CHAPTER 6

DISCUSSION

Cadmium is a non essential, non degradable, toxic pollutant in fresh water ecosystems. The factors that influence Cd absorption, distribution and elimination have not been fully understood in freshwater fishes even though a lot of work has been carried out world over. The present study is an attempt to study the effects of Cadmium on select tissues of *C. gariepinus* to give insights with regard to this fish which hasn’t been documented as yet. This work also focuses on the mitigation responses undertaken by this fish by producing detoxification enzymes and inducing the stress protein Metallothionein.

CADMIUM ACCUMULATION

The pattern of Cd accumulation in the select tissues like liver, kidney and gill of *C. gariepinus* on exposure to sub-lethal concentrations of 5, 10 and 20 ppm of Cd for 24, 48 and 72 h shows significant variations. The rate of Cd accumulation varies from tissue to tissue for various concentrations and exposure periods. The differences in accumulation may be attributed to the proximity of the tissue to the toxicant medium, the physiological state of the tissue, presence of ligands having an affinity to Cd and/or the role of tissue in the detoxification process. The availability of cadmium ions after dissociation from its compound status in aqueous medium is also known to be deciding factor. It is known that the chloride ions are soluble, while cadmium ions are not, hence the impact on the tissues even on exposure at sublethal doses for even short durations of time.

The results indicate that the Cd accumulation levels in *C. gariepinus* is clearly tissue specific and similar to many aquatic organisms such as *Rainbow trout* (Chowdhury et al., 2005), *Litopenaeus vannamei* (Wu and Chen, 2005)
and *Sinopotamon henanense* (Ma *et al.*, 2007). Cd is seen to accumulate mainly in organs such as liver that is functionally designed to store metals and to detoxify the heavy metal by synthesizing MTs (Unlu *et al.*, 1996; Romeoa *et al.*, 1999; Rao and Padmaja, 2000). The accumulation of Cd in liver and kidney is higher than gill in fishes because the liver and kidney are the major sites of Cd for detoxification and excretion by binding with MT. The gills are the major entry site of metals and act as a transient store for accumulation of metals (Soegianto *et al.*, 1999).

Aquatic animals like fish absorb heavy metals via the gills and transfer the metals to the blood and then circulated to other parts of the body. Hence Cd accumulation in gill shows a fluctuating pattern. At the initial stage of exposure, the accumulation of Cd is rapid (Ma *et al.*, 2007). According to Kent, (1998) the liver and kidney are involved in the detoxification and removal of toxic substances circulating in the blood stream. Moreover, liver and kidney, being the major organs of metabolic activities including detoxification and excretion (Klavercamp *et al.*, 1984) Cd might also be transported to these organs from other tissues, including gill and muscle for the purpose of subsequent elimination. Such transportation might lead to higher rates of accumulation in these two organs. The possibility of detoxification/elimination related mobilization of accumulated Cd may be one reason for the intermittent reductions in the quantity of accumulated Cd in gill at various stages of exposure. Further, according to Dorian and Gattone, (1992) unbound metals, such as Cd, can be reabsorbed by active transport mechanism in the cells of the proximal convoluted tubule, and once they are in the cells they bind to MT, resulting in their accumulation. All these observations justify the possible transport of trace amounts of Cd from the various tissues to kidney.

The present study shows that distribution of accumulated Cd in fishes among organs are in conformation with the results as evidenced by the works
of De Conto et al., 1999; Asagba et al., 2008. Reports have shown that during waterborne metal exposure, a high level of metal accumulation occurs in organs like liver, kidney and gill (De Conto et al., 1999).

HISTOLOGICAL ALTERATIONS IN LIVER AND KIDNEY TISSUE

The present study shows Cd induced histopathological alterations in the liver of *C. gariepinus* and the degree of morphological alterations was in dose-time dependent. It has been observed that the liver of fishes exhibited several histological alterations like hypertrophy of hepatocytes and cytoplasmic vacuolation in the hepatocytes and focal necrosis of hepatic tissue, loosening of hepatic tissue and the hepatic cells losing their original shape. Similar observations have been observed in other fishes and the hepatic cells were swollen, the cytoplasm appeared cloudy and granular (Van Dyk, 2003). Similar results have been reported by Giari et al., (2007) who studied the morphological alterations in European sea bass (*Dicentrachus labrax*) after exposing to different Cd concentrations of 4.47, 5.63, 7.08 and 8.91 ppm for 24 and 48 h respectively. These findings lend support to the observations of the present study.

The Cd induced liver toxicity is mediated by the up-regulation of reactive oxygen species (hydroxyl groups, superoxides and hydrogen peroxides) which cause oxidative damage to lipid contents of membranes (Shaikh et al. 1999, Packer and Cadenas 2002). Overproduction of ROS normally induces oxidative stress unless it was scavenged with endogenous antioxidants. Thus, overproduction of ROS could be attributed to the depletion of antioxidants or to the direct action of Cd on peroxidation reaction and iron-mediated peroxidation (Casalino et al., 2002; Pillai and Gupta, 2005). Primary injury to cells resulting from the binding of Cd to sulfhydryl groups in mitochondria and secondary injury initiated by the activation of kupffer cells have also been mentioned as a possible mechanism of toxic effect of Cd in the
liver (Rikans and Yamano, 2000). Inactivation of sulfhydryl groups causes oxidative stress, mitochondrial permeability transition and mitochondrial dysfunction (Jurczuk et al., 2004). It is also suggested that kupffer cells released proinflammatory cytokines and chemokines, which stimulated the migration and accumulation of neutrophils and monocytes in the liver (Dudley et al., 1984). Dudley also suggested that hepatocytes injury may be caused by ischemia due to sinusoidal endothelial cell dysfunction. Cd has been found to accumulate in endothelial cells, leading to necrosis and denudation of hepatic sinusoids. The hepatotoxicity of Cd has also been attributed to the formation of toxic metabolites when it is activated by hepatic cytochrome P450 (Wong et al., 1981) to a highly active metabolite N-acetyl-P-benzooquinone imine (Savides and Oehne, 1983). This could be the possible explanation to the altered histology of liver in the present study.

The histological data of the present study has revealed that Cd exposure markedly alters the renal histo-architecture of the glomerulus and renal tubules. The nephro-toxicity of Cd was indicated by changes such as cytoplasmic vacuolation. In particular glomerular degeneration, pycnotic nuclei and shrinkage, increased space of renal corpuscles, neutrophilic infiltration in tubule nucleus shows pycnotic nature with vacuolization (cloudy swelling and hyaline droplets), tubular degeneration and necrosis were seen in some areas, loosening, vocalization and degeneration of the nuclei of uriniferous tubules and narrowing of tubular lumen were common occurrence. These alterations clearly demonstrated that Cd exposure severely affects the kidney of *C. gariepinus*. Furthermore, it also activated the lysosomes of the kidney cells, leading to cell necrosis or vacuolations. Thereby it induced renal damage in both glomerulus and tubules. Similar results have been reported in Cd exposed *C. batrachus* (Bilal, 2011) and *Dicentrachus labrax* (Giari et al., 2007).
Further, the kidney of fish often shows cloudy swelling in tubule cells. This alteration can be identified by the hypertrophy of the cells and the presence of small granules in the cytoplasm, which took an appearance of a net. This initial stage of degeneration process can progress to hyaline degeneration, characterized by the presence of large eosinophilic granules inside the cells. These granules may be formed inside the cells or by the re-absorption of plasma proteins lost in the urine, indicating damage in the corpuscle (Hinton and Lauren, 1990; Takashima and Hibiya, 1995). In more severe cases, the degenerative process can lead to tissue necrosis (Takashima and Hibiya, 1995).

The excess Cd interfered with the cell signaling pathway at every stage of signal transduction and can act on receptors, secondary messengers, and transcription factors (Jing et al., 2012). The main target organelle of Cd is the mitochondria (Koizumi et al., 1996). Cd entered mitochondria through calcium channels and induced conformational changes in proteins located in the membrane by binding to thiol groups and consequently would have interfered with oxidative phosphorylation and altered its membrane permeability leading to reduction in membrane potential, decrease in cellular ATP levels, disturbances in homeostasis of calcium and leading to increased ROS, which ultimately leads to leakage of cytochrome-c. This induces DNA damage or apoptosis (Li et al. 2000; Chan et al., 2006) misfolding of the protein or eventual production of Reactive Oxygen Species ROS or Reactive Nitrogen Species RNS. High level of ROS and RNS directly or indirectly activated the Caspase 3, thereby inducing cell death.

**DNA FRAGMENTATION**

The DNA fragmentation is well studied hallmark of apoptotic cell death (Wyllie, 1980). Gel electrophoresis results revealed fragmentation in Cd treated tissues of *C. gariepinus*. Such DNA fragments were absent or minimal in the control tissues. Since the Cd treated tissues show clear fragmentation it
provides further support for its apoptotic activity. The fragments increased in a
time and dose dependent manner. The maximum fragments were observed after
72 h of Cd exposure. When compared to 24 and 48 h Cd exposed tissue, the
number of ‘ladders’ formed and observed were higher in the 72h Cd treated
tissue, revealing the extent of damage to the cellular DNA due to cadmium.

To examine whether the apoptotic pathway was involved, DAPI
staining was done on the tissues treated with 20 ppm of Cd. After 24, 48 and 72
h of treatment of Cd the various stages preluding apoptosis like, chromatin
condensation, membrane blebbing, cell shrinkage, increased number of nuclear
body fragments and irregular edges around the nucleus were observed in Cd
exposed and accumulated tissues. While the untreated control tissues exhibited
round, clear edged, uniformly stained cell nuclei, which is the characteristic
feature of normal unaffected tissues.

APOPTOSIS

Cd is known to stimulate free-radical production, resulting in oxidative
deterioration of lipids, proteins and DNA, as well as initiating various
pathological conditions in aquatic organisms and animals. In the present study
the effect of Cd on the liver and kidney tissues of *C. gariepinus* culminating in
apoptosis was studied.

Cd exposed liver and kidney of fish shows a dose and time dependent
increase in severity of alterations resulting in apoptosis. Apoptosis shows
consistent time response patterns, and it precedes necrosis (Gathwan *et al.*, 2012) as evidenced in the present study. Cd may interact with elements like
zinc, manganese, calcium, magnesium, iron, selenium and cause their
secondary deficit, thereby disrupting metabolism, resulting in the final
morphological and functional changes in many organs (Sarkar *et al.*, 2013). In
liver, exposure to different contaminant heavy metals shows histological
changes in liver cells and the present study is in continuance of earlier work of Van Dyk, (2003).

In case of chronic Cd stress, the amount of MT which is synthesized by the system may not be enough to bind and remove all the Cd, thus leading to accumulation of Cd in the liver. Excess Cd induced mitochondrial membrane lipid peroxidation, can cause damage to organelles. Cd induced oxidative stress in cells, results primarily in peroxidation damage to cell membranes has been reported by Arroyo et al., (2010) and Jomova and Valko, (2011). Furthermore, the interaction of Cd and MT leads to production of highly reactive OH$^-$ free radicals via Fenton reaction. These high reactive OH$^-$ radicals interact with plasma membrane, macromolecules and proteins before leading to cell death.

Apoptosis is characterized by cell shrinkage, cytoplasmic, nuclear and chromatin condensation, membrane blebbing, protein fragmentation, and DNA degradation, and finally breakdown of a cell into apoptotic bodies (Thompson, 1995). Although extensive research has been undertaken to elucidate signal pathways in apoptosis, at present, oxidative stress has been considered an important possible mechanism of Cd toxicity (Kim and Sharma, 2006). Accumulated evidence has also shown that Cd increased cellular reactive oxygen species (ROS) levels, lipid peroxidation and alteration in glutathione (GSH) levels in many cell types (Pathak and Khandelwal, 2006; Valko et al., 2006; Wang et al., 2009) suggesting that Cd-induced apoptosis may be connected with oxidative stress. ROS are known to be able to affect mitochondrial membrane potential and trigger a series of mitochondria-associated events including apoptosis (Pathak and Khandelwal, 2007). A high level of ROS in the mitochondria can result in free radical attack on membrane phospholipids, preceding mitochondrial membrane depolarization, which is considered as an irreversible step in the apoptosis process, which can trigger a cascade of caspases (Pelicano et al., 2003).
CASPASE-3 ACTIVITY

Apoptosis is characterized by chromatin condensation and DNA fragmentation, and is mediated by caspases (Hengartner, 2000; Elmore, 2007). The family of caspases regulates apoptosis. Caspases are normally present in the cell as proenzymes that require limited proteolysis to activate enzymatic activity (Nunez et al., 1998). Once activated, caspases cleave a variety of intracellular polypeptides, including major structural elements of the cytoplasm and nucleus, components of the DNA repair machinery, and a number of protein kinases. Collectively, these divisions disrupt the survival pathways and disassemble important architectural components of the cell, which contribute to the stereotypic morphological and biochemical changes that characterize apoptosis. Increased Caspase-3 synthesis after 72 h of cadmium exposure in the experimental fish tissues might be due to increased Cd levels as advocated by (Ma et al., 2007; Sumit et al., 2014) Among the caspases family, caspase-3 is most commonly activated in the apoptotic process (Janicke et al., 1998). Caspase-3 is a key executioner of apoptosis, its activation is mediated by initiator caspases such as caspase-9 that cleave a number of substrates which act in response to DNA strand breaks leading to apoptosis (Nicholson and Thornberry, 1997; Mancini et al., 1998; Soldani and Scovassi, 2002). This morphological and biochemical changes in apoptotic cells are chromatin condensation, cell shrinkage, plasma membrane blebbing and DNA fragmentation (Vaculova and Zhivotovsky, 2008). Thus it is evident that Cd induces apoptosis. Also quantification of caspase-3 would be a good indicator of apoptotic activity in affected cells as is the case with cadmium toxicity.

Apoptosis is mainly characterized by overall shrinkage in the volume of the cells and its nucleus, loss of adhesion to neighboring cells, blebbing, and DNA fragmentation (Susin et al., 1998). Cd exposed tissues show apoptotic cell death which can be visualized by the characteristic intense staining and
chromatin fragmentation of DAPI-stained nuclei from which it is evident that Cd has induced apoptosis in the present study. When electrophoresed the so-called “DNA ladder” pattern (oligonucleosomal multimers of ~180bp) which is the characteristic of apoptosis is observed (Borriello et al., 2002) and was also evidenced in the present study. Cd treated tissues exhibited characteristic ladder pattern of DNA fragmentation whereas the control DNA was intact. Thus it can be inferred that higher and longer Cd concentration exposed tissues in the present study showed higher degree of apoptosis which was confirmed when DAPI assay was performed. DAPI assay shows an increase in apoptotic cells in a time and concentration dependent manner in Cd treated tissues. Thus it is evident that Cd is a causative factor for apoptosis.

DETOXIFICATION ENZYMES

Three commonly studied detoxification enzymes were used for analyzes in this work. The results obtained were in conformation with previous works in similar organisms and heavy metals in aquatic environments. As expected for the dose and time dependent experimental setup, GST showed an increase in syntheise, while Catalase and SOD depreciated upto 72 h inspite of increasing Cd concentration. SOD enzymes are known for their free radical scavenging. But their activity along with other free radical scavengers and mono oxygen users enhance after certain time lag only. As this was a 72 h study, these enzymes show decrease in activity for the said period. GST action alone was immediate perhaps due to its known collation with MTs.

MT INDUCTION

MTs have been found to have four main functions in aquatic vertebrates. They are bioaccumulation of toxic metals and their detoxification, homeostatic regulation of metals, protection against oxidative stress and neuroprotective mechanism respectively.
In the present study it is seen that Cd induced MT, resulting in elevated MT levels in the liver and kidney tissues. The results clearly correlates increase in MT levels with increase in Cd concentration in the surrounding medium. The liver MT concentrations were found to be higher than kidney and gill during the study. It is opined that waterborne Cd ultimately accumulates in the liver for detoxification and accumulates in the kidney for excretion (Asagba et al., 2008) and also induces the synthesis of MT. At the initial stage of the acute waterborne Cd exposure, the tissue MT concentrations in *C. gariepinus* were in complete agreement with those of many other investigations in that the induction of MT was dose and time dependent increased this results correlate with earlier works Martinez et al., (1993) in crayfish (*Procambarus clarkia*) and Wu and Chen, (2005) in shrimp (*Litopenaeus vannamei*).

The presence of heavy metals is observed to activate the transcription of MT genes via the binding of metal binding regulatory factors to the metal-responsive elements (Roesijadi, 1992). After exposure to non-essential heavy metals like Cd, the induction of MT increases the binding of heavy metals to the protein, which serves a sequestration function to decrease the toxicity of Cd (Hogstrand and Haux, 1991).

MT was identified as a Cd binding protein. For this reason it has been considered as an important factor involved in the protection of organisms against the harmful effects of toxic heavy metals such as Cd (Dallinger et al., 2000; Dallinger et al., 2004). MT has no known catalytic function as it is non enzymatic. Hence the measurements of its concentrations are based upon the quantification assay of protein itself. In the present study the liver and kidney are the main organs of Cd accumulation followed by gill. The result of the present study falls in line with earlier works in *Rainbow trout* (Chowdhury et al. 2005) and *Litopenaeus vannamei* (Wu and Chen, 2005).
As higher concentrations of Cd was observed in the liver tissue, it is assumed that it induces synthesis of new MTs and a higher rate of Cd-MT complex and so the complex formed sequesters the cadmium (Jana et al., 2009) to other detoxification or excretory organs. Cd content and MTs concentrations increased significantly in fishes exposed to different concentrations of Cd for 24, 48 and 72 h.

Low Cd content in the liver tissue of control fishes was due to low level of Cd in the fresh water. However when the levels of Cd were increased by addition of known concentrations of Cd, significant and parallel elevations of MT up to 72 h of exposure to Cd was recorded in the present study. The highest MT induction levels were observed in liver tissues after 72 h of Cd exposure in C. gariepinus indicates the possible role of liver in the storage of Cd for detoxification which is initiated by MT synthesis and formation of Cd-MT complex. The result of the present study correlates with earlier studies with similar Cd concentrations and similar exposure periods (Mani et al., 2014) in marine catfish, A. arius.

MT is functionally a very important protein and its structural conservation is dictated by its functional requirement in mammalian tissues. However changing the length of inter domain hinge region, can lead to decrease in metal binding ability has proven by Klassen et al., (2009). Whether such mechanism in fish is possible has to be elucidated.

Thus this study helps place MT as a heavy metal biomarker. Also its highly conserved amino acid sequence provides great significance in phylogenetic studies and construction of dedrograms and defining evolution of such molecules in organisms.
CORRELATION BETWEEN CADMIUM ACCUMULATION AND MT INDUCTION

The results obtained for the study clearly indicates a correlation between Cd accumulation and MT induction in the liver and kidney tissue of fresh water catfish, *C. gariepinus*. The relationship between Cd accumulation and MT induction in the liver, kidney and gill after exposure to Cd was found to be statistically significant. The correlation is positive, which means increase in cadmium levels could induce higher levels of MT. As the present was limited to 72h, what happens on chronic exposure was not observed and forms future scope for in depth work.

Similar studies on Cd exposure on *Litopenaeus vannamei* (Wu and Chen, 2005) also exhibited a positive correlation between Cd accumulation and MT induction. Similar observations and inferences can be made in the liver, kidney and gill of *C. gariepinus* based on the results of the present study. MT concentrations increase linearly with increase Cd concentrations, indicating that MT can be used as an indicator of Cd concentration in the tissues of fishes. However further characterization and studies of the mechanism of MT are necessary.

MT induction in the study tissues increased with increase in the concentration of Cd in the external medium up to 72 h of the study. The liver and kidney Cd accumulation and MT synthesis was found to be higher than gill. These results suggest that MT played an important role in liver to detoxify high quantities of Cd and excretion of Cd in kidney. Cd accumulation in the tissues shows a positive correlation with MT induction in all the tissues. The Cd concentrations and MT concentrations in the control tissues of liver, kidney and gill were minimum as heavy metals levels were BDL. The low quantities of MT in normal tissue can be attributed to MT bound to Zn fingerlings. When cells are presented with heavy metals like Cd, MT bound to Zn displaces Zn
and binds to Cd. And the process of MT gene transcription is initiated. It is inferred that MT synthesis is dependent on Cd which is confirmed by the present study.

MT CONFIRMATION STUDIES

Western blot confirmed the induction and presence of MT in the tissues of the study fish. The results indicate that the MT expression levels were elevated in Cd exposed liver and kidney tissues of *C. gariepinus* when compared with the unexposed control tissues. This is a good indicator of the correlation between Cd accumulation and MT induction. However, Western blotting does not allow one to determine as to which specific cell-types induced MT, their localization and expression in the organs as a result of metal exposure. The use of antibodies against MT is a good approach to detect the levels of proteins (Burkhardt *et al.*, 1999; Duquesne *et al.*, 1995). This formed the basis of Immunohistochemical work in the work. Immunolocalisation helped provide better insights into patterns of MT synthesis in cells and inside cells.

The MT expression levels increased significantly after 24, 48 and 72 h of Cd exposure in the liver tissues of *C. gariepinus*. The highest MT expression observed in the present study was that after 72 h of Cd exposure. In kidney, MT protein levels were quantified to find MT expression levels which showed MT significantly increased during the 24, 48 and 72 h Cd exposure. Cd induced MT levels were quantified and compared with control kidney tissue MT levels to draw a clear correlation between Cd and MT. The highest MT expression was observed in our study for the 72 h Cd exposure of kidney tissues (Sumit *et al.*, 2014).

Western blot analyses and immunohistochemical work carried out in the present study confirmed the presence of MT and quantifying the MT
expression levels induced by Cd in treated liver and kidney tissues and their expression patterns in the tissues. The blot results conformed with previous works and results.

**MT LOCALIZATION STUDIES BY IMMUNOHISTOCHEMICAL ANALYSIS**

In liver, irMTs were mainly localized in the cytoplasm of hepatocytes and lysosomes, to a lesser extent in erythrocytes. The immunolabelling produced in hepatocytes after Cd exposure was higher than in control liver in *C. gariepinus*. The MT protein localization has been detected in several fish tissues using specific antibodies by the method of immunohistochemical techniques. MT was localized in the liver of hepatocytes. These results are in concurrence with previous study results in liver of turbot (*Scophthalmus maximus*) exposed to Cd, Cu and Zn (Amaral et al., 2002; Alvarado et al., 2005; Alvarado et al., 2006) in kidney of salmon (*Salmo salar*) exposed to Cd (Berntssen et al., 2001; Dang et al., 2001) and in the gills of brown and rainbow trouts (*Oncorynchus mykiss*) when exposed to sewage treatment plant effluents (Alvarado et al., 2006).

Cd is primarily distributed in the liver where expose to a sub-lethal dose level can result in 60 to 70% of the metal being sequestered by MTs, and to a lesser extent in the kidney (Olsson and Haux, 1986; Hogstrand et al., 1991). In the present study kidney MT protein levels were lower than that of liver tissues. This could be due to the short duration of experiments. Significant level of cadmium reaching kidney may take a longer time beyond the 72 h limit. After 72 h, MT induction may be higher but this was not carried out in this research works. The induction of fish MTs has been used as a biomarker of exposure to metals in both marine and fresh water environments (Hylland et al., 1992; Muto et al., 1999). As a general rule, most of the metal stored in the liver is within the cytosol of hepatocytes (Wicklund et al., 1992) since the
primary MTs are metal-binding protein; MT is a cytoplasmic protein and is mainly localized in the cytoplasm of hepatocytes (Olsson et al., 1998).

Immunohistochemical analysis revealed an increase in MT protein production in Cd exposed hepatocytes. In addition, together with cytosolic MT localization; the lysosomal population of hepatocytes also exhibited a strong MT labeling after Cd exposure. The lysosomes constitute a major compartment for metal accumulation and sequestration (Fowler, 1987) allowing a reduction of the availability of Cd, at least transiently. Lysosomes can contain degradation products of MTs and serve as a final storage site of degraded MTs and possibly, of other metal-binding proteins (Dallinger, 1995; Klaasen et al., 1999). Metals cannot be degraded metabolically so they have to be excreted via gills, skin, intestine liver or kidney (Filipovic and Raspor, 2003). The main cell-type of the kidney is the nephrocytes which contains numerous, invaginations of the plasma membrane, often in the form of a well-developed basal labyrinth. The presence of irMTs was mainly restricted to the basal part of the nephrocytes that form the proximal tubules in the present study. MT expression in the kidney before Cd exposure was lower when compared with Cd treated tissues in the current investigation.

MT induction and expression on exposure to Cd has be observed and confirmed using Western Blot and MT-immunohistochemical techniques in the hepatocytes of the liver and nephrocytes of the kidney tissues in *C. gariepinus* in the present study. The MT levels were increased in liver and kidney during successive exposure to Cd. Western blotting helps in conforming the induction and expression of MT in cells and tissues which are exposed to Cd. The comparison of control and treated samples demonstrates that the induction of MT on exposure to Cd and the characteristic tissue expression pattern of induction is noticable. The observation of the results clearly demonstrates that Cd is the inducing factor for MT protein (as all other heavy metals were BDL)
and this is the cellular response on initial exposure to heavy metals and subsequently the initiation of detoxification process especially in the liver. This could be the reason for elevated MTs in liver when compared to kidney. However the literature on MT expression in fish is not exhaustive and this study is the first available literature in *C. gariepinus.* (Sumit *et al.*, 2014)

**ISOLATION AND PURIFICATION OF MT**

Metal binding column was used in the present study, because the MT protein is prone to chelate transitionally to metals such as Cd. MT isolated from *C. gariepinus* liver tissue binds exclusively to nickel and is different from other metals as noted by Coyle *et al.*, (2002). Hence a Ni chelating column was used for obtaining best results.

The Ni column herein utilized was able to efficiently chelate to the MT-Cd complex. The same approach was tried in order to test the possibility of purifying the fish MT by loading the column with Cd instead of nickel. In this case, it was observed that Cd bound to the column resin was confirmed using ICP-OES after washing the column with EDTA, though the protein could not be recovered during imidazol elution step. Most probably the protein that passed through the column that did not bind to the Cd could have been attached to the resin. Current understanding shows that MT extracted from the liver of fishes could have different affinity for Cd and Ni, which could explain why it did not bind to the Cd$^{2+}$ attached to the column; however, it did bind to Ni, as confirmed by Cd$^{2+}$ and Ni$^{2+}$ determination from MT positive fractions. The results of the present study correlate with earlier of works (Romero and Vasak, 2002; Honda *et al.*, 2005). Hence a Ni chelating column was used for affinity chromatographic purification of MT in this research work.

Running the affinity chromatography with Ni chelating yielded purified protein in varying concentrations in eluted fractions from which the
elute with selected to be run on the desalting sephadex column to remove imidazole which interfered with the MT. Purified MT after removal of imidazole was run on SDS PAGE gels and the lane with no non specific bands was the pure MT. This was then used for sequence and molecular weight analysis by MALDI-TOF MS PMF.

MTs have a high content of cysteine residues that bind to various heavy metals along with the sulfadryl groups. MT contains 2 metal binding domains: 4 divalent ions are chelated within cluster A of the α-domain and are coordinated via cysteinyl thiolate bridges to 11 cysteine ligands. Cluster B, the corresponding region within the β-domain, can ligate 3 divalent ions to 9 cysteines.

The molecular weight of MT protein can be estimated using SDS-PAGE and was found to be around 6 – 7 kDa in usually isoform (MT-1 and MT-2) in liver tissues. There are references in the literature that MT from vertebrates are also found within the low molecular marker range (Olafson and Thompson, 1974; Ley et al., 1983).

The MT isolated and characterized from the liver tissue exposed to cadmium in C.gariepinus belongs to MTsuper family and has a molecular weight 6.125 kDa for the 60 amino acids. MT in C. gariepinus was confirmed by MALDI-TOF and interpretation by bioinformatic tools like MASCOT search services and NCBI protein databases. The majority of the fish MT show highly conserved amino acid sequences indicating fewer evolutionary constraints in the polypeptide chain as advocated by klassan et al 1994. In the present study the amino acid sequence of C. gariepinus shows 93% sequence similarity with C. marocephalus. The sequences vary only in four amino acids out of the sixty in the protein. Also it revealed the presence of about 20% cysteine, which is unique to MT. Which shows the amino acid sequences of fish MT are highly conserved.
The molecular weight of fresh catfish *C. gariepinus* was 6.125 kDa, indicating it’s a very low molecular weight protein and the deducted weight of the protein falls in line with earlier works of Saha *et al.*, (2013).

Some teleosts don’t have the characteristic MT I and 2 isoforms. This was reported by Bergelloni in 1999 where he found that MT in most teleosts except rainbow trout showed only a single MT form and no isomers. The MT of this fish also appears to be a single MT form with no isomers. Further work is required in the future to characterize the *C. gariepinus* MT better.

Since the protein match score of MT amino acid sequence of *C.gariepinus* with *C. marocephalus* shows a statistically calculated value of 87, which is greater than 65 (Mascot service). This value is statically significant (p<0.05). Hence the low molecular weight protein synthesized by fresh catfish *C. gariepinus* liver in response to cadmium is Metallothionein.