TOXICITY OF ROAD RUNOFF CHEMICALS TOWARDS COMMON CARP (*Cyprinus carpio*)

BY

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A thesis submitted in partial fulfillment of the requirements for the Degree of Master of Science in Environmental Science

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(2018)

CERTIFICATE

It is certified that the contents and form of thesis entitled

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Submitted by

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has been found satisfactory for the requirement of the degree

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THESIS ACCEPTANCE CERTIFICATE

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I dedicate my thesis to my beloved parents. I could not be where I am today without your sacrifices, support and love. I also dedicate this dissertation to my siblings who have been a great source of motivation.

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LIST OF ABBREVIATIONS

Cr (VI)	Hexavalent Chromium
Cd	Cadmium
RBCs	Red Blood Cells
WBCs	White Blood Cells
Hb	Haemoglobin
HCT	Haematocrit
MCV	Mean Corpusular Volume
MCH	Mean Corpusular Haemoglobin
MCHC	Mean Corpusular Haemoglobin Concentration
ROS	Reactive Oxygen Species
$CdSO_4$	Cadmium Sulphate
$K_2Cr_2O_7$	Potassium Dichromate
ENA	Erythrocytes Nuclear Abnormalities
MN	Micronuclei
APHA	American Public Health Association
AAS	Atomic Absorption Spectrophotometer
USEPA	US Environmental Protection Agency
CCC	Chronic Criterion Concentration
µg/L	Microgram per Liter
µg/g	Microgram per gram
mg/L	Milligram per Liter

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ABSTRACT

Road related contaminants are considered as a significant source of pollution, however there are only few studies conducted on the toxicity of road runoff on fresh water fish species. Fish in aquatic environment are considered as valuable indicator of aquatic pollution. This study evaluates the exposure to environmentally realistic concentrations of chromium (Cr) and cadmium (Cd) and its responses in juveniles of Common carp (*Cyprinus carpio*). To understand the toxicity of road runoff, leaching experiment was conducted to simulate road runoff processes using road dust samples. Common carp was exposed in a semi static exposure system at environmentally realistic concentrations of Cr and Cd separately and in combination. Exposure concentrations for Cr, Cd and mixture of Cr-Cd were maintained at 0.2, 2 mg/L for Cr and 0.05, 0.1 mg/L for Cd and 2 + 0.1 mg/L for Cr-Cd mixture for 45 days. The experiment was conducted in two different water qualities in two batches and toxicity of Cr and Cd was assessed by using set of biomarkers such as haematological parameters, erythrocytes nuclear and cellular abnormalities and uptake in gills tissue.

In the first batch of experiment, haematological results of Common carp revealed that exposure of Cr resulted significant ($p<0.05$) decrease in red blood cell (RBC) count, haemoglobin (Hb), haematocrit (HCT) content and significant ($p<0.001$) decrease in mean corpuscular haemoglobin concentration (MCHC), however, significant ($p<0.01$) increase in white blood cell (WBC) count and significant ($p<0.05$) increase in mean corpuscular volume (MCV) was observed among treatment groups. The frequency cellular and nuclear abnormalities in erythrocytes of Common carp revealed genotoxicity with the total percentage abnormalities of 16%, 25.6% and 66% exposed to 0.2, 2 and 17 mg/L Cr concentrations as compared to total abnormalities in control group i.e. 5.6%. The accumulation of Cr significantly ($p<0.001$) increased in gills when exposed to Cr test concentrations (0.2, 2 and 17 mg Cr/L).

In the Second batch of experiment, exposure of Cr, Cd and mixture of Cr-Cd resulted in significant ($p<0.05$) decrease in RBC count, Hb, HCT concentration and significant ($p>0.05$) decrease in MCV whereas, significant increase ($p<0.05$) in WBC count and MCHC was found. Changes in haematological indices revealed that Common carp exposed to Cr and Cd was under

stress. Nuclear and cellular abnormalities in erythrocytes of Common carp revealed genotoxicity of Cr and Cd even at the lowest concentrations (0.2 mg Cr/L and 0.05 mg Cd/L). The frequency of cellular and abnormalities in erythrocytes of Common carp revealed that maximum frequency of total abnormalities observed was 51.6% in mixture of Cr-Cd followed by total abnormalities of 24.6 and 26.3% for 0.2, 2 mg/L Cr concentration, 19.3% and 23.7% for 0.05, 0.1 mg/L Cd as compared to control i.e. 8.6%. The uptake of Cr in gills tissues increased significantly ($p<0.001$) at test concentrations singly (0.2, 2 mg Cr/L) and significantly ($p<0.01$) increased in mixture of Cr and Cd (2 + 0.1 mg/L Cr-Cd) whereas, exposure to Cd singly at a concentrations (0.05, 0.1 mg Cd/L) resulted slightly increase ($p<0.05$) uptake in gills however, Cd uptake in gills slightly decreased ($p<0.05$) when exposed to mixture of Cr and Cd. The present study gives insight of the probable harmful effects of road related contaminants to fresh water bodies.

INTRODUCTION

1.1. Background

The rapid urbanization has led to environmental pollution (Ermolon, 2018). In urban areas increasing traffic densities represents potential threat to both the environment and human health (Apeagyei, 2011). One of the major problems triggered by heavy density traffic includes the accumulation of road dust which can be deposited along the road ways. Road dust sediments acts as a sink for a variety of organic and inorganic constituents particularly, heavy metals and it is one such medium to monitor environmental quality (Liu et al., 2014).

Road traffic pollutant sources are categorized into five groups which include traffic and cargo, pavement and embankment material, road equipment and maintenance and operation (Folkeson et al., 2009). In addition, road related metals derived from vehicles are classified under various categories. Some heavy metals release from vehicular components such as brakes-linings, tires and vehicular bodies, while other derived from combustion, exhaust fumes, road surface wear and paint materials (Adamiec et al., 2016; Meland et al., 2010). For example, road related sources of chromium (Cr) in road environment originates from the chrome plating of vehicular parts and wearing of some vehicular metallic parts (Al-Shayep and Seaward, 2001). Hjortenkrans et al. (2007) reported significant amount of Cr originated from tires and brakes. Similarly, origin of cadmium (Cd) is derived from wearing of tires and combustion process in engine of cars (Hjortenkrans et al., 2007). Heavy metals primarily become attached to road dust and other particulate matter on the road surface. During the rainfall period, dissolved fraction of heavy metals being leached from road dust sediments is transported into water bodies and is bioavailable which may deteriorate ecological systems (Gunawardana, 2014). Organic and inorganic pollutants present in pore water are further moved down to the deeper soil layers or to the ground water in a process known as leaching. Water soluble and the exchangeable fractions are considered as the most mobile and bioavailable forms which may be taken up and accumulated by living organisms (Ogundiran and Osibanjo, 2009).

Generally, fresh water bodies are the ultimate recipients of toxic contaminants. Chronic heavy metal contamination may have detrimental effects on ecological balance of aquatic environment and aquatic biota diversity (Ashraf, 2005). Fish in the aquatic systems are considered to be as important indicators of environmental stressors and plays an important role in evaluating probable risk of contamination in aquatic environment (Kumar et al., 2012). Moreover, fish may accumulate metals through aquatic food chain and pass them to human beings through food which may cause chronic or acute diseases (Authman et al., 2015).

Road runoff is a major source of environmental pollution, consider to be threatening nearby aquatic habitats (Dorchin and Shanis, 2010). Metals are commonly reported contaminants in highway runoff studies leading to metal pollution in aquatic environment due to their potential toxicity towards aquatic organisms (Meland et al., 2010). In aquatic environment, the pollutants discharge can change the response of aquatic biota and also impact their growth (Woodcock and Huryn, 2004). The polluted runoff water can cause ecological impact on soil and water ecosystems and it can pose threat to aquifers and surface water (Opher and Friedler, 2010). Road runoff primarily affects aquatic organisms and their habitats located alongside road while, metals lethal effect on fish has been observed at a far distance of 8 kilometer (km) downstream from a runoff inlet (Dorchin and Shanas, 2010).

Ward, (1990) recognized eleven metals (Cu, Cr, Co, Mn, V, Ni, Zn, Br, Ce, Mo and Cd) as related to emissions from road and traffic sources. In the present study heavy metals (Cr and Cd) toxicity was assessed on Common carp (*Cyprinus carpio*) which is used as model organism, to assess the environmental impact of traffic related pollutants when exposed to Cr and Cd in the environment that can be mobilized from road runoff processes and also information is needed on the sensitivity and resistance of Common carp, when exposed to environmentally realistic concentrations of Cr and Cd.

Hexavalent Chromium, Cr (VI) is known as highly toxic metal due its ability to enter into skin or biological membranes (ATSDR, 2000). Cr can exhibit range of toxic effects in fish including hematological alterations, histological impairments, oxidative stress, growth inhibition, morphological alterations, chromosomal aberrations and impaired immune functions (Raied, 2011).

Cadmium (Cd) is a non-essential heavy metal with no biological functions in aquatic organisms (McGeer et al., 2012). Fish are generally very sensitive to cadmium poisoning with the exhibition of wide range of adverse effects even at low concentrations. Chronic Cd exposure can lead to adverse effects on growth, reproduction, immune and endocrine systems, development and behavior changes in aquatic organisms (McGeer et al., 2012). Other toxic effects of Cd in fish include adverse effects on respiratory, haematology, histopathology and alterations of antioxidant defense system and immunosuppression (Soares et al., 2003; McGeer et al., 2011; Perera et al., 2015).

According to the US Environmental Protection Agency (USEPA), Cr and Cd content of surface water for fresh water fish is 11 µg/l and 0.72 µg/L CCC (Chronic Criterion Concentration) respectively (USEPA, 2017). However, in Pakistan the average value of Cr found in Rawal lake was 0.097 mg/L and average value for Cd found was 0.025 mg/L in winter season (Iqbal et al., 2013) which was higher than permissible limits recommended by Pak EPA, (2008). In Islamabad, Pakistan, road side soil and road dust sample along Islamabad expressway were analyzed for heavy metals pollution, Cd of concentrations 5.8–6.1 and 4.5–6.8 mg/kg was found in road soil and road dust samples respectively (Faiz et al., 2009).

1.2. Significance

The scope of the present work was to identify the responses in juveniles of Common carp when exposed to environmentally relevant concentrations of Cr and Cd, being the toxic metals to fish. The effect of these two metals were identified in different water qualities and their interactive effect Cd-Cr was also observed. This study assessed the exposure of heavy metals (Cr, Cd) and its effects in Common carp by using the set of biomarkers such as nuclear and morphological alterations of erythrocytes (formation of deformities), haematological indices and the uptake in gills tissues to evaluate the stress and potential toxicity on Common carp health. Common carp is one of the widely introduced species worldwide because it can breed in confined water, its good growth rate, omnivorous specie, resistant specie and its easy adaptation to artificial feed. Therefore, this species is introduced to Asia including Pakistan. Furthermore, Common carp plays an important role in inland fish production (Khan et al., 2016). In Pakistan, studies were conducted to evaluate metals concentration in fish and Rawal lake Islamabad (Iqbal

et al., 2013; Iqbal and Shah, 2014). However, no studies on heavy metals toxicity from road contaminants such as Cr and Cd on fish are conducted so far.

1.3. Objectives

Keeping in view the scope of study, the objectives are as follow:

i. Measure the concentration of heavy metals (Cr and Cd) in road dust.
ii. Evaluate potential toxicity of heavy metals (Cr, Cd) on *Cyprinus carpio* by haematological parameters.
iii. Toxicity assessment of heavy metals (Cr, Cd) through erythrocytes cellular and nuclear abnormalities test.
iv. Monitoring the uptake of Cr and Cd in gills tissues.

LITERATURE REVIEW

2.1. Sources of metals in aquatic environments

Metals are natural components of aquatic environments and they exist as components of mixtures with other metals and or inorganic substances. Heavy metals can enter the aquatic environment by means of natural and anthropogenic sources. Natural sources include weathering of rocks, minerals and volcanic emissions whereas, anthropogenic sources of metals in aquatic ecosystems includes mining, industrial discharge, municipal discharge, agricultural runoff, road runoff, accidental spills and atmospheric deposition (Forstner and Wittmann, 2012). Of many sources road vehicles may release substantial amounts of heavy metals into the air, water and soil (Doung and Lee, 2011).

2.2. Road runoff processes

Road runoff from pervious and semi pervious surfaces may contain complex mixture of contaminants such as polycyclic aromatic hydrocarbons (PAHs), volatile organic compounds (VOCs) and inorganic pollutants such as heavy metals (Kayhanian et al., 2008). Heavy metals released from vehicular emissions can induce contamination of air, water and soil which can have adverse effects on environment including significant threats to humans (Adamiec et al., 2016). In road runoff processes, road related contaminants, both dissolved and suspended particles are transported away from road to the surrounding environment mainly via wet deposition (road runoff or spray water) and dry deposition (aerial transport) (Legret and Pagotto, 2006) and finally released to the receiving water (Watanabe, 2011). The pollutants from traffic and road in solid form can release in water by two processes:

Dissolution; chemicals are dissolved by adjacent water.

Desorption; chemicals are separated from the solids to which they are loosely bound. These two processes together are termed as leaching (Folkeson et al., 2009).

Metals in runoff can be attached to inert sediments, or exist in immiscible fluids, particles, soluble salts or insoluble compounds (Leitao, 2007). Metals dispersion among different forms in environment depends on numerous factors. Metals can be transported in soil

and aquatic environment by many processes which are regulated by chemical nature of metals, soil and sediment particles and the pH of the surrounding environment. Many heavy metals are more mobile under acidic conditions (Folkeson et al., 2009; Alloway, 2013). The principal transport mechanism involved in runoff is diffusion, advection and dispersion in saturated soil. Pollutant transport in unsaturated soil depends on soil-moisture distribution inside the pores. Chemical processes such as ion exchange, sorption and desorption, dissolution and precipitation reactions are the most significant reactions leading to contaminant transport in soils. These all factors influence the distribution of the various metal species and mobility in soils between soil matrix and soil solution (Alloway, 2013).

Characteristics of highway runoff quality in terms of contaminants depends on the several factors such as road materials, road design and traffic loads (light and heavy vehicles, driving speed, road cleaning/ road maintenance and operation etc). Other aspects such as the climatic conditions or weather (winters vs. summers, rain patterns, time between episodes) (Meland et al., 2010).

Figure 2.1: Schematic diagram representing pathways for road pollutants dispersion (Meland et al., 2010)

Researchers have already reported the highway runoff toxicity using model organisms such as Spromberg et al. (2016) demonstrated Coho Salmon spawner mortality in western US urban watersheds that untreated highway run-off was lethal to adult Coho relative to unexposed controls moreover mortality syndrome was prevented when highway run-off was pretreated by mitigation measures.

2.3. Metals bioavailability in aquatic environment

2.3.1. Metals speciation

The word speciation refers to the existence of the different forms of a metal in a system (Templeton et al., 2000). In fresh water environment, metals occur in several forms depending on fractions which comprise of free metal ions, metal complexes either dissolved in solution or sorbed on solid surfaces such as particulates, colloids and dissolved phases (Nystrand et al., 2012). The most bioavailable form of metals resulting toxicity is the dissolved ionic form. Chemical speciation of metals plays a major role in metals transport, their reactivity, fate, bioavailability and potential toxicity (Smith et al., 2015). Metals speciation is influenced by various factors which include pH, presence of organic matter, ionic strength of the medium, redox potential, water hardness and metals valence state in freshwater environment. These major factors contribute in formation of different metals species resulting toxicity and higher and lower metals bioavailability (Wang, 1987; Bjerregaard et al., 2015).

2.3.2. Metals bioavailability

There are several definition of bioavailability based on some assumptions in the literature. Fairbrother et al. (2007) define bioavailability as the fraction of a compound that absorb into or onto across biological membranes of organisms. According to Chapman, (2008) bioavailability refers to the amount of a compound that is directly available for uptake by an organism. Rainbow and Luoma, (2011) defined toxicological bioavailability as the portion of the metal that is adsorbed or absorbed by the body. The assimilated portion interacts with receptors and physiological sites essential to the body's metabolism, producing toxic effects.

There are several water quality variables that can alter metal bioavailability and toxicity such as water temperature, pH, alkalinity, salinity, hardness, presence of dissolved organic carbon (DOC), presence of competing ions, presence of organic and inorganic ligands (Total organic

carbon/Dissolved organic carbon) that bind with metals and the properties of metals (Smith et al., 2015). Major factors affecting bioavailability are described below.

2.3.2.1. Influence of pH

pH is one of the main factor which is considered to influence the behaviour of metals in environment (USEPA, 2007). pH regulates the degree of polymerization, aggregration and precipitation and proton competition for ligands (Smith, 2009). Mostly low pH increases toxicity but metal speciation in freshwater varies with the metals such as in some cases metals can be less toxic in low pH. At low pH, metals dissociate thus increasing their solubility and the free ions concentration (Wood, 2011). Some metals (Ni, Cd and Zn) toxicity was decreased at pH 6.3 for *Pimephales promelas, Hyalella Azteca* and *Ceriodaphnia dubia* (Schubauer-Berigan et al., 1993). The likely reason is that at low pH hydrogen ions (H+) competes with the metals for uptake at the gills binding sites thus protect against metal toxicity, while at greater pH, competition with H+ ions and metals ions is reduced thus metal ions is more available for uptake by the gills (Niyogi et al., 2008).

2.3.2.2. Influence of water hardness

Generally high water hardness decreases metal toxicity where ions such as calcium (Ca^{2+}) and magnesium (Mg^{2+}) competes with divalent metal ions for binding at gills surface (Kozlova et al., 2009). Calcium ions can protect the gills by decreasing gill permeability and giving the gills a positive charge causing it to repel other cations (McWilliams and Pott, 1978). Some metals such as Zn, Cd, Pb and Co are more influenced by than Mg^{2+} ions concentration because they compete with Ca^{2+} ligands therefore, higher concentration of Ca^{2+} functions as a protection factor against metals toxicity ultimately decreasing the toxicity (Niyogi and Wood, 2004).

2.3.2.3. Influence of organic matter

Dissolved organic matter is a complex heterogeneous mixture containing different chemical fractions such as a fulvic acid fraction, humic acid fraction (possesses carboxylic and phenolic functional groups) a transphilic fraction (aromatic groups) and a hydrophilic fraction (bases, amino acids and polysaccharides) (Zularisam et al., 2011).

Metal binding functional groups in Dissolved Organic Carbon (DOC) includes carboxylic groups (associated with low metal-binding affinity) phenolic groups (associated with high metal-binding

affinity) and amines and thiol functional groups. Dissolved organic matter also contain metal binding ligands (aminopolycarboxylates and sulfides) which may also form complexes with dissolved metals thus decreasing concentration of free metal cations and their bioavailability (Baken, 2011; De Schamphelaere et al., 2004).

For instance, Cu^{2+} has been directly associated to toxicity in fish while Cu bind with dissolved organic matter does not induce same degree of toxicity due to its reduced availability for uptake at gills surface (Fairbrother et al., 2007). The binding affinity of Cu to Dissolved organic carbon (DOC) is over ten folds stronger than that of Cd (McGeer et al., 2002). In an another toxicity test study, Cd toxicity to rainbow trout increased in the presence of some organic matter and Cd as compared to only Cd control groups (Schwartz et al., 2004). The elucidation of the formation of metals complexes in the presence of organic matter and the resulting changes in bioaccumulation and toxicity is a complex process as it depends on several factors such as type of organic matter, type of organism's exposed and metal concentration (De Paiva Magalhaes et al., 2015).

2.3.2.4. Influence of inorganic anions

Alkalinity is measured by carbonate, bicarbonate and hydroxide anions concentration. High alkalinity decreases metal toxicity by active competition for binding sites of tissues (Santore et al., 2001). Metals ions can form insoluble precipitates with anions thus reducing the free metal ions concentration (Wood, 2012). Metal ions can complex with other inorganic anions (phosphate, chloride, sulfate, arsenate) causing precipitation of metals thereby decreasing the bioavailability of free metal ions (Sarin et al., 2000).

2.3.3. Mechanism of metals uptake

A contaminant for example metals can be in equilibrium between a 'bound' and 'released' state in the exposure environment. The contaminant must be in the form taken up by organism and toxicity occurs when it crosses the biological membrane (Reeder et al., 2006). Generally, toxicity occurs if a toxicant from the exposure environment transfers into the sites within the organism producing toxic effect (Wood, 2011) as shown in Figure 2.2.

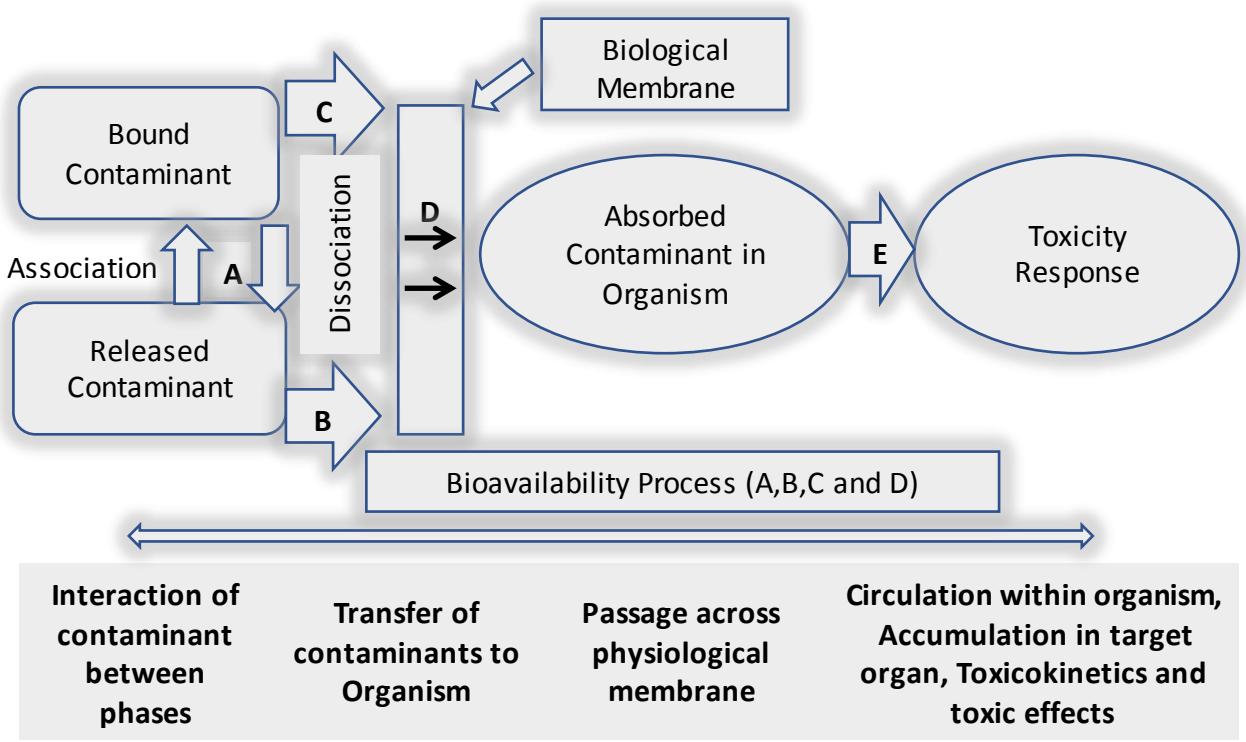


Figure 2.2: Schematic of bioavailibilty processes
(Reedar et al., 2006)

Biotic Ligand Model (BLM) explains the relationship between aquatic chemistry, biotic uptake of metal toxicants and the physiology of the effected organism (Paquin et al., 2002; As depicted in the Figure (2.3) BLM is a tool to evaluate the effects of water variables on aquatic organisms by attempting to evaluate the processes that affect metal accumulation on a target tissue (Smith et al., 2015). Metals uptake can impact multiple physiological effects in fish and toxicity commonly occurs at gills. Toxicity depends on form of metal, bioavailability, toxicodynamics (interactions with ligands) and toxicokinetics (absorption, distribution, biotransformation and excretion) (Kennedy, 2011).

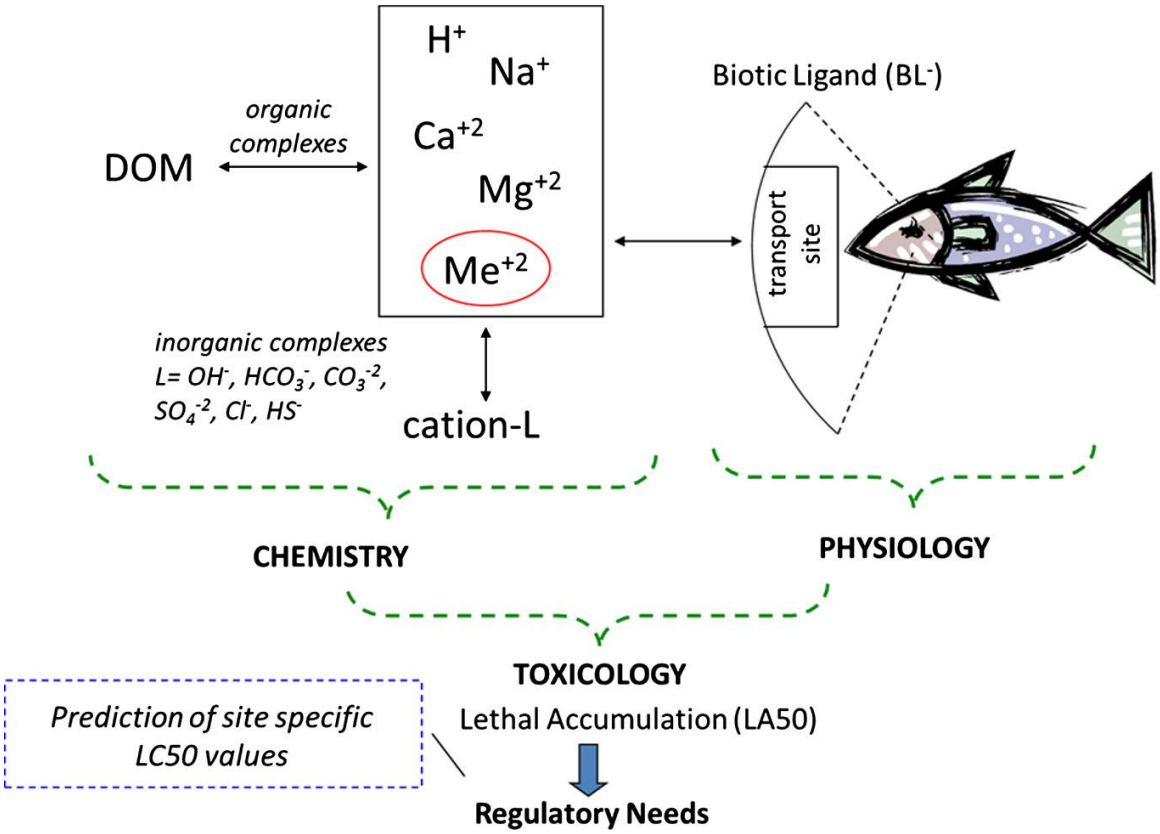


Figure 2.3: Illustration of Biotic Ligand Model (BLM)
(Smith et al., 2015)

2.4. Heavy metals toxicity towards fish

Heavy metals are among the group of contaminant that are monitored in various parts of environment due to their persistence and potential toxicity. Heavy metals are usually impacted by various surface water components that affect the heavy metals by formations of complexes and insoluble salts after entering into water. These complexes and salts are considered to be less harmful to aquatic organisms. However, when water pH declines (acidic episodes/ acid rains) heavy metals may be mobilized and released from complexes into the water causing toxicities to aquatic biota. Furthermore, heavy metals in low concentrations can cause chronic stress to fish reducing their ability to compete for food and habitat resulting in lower body weight and smaller size without directly causing mortalities in fish (Vosyliene and Jankaite, 2006). Heavy metals toxicity is associated to their ability to affect the enzymatic processes, low mobility in the

organism because of their unsaturated chemical bonds and small size causing the accumulation and subsequent alterations in the metabolism of organisms (Sanches Filho et al., 2017).

2.4.1. Chromium toxicity towards fish

Cr is a transitional metal that exists in many valence states. Among the various oxidation states trivalent Cr (III) and hexavalent Cr (VI) are predominant in environment (Towill, 1978). Hexavalent chromium Cr (VI) is highly toxic and carcinogenic. Its toxicity is related to a generation of reactive oxygen species (ROS) as a result of cellular reduction of Cr (VI) to Cr (III) which forms complexes with intracellular macromolecules such as DNA leading to oxidative stress, peroxidation of lipids and ultimately genotoxic effects in the cell (Bakshi, 2018; Ahmed et al., 2013).

Cr (VI) can enters Haber–Weiss-type reactions leading to the generation of hydroxyl radicals (•OH) therefore, Cr effects are produced by ROS generation (Lushchak, 2011; Lushchak et al., 2008). Cell reducing agents, Glutathione (GSH) and Nicotinamide adenine dinucleotide phosphate (NADPH) reduce Cr (VI) to the pentavalent state Cr (V), which can contribute in the fenton reactions to produce hydroxyl radicals (•OH) in a following reaction as described below reaction (Sevcikova et al., 2011).

$$Cr^{6+} + O_2 \bullet \longrightarrow Cr^{5+} + O_2$$

$$Cr^{5+} + H_2O_2 \longrightarrow Cr^{6+} + \bullet OH + OH^-$$

Overall reaction;

$$O_2 \bullet^- + H_2O_2 \xrightarrow{Cr^{6+}/Cr^{5+}} \bullet OH + O_2 + OH^-$$

Cr can cause abnormal physiological changes at cellular and molecular levels such as Cr induced morphological alterations in cellular and nuclear components revealed the genotoxic potential of effluents on the fish, *Oreochromis niloticus* when exposed to petroleum refinery and Cr-processing plant effluents (Cavas and Ergene, 2005). Fish blood chemistry undergoes various changes due to Cr. Cr can cause abnormal physiological changes at cellular and molecular levels such as Velma and Tchounwou, (2010) observed that Cr (VI) can induce oxidative stress due to its redox potential thus impairing the health of fish. Furthermore, exposure to Cr may also induce genotoxic, biochemical and histopathological effects in fish tissues (liver and kidney). Ahmed et al. (2013) reported genotoxicity and acute toxicity of Cr (VI) in a freshwater stinging catfish, *Heteropneustes fossilis* exposed to three sublethal Cr concentrations (1.6, 3.5 and 9 mg/L)

resulting concentration dependent increase in MN frequency and tail DNA in whole blood, gills and liver tissue.

2.4.2. Cadmium toxicity towards fish

Cd is genotoxic, mutagenic, carcinogenic and teratogenic metal which is considered as extremely toxic for fish and other aquatic organisms (Cavas et al., 2005). Cd can induce oxidative stress resulting to lipid peroxidation (LPO) and protein carbonyl (PCO) formation which are the two important indicators of oxidative damage of macromolecules induced by ROS (Cao et al., 2010). Cd exposure is also linked with the disruption of sodium balance and Na+/K+ ATPase activity (Atli and Canli, 2007). Fish exposed to Cd can cause alteration in blood constituents and differential blood count such as destruction of erythrocytes, decrease hemoglobin and hematocrit concentrations leading to anemic conditions (Gill and Apple, 1993). Cytogenicity and genotoxicity studies revealed Cd induced nuclear and cellular anomalies such as chromatin condensation, nuclear malformations, swelling and haemolysis and cytoplasm vacuolization in erythrocytes of carp (Witeska et al., 2011). Jia et al. **(**2011) revealed that exposure of Cd (0.41, 0.52, 0.69, 1.03 and 2.06 mg/L) induced oxidative stress and DNA damage in Common carp (*Cyprinus carpio*). DNA damage percentage was increased when the Cd concentration was greater than 0.41 mg/L. Souid et al. (2013) observed that exposure of Gilt-head bream (*Sparus aurata*) exposed to Cd (0.5 mg/L) induced metal accumulation and oxidative stress response. Cd can bioaccumulate in aquatic organisms with its total uptake depending on the concentration in environment, exposure route and the duration of exposure (Annabi et al., 2013).

2.4.3. Metals in mixture/ Multiple Stressor

In natural environment, contaminants are usually present in mixtures form so fish are usually exposed to mixture of metals. Toxicity of mixtures of metals is usually different from those of single metal exposure and their performance may be additively (1 + 1 = 2), synergistically (1 + 1 > 2) or antagonistically (1 + 1 < 2) (Salbu et al., 2005; Jezierska and Sarnowski, 2002). Metals behaviour in mixture exposure is quite different from that during single metal exposure therefore, it could not be explained based on single metal accumulation. Mode of action of metals in a mixture even in the same fish species may also differs ranging from antagonistic, synergistic or additive depending on the multimetals in the mixture (binary, ternary, etc.) and their concentration ratio. Moreover, physiochemical properties of water may

also play vital role among metal interactions and may probably affect the final response (Sauliute and Svecevicius, 2015).

Svecevicius, (2014) assessed heavy metals uptake in various fish tissues of Atlantic Salmon (*Salmo salar L*) when exposed to a model Mixture (Ni, Cr, Zn, Cu, Pb, Cd) and singly to Ni, Cr and Pb at a concentration equivalent to Lithuanian inland water standards for 14 days. It was revealed that presence of metal mixture in the water partly affected the accumulation of single metals in tissues possibly due to synergistic interactions between metals. Palaniappan and Karthikeyan, (2009) assessed the impact of Cr–Ni binary mixture on the bioaccumulation in some tissues of freshwater fish (*Cirrhinus mrigala*). Binary mixture of Ni and Cr at sub lethal concentrations of 2.9 mg/L for each metal over seven days exposure resulted in 10–28% higher Ni accumulation in presence of Cr and 7–12% increase in Cr accumulation in the presence of Ni in fish tissues representing synergistic interactions between these two metals.

2.5. Erythrocytes cellular and nuclear abnormalities

Various biomarkers have been used for the assessment of effects by genotoxic pollutants. These biomarkers consist of various tests including the assessment of DNA adducts and breaks, chromosomal abnormalities, Comet assay, measurement of micronucleus frequency and other chromosomal abnormalities (Bombail et al., 2001). Among mutagenicity assay, piscine micronucleus and erythrocytes nuclear abnormalities (ENA) test has been widely applied extensively since it is safe, simple and sensitive method (Minissi, et al., 1996). Micronucleus test has been carried out to evaluate the condition of aquatic organisms particularly fish exposed to different toxicants in field and laboratory studies in order to measure the genotoxic potential of toxicants (da Silva Souza and Fontanetti, 2006).

For the first time nuclear and morphological alterations have been described in fish peripheral blood erythrocytes (Carrasco et al., 1990). Micronuclei (MN) are produced as a result of chromosome fragments or whole chromosomes that lag at cell division due to lack of centromere, damage in centromere or defect in cytokinesis. In tissues with actively dividing cells micronuclei formation reflects the action of aneugenic or clastogenic compounds (Stankeviciute et al., 2016). The analysis of erythrocyte nuclear abnormalities is one another alternative of the standard MN test which has been also commonly used in fish toxicology (Ergene et al., 2007). Nuclear abnormalities were manifest as alterations in the normal elliptic shape of nuclei (Ferraro

et al., 2004). The formation of morphological alterations in the nuclear envelop are described by Carrasco et al. (1990) such as blebbed (nuclei that present a fairly small evagination from the envelope which seems to contain euchromatin), lobed (nuclei showing evaginations larger than those from blebbed nuclei) and notched (nuclei that shows a remarkable notch containing nuclear material). These types of abnormalities occurrence in fish erythrocytes are considered to be indicators of genotoxic damage as a result of exposure in environment to chemical contaminants of genotoxic, mutagenic and carcinogenic nature however, the underlying mechansims triggering these types of abnormalities have not been entirely explained so far (Muranli and Guner, 2011).

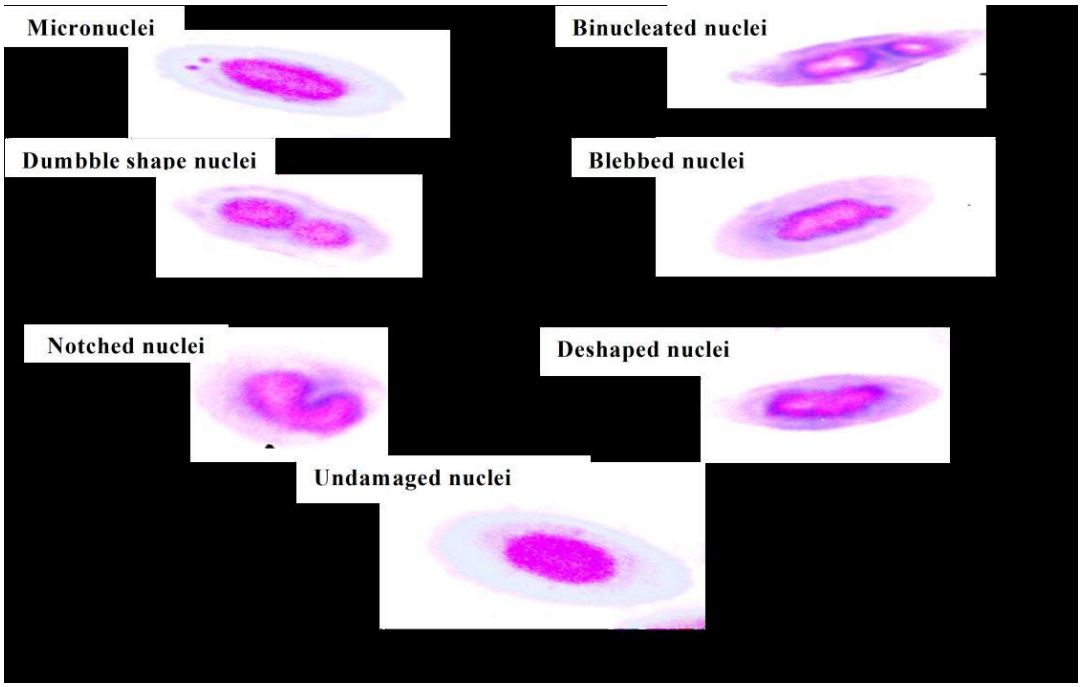


Figure 2.3: Metals exposure induces nuclear abnormalities in the fish blood (Kousar et al., 2015)

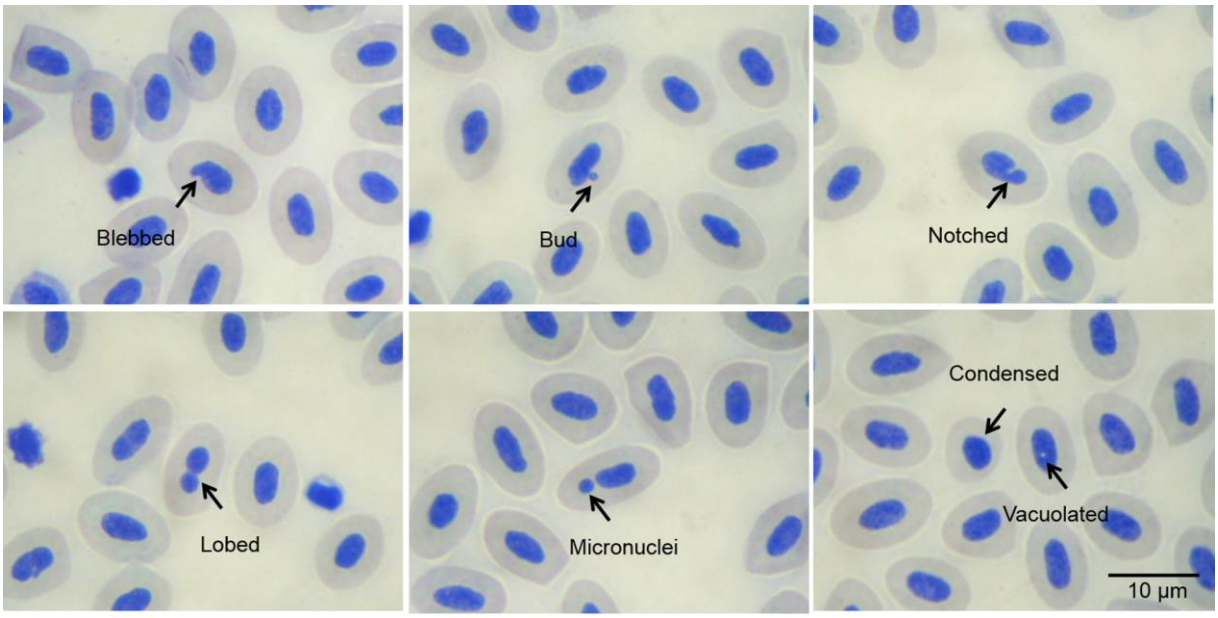


Figure 2.4: Types of abnormalities in erythrocytes of fish (Gomes et al., 2015)

2.5.1. Oxidative stress/ DNA damage

Metals ions are well known inducers of oxidative stress (Lushchak, 2011). Oxidative stress is defined as an imbalance between ROS and the antioxidant defense mechanism which includes a battery of enzymes and repair mechanisms. This imbalance is explained either as an excess of ROS or as a deficiency of antioxidants, which may cause oxidations of DNA, membrane lipids, proteins leading to cell damages or even cell death (van der Oost et al., 2003). ROS are generated by substances such as pesticides, transitional metal ions and petroleum pollutants. (Slaninova et al., 2009; Sevcikova et al., 2011). As the ROS levels increases, aerobic organisms use body defense mechanism by modulating the activities of antioxidants such as superoxide dismutase (SOD), catalase (CAT), metallothioneins (MT) and glutathione related enzymes but when ROS generation exceeds the capability of the cellular antioxidants, it will cause oxidative stress and oxidative damage (Roberts and Oris, 2004). Large concentration of free radicals induce oxidative stress which decreases the effectiveness of antioxidant defense system hence damages proteins, lipids and cells.

2.6. Haematological parameters

Haematological parameters are widely used indices to determine the physiological changes in fish species during stress conditions. Al Asgha et al. (2015) studied haematological parameters in *Oreochromis niloticus* exposed to cadmium chloride various concentrations (1.68, 3.36 and 5.03 mg/l). RBC count decreased significantly in all treatments of $CdCl_2$ over a period of 10 days with RBC counts of 1.82 ± 0.06 (10^6 mm^3), 1.76 ± 0.07 (10^6 mm^3), 1.65 ± 0.03 (10^6 mm^3) and 1.30 ± 0.05 (10^6 mm^3) for Control T1, T2 and T3 respectively. Similarly, Hb concentration was decreased significantly ranging from 6.00 ± 0.71, 5.29 ± 0.18, 5.11 ± 0.41 and 4.96 ±0.22 (g/100 mL) for the control, T1, T2 and T3 respectively. HCT (%) showed a significant difference between exposed fish and the control such as 33.26 ± 0.32 31.70 ± 0.32, 28.41 ± 0.62 and 27.58 ± 0.53 (%) for control, T1, T2 and T3 respectively. Haematological parameters were significantly reduced in fish exposed to Cd at all periods.

Hassan et al. (2018) conducted a study on the assessment of heavy metals toxicity in *Catla catla* through haematological and biochemical blood markers. The experimental fish were treated with Cd and Cu water born lethal concentrations (LC50) for 24, 48, 72 and 96 hours. The hematological parameters of exposed fish at all exposure durations showed significant decrease in RBC count, Hb and HCT content whereas, increase in WBC count, MCV, MCH and MCHC was noticed in comparison to control.

2.7. Uptake of metals in gills tissues

Fish gills are considered as the significant site for uptake of metals directly from the water (Afshan et al., 2014). Gills serve as a useful organ for assessing bioavailable pollutants in water due to their bioaccumulation (Heier et al., 2009). The uptake routes of metals in fish primarily occurs through the gills and partly by body surface and via gut through food, then metals enters into the blood stream and finally accumulated in different organs (Kamunde et al., 2002). During aqueous metal exposures, acute toxicity normally occurs at fish gills producing ionoregulatory impairments because gills are the first targeted organs which come in contact with toxicants (Rosseland et al., 2007; Smith et al., 2015). Metal ions are normally absorbed through passive diffusion or carrier mediated transport over the gills while metals associated with organic materials are ingested and absorbed by endocytosis through intestine (Kumar and

Singh, 2010). Dissolved pollutants in water including metals enter to fish primarily via gills through ion channels of respiratory epithelium or protein complex of the chloride cells located in gills (Kondera et al., 2014). Gills surface serves as metal-binding ligands sites and metal bioaccumulation can occur due to positively charged metal species in the water to negatively charged sites on the gills (Terra et al., 2008).

Cr accumulation pattern conducted on *Labeo rohita* exposed to 48.3 ppm (1/3rd of LC50 of potassium dichromate) in the long term exposure (15 days) showed that accumulation of Cr in *Labeo rohita* were greater in gills, liver, muscle and brain respectively (Kumari, 2014). Tissue-specific accumulation of Cd in juveniles of Japanese flounder following Cd exposure for 28 days resulted dose-dependent accumulation of Cd in liver, followed by kidney, gills and muscles. (Cao et al., 2012).

MATERIALS AND METHODS

Toxicological Assays were carried out in Environmental Toxicology Laboratory, IESE, SCEE, National University of Sciences and Technology (NUST) Islamabad. The study was conducted to evaluate the exposure of environmentally relevant concentration of Cr and Cd towards Common carp. The exposure containing fish was planned following APHA, (2017).

3.1. Concentration of Cr and Cd in road dust

Road dust samples were collected in order to determine the environmentally relevant concentrations of Cr and Cd. Field sampling was conducted during the dry period in month of October and November 2016. Sampling locations are shown in Figure (3.1). Road dust samples were collected from selected points of Islamabad and Rawalpindi which has an estimated average daily traffic density of about 48,000 vehicles. The selected roads were H9 Service road, Kashmir Highway, Murree road and G9 from Islamabad whereas, IJP and Faizabad roads were selected from Rawalpindi. From every location, a representative composite samples of road dust were collected from three points, with a one kilometer (km) distance between each road points and then mixed thoroughly to make a composite sample (Suryawanshi et al., 2016). The samples were collected from the road curb with the help of a clean brush by sweeping road surface and collected in self-sealed clean polyethylene bags. The samples were transported to the laboratory immediately, kept and stored at room temperature until further analysis.

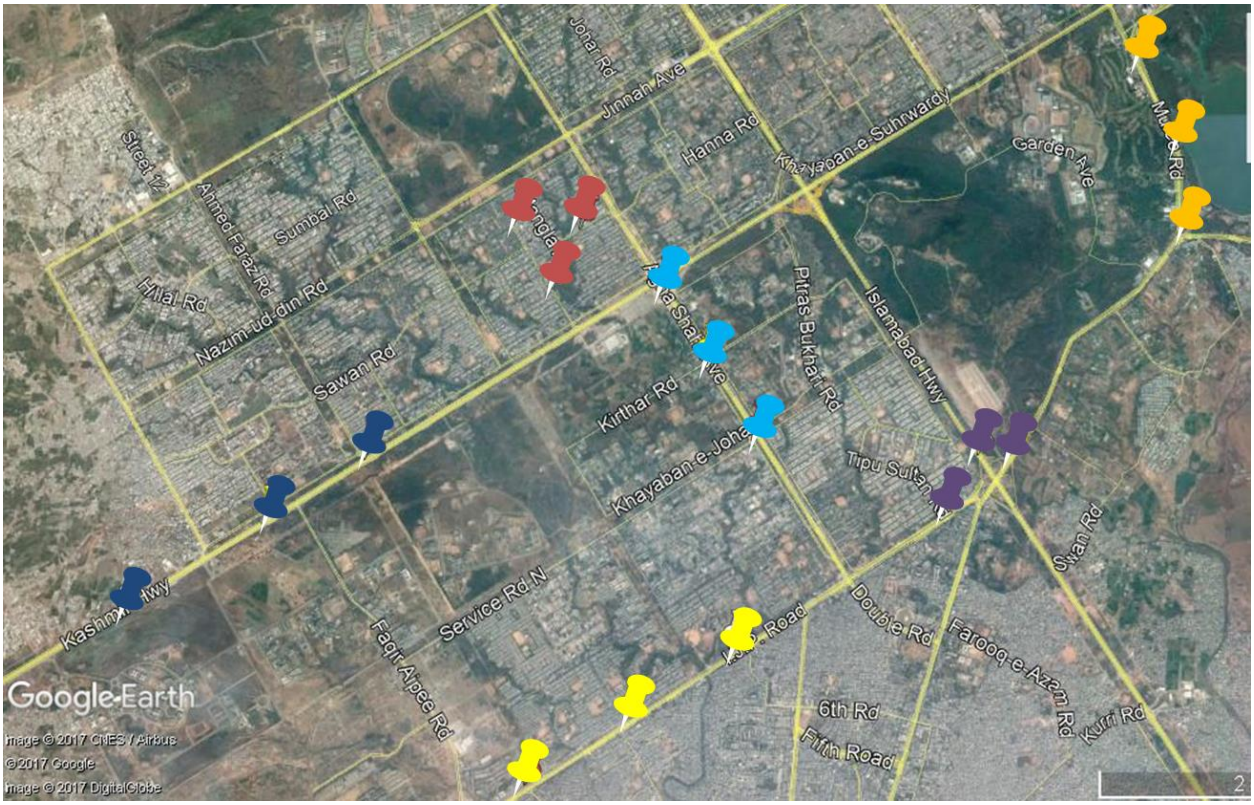


Figure 3.1: Map of the study area showing sampling locations.

3.1.1. Lab scale setup for leaching experiment

Leaching experiments was conducted to simulate the realistic road runoff episodes from road dust samples. Fifteen grams (g) of road dust was added in 150 ml distilled water and placed on shaker (LSI-3016A) for 24 hours. Samples were centrifuged (Refrigerated centrifuge, K3 series) at a speed of 5000 rpm for 15 minutes using centrifuge. The supernatant was separated, filtered and acidified by 2% nitric acid (HNO_3) for total Cr and Cd analysis by Atomic Absorption Spectrophotometer (AAS) as per method described by Mahrosh et al. (2014).

3.2. Experimental setup

3.2.1. Fish acclimatization and maintenance

Juveniles (six months old) specimens of Common carp (*Cyprinus carpio*) were purchased from Punjab Hatchery, Rawal Town, Islamabad and transported to the laboratory in plastic bags

filled with oxygenated water. Fish specimens were immediately transferred into experimental tanks of dimension (3 X 1.5 X 1.5 ft) with continuous aeration. The experiment was conducted in two batches in a semi-static exposure system. In the first batch, twenty six (26) fishes of an average size (14.9 ± 0.8 cm) and average weight (53.2 ± 8.4 g) were allowed to acclimatize for one month prior to exposure. In the second batch, forty specimens (40) of Common carp, with an average weight (38.7±8.9) and length (14.8±1) were allowed to acclimatize in the experimental tanks for two weeks. During the acclimatization period fish were fed daily with commercial dry food pellets. Feed formulations usually contains fish meal (0-10 percent), soybean meal (10-30 %), rapeseed meal (10-30 %), rice bran, corn, wheat and other agricultural by-products. Vitamin and mineral premix and feeding attractants comprise around 2-5 % of the feed. Water was changed every alternate day to remove leftover food and excretory waste in tanks. Dead fish were removed immediately. Furthermore, water quality was monitored throughout the experiment.

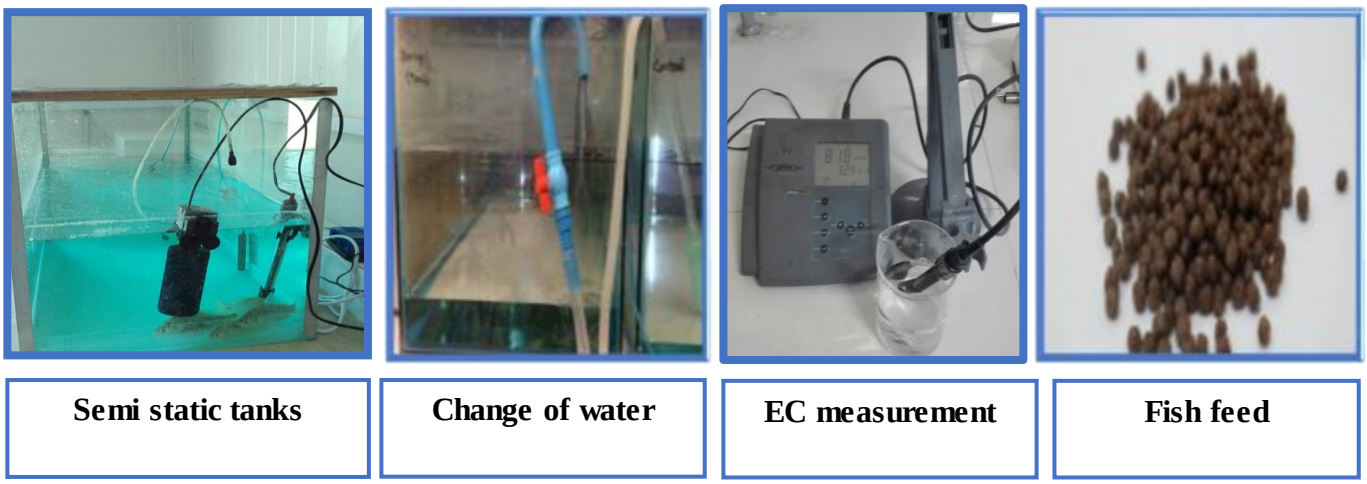


Figure 3.2: Maintenance measures during experiment

3.2.2. Exposure concentrations for tap water experiment

First batch of fish were divided into four treatment groups with six fish in each aquarium containing 100 L tap water. Following treatments were included with exposure concentrations such as:

1. Control (no metal added in NUST tap water)

2. 0.2 mg Cr/L
3. 2 mg Cr/L
4. 17 mg Cr/L

The exposure period was 3 months from 26 December, 2016 – 25 March, 2017. First group served as control, second and third treatment groups contain Cr concentrations (0.2 and 2 mg/L). These values were selected based on the environmentally relevant concentrations determined in the leaching experiment, while the highest dose i.e. 17 mg/L was reported as a lethal concentration dose (LC50-96 h) for Common carp, this dose was selected on the basis of previous lab based study (Abedi et al., 2012). Analytical grade potassium dichromate ($K_2Cr_2O_7$) was used as a source of Cr metal. Cr concentrations were produced by dissolving $K_2Cr_2O_7$ in distilled water to produce stock solution (1000 mg/L) and further dilution of freshly prepared $K_2Cr_2O_7$ was added in the tanks to achieve the respective dose concentrations. Experiment exposure type was semi-static renewal systems. The water was changed weekly with the fresh doses of Cr in order to maintain the Cr concentrations constant.

3.2.3. Exposure concentrations for lake water experiment

The second experiment was carried out in Rawal lake water. Initially 1500 liters of lake water was brought and stocked in water containers, which was supplied to the Laboratory. This experiment included treatments based on both Cr and Cd exposure. This experiment also evaluated the combined effect of Cr and Cd. Fish were divided in six experimental tanks. Each experimental tank contained 70 liters of Rawal lake water containing six fish per tank. Fish were exposed to test concentrations based on the leaching experiments values obtained for Cr and Cd. The experiment was conducted for 45 days from 11 May, 2017- 26 June, 2017. Following test concentrations were included;

1. Control water (no metal added in Rawal lake water)
2. 0.2 mg Cr/L
3. 2 mg Cr/L
4. 0.05 mg Cd/L
5. 0.1 mg Cd/L
6. 2 mg Cr/L + 0.1 mg Cd/L (Mixture of Cr and Cd)

Cr and Cd stock solutions were prepared by adding Potassium dichromate ($K_2Cr_2O_7$) and Cadmium sulphate ($CdSO_4$) salts respectively. The stock solutions were prepared in distilled water. Cr and Cd test concentrations were delivered to experimental tanks to achieve the desired test concentrations as mentioned above.

3.3. Water quality parameters

3.3.1. Tap water quality parameters

Temperature and electrical conductivity (EC) were measured three times in a week by using 720 WTW probe, pH and dissolved oxygen (DO) were measured by using multimeter (156 Hach sension, Germany). Water total hardness was measured weekly by EDTA (Ethylene Diamine tetra acetic acid) titration method according to the standard procedures. Ammonia was determined weekly by using kjeldahl nitrogen test. (APHA, 2017).

3.3.2. Rawal lake water quality parameters

The physicochemical parameter of Rawal lake water include temperature and pH which were measured by multimeter (156, Hach sension, Germany). Electrical conductivity was measured by using 720 WTW probe. Turbidity was measured by portable turbidity meter (2100P, Hach). Water hardness was measured weekly by EDTA (Ethylene Diamine tetra acetic acid) titration method. Total alkalinity was measured by titration method. Dissolved oxygen was measured by winkler titration method. All these parameters were measured twice a week. Chemical oxygen demand (COD) was measured before the start of experiment by open reflux titrimetric method (APHA, 2017).

3.4. Study design

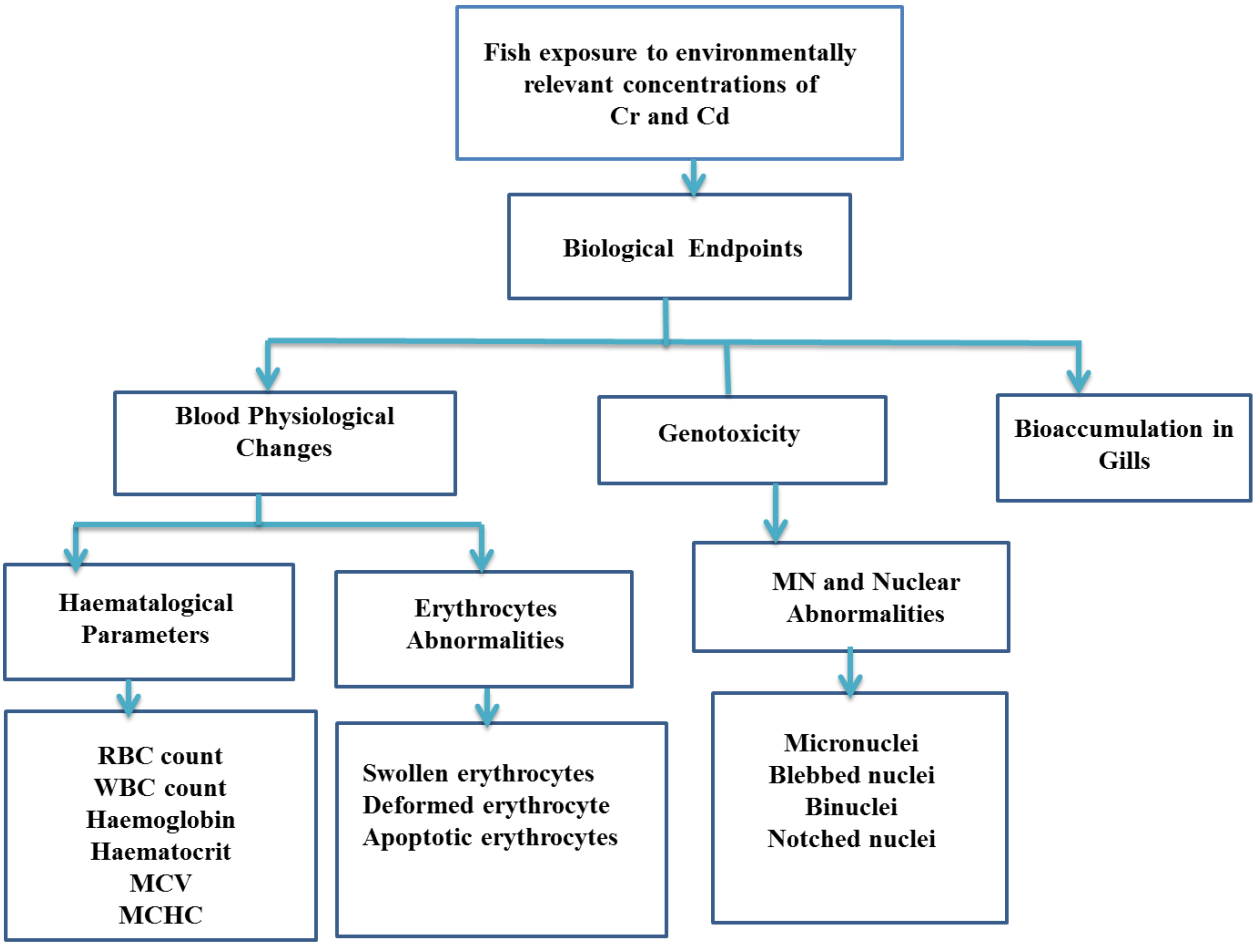


Figure 3.3: Study design for experimental work

3.5. Genotoxicity test

3.5.1. Micronucleus (MN) and nuclear abnormalities assay

Micronucleus (MN) test is known as one of the most convenient methods for assessing genotoxicity in aquatic organisms. Upon the completion of exposure period peripheral blood samples from 3 fish specimens were withdrawn from caudal vein puncture by using a 1mL syringe. A drop of blood was carefully smeared on clean microscope slides then dried smears were fixed in absolute methanol for 10 min, slides were air dried and later the smears were

stained with 10% Giemsa solution for 8 minutes and left for air drying. (Barsiene et al., 2004). Slides were observed by using Leica CME light microscope at final magnification of 1000x. The erythrocytes were randomly scored for micronucleus and other nuclear and cellular abnormalities Mean frequencies of MN and erythrocytes cellular and nuclear abnormalities observed in each treatment groups were calculated and expressed per 100 cells counted.

$$MN = \frac{\text{Number of cells containing micronucleus}}{\text{Total number of cells counted}} \times 100$$

3.5.1.1. Scoring criteria

MN were recognized according to criteria described by Fenech et al. (2003). Only the cells clearly separated from the surrounding cells were scored. The criteria for the scoring of micronuclei were as follows:

(1) MN must be smaller than one-third of the main nuclei.

(2) MN must be clearly separated from the main nuclei.

(3) MN must be on the same plane of focus and have the same color.

Other cellular and nuclear abnormalities were recorded separately according to criteria described by Ergene et al. (2007) and Cavas et al. (2005).

Figure 3.4: Fish blood sample collection from caudal vein.

3.6. Haematological analysis

Upon completion of exposure period for each experimental phase, samples of blood were collected from the three fish specimens in each treatment group. Fish were starved for 24 hours before blood sampling. Fish were captured with net by minimum disturbance and were not anesthetized. Whole blood samples were withdrawn from the fish caudal vein in a 1 mL clean disposal syringes and transferred to vacutainer tubes coated with EDTA as anti-coagulant. Blood samples were shaken several times by thorough mixing in EDTA tubes. Whole blood was analyzed for red blood cells count (RBC), white blood cell count (WBC) hemoglobin content (Hb), hematocrit level (Ht), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) by using an automatic blood Cp machine (Sysmex XP-1000). Common haematological parameters measured during stress are red blood cell count, white blood cell count, haemoglobin concentration, hematocrit value and red blood cell indices (Javed and Usmani, 2013).

Figure 3.5: Automated Blood Analyzer, Sysmex XP-100 (Singapore).

3.7. Determination of metals (Cr, Cd) in gills tissue

Fish were decapitated by giving a blow on head. Gills tissues were dissected (excised second arch of gills for metal analysis) and freezed until analysis (Emerge protocol).

Each thawed frozen tissue sample was weighed in a beaker. The actual weight of the gills was the difference between the weight of the empty beaker and weight of the beaker with gills (Weight of the gills = Weight of the beaker with gills - weight of the empty beaker).

Gills tissues were processed by digestion method according to Du Preez and Steyn, (1992). During gills digestion process, 5mL HNO_3 (55%) and 1mL $HClO_4$ (70%) were added and left overnight. Furthermore, 5mL HNO_3 (55%) and 4mL $HClO_4$ (70%) were added in a 100 mL beaker and heated on a hot plate at 200 to 250 °C. Transparent clear solution was then achieved as brown fumes transformed to dense white fumes which indicated complete digestion. Finally samples were cooled, diluted by adding 10 mL distilled water with proper rinsing of beakers. Samples were stored at 4°C until analysis. Cr and Cd uptake by gills were examined by using Atomic Absorption Spectrophotometer (Shimadzu, 7000 JAPAN).

The following equation was used for the uptake of metals in gills tissues

$$\mu g/g \text{ wet weight} = (A \times V) / W$$

A = Absorbance value in µg/L

V= Final dilution volume

W= Wet weight of sample

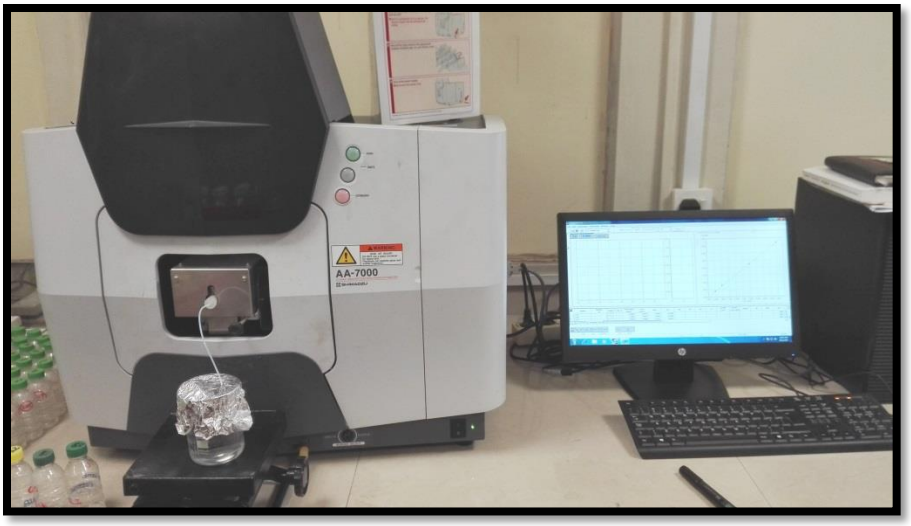


Figure 3.6: Atomic Absorption Spectrophotometer (Shimadzu, 7000 JAPAN)

3.8. Statistical analysis

Statistical analysis of all the parameters was completed in triplicates. All values were presented as mean ± SD. Values of water quality parameters, haematological parameters, nuclear and cellular abnormalities and bioaccumulation in gills were compared statistically by one way ANOVA for determination of significant difference among control treatment groups. Significant difference was defined by using the standard, $p<0.05$ as significance level. All the data obtained has been statistically analyzed by using Microsoft excel 2010 and Prism Graph Pad 7 software.

RESULTS AND DISCUSSION

4.1. Water quality parameters

For the first phase of experiment, laboratory tap water was utilized for exposure. During experimentation, the mean value for pH measured was within 8.2-8.3 mg/L in all treatment groups. Independent of the Cr treatment groups as such no significant difference in pH (p=0.38) was observed. Similarly, for temperature (p=0.56), no significant difference was found. Temperature mean value was 19°C for all the treatment groups that remained same since it was maintained during winter period by using heaters. Furthermore, the concentration of dissolved oxygen mean value ranged within 8.2-8.3 mg/L (p=0.87), while the addition of Cr in treatment groups cause no significant difference in EC (p=0.05) between different treatment groups. Similarly, for total hardness (p=0.42), no significant difference was found. Ammonia concentration remained below the detection limit (BDL) for all treatment groups. All measured parameters were within the range of standards (USEPA, 2017; APHA, 2017).

Table 4.1: Physiochemical analysis of tap water quality parameters obtained from the tanks during the experimental period

Parameters	Units	Control	Cr Concentrations (mg/L)			Standard values
			0.2	2	17	
pH		8.3±0.2	8.2±0.2	8.3±0.1	8.3±0.2	18-24 (APHA, 2017)
Temperature	(^{0}C)	19±2	19±1.4	19±1.5	19±1.4	6.5-9 (US.EPA,2017)

Parameters	units	Control	Cr Concentrations (mg/L)			Standard Values
			0.2	2	17	
Electrical Conductivity (EC)	(µS/cm)	745±37	679±80	701±74.8	752±54	1000-2000 (US.EPA,2017)
Dissolved Oxygen (DO)	(mg/L)	8.2±0.8	8.2±0.7	8.2±0.7	8.3±0.6	>5mg/L (US.EPA,2017)
Total Hardness	(mg/L)	314±33.4	280.3±48.3	294.7±34.5	304.8±23.3	<250 mg/L (APHA,2017)
Ammonia	(mg/L)	BDL	BDL	BDL	BDL	1.9 mg TAN/L (US.EPA,2017)

BDL: Below Detection Limit
TAN : Total Ammonia Nitrogen

In the second experimental phase, Rawal lake water was utilized for fish exposure to simulate the natural lake water conditions. The experiment was based on Cr, Cd and the interactive effect of Cr-Cd toxicity. The two test concentrations of Cr were repeated as in the first experiment, two test concentrations of Cd and Cr-Cd combined toxicity was included. Before the start of experiment the Rawal lake water, Chemical oxygen demand (COD) was tested immediately and its values was reported as 43.2 mg O_2/L. Independent of the Cr treatment groups as such no significant difference in pH ($p=0.98$) was observed. Similarly, for temperature ($p=0.56$), EC ($p=0.9$), turbidity ($p=0.93$), DO ($p=0.99$), hardness ($p=0.99$) and alkalinity ($p=0.55$). Results of One-way ANOVA showed that there was no statistical significant difference in measured water quality parameters during the whole experimental period. The measured parameters were within the range of acceptable limits (USEPA, 2017; APHA, 2017).

Table 4.2: Physiochemical analysis of lake water quality parameters obtained from the tanks during the experimental period

Parameters	Units	Control	Chromium (mg/L)		Cadmium (mg/L)		Mixture (mg/L)	Standard Values
			0.2	2	0.05	0.1	2 + 0.1 (Cr-Cd)	
pH	(mg/L)	8.2±0.3	8.2±0.2	8.2±0.2	8.2±0.3	8.2±0.2	8.2±0.2	6.5-9 (US.EPA,2017)
Temp	(^{0}C)	28±2	28±2	28±2	28±2	28±2	28±2	18-24 (APHA, 2017)
EC	(µS/cm)	533±119	556±105	561±104	518±118	538±108	523±101	1000-2000 (US.EPA,2017)
Turbidity	(NTU)	4.8±2	4.3±2	3.8±2	3.8±2	3.7±2	4.1±2	5 NTU (US.EPA,2017)
DO	(mg/L)	7.2±0.9	7.1±0.9	7.3±0.9	7.1±0.9	7.2±0.9	7.3±1	>5 mg/L (US.EPA,2017)
Hardness	(mg/L)	205±52	211±49	207±50	206±50	197±53	200±53	<250 mg/L (APHA,2017)
Alkalinity	(mg/L)	18.2±1.7	18.4±1.5	18.9±1.6	17.3±1.9	17±2.4	17.4±2.1	20000 (US.EPA,2017)

4.2. Cr and Cd concentrations by leaching experiment

The mean concentration of Cr and Cd in road dust collected from six sites is shown in the figure (4.1). The sample collected from the Faizabad road showed the highest mean concentration for Cr (2 mg/L) and Cd (0.1 mg/L). The origin of Cr and Cd is probably due to the heavy traffic density moreover, this area is a well-known commercial site. Least concentration of Cr (0.2 mg/L) coupled with no detection of Cd was observed along the H9 service road Islamabad. Our results are in agreement with the findings of Dounge and Lee, (2011) that the accumulation of Cr and Cd is attributed to vehicular depositions. The pollution level of heavy metals in the road dust could be attributed to traffic density and emissions, vehicular speed and atmospheric dispersion in the road environment. Similarly, Domekpor et al. (2016) reported that Cd in road environment may be due to the leakage of lubricating oils and wearing of old tyres and Cr is attributed to vehicular depositions and constructional activities nearby roads may also contribute Cr in road environment.

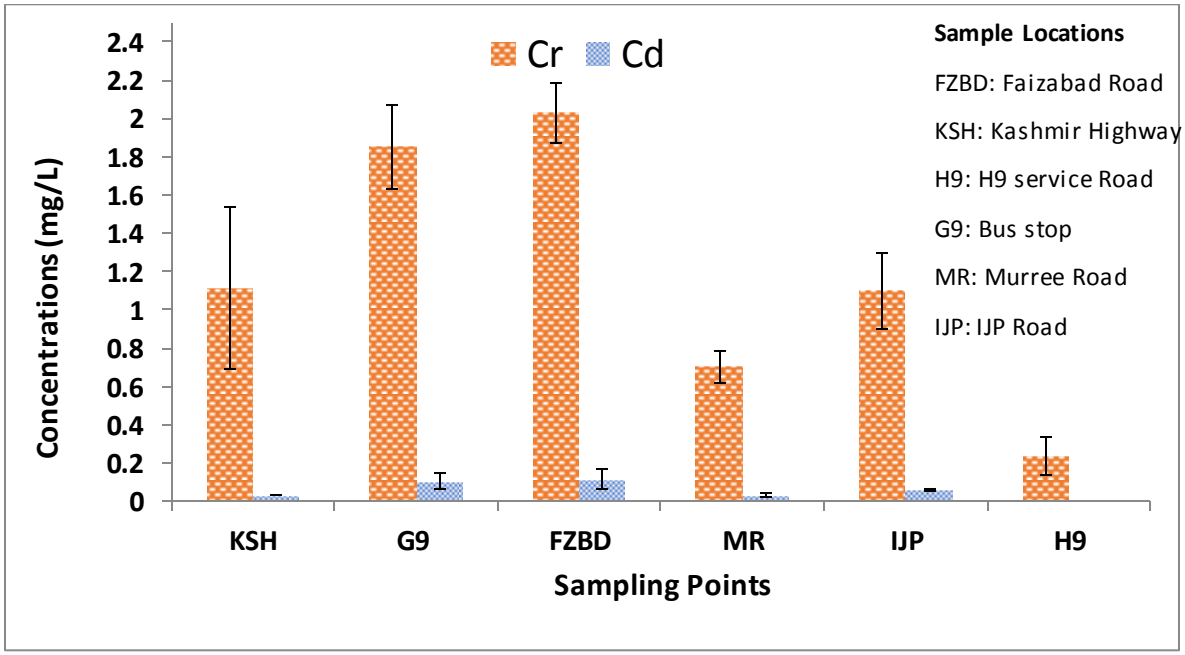


Figure 4.1: Cr and Cd concentrations in road dust samples

4.3. Haematological parameters

Haematological parameters are considered as important indicators to monitor physiological status of fish heath when exposed to toxicants which consequently cause deformities in blood and other vital organs (Desai and Bhilave, 2018). Evaluation of haematological indices has been used effectively in monitoring the responses of fishes under stressors or adverse conditions (Vazquez and Guerrero, 2007). In addition, blood parameters contribute as suitable biomarkers to assess the in vivo environmental exposures (Zutshi et al. 2010; Gaber et al., 2013).

4.3.1. Red blood cell count

4.3.1.1. Exposure in tap water

At the end of exposure period the total red blood cells (RBC) count measured in 10^6 per microliter of blood *(*$x10^6$/µL) was significantly ($p<0.05$; $p=0.0160$) reduced in Cr treatment groups as compared to control as shown in Figure 4.2 (A). Red blood cells count decreased gradually when subjected to Cr treatments, with the RBC count of 0.73 ± 0.02 *(*$x10^6$/µL) ,0.70 ± 0.07 *(*$x10^6$/µL) and 0.65 ± 0.04 ($x10^6$/µL) for 0.2, 2 and 17 mg Cr/L respectively, when compared with the control RBC count of 1 ± 0.2 ($x10^6$/µL).

4.3.1.2. Exposure in lake water

Red blood cells count were reduced in all treatment groups with a significant difference ($p<0.05$; $p=0.0262$) among treatment groups as shown in Figure 4.3 (A). RBC count decreased in Cr concentrations of 0.2 and 2 mg Cr/L with a count of 0.73 ± 0.1 ($x10^6$/µL) and 0.70± 0.1 ($x10^6$/µL) respectively, Similarly, Cd concentrations of 0.05 and 0.1 mg Cd/L showed a decline with a RBC count of 0.72 ± 0.04 ($x10^6$/µL) and 0.70 ± 0.1 ($x10^6$/µL) respectively. Mixture of Cr-Cd (2mg Cr/L + 0.1 mg Cd/L) reduced RBC count to 0.67 ± 0.1 ($x10^6$/µL) as compared with control RBC count of 0.9 ± 0.04 *(*$x10^6$/µL). It was observed from the treatment groups that mixture of Cr-Cd showed highest decrease, however slight variations were observed among other treatment groups.

In the present study, fish exposed to test concentrations of Cr and Cd causes decline in RBC count. In support to the present work, Shaheen and Akhtar, (2012) found similar declining trend in RBC and Hb in fresh water fish (*Cyprinus carpio*) when exposed to Cr (VI). This

decline in RBC count may be possibly because of decreased rate of RBC synthesis or an increased loss of erythrocytes due to hemolysis (Vosyliene and Jankaite, 2006). Fish (*Channa punctatus*) exposed to metal effluents containing heavy metals resulted in reduced RBC count and haemoglobin content whereas, WBC count increased (Javed & Usmani, 2013).

4.3.2. White blood cells count

4.3.2.1. Exposure in tap water

White blood cells (WBC) count measured in (10^3/µL) showed statistically significant difference ($p<0.01$; $p=0.0025$) among treatment groups as shown in Figure 4.2 (B). Control group WBC count measured to 231 ± 14.7 (x10^3/µL) whereas, chromium treated groups showed increased WBC count to 244 ± 5.8 (x10^3/ µL), 264 ± 4.8 (x10^3/µL) and 269 ± 6 (x10^3/µL) for 0.2, 2 and 17 mg Cr/L respectively. The overall WBC count observed showed increased trend as compared to control.

4.3.2.2. Exposure in lake water

WBC count in all treatment groups increased with a significant difference ($p<0.05$; $p=0.0272$) among treatment groups as shown in Figure 4.3 (B). The WBC count in Cr test concentrations increased 249.4 ± 6.4 (x10^3/µL), 252 ± 6 (x10^3/µL) for 0.2 and 2 mg Cr/L. Likewise, WBC count in 0.05 and 0.1 mg Cd/L was increased with a count of 248 ± 6.6 (x10^3/µL) and 258 ± 3.3 (x10^3/µL). WBC count for mixture of Cr-Cd (2mg Cr/L + 0.1 mg Cd/L) was found to be 262 ± 4.5 (x10^3/µL) when compared with control WBC count of 249.4 ± 6.4 (x10^3/µL).

In our study, exposure to test concentrations of Cr and Cd caused increase in WBC count that may indicates stress conditions in fish. Our results are in line with the findings of previous authors such as Remyla et al. (2008) who also found significant increase in the WBC count under Cd exposure probably due to leucocytosis (an adaptation made to overcome the stressful conditions). Moreover, increase in WBC count may be related with an increase in antibody production in fish under stress conditions which helps in recovery and survival of fishes exposed to toxicant. Increase in total WBC count might also be resultant of direct stimulation for its defense from disease conditions due to the presence of heavy metals. Other studies also reported the increased level of WBC count due to exposure of heavy metals such as *Clarias gariepinus*

showed increase WBC count when exposed to metal finishing company effluents (Adakole, 2012).

4.3.3. Haemoglobin

4.3.3.1. Exposure in tap water

Hb measured in (g/dL) was significantly ($p<0.05$; $p=0.0109$) decreased in Cr treatment groups as shown in Figure 4.2 (C). In control Hb value measured was found 9.8 ± 1.3 (g/dL) whereas Hb values observed were 9.7 ± 1, 7.5 ± 0.4 and 6.7 ± 1.2 (g/dL) for 0.2, 2 and 17 mg Cr/L respectively. This shows decreased Hb concentrations in exposed fish as compared to control

4.3.3.2. Exposure in lake water

Concentration of haemoglobin (g/dL) measured for control (8.9 ± 0.1) reduced to 7.7 ± 0.2, 7.0 ± 1.1 for 0.2 , 2 mg Cr/L, 7.4 ± 0.3, 7.1 ± 0.8 for 0.05, 0.1 mg Cd/L and 6.5 ± 1.1 for mixture of Cr-Cd (2 mg Cr/L + 0.1 mg Cd/L) with a significant difference ($p<0.05$; $p=0.0109$) among treatment groups as shown in Figure 4.3 (C).

Haemoglobin is a very important component of erythrocytes which plays important role in transport of the oxygen in blood (James and Sampath, 1995). In the present study decrease in total erythrocytes count and haemoglobin was observed which indicates anemic conditions. Gill and Epple, (1993) attributed anaemia to damage of erythropoiesis due to direct consequence of metals on hematopoietic centers (kidney and spleen) or enhanced erythroclasia as a result of altered membrane permeability. Other probable reasons of decreased haemoglobin may be increased mechanical fragility and defective iron (Fe) uptake in red blood cells. Decrease in haemoglobin is reported in previous studies such as treatment to a mixture of heavy metals (Cd + Pb + Cr + Ni) metals solution caused reduction in the values of RBC, Hb and haemotocrit in *Cyprinus carpio* (Vinodhini and Narayanan, 2009). Javed & Usmani, (2013) revealed that fish (*Channa punctatus*) when exposed to metal effluents containing heavy metals resulted in reduced RBC count and Hb content.

4.3.4. Haematocrit

4.3.4.1. Exposure in tap water

Haematocrit measured values expressed in (%) were statistically significant ($p<0.05$; $p=0.0461$) among treatment groups as shown in Figure 4.2 (D). Haematocrit measured values in control group was 16.3 ± 3.5 (%), whereas, it was gradually reduced to 13.1 ± 0.6 (%), 11.5 ± 0.6 (%) and 10.7 ± 1.5 (%) for 0.2, 2 and 17 mg Cr/L Cr respectively. This shows a significant low values for haematocrit level in Cr treatment groups as compared to control.

4.3.4.2. Exposure in lake water

The level of haematocrit (%) measured for control was 12.9 ± 0.5 which reduced to 10.9 ± 1.8, 10.2 ± 1.7, 9.6 ± 0.6, 9.4 ± 1.6 and 9.2 ± 0.7 for 0.2, 2 mg Cr/L, 0.05, 0.1 mg Cd/L and mixture, Cr-Cd (2mg Cr/L + 0.1 mg Cd/L) with a significant difference ($p<0.05$; $p=0.0185$) among treatment groups as shown in Figure 4.3 (D).

The decrease in haematocrit level may occur because low RBC's count usually leads to low haemoglobin and haematocrit levels. This indicates anemic conditions that could be due to the impaired haemopoietic tissue or inhibition of erythropoiesis and transferrin dysfunction (Javed et al., 2016).

4.3.5. Mean cell volume

4.3.5.1. Exposure in tap water

Mean corpuscular volume (MCV) expressed as femto liters (fL) were statistically significant ($p<0.05$; $p=0.0163$) among treatment groups as shown in Figure 4.2 (E). The MCV mean values measured were 171.6 ± 4.3 (fL), 178.6 ± 6.8 (fL), 179.7 ± 5.8 (fL) and 189.9 ± 2.9 (fL) for control, 0.2, 2 and 17 mg Cr/L respectively. This showed that MCV values increased in Cr treatment groups as compared to control.

4.3.5.2. Exposure in lake water

MCV showed a non-significant ($p=0.0715$) decrease among treatment groups. The values found was 168 ± 6.1, 155.2 ± 7.4, 152 ± 2.9, 156 ± 6.9, 153.8 ± 8.2 and 150.5 ± 6.1 for control, 0.2, 2 mg Cr/L, 0.05, 0.1 mg Cd/L and Cr-Cd (2mg Cr/L + 0.1 mg Cd/L) respectively as shown in Figure 4.3 (E). This showed that MCV values decreased in Cr treatment groups as compared to control.

MCV and MCHC are important erythrocytes constant indices which indicate the size and haemoglobin content of erythrocytes. These blood indices reflects the morphological anemia types such as Normocyte, Macrocyte or Microcyte anaemia. The exposure of Cr in the first experiment showed increase in the MCV coupled with the decrease in MCHC. Our study findings are in accordance with Shalaby, (2001) who also described significant increase in MCV followed by decrease in MCH and MCHC values in fish, *Oreochromis niloticus* when exposed to mercury. Increase in MCV and WBC is attributed to macrocytic anemic condition (Praveena et al., 2013; Afaq and Rana, 2009). The increase in MCV concentrations in fish may be due to the swelling of erythrocytes as a result of macrocytic anemia or hypoxic conditions (osmotic stress) exposed to metal pollution reported previously by Praveena et al. (2013). Contrary to the tap water experiment, the lake water experiment revealed the decrease in MCV. Our study finding corroborates with Al-Attar, (2005) who reported the decrease in MCV in the *Oreochromis niloticus* treated with sublethal concentrations of Cd. The decrease in the MCV indicates microcytic anemic condition due to release of immature erythrocytes from the haemopoetic tissue in order to compensate for the loss of erythrocytes.

4.3.6. Mean corpusular haemoglobin concentration

4.3.6.1. Exposure in tap water

Mean corpuscular haemoglobin concentration (MCHC) measured in grams per deci liter (g/dL) showed significant difference ($p<0.001$; $p<0.0001$) among treatment groups as shown in Figure 4.2 (F). MCHC decreased with the increasing Cr concentrations. MCHC values found to be 74.5 ± 2.6 (g/dL), 68.4 ± 2.9 (g/dL) and 42.5 ± 2.6 (g/dL) for 0.2, 2 and 17 mg Cr/L respectively. MCHC values showed gradual decrease with the reduction of MCHC values as compared to control MCHC values, 76.8 ± 3.5 (g/dL).

4.3.6.2. Exposure in lake water

MCHC showed the decreasing trend with the significant difference ($p<0.05$; $p=0.0429$) in the measured mean values such as 68.9 ± 4.3, 66.8 ± 5.4 (g/dL) for 0.2 and 2 mg Cr/L. Similarly, MCHC mean values found to be 68 ± 1.3, 65 ± 6.7 and 63 ± 6.7 (g/dL) for 0.05 mg Cd/L, 0.1 mg Cd/L and Cr-Cd (2 mg Cr/L + 0.1 mg Cd/L) when compared with control MCHC value i.e. 77.8 ± 5 as shown in Figure 4.3 (F).

MCHC expresses the concentration of haemoglobin per unit volume of red blood cells. The decreasing trend of MCHC is in agreement with the findings of Praveena et al. (2013). This decrease in MCHC in the present study may indicate that the concentration of hemoglobin in RBC may be reduced due to decrease in haemoglobin synthesis (Javed et al., 2016). Contrary to our study findings Madhavan and Elumalai, (2016) noticed increased MCH and MCHC in *Clarias batrachus* after exposure to sub lethal concentration of chromium for 28 days which could be due to a compensatory erythropoiesis

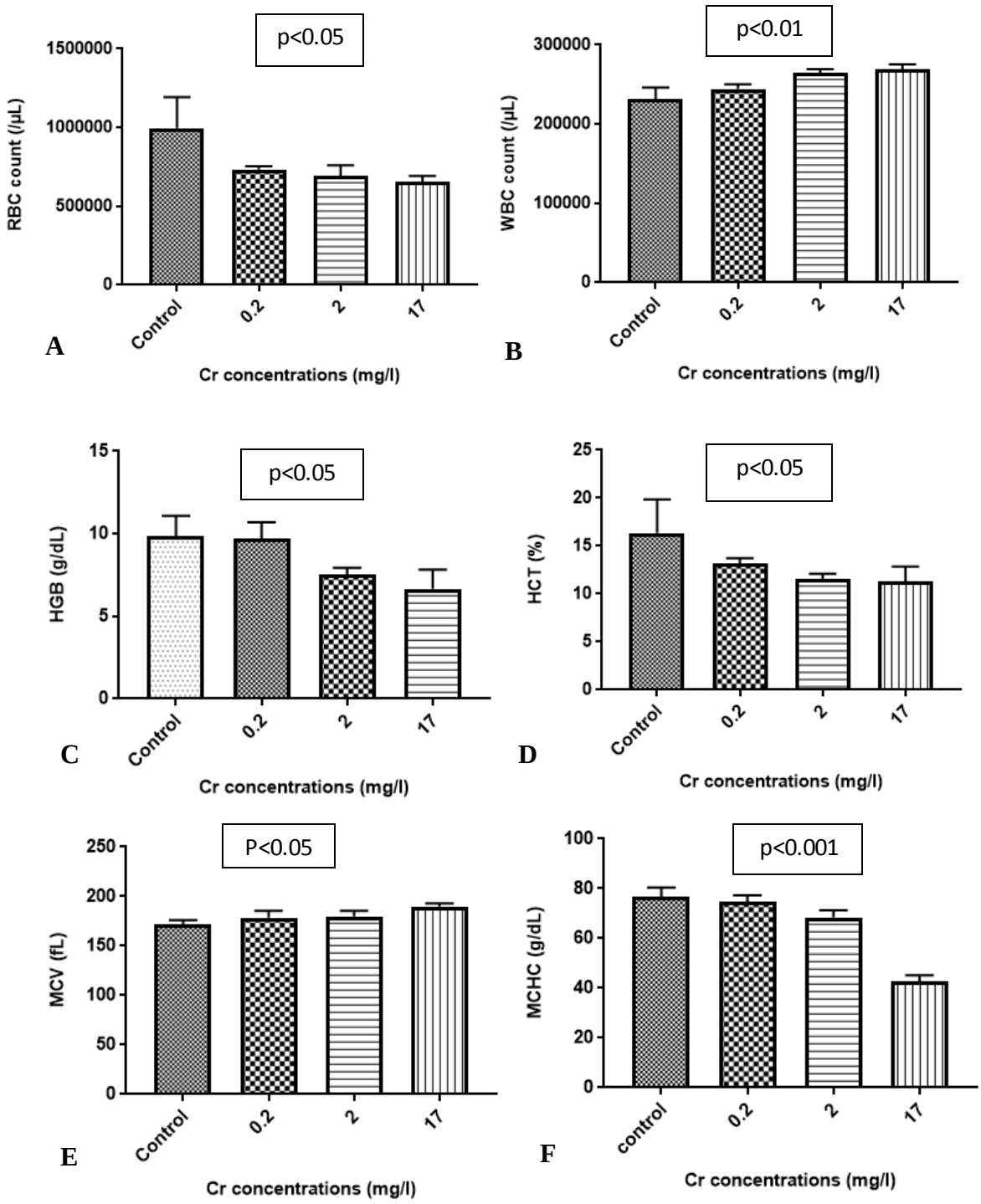
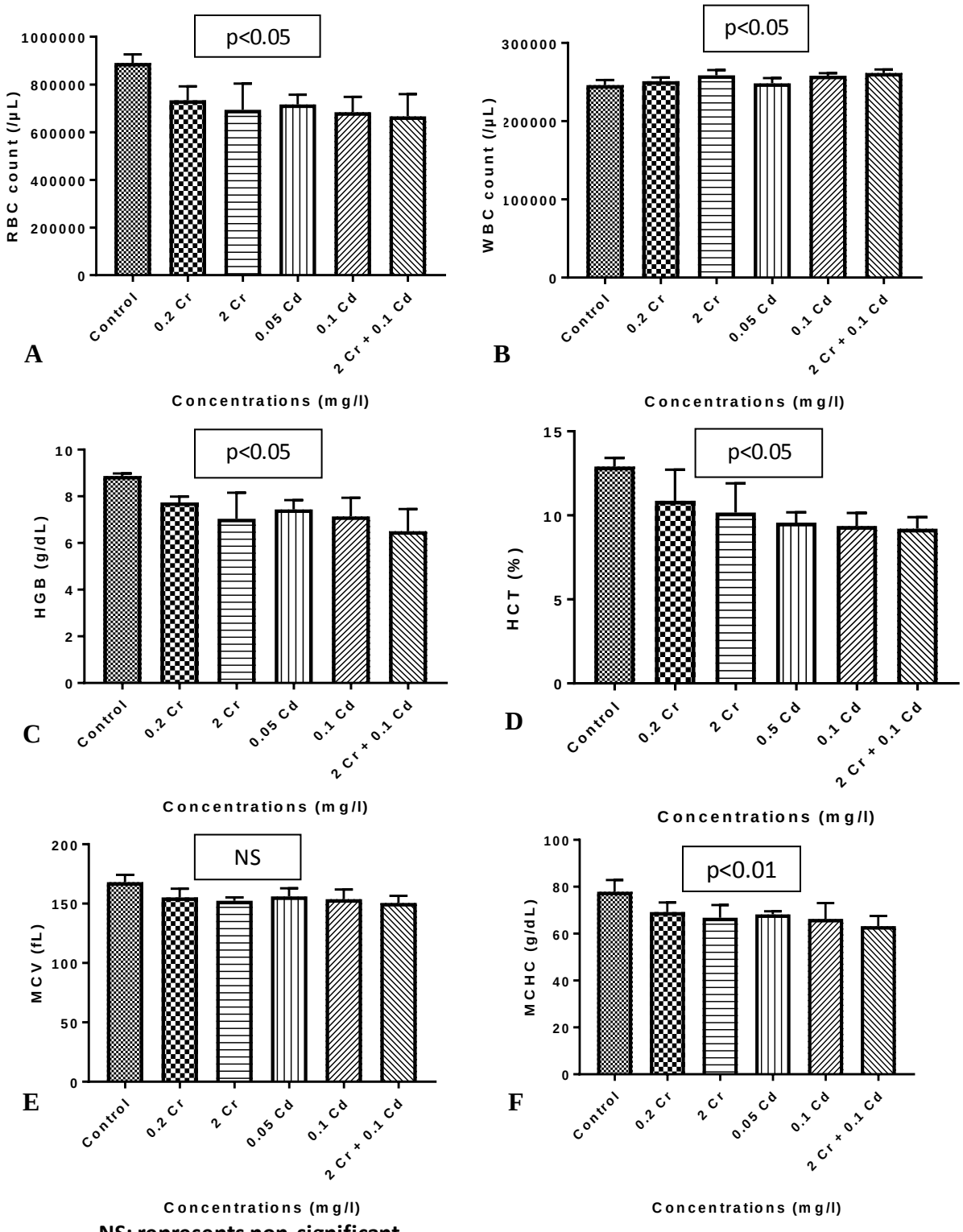


Figure 4.2: Changes in blood parameters exposed to Cr concentrations

(A) RBC Count (B) WBC count (C) Hb (D) HCT (E) MCV (F) MCHC



NS: represents non-significant

4.3: Changes in blood parameters exposed to Cr, Cd and mixture of Cr-Cd

(A) RBC Count (B) WBC count (C) Hb (D) HCT (E) MCV (F) MCHC

4.4. Micronucleus (MN) and nuclear abnormalities assay

MN test is the most widely used test to detect genotoxicity including clastogenic and aneugenic effects of the toxicants (Cavas et al., 2005) In the present study induction of micronuclei and erythrocytes cellular and nuclear abnormalities both were used to evaluate the genotoxic potential of Cr and Cd. Various nuclear and cellular abnormalities were observed in *Cyprinis carpio* erythrocytes including the formation of micronuclei, binuclei, notched nuclei, blebbed nuclei, swollen cells and deformed cells and apoptotic cells as shown in the Figure (4.4).

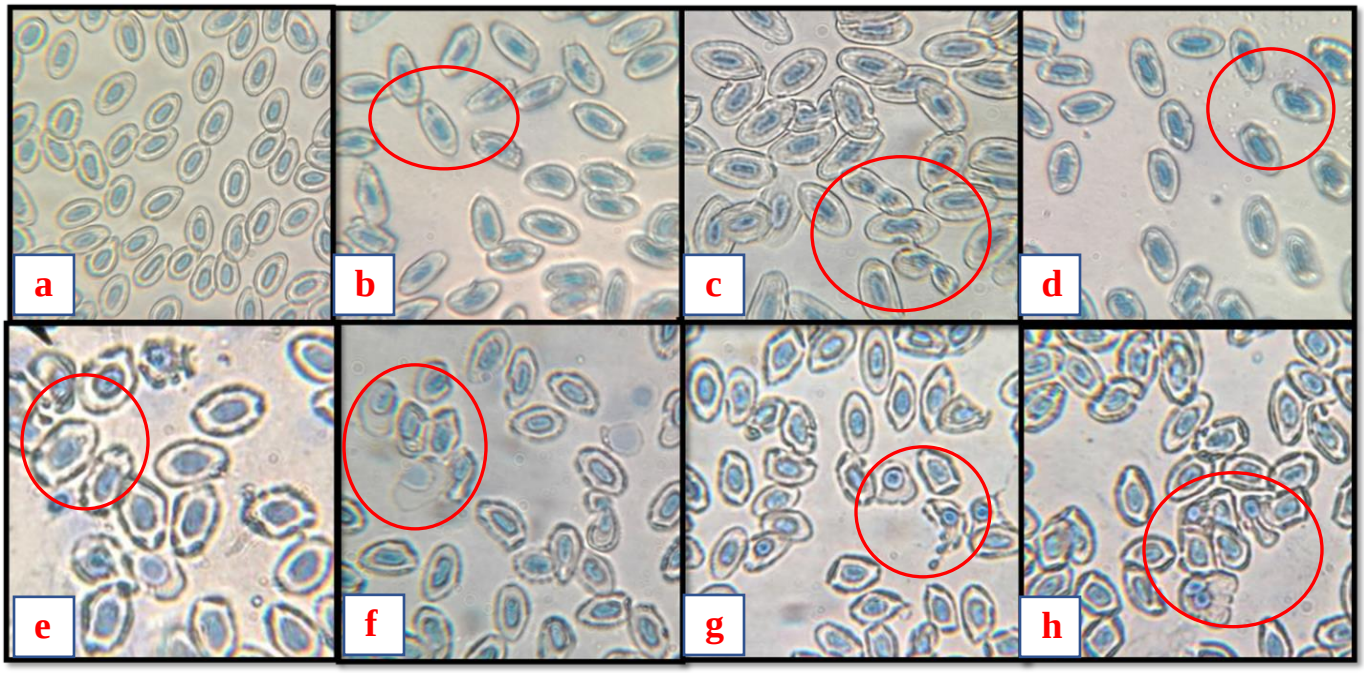


Figure 4.4: Illustration of the induction of abnormalities in erythrocytes of Common carp exposed to Cr and Cd

(a) Normal erythrocytes (b) Micronuclei (c) Binuclei (d) Notched nuclei (e) Blebbed nuclei (f) Swollen Cell (g) Apoptotic cell (h) Deformed cells

Micronucleus formation is assume to be produce as a result of disrepair of DNA double-strand breaks resulting to symmetrical and asymmetrical chromatid and chromosome fragments that fail to be included in the daughter nuclei at the completion of telophase during mitosis because of spindle attachment during the segregation process in anaphase (Fenech et al., 2003). The number of micronuclei is a highly variable feature and different authors report different numbers of micronuclei for the same species in fish cells but the majority of authors report a very low MN frequency. The low MN frequency in fish erythrocytes represents the main limitation of the MN test used, demanding the scoring of a large number of cells from the test organism (Ferraro et al., 2004; Bolognesi et al., 2006). In another study, MN assay undertaken by using *H. malabaricus* showed the absence of MNs, and only nuclear morphological alterations were found (Ramsdorf et al., 2009) therefore, morphological nuclear alterations is another alternative method to detect genotoxicity (Ayllon and Garcia-Vazquez, 2000; Caressco et al., 1990). It has been recommended that in genotoxicity studies using the Piscine micronucleus test on fish as a model organism, micronuclei number and morphological changes in nuclei both should be considered (Ayllon and Garcia-Vazquez, 2000; Caressco et al., 1990).

Types of Nuclear abnormalities such as nuclear buds, blebbed nuclei, 8-shaped nuclei, vacuolated nuclei and bi-nucleated cell and fragmented-apoptotic has been considered in order to evaluate cytotoxicity and genotoxicity in aquatic organisms (Barsiene et al., 2004). The formation of MN, notched nuclei and blebbed nuclei is due to inability of organisms to eliminate damaged nuclei, failed DNA replication or improperly condensed chromatin, chromosome fragments deprived of telomeres and centromeres from the nucleus due to clastogenic action of the toxicant. Bi-nucleated cells are formed in abnormal cell division due to blocking of cytokinesis (Cavas and Ergene-Gozukara, 2005). Morphologically altered erythrocyte is taken as an index of cytotoxicity. The swelled blood cells were recorded as signs of necrosis (Ateeq et al., 2002).

The results of erythrocytes nuclear and cellular abnormalities test for two experiments obtained by analyzing the erythrocytes of *Cyprinus carpio* exposed to Cr and Cd concentrations are shown in the Figure (4.5) and Figure (4.6). The erythrocytes containing MN and other nuclear and cellular abnormalities demonstrated the cytogenicity and genotoxicity of Cr and Cd. Mean percentage in erythrocytes abnormalities increased with the increased exposure

concentration. Total erythrocytes abnormalities increased steadily from 5.6, 16, 25.6 and 66% for control, 0.2, 2 and 17 mg/L Cr. Similarly, in lake water experiment total erythrocytes abnormalities percentage increased such as 8.6% for control, 24.6%, 26.3% for 0.2 and 2 mg/l Cr, 19.3%, 23.7% for 0.05 and 0.1 mg/L Cd. 51.6 % for mixture.

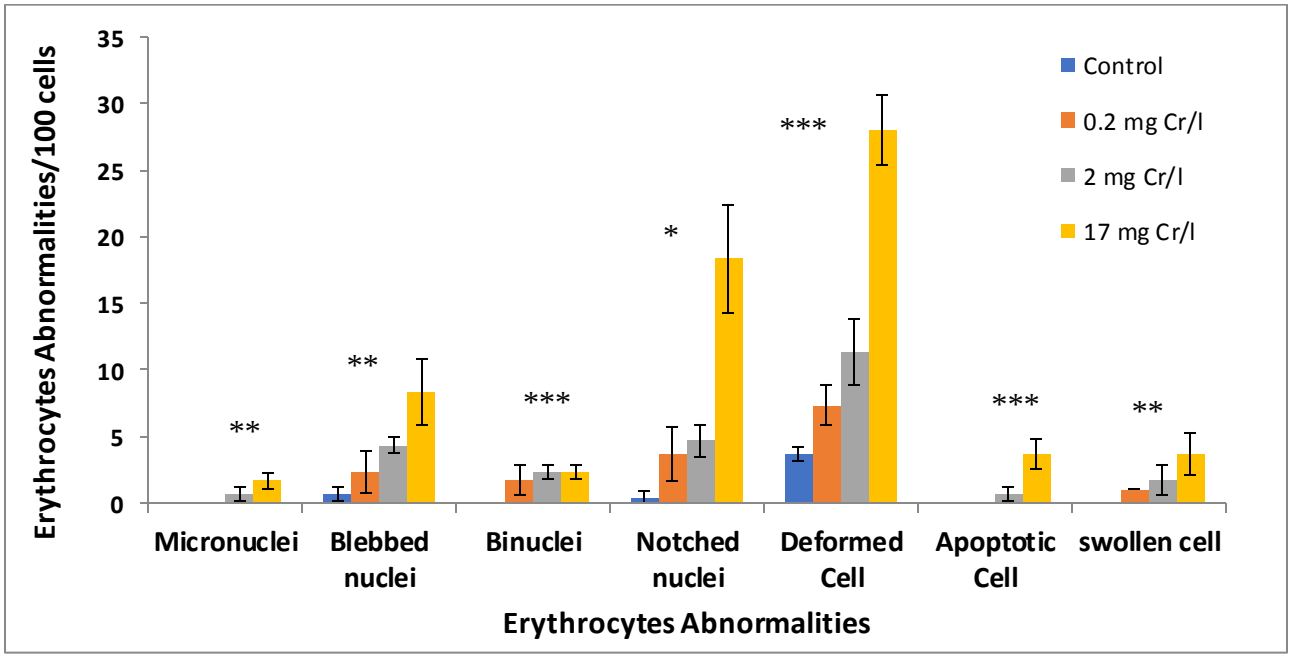


Figure 4.5: Frequency of erythrocytes abnormalities exposed to Cr concentrations (mg/L) Significance level (* = p< 0.05, ** = p< 0.01, *** = p< 0.001).

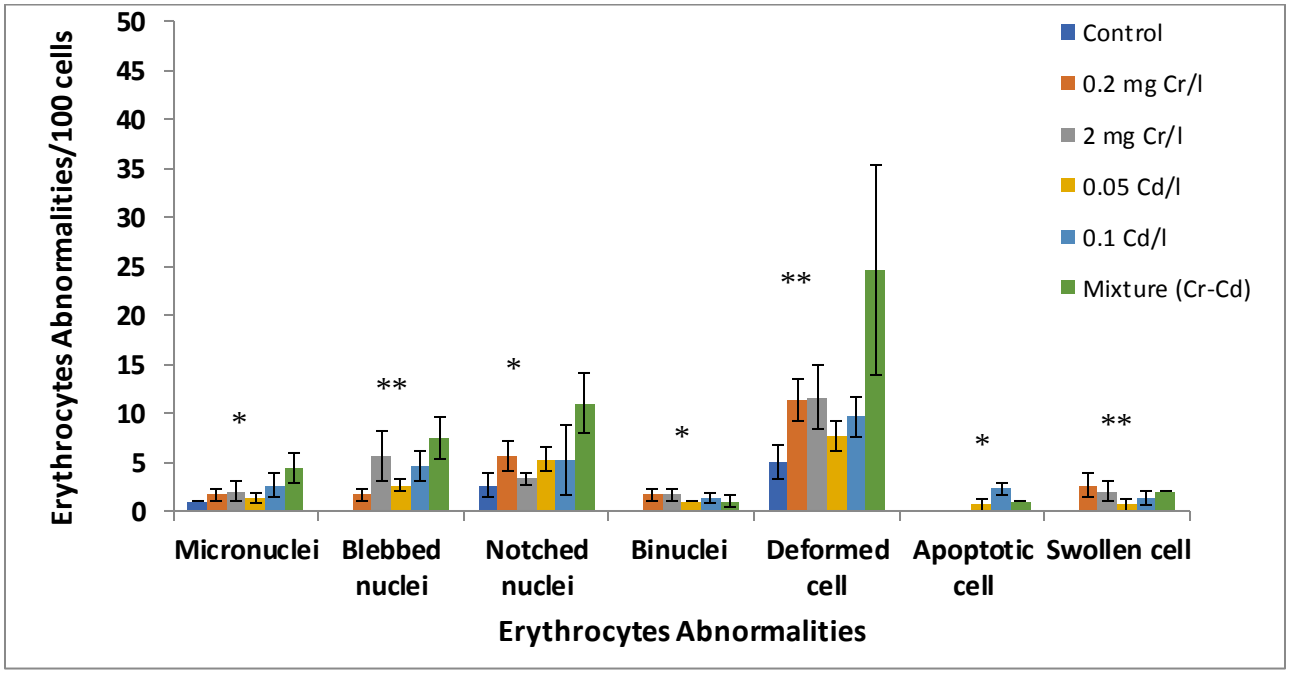


Figure 4.6: Frequency of erythrocytes abnormalities exposed to Cr, Cd and mixture of Cr-Cd concentrations; Significance level (* = p< 0.05, ** = p< 0.01, *** = p< 0.001)

Several researchers stated that genotoxic potential of Cd and Cr has been attributed to oxidative stress and ultimately the production of ROS. Increase ROS production induce lipid peroxidation by acting on plasma membrane or directly cause DNA damage and damage to the nuclear bases consequently causing DNA strand breaks. (Cavas et al., 2005; Kumar et al., 2012; Ahmad et al., 2006). Genotoxic effect of Cd and Cr analysed by the induction of nuclear abnormalities including micronuclei and cellular abnormalities are consistent with the previous findings of Jindal & Verma, (2015) who showed that frequencies of nuclear and cellular abnormalities in *Labeo rohita* increased significantly as the concentrations of Cd increased concluding that Cd is genotoxic and cytotoxic even at low concentrations (0.37 and 0.62 mg/L of $CdCl_2$).

4.5. Heavy metals uptake in gills

Gills are important organs for respiratory and osmoregulatory functions therefore, cellular impairment caused by the metals might damage the respiratory function of the fish by decreasing the respiratory surface area (Vutukuru, 2005).

4.5.1. Cr uptake in gills in tap water

The uptake of Cr (µg/g wet weight) in gills tissues of Common carp was observed after the end of the exposure period at all experimental concentrations as shown in Figure (4.7). The present results showed that accumulation in gills were 2.8 ± 0.8, 5.8 ± 0.6, 14.5 ± 17.2 and 47.1 ± 14.6 µg/g wet weight for the control, 0.2, 2 and 17 mg/L Cr concentrations respectively. Accumulation in gills showed a statistically significant difference ($p<0.001$; $p=0.0006$) among treatment groups. The concentration of Cr in gills tissues increased as the concentration of Cr doses in exposure water was increased. Similar findings were observed by Mallesh et al. (2015) when *Cirrhinus mrigala* was exposed to sublethal concentrations of Cr.

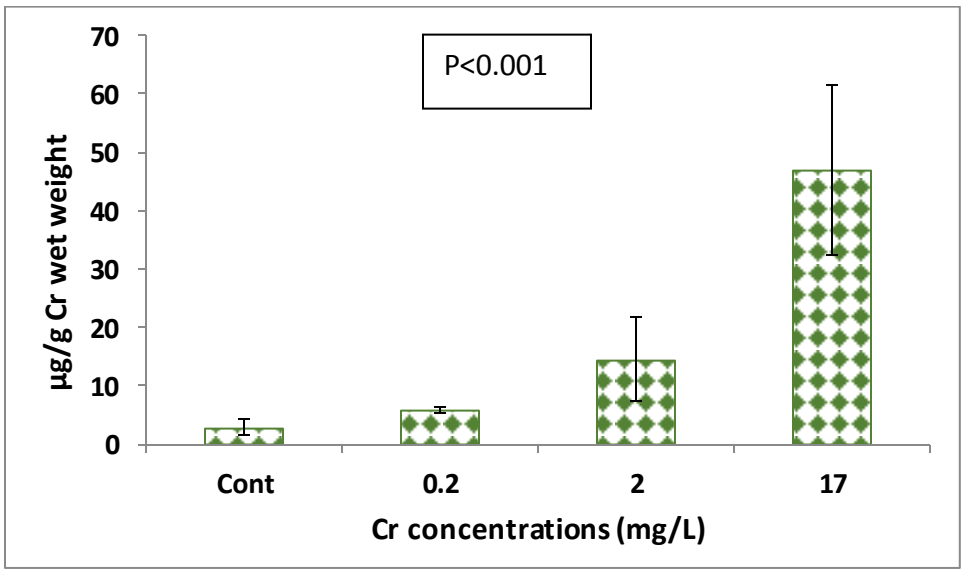


Figure 4.7: Concentration of Cr (µg/g wet weight) in gills tissues
Values are given as mean ± SD (n=3).

4.5.2. Cr uptake in gills exposed to Cr and mixture (Cr-Cd) in lake water

In the lake water experiment, Cr accumulation increased significantly (p=0.001) in the gills of the fish at test concentrations singly (0.2, 2 mg Cr/L) and in combination (mixture of Cr and Cd; 2+ 0.1 mg/L Cr-Cd). The mean values were 56.4 ± 2.6 µg/g, 68 ± 3.3 µg/g for 0.2 mg Cr/L, 2 mg Cr/L whereas, 114 ± 4.2 µg/g for mixture (2+ 0.1 mg/L Cr-Cd) as compared to control means value (16 ± 2.6 µg/g). The results clearly indicated that when fishes were exposed to binary mixture of Cr and Cd concentrations, Cr accumulation was increased in mixture compared to singly exposed concentrations. It was found that accumulation of Cr in gills tissues increased in the presence of Cd at the highest concentrations of the Cr + Cd mixture. Moreover, the synergistic effect of Cr in the presence of Cd was observed (Figure 4.8).

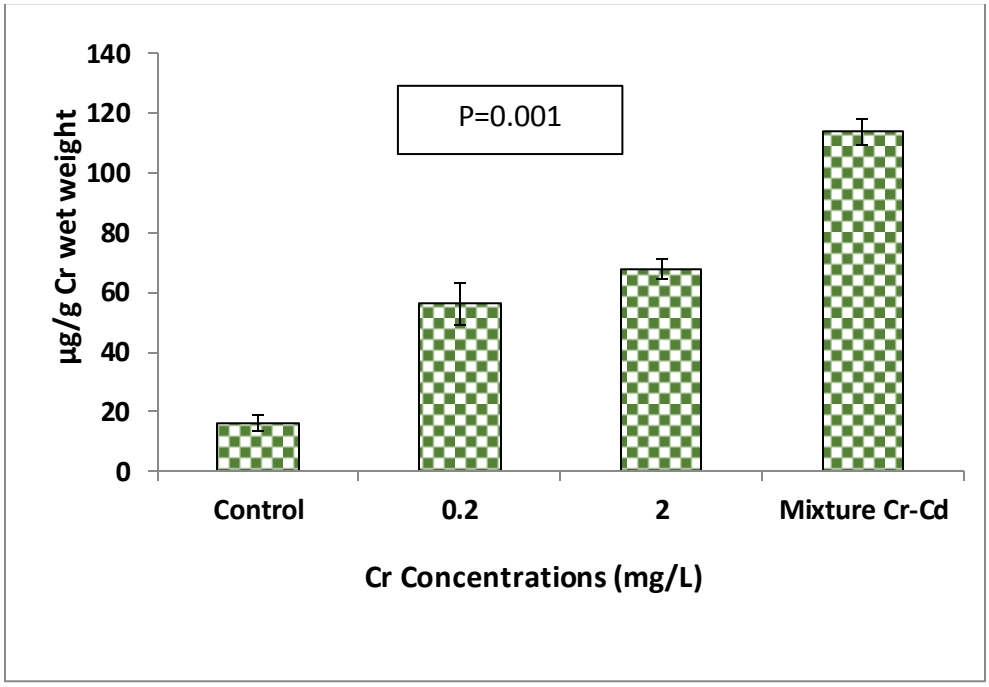


Figure 4.8: Concentration of Cr (µg/g wet weight) in gills exposed to Cr concentrations and mixture of Cr + Cd. Values are mean ± SD (n=3).

The present results indicate that the uptake of Cr in the gills tissue was increased as the Cr concentrations in water was increased. Our study findings corroborates with Rajeshkumar et

al. (2017) who studied the effects of exposure to Cr, Cd and Lead (Pb) mixtures using environmentally relevant concentrations and found significantly increased accumulation in fish tissues (gills, liver, kidney, muscle and intestine) of common carp as compared with control group. In another study, Palaniappan and Karthikeyan, (2009) demonstrated Cr and Nickel (Ni) bioaccumulation in *Cirrhinus mrigala* tissues separately and in mixture of Cr and Ni which indicated that Cr–Ni binary mixture at sublethal concentrations of 2.9 mg metal/L for each metal induced a 10–28% higher Ni accumulation in fish tissues. As a result, 7–12% increase in Cr accumulation in the presence of Ni was observed that revealed metal accumulation in binary mixtures of Cr and Ni was considerably higher than those of the single metals, indicating synergistic effect.

4.5.3. Cd uptake in gills exposed to Cd and mixture (Cr-Cd) in lake water

Exposure to Cd concentrations singly at a concentration of 0.05 and 0.1 mg/L Cd has resulted in increased accumulation of Cd in gills. The accumulation increased significantly ($p<0.05$; $p=0.031$) as the Cd concentrations increased. The mean value observed for Cd uptake were 15.3 ± 2.7 µg/g, 17.3 ± 1.8 µg/g for 0.05 mg Cd/L and 0.1 mg Cd/L compared to control value (7.5 ± 2.1 µg/g). However, when fishes were exposed in combination (mixture of Cr-Cd; 2 + 0.1 mg/L Cr-Cd) the accumulation of Cd in gills decreased significantly ($p<0.05$; $p=0.031$). The uptake of Cd (16.1 ± 2.5 µg/g) in mixture showed the antagonistic effect of Cr on Cd accumulation (Figure 4.9).

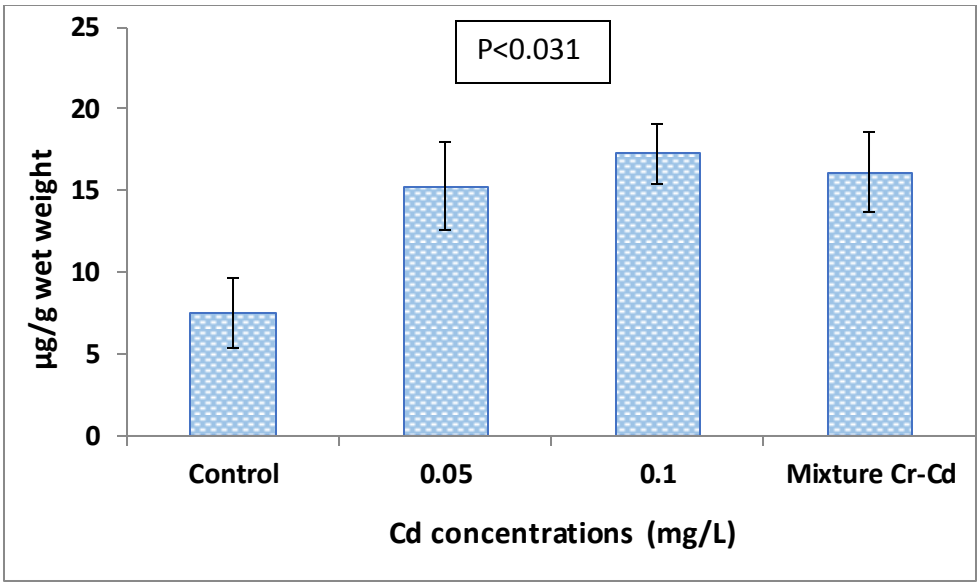


Figure 4.9: Concentration of Cd (µg/g wet weight) in gills exposed to Cd concentrations and mixture of Cr + Cd. Values are mean ± SD (n=3).

Decreased uptake of Cd by Cr observed in this experiment may consistent with the findings observed by Niyogi et al. (2015) that aqueous exposure to Zinc (Zn) decreased Cd binding at gills in freshwater, rainbow trout (*Oncorhynchus mykiss*) when exposed to binary mixture (Cd and Zn). The possible reason behind decrease is that the pool of Cd binding sites in the rainbow trout gills is much smaller than the pool of Zn binding sites. Inaddition, both Cd and Zn in mixture showed inhibitory effect on branchial Ca^{2+} uptake which was significantly greater than that of Cd or Zn singly. This probable reason is that the Cd and Zn are Ca^{2+} analogs, which are believed to compete with Ca^{2+} for uptake via apical voltage independent Ca^{2+} channels on the gills epithelium. Another study reported that Cd and Pb uptake is affected by the presences of other metals. The Cd uptake rate was suppressed by Zn, Cu, and Pb whereas, enhanced by Ni (Komjarova and Blust, 2009). Heavy metals accumulation in fish via direct route is a highly complex process, which depends on many external and internal biotic and abiotic factors. Metal interactions depend mainly on a fish species, stage of development, gender, etc. Sauliute and Svecevicius, (2015) stated that interactions among metals usually exhibit both competitive and cooperative models depending on the chemical origin of the metal, the tissue/organ analyzed, relative exposure concentrations and duration of exposure to metals. Our study findings are in agreement with the Lopa et al. (2010) who studied the accumulation of very low concentrations

of Pb (0.0001 mg/L) singly and in a binary mixture of Pb and Cr (0.0001 mg Pb/L + 0.00015 mg Cr/L) in *Labeo rohita* tissues (gills, liver, kidneys, muscles and skin). It was observed that exposure of Pb in combinations with Cr for 60 days resulted in significant decrease accumulation in all fish tissues compared to singly Pb exposed fish.

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CONCLUSION AND RECOMMENDATIONS

I. Substantial amount of Cr (0.2 - 2mg/L) and Cd (0.05 - 0.1 mg/L) was detected in road dust that can be mobilized via road runoff into surface water representing risks to fresh water fish.

II. The haematological parameters results of Common carp revealed that exposure of Cr resulted in significant ($p<0.05$) decrease in red blood cell count, haemoglobin, haematocrit content and significant ($p<0.001$) decrease in MCHC was observed, however significant ($p<0.01$) increase in white blood cell count and significant ($p<0.05$) increase in MCV was observed among treatment groups. Exposure of Cr, Cd and mixture of Cr-Cd resulted in significant ($p<0.05$) decrease in red blood cell count, haemoglobin and haematocrit content and significant ($p>0.05$) decrease in mean corpuscular volume (MCV) and significant decrease ($p<0.01$) in MCHC was observed whereas, significant increase in white blood cells count (WBC) was found. Increase and decrease in haematological indices revealed that Common carp exposed to heavy metals was under stress.

III. Nuclear and cellular abnormalities in erythrocytes of Common carp revealed the genotoxicity of Cr and Cd to common carp even at the lowest concentrations (0.2 mg Cr/L and 0.05 mg Cd/L). Frequency of erythrocytes abnormalities for Cr treatment increased in the concentration dependent manner from 5.6, 16, 25.6 and 66% for control, 0.2, 2 and 17 mg/L Cr. Exposure of Cr, Cd and mixture of Cr-Cd showed frequency of erythrocytes abnormalities that increased steadily from 8.6% in control to 24.6 and 26.3% for 0.2 and 2 mg Cr/L. 19.3 and 27.3 % for 0.05 Cd and 0.1mg Cd/L and 51.6% for mixture of Cr-Cd.

IV. The uptake of Cr increased significantly ($p<0.001$) in gills exposed to Cr concentrations singly (0.2, 2 and 17 mg Cr/L) and significantly ($p<0.01$) increased in gills when exposed to mixture (2 + 0.1 mg/L Cr-Cd) whereas, exposure to Cd singly at concentrations (0.05, 0.1 mg Cd/L) resulted increase ($p<0.05$) Cd uptake in gills however, Cd uptake in gills

decreased significantly ($p<0.05$) when exposed to mixture (2 + 0.1 mg/L Cr-Cd). Binary mixture of Cr-Cd increased the accumulation of Cr in gills, however, it decreased the Cd accumulation in gills.

Followings recommendations are drawn for future studies

I. More research is needed in evaluating potential threats regarding traffic derived contaminants such as poly aromatic hydrocarbons (PAH) on health of fish.
II. Alternative biological endpoints incorporating biomarkers such as antioxidant cellular defense mechanism and histopathological studies are required.
III. Mitigation measures to prevent road runoff into fresh water are needed.

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