione (GSH)

Glutathione (L-alpha-glutamyl-cysteinyl-glycine) is a tripeptide which is present in reduced form (GSH). It acts as an intracellular reductant and nucleophile. Copper and Cadmium predominantly bind to the thiol group of glutathione. As evidence, inhibition of liver GSH was seen in three spined sticklebacks (*Gasterosteus aculeatus*) upon exposure to copper sulfate (Sanchez *et al.,* 2005). In support of this study, Jena *et al.,* 2009 showed the depletion of muscle GSH after copper sulphate exposure. The significant reduction in the GSH level is mainly due to direct copper interference with GSH synthesis by inhibition of glutathione reductase. The effects of cadmium on GSH levels is unstable as both increase and decrease in GSH has been observed, based on fish species, duration of exposure and experimental conditions. GSH reduces the chromium (Cr) tetravalent state (VI) to pentavalent state (V), to participate in the Fenton reaction resulting in the production of OH- radical. Many researchers have observed a reduction in GSH level by mercury exposure. Mieiro *et al.,* (2010) explained that the decline in the GSH level is due to direct binding of Hg to SH moiety of GSH and formation of metal-SG complexes or oxidation of thiol group of GSH. These studies show that GSH has a key role in mercury induced oxidative stress. GSH also plays an important role in redox state produced by arsenic. GSH donates an electron to arsenate that results in the production of arsenite. This increase in GSSG/GSH ratio was also observed in the Indian catfish (*Clarias batrachus*) upon exposure to arsenic (Bhattacharya and Bhattacharya, 2007). In many situations, GSH indicates oxidative stress by a decline in its activity. But on the contrary, lead caused an increase in hepatic glutathione concentrations. This may be due to lead mediated synthesis of GSH.

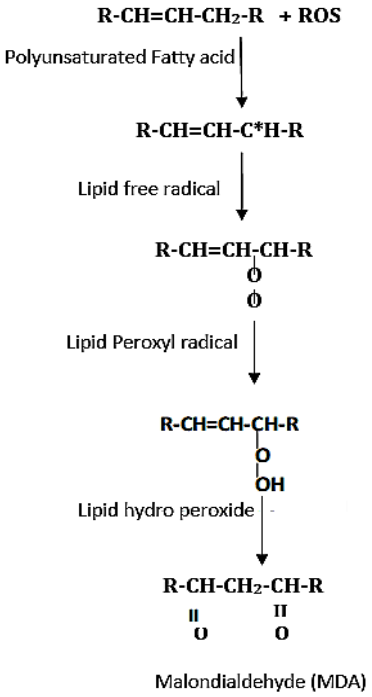
$$2GSH + H_2O_2 \xrightarrow{\text{Peroxidase}} GSSSG + 2H_{2ss}O$$

Malondialdehyde (MDA)

Transition metals (such as Cd, Co, Cu, Hg, Ni, Pb, Fe, Sn, and V) cause peroxidation of membrane polyunsaturated fatty acids. This results in the synthesis of lipid peroxy radicals which in turn produce many lipid degradation products such as Bis (dimethyl acetal) also called Malondialdehyde (MDA). MDA is highly reactive three carbon dialdehyde. It has a high affinity for thiol group and amino group of proteins and nucleic acid. Maiti *et al.,* (2010) carried out a 60-day lead exposure to walking catfish (*Clarias batrachus*) and observed the elevated MDA level. Doherty *et al.,* (2010) analyzed the metal contamination in 4 fishes collected from contaminated lagoon. They observed the 15.22%, 12.6%, 13.1% and 6.98% increased MDA level in the liver of *Tilapia guinensis*, liver of *Chrysichthys nigrodigitatus*, gills of *Tilapia guinensis* and gills of *Chrysichthys nigrodigitatus* respectively in comparison to a reference site. In support of this, Ghada *et al.,* (2013) exposed *Sparus aurata* to 0.5 mg/L of Cadmium (Cd) for 2 to 24 hours. Ghada *et al.,* found a significant increase in MDA after 24 hours of exposure. The thiobarbituric acid (TBARS) method is the commonly used method for MDA quantification. But this reaction is nonspecific as both free as well as protein-bound MDA can react to TBARS and give false positive results (Canli *et al.,* 2003).

Each oxidative stress parameter is specific for some of the heavy metals. But Atomic absorption spectrophotometric analysis of fish tissue, water and sediments for heavy metals is recommended

prior to the selection of oxidative biomarkers. Because, it shows which heavy metal is in high quantity. Based on this information, selection of specific oxidative biomarkers has to be done for accurate analysis.



Genotoxic parameters

The exposure of fishes to different heavy metals exerts a cascade of genotoxic effects. Several studies observed that the heavy metals induced pre-mutagenic lesions such as, DNA adducts, base modifications, DNA–DNA and DNA–proteins cross-linking, DNA strand breaks, chromosomal aberrations, alterations of genetic frequency and micronucleated blood cells in fish species (Liney *et al.,* 2006). Many researchers observed the positive correlation between sediment levels of chromium and nickel and DNA damage in mussels collected from coastline polluted by heavy metals. Sanchez-Galan *et al.,* (2001) found DNA damage in Eel (*Anguilla anguilla* L.) by cadmium and mercury. The hepatic DNA adducts are the indication of long exposure of fishes to heavy metals. On the other hand, DNA adduct in GI tract indicates the recent exposure (Shugart, 1996). Heavy metals are the good oxidative stress inducer which may result in secondary concomitant DNA alterations such as single or double strand DNA break (ssDNA and ds DNA break), changes in base composition etc. Genotoxic lesions exert genetic instability in the call resulting in Apoptosis or necrosis. Piechotta *et al.,* 1999 demonstrated cadmium induced apoptosis in Dad (*limanda limanda*). Some of the analysis like standard chromosomal analysis, sister-chromatid exchange (SCE) and erythrocitic nuclear abnormalities are employed to analyse the genetic aberrations. SCE assay is helpful in detecting the exchange of chromosomal fragments between sister chromatids which follow DNA strand breakage. Comet assay is the widely used, sensitive and reliable tool for detection of genotoxicity in aquatic environments (Frenzilli *et al.,* 2009).

Matsumoto *et al.,* (2006) exposed the Nile tilapia (*Oreochromis niloticus*) to chromium and found the significant increase in DNA breaks of peripheral blood cells. This clastogenic activity of chromium was detected by comet assay. Single-cell gel electrophoresis assay detects the DNA damage and repair at single cell level detecting one break in $1x10^{10}$ Da. This assay includes the lysis of cells with a detergent and high salt and relaxation of supercoiled DNA in agarose-embedded nucleoids. The DNA moves towards anode under electrophoresis, forming comet-like appearance which can be seen under fluorescence microscopy. The tail lengths, the percentage of DNA in tail and Olive tail movements are directly proportional to DNA damage and are the main parameter of comet assay which gives an idea on the extent of DNA damage. The neutral comet assay helps in distinguishing apoptosis from necrosis by analyzing comet score. There is an increased comet score in apoptotic cells and the almost zero comet score in necrotic cells. But this technique is incapable of detecting the base oxidation and DNA adduct. In fishes, comet assay is applied to numerous cell types: erythrocytes and cells of gill, liver, kidney, and gut.

Fishes have a low amount of DNA per cell and the large numbers of small chromosomes, so metaphase analysis of chromosomes is not a suitable test for fishes (Ojima *et al.,* 1976). Micronucleus (MN) test utilizes the interphase cells of proliferating cells irrespective of its karyotypes. The gills, fins, kidney and hepatic cells and peripheral erythrocytes of fishes are mainly employed for MN assay. Nucleated erythrocytes are the most commonly used cells in the piscine MN test on chronic exposure to heavy metals. Various kinds of nuclear abnormalities were found during MN test of fish erythrocytes. Some of them were buds, broken eggs, lobed, notched, vacuolated and karyolitic nuclei. Zhu *et al.,* (2004) exposed *Cyprinus carpio* to hexavalent chromium and found the MN in erythrocytes. Matsumoto *et al.,* (2006) concluded that chromium at a concentration of 0.01 mg/mL is capable of producing micronucleated erythrocyte indices in *O. niloticus*. On the other hand, negative results were reported by many researchers. Cavas *et al.,* (2005) concluded that subchronic exposures of hexavalent chromium to three fish species did not induce micronucleus formation. Same results for the absence of MN were noticed when *Salmo trutta* and *Phoxinus phoxinus* were intraperitoneally injected with zinc and copper (Sanchez-Galan *et al.,* 1999). Furthermore, some authors have reported that the prolonged exposure of heavy metals results in a

decrease of micronuclei frequency in fishes (De Lemos *et al.,* 2001). This is because the presence of heavy metals like Cadmium, Copper and Zinc, individually or in combination with other metals, may exert a strong inhibitory effect on the cell division (Unyayar *et al.,* 2006). Another reason for the decrease in micronuclei frequency maybe due to the development of DNA repair mechanism or apoptosis of genetically unstable cells. So, in comparison to Comet assay, MN is less sensitive. Furthermore, the use of DNA-reacting fluorescent dye is useful in detecting the small MN.

Advantages

a) In fishes, DNA serves as the main genetic material and the analysis of genetic aberrations in fishes is important as these aberrations may result in a reduction of population size and even to the extinction of the present generation.
b) Through the genotoxic analysis, the premutagenic lesion can be studied.
c) The main advantage of using these biomarkers is that they have well-developed techniques for studying these genotoxic biomarkers.
d) Micronucleus test detects irreversible aberrations such as clastogenic and aneugenic aberrations. But COMET assay helps in the early detection of reversible lesions such as DNA break and alkali labile site.
e) Many of the biomarkers such as metallothioneins, oxidative stress parameters, and histological aberrations fluctuate depending on temperature, season, endogenous or exogenous factors of fish like hormones, vitamins etc. But genotoxic biomarkers are more stable biomarkers as they depend on the DNA, whose integrity does not get altered by mild variations of those factors.

Disadvantages

a) The 32P-postlabeling assay for DNA adduct analysis is time consuming and expensive, so this biomarker needs better immunological detection techniques.
b) This biomarker is an effect biomarker and it depends on genetic instability/aberrations which are destructive.
c) Many of the genotoxic lesions are general to many of toxicants such as pesticides.
d) DNA repair mechanism repairs many of these lesions before they get prominent.

Histological parameter

The histological analysis comprises of microscopic semi-quantitative evaluation of tissue or organ abnormalities. The exposure of heavy metals to fishes causes multiple lesions and injuries to various fish organs, but liver and gills are suitable organs for histological analysis. Vinodhini *et al.,* (2008) observed heavy metal induced gill epithelial lesions, filamental edema, intense lamellar vasodilatation in common Carp (*Cyprinus carpio* L.). De Boeck *et al.,* in 2001 reported the trace metal induced gill epithelial hyperplasia in various species of fishes. But Oropeca *et al.,* in 2005 refuted the above report and showed gill epithelial hyperplasia in fishes exposed to other pollutants such as pesticides. Thophon *et al.,* (2003) observed the cadmium induced gills lamellar aneurysms in which vasodilatation occur at lamellar axis, which alters the structure of pillar cells. There is an immense amount of literature on various histological alterations in the fishes due to heavy metal perturbation both in invitro and invivo studies and also after acute or chronic exposure of heavy metal at various concentrations. But it is hard to determine whether histological alterations are adaptive or destructive. In addition to this, heavy metal exposure causes epithelial degradation and necrosis in gills, but these negative impacts can be restored by the fish defense system. Velmurugan *et al.,* (2009) stated that the secondary lamella length (SLL), width (SLW) and interlamellar distance (ILD) in gills are the prominent biomarkers for heavy metal contamination. Along with this, Monteiro *et al.,* (2005) concluded that aneurysms can be used as a reliable biomarker for copper acute exposure. Ribeiro *et al.,* (1995) studied the effect of water-borne inorganic Hg on the olfactory epithelium of *Trichomycterus brasiliensis* and observed the partial as well as complete loss of sensory cells on sub-lethal exposure to inorganic Hg. The peculiarity in observation was that the sensory cells did not heal after the recovery of the epithelium.

In the liver, many histopathological lesions such as inflammatory lesions, preneoplastic lesions, neoplastic lesions and hepatocellular fibrillar inclusions are high in fishes upon exposure to heavy metals. As many toxicants can alter the histology of liver, so liver histological biomarkers are not as specific as gill histological biomarkers. Yancheva *et al.,* (2015) studied hepatocytes of fishes collected from a heavy metal contaminated lake. They found the morphologically altered hepatocytes with flattened nucleus located on the periphery of the cells. The alterations in size, shape and number of hepatocyte nuclei are mainly due to heavy metal induced toxicity Paris. -Palacios *et al.,* (2000) exposed Zebrafish (*Brachydanio rerio*) to copper sulfate at sub-lethal concentration and found the huge variations in hepatocyte nuclei. Furthermore, the metal accumulation in the liver causes hepatocyte lysis and cirrhosis. As an evidence for this concept, Varanka *et al.,* (2000) showed heavy metal induced hepatocyte lysis and cirrhosis in common carp (*Cyprinus carpio*). Yancheva *et al.,* (2014) demonstrated the metal induced interruption in the hepatic blood circulation, which results in venous hyperaemia. The loss of stored lipid in hepatocyte is a reliable marker for acute water-borne and trophic doses of inorganic Hg. But some physiological process such

as vitellogenesis in females can also reduce the lipid content so only immature fishes should be used for the above analysis.

In line with these, kidneys also provide well established histological aberrations upon exposure to heavy metals. The most common heavy metal induced histological catastrophes in the fish kidneys are vasodilatation, mainly capillary dilation in the glomerulus, bowman capsule shrinkage and tubular degeneration due to swelling and hyaline droplet accumulation (Takashima and Hibiya, 1995). Hadi and Alwan (2012) exposed freshwater fish *Tilapia zillii* to Aluminum and found the tubule degeneration but failed to find necrosis. They concluded that long time exposure to Aluminum is required for necrotic studies. They also confirmed the shrinkage of glomeruli and blood hemorrhage, at elevated doses of Aluminum. In support of this study, Abdelhamid and El-Ayouty (1991) found congestion and hemorrhages in catfish's (*Clarias lazera*) kidneys and gastrointestinal tract upon exposure to Aluminium. Athikesavan *et al.,* (2006) exposed silver carp (*Hypophthalmichthys molitrix*) to sub-lethal concentrations of Nickel for 30 days and detected vacuolization, hypertrophy, karyolysis, hyperplasia, haemolysis, ruptured cell and pyknotic nuclei in the kidneys of the fish.

Advantages

a) Histological parameters help to analyse the effects of each heavy metal on individual organs mainly in invitro studies.
b) It correlates the duration, dose, and route of exposure of heavy metals due to biological consequence in the form of histological aberrations.
c) Histological analysis also provides insight on other alterations such as behaviour, reproduction, metabolism, growth and physiology of fish. For instance, Pevzner *et al.,* (1986) studied alterations in taste buds of *Alburnus alburnusi* on exposure to inorganic Hg. They observed changes microvillar structures of fish which resulted in alteration of feeding behavior. Furthermore, olfactory epithelium of *S. alpinus* was damaged by exposure to Hg, which resulted in variations in fish reproductive behavior, feeding, and avoidance of predators. (Ribeiro *et al.,* 2002)

Disadvantages

a) Histopathological analysis demands good laboratory instrumentation and experienced manpower for proper analysis. But it is non-specific and time-consuming.
b) Histological biomarker lacks information on the effect of individual heavy metals on various organs. So, no one can predict the specifics of the exposed heavy metals. As a consequence, one needs to depend on some qualitative chemical analysis for identification of heavy metals.
c) Many researchers observed that all histological aberrations are not specific for heavy metals. Some of them can be seen upon exposure to pesticides, PCB's etc. For example, the interstitial edema in gill epithelium was considered as a specific aberration for heavy metal contamination, until Nowak (1992), Banaee (2013), Schwaiger *et al.,* (2004), and many others showed that this aberration is observed even upon exposure to endosulfan, paraquat, and drugs respectively.
d) Histological biomarker failed to be considered as early warning signal because this biomarker can be studied once heavy metals adversely affect the fish. Many of these adverse effects cannot be healed and results in fish death.

According to the above disadvantages, a proper study indicating the histological response of specific organs for particular heavy metal is very much desired. Thus, further studies need to be undertaken for detecting pollutant specific histological alteration.

Conclusion

Biomarkers have pronounced importance in evaluating the exposure to and the effects of heavy metals in organisms. They show a specific interaction between the heavy metals and organisms with which they provide significant information for temporally and spatially integrated measure of heavy metals. But traditional quantitative methods reveal nothing about adverse effects of heavy metals. Biomarkers provide valuable early warning signal so that remedial or preemptive action can be employed. Biomarker analysis is compatible with both laboratory and field studies. The investigation and development of new biomarkers need extensive laboratory experimentation to detect contaminant specific, reliable and prominent biological response in organisms. But fluctuations of biomarkers due to intrinsic and extrinsic factors provide trivial information on the quantity of heavy metals in the ecosystem. So integrative biomarker analysis along with traditional quantification methods give complete information on the level of heavy metal contamination and its biological consequences.

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