CHAPTER 2

REVIEW OF LITERATURE

2.1. Defining Toxicology

Toxicology can be defined as that branch of science that deals with any substance that causes a harmful effect when administered, either by accident or design, to a living organism. Broader definitions of toxicology, such as "the study of the detection, occurrence, properties, effects, and regulation of toxic substances". Toxicology is preeminently an applied science, dedicated to the enhancement of the quality of life and the protection of the environment. The contributions of toxicology to environmental studies has become increasingly important in recent years. The disconcertion of normal life processes by toxic chemicals enables us to learn more about the life processes themselves. The field of toxicology has expanded immensely in recent decades, both in numbers of toxicologists and in accumulated knowledge. This expansion has brought a change from a primarily descriptive science to one which utilizes an extensive range of methodology to study the mechanisms involved in toxic events.

Toxicity is a relative event that depends not only on the toxic properties of the chemical and the dose administered but also on individual and interspecific variation in the metabolic processing of the chemical. Toxicity itself can rarely, if ever, be defined as a single molecular event but is, rather, a cascade of events starting with exposure, proceeding through distribution and metabolism, and ending with interaction with cellular macromolecules (usually DNA or protein) and the expression of a toxic end point.

The definition of a poison or toxicant involves a qualitative biological aspect because a compound, toxic to one species or genetic strain, may be relatively harmless to another. The importance of dose is well explained by metals that are essential in the diet but are toxic at higher doses. Thus iron, copper, magnesium, cobalt, manganese, and zinc can

be present in the diet at too low a level (deficiency), at an appropriate level (maintenance), or at too high a level (toxic). Compounds may be toxic under some circumstances but not others or, perhaps, toxic in combination with another compound but nontoxic alone. The measurement of toxicity is also complex. Toxicity may be acute or chronic, and may vary from one organ to another as well as with age, genetics, gender, diet, physiological condition, or the health status of the organism. Exposure of humans and other organisms to toxicants may result from many activities such as occupational exposure, environmental exposure and accidental exposure. The toxicity of a particular compound may vary with the portal of entry into the body, whether through the alimentary canal, the lungs, gills or the skin. Thus toxicity may vary as much as tenfold with the route of administration.

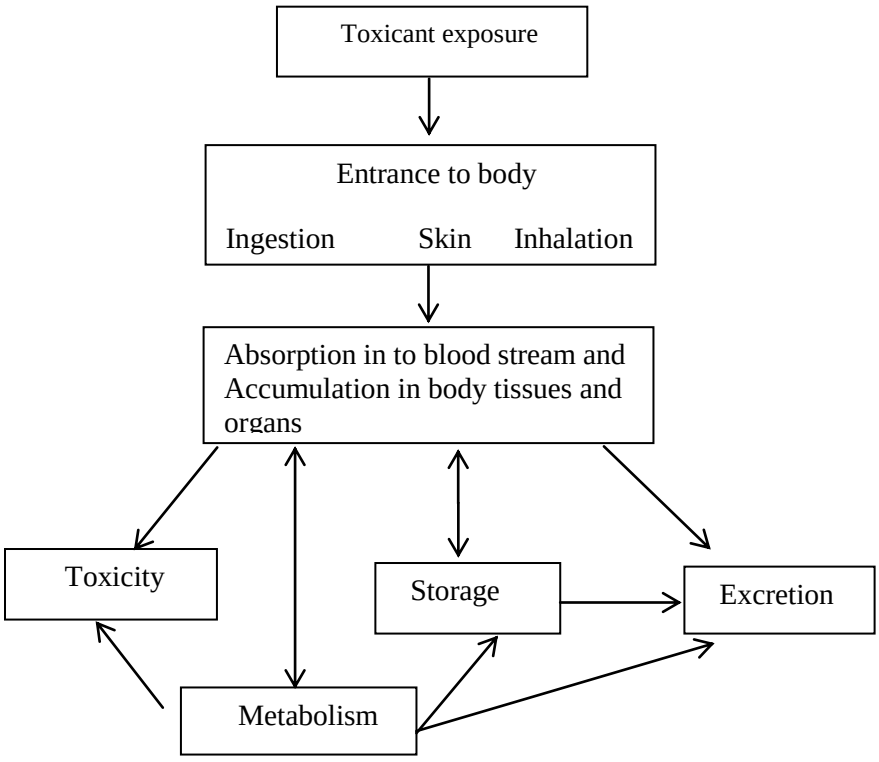

Fig.2.1: Effect of toxicants in the body

2.2. Heavy metal toxicity

Trace heavy metals, such as arsenic, cadmium, lead, chromium, nickel, and mercury, are important environmental pollutants, particularly in areas with high anthropogenic pressure (Waseem *et al*., 2014). Some of the heavy metals are considered to be xenobiotics because these have no beneficial role in body functioning and are even very harmful in minor concentrations. Cadmium, beryllium, aluminum, uranium, mercury, lead, bismuth, barium, antimony, arsenic, and so forth are included in toxic metals. Higher levels of these metal ions are highly toxic to animals including humans and plants, and their solubility in water is considered to be one of the major environmental issues (Azizullzh *et al*., 2011) (Wei *et al*., 2010) (Giller *et al*., 1998). Along with global environmental pollution resulting from economic development, heavy metal poisoning has become an increasingly serious health problem in the world. Industry, mining, advanced agriculture, household waste and motor traffic are all among activities considered to be major sources of metal pollution. Heavy metals such as cadmium, lead, cupper and more specifically mercury are potentially harmful to most organisms even in very low concentrations and have been reported as hazardous environmental pollutants able to accumulate along the aquatic food chain with severe risk for animal and human health (Kaoud *et al*., 2010). The presence of these heavy metals have been associated with decreased fertility and other reproductive abnormalities in birds, fish, shellfish and mammals and also altered immune function. Heavy metals like mercury and cadmium are known to accumulate in marine organisms and cause rapid genetic changes (Nimmo *et al*. 1978, Nevo *et al*. 1986). Toxic heavy metal can cause dermatological diseases, skin cancer and internal cancers (liver, kidney, lung and bladder), cardiovascular disease, diabetes and anemia as well as reproductive, developmental, immunological and neurological affects in the human body. The toxicity of these elements is due to

their ability to cause, oxidative damage to living tissues. Damage includes enhanced lipid peroxidation, DNA damage, enzyme inactivation and the oxidation of protein sulfydryl groups (Taiz and Zeiger 1998).

The heavy metals, lead mercury and cadmium, all have electron sharing affinities that can result in formation of covalent attachments (Bondy, 1996). These attachments are mainly formed between heavy metals and sulfydryl group of proteins (Quig, 1998). Detailed studies in past two decades have shown that metals like iron, copper, cadmium, chromium, mercury, nickel, vanadium possess the ability to produce reactive radicles, resulting in DNA damage, lipid peroxidation, depletion of protein sulfydryls and other effects. Reactive radical species include a wide range of oxygen-, carbon-sulfur- radicals originating from superoxide radical, hydrogen peroxide and lipid peroxides but also in chelates of amino acids, peptides and proteins complexed with the toxic metals. The toxic effects of metals involve hepatotoxicity, neurotoxicity and nephrotoxicity (Valko *et al.,* 2005)

Heavy metals are non-biodegradable and once they enter the environment, bioaccumulation occurs in the fish tissue in the case of aquatic environment, by means of metabolic and biosorption processes (Wicklund-Glynn1991). It is also possible that environmental toxicants may increase the susceptibility of aquatic animals to various diseases by interfering with the normal functioning of their immune, reproductive and developmental processes (Couch and John, 1978). Prolonged exposure to water pollutants even in very low concentrations have been reported to induce morphological, histological and biochemical alterations in the tissues which may critically influence fish quality. Fishes are important component of human nutrition, and those from contaminated sites present a potential risk to human health. Since fish occupy the top of the aquatic food chain, they are suitable bioindicators of metal contamination. Metals

are well known inducers of oxidative stress and assessment of oxidative damage and antioxidant defenses in fish can reflect metal contamination of aquatic environment (livingstone, 2003).

2.3. Mercury

2.3.1. Properties of Mercury (Hg)

Mercury is a chemical element with symbol Hg and atomic number 80. It is commonly known as quicksilver and was formerly named hydrargyrum. A heavy, silvery d-block element, mercury is the only metallic element that is liquid at standard conditions for temperature and pressure. Mercury has a freezing point of −38.83 °C and a boiling point of 356.73 °C.

Table 2.1: Mercury in the periodic table:

Atomic number	80
Standard atomic weight	200.592(3)
Element category	transition metal
Group, block	group 12, d-block
Period	period 6
Electron configuration	[Xe] $4f^{14}$ $5d^{10}$ $6s^{2}$
per shell	2, 8, 18, 32, 18, 2

2.3.2. Sources and Uses of Mercury

Mercury is a naturally occurring element that is found in air, water and soil. It exists in several forms: elemental or metallic mercury, inorganic mercury compounds, and organic mercury compounds. Mercury is an extremely rare element in the Earth's crust,

having an average crustal abundance by mass of only 0.08 parts per million (ppm) (Ehrlich *et al*., 2008). The most toxic forms of mercury are its organic compounds, such as dimethylmercury and methylmercury. Mercury can cause both chronic and acute poisoning. Humans cannot create or destroy mercury. Pure mercury is a liquid metal, sometimes referred to as quicksilver that volatizes readily. It has traditionally been used to make products like thermometers, switches, and some light bulbs. Mercury is found in many rocks including coal. When coal is burned, mercury is released into the environment. Burning hazardous wastes, producing chlorine, breaking mercury products, and spilling mercury, as well as the improper treatment and disposal of products or wastes containing mercury, can also release it into the environment. Mercury is used primarily for the manufacture of industrial chemicals or for electrical and electronic applications. Mercury is used in thermometers, barometers, manometers, sphygmomanometers, float valves, mercury switches, mercury relays, and fluorescent lamps. Mercury remains in use in scientific research applications and in amalgam material for dental restoration in some locales.

2.3.3. Distribution in the Environment

Mercury enters into the environment through the improper disposal (e.g., land filling, incineration) of certain products. Products containing mercury include: auto parts, batteries, fluorescent bulbs, medical products, thermometers, and thermostats (EPA 2007). Mercury in the air eventually settles into water or onto land where it can be washed into water. Once deposited, certain microorganisms can change it into methylmercury, a highly toxic form that builds up in fish, shellfish and animals that eat fish. The methylation of inorganic mercury in the sediment of lakes, rivers and other waterways, as well as in the oceans, is a key step in the transport of mercury in aquatic food chains. Mercury accumulated in the tissues of fish is usually in the form of

methylmercury, while the source is usually inorganic mercury (Huckabee *et al*., 1979). Several hypotheses of how and where methylation occurs have been proposed. The main hypotheses are:

- Biological methylation, bacterial in origin, which produces methylmercury in the environment (methylmercury is taken up by fish more readily than inorganic mercury),
- Methylation by microorganisms associated with branchial mucus of the fish or in the fish gut
- Methylation in the fish's liver (Thellen *et al*, 1981).

Birds and mammals that eat fish are more exposed to mercury than other animals in water ecosystems. Similarly, predators that eat fish-eating animals may be highly exposed. At high levels of exposure, methylmercury's harmful effects on these animals include death, reduced reproduction, slower growth and development, and abnormal behavior. Fish and shellfish have a natural tendency to concentrate mercury in their bodies, often in the form of methylmercury, a highly toxic organic compound of mercury. Species of fish that are high on the food chain, such as shark, swordfish, king mackerel, bluefin tuna, albacore tuna, and tilefish contain higher concentrations of mercury than others. As mercury and methylmercury are fat soluble, they primarily accumulate in the viscera, although they are also found throughout the muscle tissue (Cocoros *et al*., 1973). When this fish is consumed by a predator, the mercury level is accumulated. Since fish are less efficient at depurating than accumulating methylmercury, fish-tissue concentrations increase over time. Thus species that are high on the food chain amass body burdens of mercury that can be ten times higher than the species they consume. This process is called biomagnification. Mercury poisoning happened this way in Minamata, Japan, now called Minamata disease.

2.3.4. Health effects of mercury

Mercury exposure at high levels can harm the brain, heart, kidneys, lungs, and immune system of people of all ages. Mercury poisoning can result in several diseases, including acrodynia (pink disease) (Bjorklund 1995), Hunter-Russell syndrome, (Tokuomi *et al*., 1977) and Minamata disease (Davidson *et al*., 2004). Scientists today are concerned that people who eat fish contaminated with mercury will be put at risk – particularly pregnant mothers and children, as mercury poisoning has been shown to adversely affect brain development. However it is also being linked to a higher risk of heart disease and stroke in men as well as memory loss, and central nervous system damage. In humans, approximately 80% of inhaled mercury vapor is absorbed via the respiratory tract, where it enters the circulatory system and is distributed throughout the body (Cherian *et al*., 1978). Acute inhalation of high concentrations causes a wide variety of cognitive, personality, sensory, and motor disturbances. The most prominent symptoms include tremors (initially affecting the hands and sometimes spreading to other parts of the body), emotional liability (characterized by irritability, excessive shyness, confidence loss, and nervousness), insomnia, memory loss, neuromuscular changes (weakness, muscle atrophy, muscle twitching), headaches, polyneuropathy (paresthesia, stocking-glove sensory loss, hyperactive tendon reflexes, slowed sensory and motor nerve conduction velocities), and performance deficits in tests of cognitive function (ATSDR 1999).

2.3.5. Mode of action of mercury

- Dissociation of salts precipitates proteins and destroys mucosal membranes
- Necrosis of proximal tubular epithelium
- Inhibition of sulfhydryl (-SH) group containing enzymes

- Inhibition of mitochondrial oxidative phosphorylation

2.3.6. Toxicokinetics of Mercury

2.3.6.1. Absorption

Absorption of Mercury Species occurs through the three major exposure routes- inhalation, oral and dermal. 75-85% of an inhaled dose of the vapor is absorbed by the body. 97% of absorption occurs through the lungs. Absorption from the gastrointestinal tract (GI) tract following oral dose is estimated at 7-15%. < 3% of total amount of elemental Hg absorbed by the body is from dermal exposure (Clarkson *et al*., 2002).

2.3.6.2. Distribution

Absorption results in rapid diffusion across the lungs and entrance into the bloodstream, where it is distributed throughout the body (because it is lipophilic), including the blood-brain barrier and the placenta. The ingested dose is rapidly distributed from the GI tract to the blood and organs. Divalent ionic (mercuric) mercury has a high affinity for sulfhydryl groups in the RBCs and plasma. The highest concentration is in the kidneys. Mercuric mercury induces metallothionein production in the kidneys, which may contribute to the kidney's accumulation of mercuric mercury. It does not cross readily cross the blood-brain barrier or the placenta because of its ionic charge (Clarkson *et al*., 2002).

2.3.6.3. Metabolism

Elemental Hg is oxidized in the red blood cells by catalase and hydrogen peroxide to divalent ionic (mercuric) Hg. Mercuric Hg is unstable in vivo and has been shown to convert to elemental Hg (rat study). It can also be methylated by intestinal flora, but cannot be methylated in body tissues. Methylmercury is stable in the body compared to other species. It is slowly demethylated to mercuric Hg in tissue macrophages, intestinal flora, and the fetal liver. Although these sites of demethylation are known, the enzymes

in mammalian tissues responsible for the biotransformation have not yet been identified. It is metabolized to ionic mercury at a rate of around 1% of the body burden per day. The mercuric Hg resides for long periods of time in the CNS, probably in an inert form (NAS 2000).

2.3.6.4. Elimination

The major routes of excretion are bile and feces. Methylmercury undergoes enterohepatic cycling where it is secreted into bile, and then partly reabsorbed and returned to the liver. Most Methylmercury is eliminated by demethylation and then excretion of the ionic form in the feces (~90% in feces as mercuric Hg). Most of the absorbed ionic Hg is excreted in urine. Smaller amounts are excreted in saliva, bile, sweat, exhalation, and breast milk (USEPA 1997).

2.4. Cadmium

2.4.1. Properties of cadmium (Cd)

Cadmium is a chemical element with symbol Cd and atomic number 48. It is a lustrous, silver-white, ductile, very malleable metal. Melting point 320.9°C and Boiling point 765°C at 100 kPa.

Table 2.2: Cadmium in the periodic table:

Atomic number	48
Standard atomic weight	112.414(4)
Element category	transition metal, alternatively considered a post-transition metal
Group, block	group 12, d-block
Period	period 5
Electron configuration	[Kr] $4d^{10}$ $5s^2$
per shell	2, 8, 18, 18, 2

2.4.2. Sources and uses of cadmium

Cadmium can mainly be found in the earth's crust. It always occurs in combination with zinc. Cadmium also consists in the industries as an inevitable by-product of zinc, lead and copper extraction. After being applied it enters the environment mainly through the ground, because it is found in manures and pesticides. About three-fourths of cadmium is used in Ni-Cd batteries, most of the remaining one-fourth is used mainly for pigments, coatings and plating, and as stabilizers for plastics. Cadmium has been used particularly to electroplate steel where a film of cadmium only 0.05 mm thick will provide complete protection against the sea. Cadmium has the ability to absorb neutrons, so it is used as a barrier to control nuclear fission. Cadmium is used to manufacture pigments and batteries and in the metal-plating and plastics industries (ATSDR 1997).

2.4.3. Distribution in the environment

Naturally a very large amount of cadmium is released into the environment, about 25,000 tons a year. About half of this cadmium is released into rivers through weathering of rocks and some cadmium is released into air through forest fires and volcanoes. The rest of the cadmium is released through human activities, such as manufacturing. Cadmium waste streams from the industries mainly end up in soils. The causes of these waste streams are for instance zinc production, phosphate ore implication and bio industrial manure. Cadmium waste streams may also enter the air through (household) waste combustion and burning of fossil fuels. Another important source of cadmium emission is the production of artificial phosphate fertilizers. Part of the cadmium ends up in the soil after the fertilizer is applied on farmland and the rest of

the cadmium ends up in surface waters when waste from fertilizer productions is dumped by production companies.

Cadmium can be transported over great distances when it is absorbed by sludge. This cadmium-rich sludge can pollute surface waters as well as soils.

Cadmium strongly adsorbs to organic matter in soils. When cadmium is present in soils it can be extremely dangerous, as the uptake through food will increase. Soils that are acidified enhance the cadmium uptake by plants. This is a potential danger to the animals that are dependent upon the plants for survival. Cadmium can accumulate in their bodies, especially when they eat multiple plants. Cows may have large amounts of cadmium in their kidneys due to this.

In aquatic ecosystems cadmium can be bioaccumulated in mussels, oysters, shrimps, lobsters and fishes. The susceptibility to cadmium can vary greatly between aquatic organisms. Salt-water organisms are known to be more resistant to cadmium poisoning than freshwater organisms. Animals eating or drinking cadmium sometimes get high blood-pressures, liver disease and nerve or brain damage.

2.4.4. Toxicokinetics of cadmium

2.4.4.1. Absorption

- Inhalation route: Inhalation exposure primarily occurs in the workplace. Cadmium compounds are inhaled as particulate matter, either as fumes with very small particle size or as dust. Thus particle size, which controls alveolar deposition, is a key determinant of cadmium absorption in the lung. The respiratory Cd intake can be diverted to the gastro-intestinal tract due to the

clearance of Cd deposited on the mucosa of nasopharynx, trachea or bronchi. It can also be deposited in the alveoli and from there be absorbed into the blood.

- Oral route: For a given individual, the absorption following oral exposure to cadmium is likely to depend on physiologic status (age; body stores of iron, calcium, and zinc; pregnancy history; etc.) and, also, on the presence and levels of ions (Zn) and other dietary components ingested with the cadmium.
- Dermal route: Absorption of cadmium through the skin is extremely low (0.5%) and would be of concern only in situations where concentrated solutions would be in contact with the skin for several hours or longer (ATSDR 1997).

2.4.4.2. Distribution

Cadmium is widely distributed in the body, with the major portion of the body burden located in the liver and kidney. Liver and kidney cadmium concentrations are comparable after short-term exposure, but the kidney concentration exceeds the liver concentration following prolonged exposure.

The concentration of cadmium in the liver of occupationally exposed workers generally increases in proportion to intensity and duration of exposures to values up to100 µg/g. The concentration of cadmium in the kidney rises more slowly than in the liver after exposure and begins to decline after the onset of renal damage at a critical concentration of 160-285 µg/g.

2.4.4.3. Metabolism

The most hazardous characteristic of cadmium is that it accumulates throughout lifetime. Cadmium accumulates in the liver and kidneys and has a long biological half-life, from 17-30 years in man (Goyer, 1997). After uptake from the lung or the

gastrointestinal tract, cadmium is transported in blood plasma initially bound to albumin, as shown in experimental animals. Cadmium bound to albumin is taken up by the liver. In the liver, cadmium induces the synthesis of metallothionein and a few days after exposure metallothionein-bound cadmium appears in the blood plasma. Because of its low molecular weight, cadmium-metallothionein is competently filtered through the glomeruli and thereafter taken up by the tubules. Cadmium accumulates in the human kidney over the entire lifetime (Nordberg, 1992).

2.4.4.4. Elimination

Most cadmium that is ingested or inhaled and transported to the gut via mucociliary clearance is excreted in the feces. Almost all fecal cadmium represents material that was not absorbed from the gastrointestinal tract. Most absorbed cadmium is excreted very slowly, with urinary and fecal excretion being approximately equal. Cadmium is also eliminated through hair and breast milk, but these routes are of limited importance for total excretion and do not significantly alter the biological half-time. Several studies have shown that in the general population urinary cadmium excretion increases with age, this increase coinciding with the increased body burden. Smokers have higher urinary excretion than non-smokers. The amount of cadmium excreted represents only a small fraction of the total body burden unless renal damage is present (Chang 1996).

2.4.4.5. Mode of action of cadmium toxicity

Cadmium is known to increase oxidative stress by being a catalyst in the formation of reactive oxygen species, increasing lipid peroxidation, and depleting glutathione and protein-bound sulfhydryl groups. Cadmium also can stimulate the production of inflammatory cytokines and downregulates the protective function of nitric oxide formation (Navas *et al*. 2004).

2.4.4.6 Health effects of cadmium

The chief organs acted upon by cadmium with its chronic toxic effects are kidneys, and bone. The lungs are a target organ in acute high-dose exposures to inhaled cadmium fumes.

Human uptake of cadmium takes place mainly through food. Foodstuffs that are rich in cadmium can greatly increase the cadmium concentration in human bodies. Examples are liver, mushrooms, shellfish, mussels, cocoa powder and dried seaweed. An exposure to significantly higher cadmium levels occurs when people smoke. Tobacco smoke transports cadmium into the lungs. Blood will transport it through the rest of the body where it can increase effects by potentiating cadmium that is already present from cadmium-rich food.

Cadmium is first transported to the liver through the blood. There, it is bond to proteins to form complexes that are transported to the kidneys. Cadmium accumulates in kidneys, where it damages filtering mechanisms. This causes the excretion of essential proteins and sugars from the body and further kidney damage. It takes a very long time before cadmium that has accumulated in kidneys is excreted from a human body (ATSDR 2008).

Other health effects that can be caused by cadmium are:

- Diarrhea, stomach pains and severe vomiting
- Bone fracture
- Reproductive failure and possibly even infertility
- Damage to the central nervous system
- Damage to the immune system

- Psychological disorders
- Possibly DNA damage or cancer development

2.5. A comparative study of heavy metals, mercury (Hg) and cadmium (Cd) toxicity in different species

Das *et al*., (2001) evaluated biochemical changes induced by mercury in the liver of penaeid prawns *Penaeus indicus* and *P. monodon* (Crustacea: Penaeidae) from Rushikulya estuary, east coast of India and found significantly decreased level of biochemical component viz., protein, lipid, carbohydrate following six days of exposure to 0.005 ppm to 0.01 ppm $HgCl_2$ during various reproductive stages.

Sarmento *et al*., (2004) evaluated the effect of mercuric chloride on the function and integrity of Sea bass (*Dicentrarchus labrax*) head kidney macrophages (S-HKM), and the response of $HgCl_2$-exposed cells to macrophage activating factor(s) (MAF) produced by sea bass head kidney leukocytes and showed a decrease in ROS production as compared to cells incubated with medium alone.

Ovigerous females of the estuarine crab *Chasmagnathus granulatus* showed a delay in the egg incubation period and some morphological abnormalities were detected in larvae hatched after the exposure to mercury (0.1mg/L) during the entire, early, or late embryonic development(Sánchez *et al*., 2005).

Velyka *et al*., (2014) evaluated the catalase and glutathione peroxidase activity within rat kidneys 72 hours after mercury dichloride intoxication in the ratio of 5 ml per 1 kg of the animal weight and found Decreased glutathione peroxidase activity in cortical and cerebral substances and renal papillae were accompanied by increased contents of

oxidative modified proteins and lipids and morphological changes in renal tissue under salt and water loading after mercury dichloride poisoning.

Cadmium (Cd) is a nonessential heavy metal causing great toxicity. Robohm (1986) found that Cd treatment inhibited the antibody levels in cunners (*Tautogolabrus adspersus*) and enhanced the antibody levels and chemotactic activity of peritoneal exudate cells in striped bass (*Morone saxatilis*). In rainbow trout exposed to 2 ppb of Cd, the same level found in some contaminated waters, the lysozyme activity was unaffected while the macrophage functions, phagocytosis and production of ROS, were significantly impaired (Zelikoff *et al*., 1995). These authors also demonstrated that Japanesemedaka (*Oryzias latipes*) leucocytes increased their production of ROS and phagocytic functions without any change in many haematological parameters or antibody levels (Zelikoff *et al*., 1996). In the European sea bass, while in vivo exposure had a similar inhibitory effect on phagocytic functions the in vitro treatment produced an increment (Bennani *et al*., 1996). In the case of juvenile common carp experimentally infected with the blood parasite, Sanguinicola inermis (Trematoda: Sanguinicolidae) there were tissue changes and while the counts of neutrophils, eosinophils and thrombocytes increased in the thymus the number of neutrophils in the pronephros was reduced due to Cd2+ treatment (0.1 mg/L) (Schuwerack *et al.,* 2003). In the hybrid tilapia (*Oreochromis niloticus* × *O. aureus*), the Cd exposure increased the lysozyme activity but greatly reduced the alternative complement activity (Wu *et al*., 2007).

Heavy metals may affect the immune system of cetaceans. Pellisso *et al*., (2008) studied the effects of Hg (1, 5 and 10 mg/L), Al (2,5, 25 and 50 mg/L), Cd (1, 10, 20 and 40 mg/L), Pb (1, 10, 20 and 50 mg/L) and Cr (1 and 10 mg/L), on the function of phagocytes and lymphocytes isolated from the peripheral blood of bottlenose dolphins *(Tursiops truncatus)*

under *in vitro* conditions and evaluated Cell viability, apoptosis, lymphocyte proliferation and phagocytosis. Viability and lymphoproliferation were measured with Alamar Blue assay, and apoptosis and phagocytosis were evaluated with flow cytometry. A significant reduction in the lymphoproliferative response was registered by exposure to 1 mg/L of mercury, 10 mg/L of cadmium and 50 mg/L of lead. Decreased phagocytosis was also observed at 5 mg/L of mercury, 50 mg/L of aluminium and 10 mg/L of cadmium. More recently, the Cd exposure has been related to the increase of melano-macrophage centres on several fish tissues (Suresh, 2009).

Tawwab and Wafeek (2014) evaluated the effects of water temperature, Cd toxicity and their interaction on fish performance as well as metallothionein (MT) and Cd distribution in different fish organs of Nile tilapia, *Oreochromis niloticus* (L.) and found that fish reared in Cd-free group showed the optimum growth and feed intake, while Cd-exposed fish showed low growth and feed intake irrespective to water temperature. A synergetic relationship between water temperature and Cd toxicity was observed where Cd toxicity increased as water temperature increased and the worse growth was obtained in Cd-exposed fish reared at 32°C.

2.6. Heavy metals (mercury and cadmium) and oxidative stress

Heavy metals are ubiquitous environmental toxicants that have been shown to exert oxidative stress on living systems through the production of reactive oxygen species (ROS), which overwhelm the cell's capacity to maintain a reduced state (Ercal *et al*., 2001; Stohs and Bagchi, 1995). Metal-induced ROS cause damage to cellular proteins, nucleic acids and lipids, leading to a variety of cellular dysfunctions including cell death. Oxidative stress occurs when generation of free radicals (i.e. substances with one

or more unpaired electrons) exceed the capacity of antioxidant defense mechanisms (that is, pathways that provide protection against harmful effect of free radicals).

Fish as other animals may accumulate higher concentration of Hg (Salonen *et al*., 1995; Guallar *et al*., 2002). In experiments Atlantic salmon exposed for four months to mercuric chloride, methylmercury was accumulated significantly in brain and did not cause mortality or growth reduction (Berntssen *et al*., 2003). But it significantly increased levels of lipid peroxidation products (evaluated as TBARS) and decreased the activities of SOD and glutathione peroxidase.

Soares *et al.,* (2008) analyzed and compared the oxidative stress responses induced by an acute intravenous exposure (1 and 7 days) to a sub-lethal concentration (5 mM) of two vanadium solutions, containing different vanadate oligomers (n=1–5 or n=10), and a cadmium solution on the cardiac muscle of the marine teleost *Halobatrachus didactylus* (Lusitanian toadfish). It was observed that cadmium increases cytosolic catalase (+111%) and glutathione peroxidases (+50%) activities.

Senthamilselvan *et al*., (2012) evaluated the effects of toxic metals (cadmium plus mercury) on the haematology and DNA damage of fish, lates calcarifer for every 24 h up to 96 h. A significant gradual decrease of hemoglobin (-35.01 to -24.11) and hematocrit (-35.01 to -31.78) was noted at acute concentration of cadmium plus mercury (3.0 ppm) as compared to that of control. The comet assay of fish blood cells exhibited a significant higher DNA damage and the highest OTM was observed in 96 h of acute mixed metals concentration.

Kim *et al*., (2012) studied the effects of inorganic mercury on hematological parameters and hepatic oxidative stress enzyme activity in olive flounder *Paralichthys olivaceus* and observed significant decreases in the red blood cell count, hematocrit value. A

significant increases in glutathione peroxidase, catalase activity, glutathione reductase and glutathione S tranferase were also observed above 4 mg Hg/kg BW and 8 mg Hg/kg BW respectively.

Ahmed *et al*., 2014 investigated the effects of cadmium (Cd) to induce oxidative stress and biochemical perturbations in Nile tilapia liver and gills and the role of Vitamin C (Vit. C) in alleviating its toxic effects and observed that after exposure to Cd, caused increase in Liver aminotransferases (AST and ALT), elevation in lipid peroxidation (LPO), activity of catalase (CAT) enzyme, and the activity of glutathione S-transferase (GST). However, reduction in the activity of glutathione peroxidase (GPx) was also observed.

Vinodhini and Narayanan (2008) evaluated the effect of heavy metal pollutants such as cadmium, chromium, nickel and lead in aquatic system on common carp (*Cyprinus carpio L*.) by using a set of biochemical parameters. The experimental group of fish was exposed to a sublethal concentration of 5 mg/L of combined (Cd+Pb+Cr+Ni) metal solution containing 1.25 mg/L of each metal ion (1/10th of LC 50/48 h) for a period of 32 days. The results showed the decreased activity of vitamin C during chronic exposure to toxic heavy metals, which indicates the presence of reactive oxygen species, induced peroxidation.

2.7. Role of mercury and cadmium on antioxidant system and immune defenses

2.7.1. Effect of mercury exposure

Usually the deleterious effects of oxidative stress are counteracted by endogenous antioxidant enzymes, mainly superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) (Winterbourn, 1993). SOD and catalase are considered primary

enzymes since they are involved in direct elimination of ROS. SOD plays an important role in protecting the cells against the toxic effects of O_2- by catalyzing its dismutation reactions.

Mercury (Hg), and derivatives such as methylmercury, are important contaminants in aquatic environments inducing organ lesions, neurological, haematological and immunological disorders (Sweet & Zelikoff, 2001). Experiments showed that in rainbow trout, there is a decrease in the number of mucous-producing cells and mucus production after exposure to mercury and methylmercury, which can be associated to impaired immunity (Lock & Overbeek, 1981). Afterwards, serum C-reactive protein was increased in freshwater murrel (*Chana punctatus)* (Ghosh & Bhaattacharya, 1992) and major carp (*Catla catla*) (Paul *et al.,* 1998) by exposure to mercury. However, plasmatic lysozyme of plaice was decreased after exposure to sub-lethal doses of mercury (Fletcher, 1986). Blue gourami (*Trichogaster trichopterus*) showed increased kidney and plasma lysozyme activity, but at the same time reduced the production of agglutinating specific antibodies after chronic exposure to mercury (Low & Sin, 1998). In the marine fish *Sciaenops ocellatus*, mercury treatment (≤10 μM) produced a high-dose inhibition and a low-dose activation of leukocytes as determined by Ca-mobilization and tyrosyne phosphorilation of proteins (MacDougal *et al*., 1996). More recently, in the European sea bass (*Dicentracrchus labrax*), *in vitro* treatment with $HgCl_2$ induced apoptosis in head-kidney macrophages as well as reduced the ROS production and the benefits of macrophage-activating factors (MAF) (Sarmento *et al*., 2004).

2.7.2. Effect of cadmium exposure

As a nonessential element, cadmium may endanger the growth and development of aquatic life. For example, cadmium may inhibit the bioluminescence of bacteria (Ishaque *et al*., 2006), cause limited activity even death in daphnias (Canizares-Villanueva *et al*., 2000), and induce oxidative stress in fish (Livingstone, 2001). A number of investigations have suggested that cadmium may exert immunosuppressive effects both fishes and mammals (Zellkoff *et al*., 1995; Kim *et al*., 2000 and Giari *et al*., 2007). Recent reports suggests that cell mediated immunity is most affected (Kumar *et al*., 2008) and phagocytosis, natural killer cell activity and host resistance towards experimental infections are markedly impaired in cadmium toxicity. Sovenyl and Szakolczal (1993) also reported marked immunosuppressive effects of cadmium exposure on common carp in terms of lowered antibody response, lysozyme level and microcidal capacity of phagocytes. Some reports suggest that cadmium enhances humoral immune response at low level of exposure (Descotes, 1992; Krumschnabel *et al*., 2010).

2.8. Molecular basis of heavy metal induced toxicity on the dual defenses in the body

2.8.1. Antioxidant defenses

Heavy metals have electron-sharing affinity that can result in formation of covalent attachments (Bondy, 1996). These attachments are mainly formed between heavy metals and sulfhydryl groups of proteins. Several enzymes in antioxidant defense system become inactive or non-functional due to direct binding of the metal to the enzymes' active sites, if the sites contain sulfhydryl groups (Quig, 1998). There are a number of molecules that function as scavengers of free radicals. The most abundant

and most important molecular antioxidants in cellular cytoplasm is glutathione (GSH). GSH, along with metallothionein (MT), are the two most well studied antioxidant molecules in fish. GSH is a low molecular weight thiol. It can react directly with ROS species, thereby detoxifying them. In addition, GSH is used as a conjugating molecule by GST to ease excretion of xenobiotics. GSH is also used as a reducing equivalent in the metabolism of reactive intermediates, for example reduction of lipid peroxides by the action of glutathione peroxidase (GPx). These reactions can result in the production of oxidized glutathione (GSSG). The balance between GSH and GSSG can be restored by glutathione reductase (GR). Levels of glutathione within the cell are regulated via the activity of GR and via de novo synthesis of GSH, which occurs in a two-step process involving glutathione synthetase (GS) and γ-glutamylcysteine synthetase (GCS). Decreased levels of glutathione in the cytoplasm or an increase in the ratio of GSSG to GSH resulting from an increase in ROS can lead to transcription of redox sensitive genes. This occurs via initiation of a signal cascade resulting in assembly of transcription factor subunits in the nucleus. Initiation of transcription of such genes as SOD and CAT are known to occur in this manner (Thannickal and Fanburg 2000, Dröge 2002). Metallothioneins (MTs) are small ubiquitous proteins known to be involved in metal homeostasis, including metabolism and detoxification. Free metals entering into the cell, generally via transport proteins or ion channels, can interact with ligands or MTs. Synthesis of MT is in part regulated by free metal ions, which bind to MT transcription factors which in turn bind to metal-responsive elements in the promoter region of the MT gene (Di Giulio *et al.*, 1995). MT can exhibit antioxidant capacities indirectly through chelation of heavy metals that otherwise have the potential to interact with enzymes and respiratory protein complexes within mitochondria, thereby disrupting their function, uncoupling oxidative phosphorylation and causing

oxidative stress (Di Giulio *et al*., 1995). Metallothionein was first noted for its metal scavenging capacity, but has more recently been recognized as an important antioxidant (Sato and Kondoh 2002). This is in part due to its many cystein residues which can readily be oxidized (Palmiter 1998).

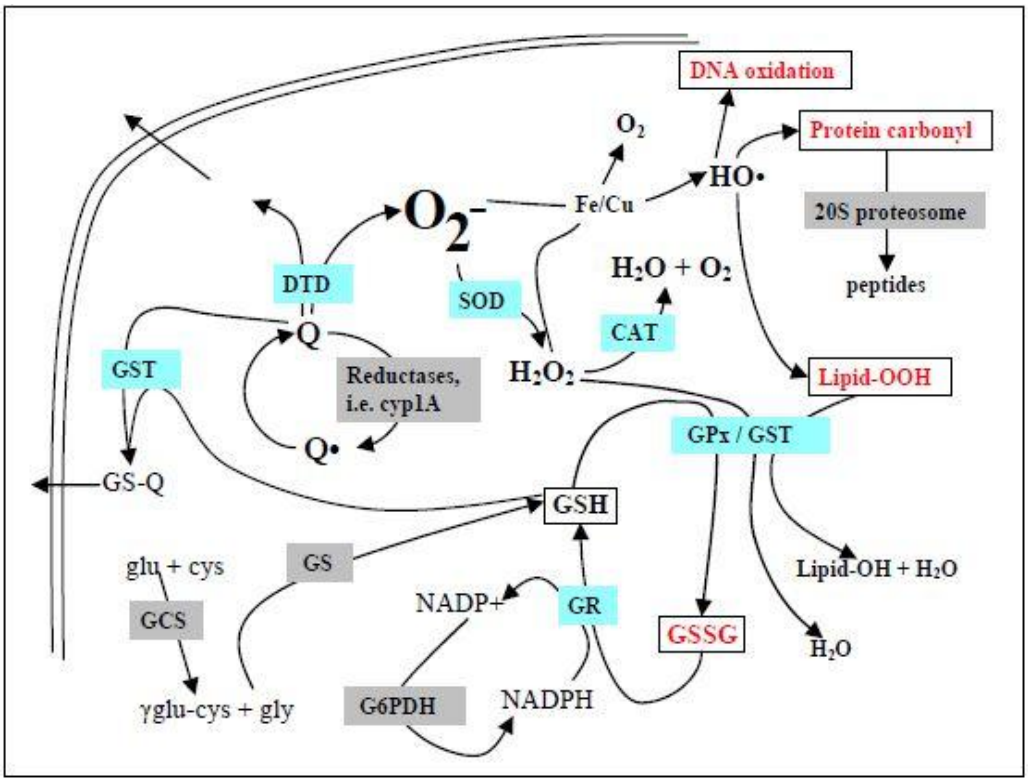

Fig. 2.2: Schemetic diagram of oxidative stress. Antioxidant enzymes are shown in the blue boxes, other enzymes in grey, oxidized molecules are shown in red text. Q represents a redox cycling compound (Almroth 2008).

Antioxidant enzymes which remove peroxides, and superoxide radicals including glutathione peroxidase (GPX), catalase (CAT) and superoxide dismutase (SOD), are potential targets for heavy metals like lead, arsenic, cadmium and mercury. Because GPx requires selenium for its activity, when lead forms a complex with selenium, GPx activity decreases (Howard, 1974; Whanger, 1992). One of the most important mechanisms for mercury induced oxidative damage is its sulfhydryl reactivity. Hg^{2+} and

Methylmercury (MeHg) form covalent bonds with GSH and cysteine residues of proteins (Quig, 1998). Inorganic mercury is suggested to increase H_2O_2 production by impairing the efficiency of oxidative phosphorylation and electron transport chain (Lund *et al*., 1991; Chavez and Holguin, 1988; *Nath et al*., 1996). It has been postulated that cadmium too overwhelms the defense system by challenging the thiol status of cells. Cadmium induced disturbances in GSH and metallothionein levels allow free radicals to attack double bonds in membrane lipids and result in an increase in lipid peroxidation.

2.8.2. Innate immune responses

The immune system is a host's last line of defense against any infectious agents and neoplasms (Luster *et al*., 1988) and is highly combined with other organs and their functions, including the central nervous system (CNS). It is a complex network of cells in a constant state of proliferation and differentiation, which is highly regulated by numerous soluble factors such as antigens, mitogens, cytokines, neurotransmitters and hormones. These characteristics make the immune system extremely vulnerable to insult from heavy metal stress and xenobiotic (Dean and Murray, 2001).

The innate response is mediated by white blood cells such as neutrophils and macrophages, cells that phagocytose and kill the pathogen or foreign body, and that concurrently coordinate additional host responses by synthesizing a wide range of inflammatory mediators and cytokines which reflect the encounter between the host and the invading antigen. In macrophages, the infectious agent is killed and degraded within the maturing phagosome, and components of the antigen are presented to T cells, resulting in the activation of the adaptive immune response and the establishment of protective immunity (Aderem and Underhill, 1999). A primary challenge to the innate

immune system is the discrimination of a large number of potential pathogens from self, with the use of a restricted number of receptors. This challenge has been met by the evolution of a variety of receptors that recognize conserved motifs on pathogens that are not found in higher eukaryotes. These motifs have essential roles in the biology of the invading agents, and are therefore not subject to high mutation rates. Janeway and Medzhitov (1998) have provided a set of definitions to formalize a description of the components of the innate immune system. They propose calling the motifs pathogen associated molecular patterns (PAMPs), and their cognate binding partners on the phagocytes pattern-recognition receptors. The innate immune system provides protection, in part, due to the synthesis of potent antimicrobial peptides. These peptides are induced in response to signalling pathways activated by members of the TLR family. During phagocytosis, TLRs are recruited to the phagosomes, where they sample the contents and determine the nature of the antigen (Underhill 1999). Thus specific TLRs might distinguish between components in the phagosome and participate in the formulation of an inflammatory response appropriate for defense against a specific pathogen.

Omima (2010) evaluated of the effects of lead, mercury and cadmium on both humoral and cellular immune response of *Oreochromis niloticus* "T. nilotica" and was carried out towards an important fish pathogenic bacteria "Pseudomonas flourscens". The results revealed that, lead, mercury and cadmium have inhibitory effect on phagcytic acgivity of fish macrophages and so having an inhibitory effect on cell mediated immune response.

Exposure of cadmium to the body, especially chronic exposure, leads to mal-functioning of the immune system. Because the target cells of cadmium are T cells [cytotoxic K (killer) cells], macrophages, B cells and NK (natural killer) cells. It shows

that the direct immunotoxicity by cadmium is a modification of the immune responses of both- cell mediated and humoral immunity (Krocova 2000: Marth 2000).

2.9. Ultrastructural changes due to heavy metal stress

The histological changes on fish is a significant and promising field to understand the extent to which changes in the structural organization are occurring in the organs due to environmental pollution. Srivastava *et al*. (1982) have observed the hisotopathological changes and accumulation potential in the different tissues of fishes under chromium stress. Gill hyperplassia, , fat infiltration of liver parenchyma cells, necrosis of intestinal mucosa were observed due to ammonia poisoning in the kidney, gills and intestinal tract of juvenils *Sparus auratus* (Zaki *et al*., 1987)

Cadmium exposure induces the appearance of granular deposits in the liver, increases in chloride cell turnover at the gills and atrophy of the proximal renal tubules, (Pratap and Bonga, 1993). Heavy metal toxicant leads to many pathological changes in different tissues of fishes and have been reported for *Labeo rohita* exposed to mercurichloride and *Chana punctatus* exposed to phenyl mercuric acetase (Karuppasamy, 2000).

Selvanathan *et al*., (2013) investigated the effect of heavy metals like mercury and cadmium on Fresh water fish *Clarias batrachus*. The result showed that the degree of distortion of the gill, liver was proportional to the exposure period and concentration of the metals was found to be dose and time dependent.

2.10. Stress related cytokine gene expression in fish

Under stressed condition, fish may cause many different alterations in the immune system affecting performance, health and welfare. Several fish cytokine genes have been isolated and characterized in recent years and researchers have used its mRNA

expression as a tool for measuring immune responses (Castillo *et al.*, 2009). In particular, pro-inflammatory cytokines, including interleukin-1β (Zou *et al*., 1999), TNF-α (Laing et al., 2001), and IL-6 (Kishimoto and Hirano1988) are commonly used immune-regulatory genes in fish. TNF-α is produced by macrophages, neutrophils, monocytes, natural killer cells and T cells after their stimulation by bacterial lipopolysaccharide. It is probably the most studied cytokine in fish, in species such as rainbow trout (Laing *et al*., 2001), carp (Saeij *et al*., 2003), catfish (Zou *et al.*, 2003), red seabream (Cai *et al*., 2003), Atlantic salmon (Ingerslev *et al*., 2006), Gilthead seabream (Castillo *et al*., 2002) and the mandarin fish (Xiao *et al*., 2007). IL-6 is mainly secreted by T cells and macrophages to stimulate immune response to inflammation (Castillo et al., 2009) and has been described in Fugu (Bird et al., 2005), rainbow trout (Iliev *et al*., 2007) and in the seabream (Castellana *et al*., 2008). TGF-β1, on the other hand, is a multifunctional peptide controlling proliferation, differentiation, and other functions in many cell types, described in carp (Zhan and Jimmy 2000), seabream (Tafalla *et al*., 2003) and recently in grass carp (Yang and Zhou 2008).