

**ANALYSIS OF HEAVY METALS IN THE KIDNEY,
MUSCLE TISSUE AND BLOOD OF INDIAN BULLFROG
SPECIES (*Hoplobatrachus tigerinus*) COLLECTED FROM
SIALKOT AND GUJRANWALA DISTRICTS**



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IN
ZOOLOGY**

By

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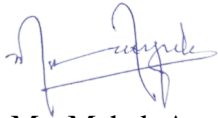
**DEPARTMENT OF ZOOLOGY
KINNAIRD COLLEGE FOR WOMEN, LAHORE
2018-2022**

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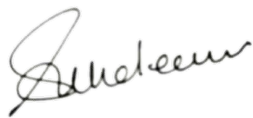


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ABSTRACT

Amphibians include frogs, salamanders, and caecilians, which are vertebrate tetrapods. Frogs are known as "indicator species" because they are the first to be affected by environmental degradation. They are particularly sensitive to environmental changes and can provide scientists with significant information on how an ecosystem functions. The research was focused on analysis of heavy metals in the kidney, muscle tissue and blood in Indian Bullfrog (*Hoplobatrachus tigerinus*) specie collected from the selected areas of Gujranwala and Sialkot Districts. Their significance in regard to the role they play as pollution indicators was observed. Samples from both study areas were collected by using sweep nets. Frogs were dissected humanely and organ specimens were preserved in 10% buffered Formalin Solution. Blood samples were collected by cardiac puncture and acid digested with 1HNO_3 : 0.3HClO_4 on hot plate. For heavy metal detection in the samples of kidney and muscle tissues of frogs were burnt to ashes in the muffle furnace at 600°C (1hour for kidney and 2hours for muscle tissue) respectively and then acid digested with 1HNO_3 : $1\text{H}_2\text{SO}_4$. Atomic Absorption Spectrophotometer was used to analyze the digested samples after filtering them to remove any solid particles. The results obtained were compared with standard stock solution values. The analyzed values were then matched with the standard permissible limits recommended by WHO. Experimental results further showed that all the studied metals Cadmium, Chromium, Nickel, Copper, Cobalt, Manganese, and Zinc were present within the permissible limits. The values of heavy metals [Nickel, Zinc, Manganese and Copper] were negligible in muscle tissue, kidney and blood samples and the concentrations of cadmium, chromium and cobalt were notable but within the WHO permissible limits. It indicates that quality of land is better and water quality is above average in both of the study areas. Though some concentrations of heavy metals are naturally present in the groundwater and the tap water sources, the prevalence of these heavy metals could be due to the long-term overuse of ground water as a source of irrigation therefore, the industrial effluent waste and sewage must have leached into

the soil and contaminated the groundwater in the nearby fields from where frogs were collected. This research would be helpful in providing baseline information on pollution status as well as defining the heavy metals' point sources in the study sites.

LIST OF ABBREVIATIONS

AAS	Atomic Absorption Spectroscopy
Cd	Cadmium
Co	Cobalt
Cr	Chromium
Cu	Copper
g	Gram
GDP	Gross Domestic Product
Hb	Haemoglobin
Hg	Mercury
Ht	Haematocrit
kg	Kilogram
Pb	Lead
ml	Milliliter
Mn	Manganese
MoM	Metal-on-Metal
Ni	Nickel

PIXE

Particle-Induced X-ray Emission

WHO

World Health Organization

Zn

Zinc

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CHAPTER 1

INTRODUCTION

Vertebrates are animals with a backbone, a notochord, a dorsal hollow nerve cord and a post anal tail. Fish, amphibians, reptiles, birds, and mammals are the primary groups under the subphylum vertebrate. These have well-developed organs and organ systems adapted to their lifestyles. Vertebrae and vertebral column are the characteristic features of vertebrates, which in ancient species were not present but notochord was there in their place [1]. Central nervous system, digestive system, excretory system and heart are well-developed and functioning in vertebrates [2]. Vertebrates have evolved themselves to sustain in a variety of environments, ranging from below the ground, terrestrial, aerial or aquatic. They hold prominent place in the food chains and food webs either as feeders or the food. [1].

Amphibians including frogs, salamanders, and caecilians, are vertebrate tetrapods with four limbs. Because most frogs and toads spend part of their lives in water and part on land, alterations in either environment could have an impact. Toads and frogs consume water by absorbing it via their skin. As a result, they are more likely to absorb hazardous substances or microbes through their skin. Frog and toad eggs usually float in a jelly-like mass at the water's surface [3]. Amphibians' complicated life cycles also aid in the transfer of pollutants from the aquatic to the terrestrial food web. Amphibians are known for their ravenous appetites and great mass conversion efficiency favors contaminant bioaccumulation at a faster rate [4]. Frogs are important indicator species of amphibians [5].

The Indian bullfrog, *Hoplobatrachus tigerinus* primarily a freshwater aquatic species that is mostly solitary and nocturnal. Invertebrates, small mammals and birds make up its diet [5]. Their ecological linkages with the area's biota reveal that amphibians are more susceptible to pollution because their skin is more permeable than other groups. This is why toxins can invade their critical organs and can be detected in species' tissues and blood. The presence of more amphibian

species in an environment indicates that the pollution level of that area is low. So, if healthy amphibian specimens are collected from an area, it indicates that the pollution index of that area is low and vice versa [6].

Any toxic metal, regardless of atomic mass or density, can be referred to as a heavy metal [7]. Heavy metals are naturally occurring elements in the earth's crust, but human activity has drastically disrupted their geochemical cycles and biological balance. Heavy metals such as Cadmium, Copper, Lead, Nickel, and Zinc can be hazardous to human health if exposed for an extended period of time [8]. The significant ability of heavy metals is a source of concern as they accumulate in biological systems where they cause severe toxicity, leading in a variety of negative consequences, including immunosuppression, induction of stress proteins, oxidative stress, histopathological damage, disturbance in reproductive potential and seizures and mortality/acute toxicity [9].

Cadmium (Cd) is a heavy metal that is recognized to be a ubiquitous environmental hazard as well as a possible poison that might harm human health. Cadmium exposure occurs when contaminated food (e.g., crustaceans, organ meats, and green vegetables, rice from specific places or water) is consumed. Drug and dietary supplement contamination may potentially be a source of contamination. Cadmium toxicity was initially reported in the nineteenth century by workers in zinc smelts. Cd is a pollutant found in zinc and lead-containing ores, and chronic Cd toxicity is most common among industrial workers or in people who have been exposed to Cd who live in strongly contaminated areas [10]. Cadmium is released in increasing amounts as a result of different natural and manmade activities into the environment [6].

Chromium in its hexavalent form, the most hazardous type of chromium, is widely employed in certain industries such as leather manufacturing. Chromium's toxicity originates from its corrosive nature and proclivity to produce allergic reactions. Chromium is a carcinogen, especially to the lungs when inhaled. Heavy metal ion-induced cell toxicity is due to oxidative and nitrosative stress, which is defined as

an excess of oxidants over antioxidants [11]. Chronic human inhalation or oral exposure to high amounts of hexavalent chromium may have consequences on the liver, kidney, gastrointestinal, and immunological systems, as well as the blood. Contact dermatitis, sensitivity, and skin ulceration may result from dermal exposure to hexavalent chromium.

Nickel is a metal that is widely distributed in the environment: it is an essential ingredient of about 100 minerals, all of which have a wide range of industrial and commercial uses. Nickel (Ni) is the 24th most common element in the Earth's crust, accounting for about 3% of the planet's composition. Nickel and nickel compounds are classic toxic agents found in industry, but they're also known to cause harm to people who aren't working with them. Nickel can be present in the air, water, and food, among other places. When it comes to occupational nickel exposure and health hazards, inhalation is a prevalent method [12]. Acute exposure to Nickel has been linked to liver, kidney, spleen, brain, and tissue damage in humans, as well as vesicular eczema, lung, and nasal cancer. [13].

Cobalt (Co) and its derivatives are found in abundance in nature and are used in a wide range of human activities. Oral Co supplementation and internal exposure through metal-on-metal (MoM) hip implants provide the highest systemic Co concentrations [18]. Effects of excess cobalt include skin and respiratory problems. Wheezing, asthma, respiratory irritation, decreased lung function, pneumonia, fibrosis, cardiac consequences, liver and kidney congestion, conjunctivitis, cardiomyopathy. Other health risks include nausea and vomiting, diarrhoea, allergic dermatitis and liver disease [19].

Copper is both a necessary mineral for human health and a potentially toxic substance, depending on the amount consumed. Copper has been associated to changes in bone health, immunological function and infection risk, as well as cardiovascular risk and cholesterol metabolism. Its metabolism is intricately connected to that of other micro minerals, and a lack of it has been demonstrated to affect iron mobilization, leading to secondary iron insufficiency. Because of a lack

of understanding of the limitations, the consequences of moderate excess copper exposure, and the indications to detect early adverse effects, determining the copper dose and administration regimen for safe human consumption is difficult [17].

Manganese in your water can stain your clothes, cause plumbing to scale, and stink, look, and taste bad. Drinking water with a manganese level above the Michigan Department of Health's recommendation limit is potentially detrimental to your health, however bathing or showering in it is not. While manganese is safe in adequate doses, persons with iron deficiency anaemia, liver or renal problems, or who inhale the mineral should exercise caution. Manganism, a sickness comparable to Parkinson's disease, is caused by excessive Mn exposure, which occurs most commonly in the workplace. The early stage, often known as "manganese mania," has a negative impact on mental health, causing irritation, apathy, aggression, hallucinations, and psychosis [20].

In 1961, zinc was detected in an Iranian guy. It is more common in places with high cereal consumption and low animal food intake [14]. Zinc toxicity has been linked to the same symptoms as lead poisoning, making it easy to confuse the two. Zinc is generally considered to be safe, especially when used orally. Excessive amounts, on the other hand, can disrupt the system, preventing development and reproduction. Symptoms of zinc toxicosis include vomiting, diarrhoea, bloody urine, icterus (yellow mucus membrane), liver failure, renal failure, and anaemia [15].

Frogs are known as "indicator species" because they are the first to be harmed by environmental degradation. They are especially sensitive to environmental changes and can give scientists a lot of information about how an ecosystem works. Adult frogs are more sensitive than frog eggs and tadpoles. Chemical contamination is particularly harmful to frogs, because they breathe and absorb water through their skin [23]. Heavy metals are naturally occurring, they are widely disseminated in the environment due to their numerous industrial, residential, agricultural, medical, and technical applications, raising concerns about their potential consequences on

human health and the environment. [24]. Atomic spectrometry equipment are the most commonly used tools for trace metal analysis.

1.1 Study Area

The study areas comprise of Sialkot and Gujranwala Districts, Punjab Pakistan.

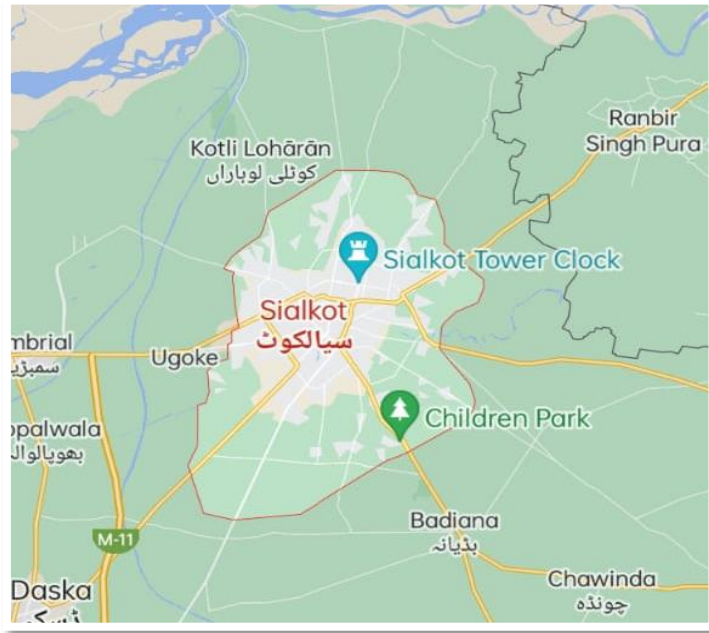


Figure 1.1: Map of Sialkot, Punjab, Pakistan.

Sialkot:

In Pakistan's Punjab province, Sialkot is a big metropolis. It's close to the famed Chenab River and near the Kashmir Mountains. It is the country's cultural and historic hub. Sialkot is 32.497223 degrees North latitude and 74.536110 degrees East longitude. Sialkot's soil is clay loam, with an average pH of around 8, and a field capacity of more than 60%. During the summer, Sialkot is hot and humid, while the winter is frigid. The warmest months are June and July. Winter temperatures can plummet to 2°C (28°F). In general, the ground is flat and fruitful. The annual rainfall averages around 1000 millimetres. The district's population is urbanized by over 25.82 percent.

Jammu district is located in Jammu and Kashmir, India, to the southwest, with Narowal District to the southeast and Gujranwala District to the west. The district of Sialkot covers a total size of 3,016 square kilometers. More than 264 small and medium-sized tanneries have been developed in the city and its suburbs, with more planned to be built in the future.

Surgical equipment developed in Sialkot were utilised throughout British India. Sialkot's contribution to agriculture is comparable to that of any other district. It is most renowned for producing Basmati rice, wheat, and sugarcane

The significant environmental pollution created by tanneries poses serious health risks to humans and other animal life forms in Sialkot. Heavy metals leak into the water through industrial effluents, which then leach into the groundwater. Near Sambrial-Daska, Zafarwali-Sahowala, Sialkot, Sambrial-Wazirabad Road, Sialkot-Daska Road, and Sialkot Head Marala Road, the majority of tanneries are located. The tanneries discharge untreated water into seasonal nullahs, watercourses, and neighbouring agricultural fields on a regular basis, causing environmental deterioration and poisoning the area's water table.

Every day, tanneries in the Sialkot district process approximately 350, 000 ft² of finished leather. Tanneries in Sialkot process 200,000 square feet of this. The total volume of water emitted by tanneries is estimated to be around 1.1 million L per day.

Gujranwala:

Gujranwala is a city in Punjab, Pakistan. It's known as the "City of Wrestlers" and is well-renowned for its cuisine. The city is the fifth most populated municipal region in Pakistan, as well as the fifth most populated city. Gujranwala is located in the Rachna Doab, which stretches between the Chenab River in the north and the Ravi River in the south. Gujranwala is also located in the old Majha area in northern Punjab. The city was developed on Punjab's plains, and the surrounding area is a flat plain with little topographical diversity.



Figure 1.2: Map of Gujranwala, Punjab, Pakistan.

Gujranwala, Punjab, Pakistan's coordinates are 32.166351 degrees North latitudes and 74.195900 degrees East longitudes. The terrain is mostly flat and fertile. The average rainfall during the other months is around 25 mm (0.98 in). There is little rain from October to May. Gujranwala, after Karachi and Faisalabad, is presently Pakistan's third largest industrial city, accounting for 5% to 9% of the country's GDP. The city is part of a network of significant metropolitan centers in Pakistan's north-east Punjab province, which is one of the country's most industrialized areas. The most recent studies in Pakistan have focused on calculating frog species abundance. Bull Frog (*Hoplobatrachustigerinus*) and Common Skittering Frog (*Euphlyctiscyanophlyctis*) had mean population densities of 25.07 frogs ha⁻¹ in the paddy fields of Gujranwala, Punjab, while the mean population density of Common Skittering Frog 0.46 0.11 frogs. The species has a diverse spectrum of body types. Light grey, light brown, and olive-green hue with irregular black dots Heavy metals seep into groundwater from industrial effluents [61].

AIM AND OBJECTIVE

The aim of the research was:

- To analyze heavy metals (Cadmium, Chromium, Manganese, Nickel, Cobalt, Copper and Zinc) in the Blood, Kidney and Muscle Tissue of Indian bullfrog species present in the selected areas.

RATIONALE

Amphibians are present in aquatic and terrestrial habitats. They are considered as indicators of pollution and help in assessing the quality of water. There has not been enough research on the concentration of heavy metals in the blood, kidney and muscle tissues of Indian Bullfrog species present in the selected study areas of Gujranwala and Sialkot, therefore the current study will provide future researchers with a benchmark of knowledge on the status of these species in the selected areas and will further benefit them in developing sustainable strategies for the maintenance of the Indian Bullfrog population, since they are considered as indicator species, by reducing heavy metal pollution in the selected areas.

CHAPTER 2

LITERATURE REVIEW

Amphibians are ectothermic, tetrapod vertebrates inhabiting wide variety of habitats on both land and water. The initial embryo and larva stages occur in aquatic environment and then they reside on land after metamorphosis, before returning to water bodies to reproduce [26]. Their life cycle includes both aquatic and land environments, therefore amphibians are ideal pollution indicators. The evaluation of biological effects on amphibian species is commonly used as an indicator of pollution because it renders valuable bioavailability data and levels of absolute concentration levels of heavy metals in blood and tissue of the sample species [27]. In 2019 together with other researchers Mustafa Soliman and Mohamed El-Shazly studied changes in heavy metal concentration between the food web trophic levels in two agro ecosystems. Excessive accumulation of soil and water trace and heavy metals caused by industrial and sewage facilities has led to growing environmental issues posing several risk factors to biota, humans and ecosystems. The levels of Copper, Cadmium, Zinc and Lead in the soil and the ecological webs in two Egyptian agricultural ecosystems were identified, namely El-Mansala, a rural area northeast of Delta of the Nile River and El-Tebbin which is a commercial area situated in South Cairo. Amphibians, birds, arthropods, reptiles and small mammals were representatives of the trophic levels. The results showed that amphibians can absorb maximum amounts of heavy metals because of heavy metal contamination, since population index of these metals represents environmental pollution indices [28].

In a significant research of 2007, Barni, Boncompagni *et al.* studied the blood cell response of *Rana snk esculenta* to chemical stressors in the surroundings in the larval as well as the adult phase. Tadpoles and adults of *Rana snk esculenta* (Green frog) were gathered from two rice fields in order to investigate the alterations in various characteristics of circulating blood cells. One site was extremely polluted

while the other was relatively unpolluted. The changes in various hematological parameters were investigated to determine the toxicity due to Cadmium, in their blood samples. The eosinophilic white blood cells, anucleated, micronucleated and mitotic erythrocytes frequency, as well as haemopoietic and Nitric Oxide Synthase expression of thymocytes, were all assessed. A significant decrease in red blood cells as well as a decrease in hemoglobin and the hematocrit values was observed in animals given the highest Cadmium dosages, implying sub-lethal health alterations. The importance of peripheral blood as an indicator of pollution was highlighted by the usual nature of amphibian responses to stress in water habitats [29].

A study was carried out by Ramiro and Aurelio for the analysis of heavy metal concentrations of Nickel, Cadmium, Lead, Zinc and Chromium in sediment, water and the intestinal, gill and liver tissues of the anuran amphibian *Dryophytesplicatus*. For this study, tadpoles were gathered from a lake in Mineral El Chico, Hidalgo and Mexico. Spectrochemical analysis was used to determine the metal concentrations. Cadmium concentrations in water above the maximum permissible concentration stipulated in the Mexican Official Water Quality Standard. The observed sediment Cadmium concentrations exceeded Environmental Protection Agency guidelines. Cadmium and Zinc concentrations in tadpole tissues were higher in the gills, while Lead, Chromium and Nickel concentrations were higher in the liver tissue. All five metals were observed to have the inflated concentrations in sample tissues of tadpoles, indicating that a bioamplification process is taking place. Thus, it was deduced that heavy metal concentrations are putting this species' survival in jeopardy [30].

In another research of 2015, Qureshi, Kashif *et al.* conducted a heavy metal assessment for Nickel, Iron, Zinc, Copper, Cobalt, Cadmium, Manganese, Lead, Chromium and Metalloids in major body tissues and organs including liver, kidney, stomach, lungs, heart and skeletal muscles of the chosen species of frogs; *Euphlyctiscyanophlyctis* and *Rana tigrina* of the area of Sialkot, Pakistan, the city

famous for its tanning industry worldwide. Both species taken as samples were observed to have darker skin, varying snout-vent lengths and altered wet body weights. Findings showed higher heavy metals levels in sample specimen of the selected industrial sites. Few prominent differences among the tissue samples of both species from both sites were observed. Cadmium, Iron, Nickel, Manganese, Copper and Chromium were higher in *Rana tigrina* tissues, while Lead and Cobalt concentrations were observed to be higher in *E. cyanophlyctis*. Copper and Cadmium concentrations in the kidney and liver were found to be higher in both sample species as compared to the rest body organs. Observations showed that morphologically, frogs inhabiting industrial sites had shorter and heavier bodies, as well as more metal accumulation [31].

In another research of 2013, Shaapera, Nnamonu *et al.* used an Atomic Absorption Spectrophotometer to find the accumulated levels of seven heavy metals (Copper, Lead, Chromium, Zinc, Iron, Cadmium, and Manganese) in the frog *Rana esculenta*'s organs collected from Guma River of Benue State, Nigeria. All of the metal concentrations in the frog's skin, intestine and liver were found to be significant statistically. Results indicated that the intestine had the lowest, skin followed it and the highest concentration of all the heavy metals were detected in the liver samples. The Lead, Copper, Zinc, Iron, chromium, Cadmium, and Manganese concentrations in all of the samples were generally found to be above the World Health Organization allowable limits, except of Copper, Cadmium and Zinc, which were found to be within the permissible limits in the samples in the pattern of $Fe > Mn > Pb > Zn > Cu > Cr > Cd$ [32].

Another research in 2008 was undertaken for the assessment of heavy metals for seasonal and spatial variations in the liver samples of frog *Rana ridibunda* collected from both from urban and rural site in Western Ukraine. The weight of the liver increased from spring to summer-autumn for which the accumulation of metabolites, notably fat and glycogen, is most likely to be the major cause to be considered. The metal concentrations in the frog's liver were in the following order:

Fe>Cu>Zn>Mn>Cd. The frogs living in the urban area had the highest concentrations of most metals. In the agricultural area, the highest index of Copper in the liver was found in the spring, while of other metals was found in the summer because of discharged effluents from fungicides that contained Copper in their formula in the wetland. Other than the fact that the very low concentration of Cadmium found in the water, this indicates that tissues accumulate Cadmium. It further indicated that frogs were being exposed to Cadmium and possibly other heavy metals on a regular basis [33].

Accumulation of Mercury along a toxicity gradient and nondestructive indexes of net bioaccumulation in amphibians was indicated by Bergeron and Bodinof in 2010. Adult amphibian samples from three species, *Plethodon cinereus*, *Eurycea bislineata*, and *Bufo americanus* and larvae stage samples from the latter two species were collected along a contamination gradient on the South River to see if species and life stages had any effect of Mercury bioaccumulation. The contaminated site's total Mercury (Hg) concentrations were prominently higher than that of the reference site. Total Mercury concentrations in amphibians differed in ways that coordinated with their feeding priorities and habitat requirements. The findings suggested the risk of Hg exposure to the amphibians and their terrestrial predators of this system and that nondestructive sampling methods could be a viable sampling option with fewer negative consequences for local populations [34].

In 2014, notable researchers Zocche, Da Silva *et al*, carried out the analysis of heavy metal levels in tissues and DNA damage caused by it in samples of *Hypsiboas faber*, an anuran amphibian, in open-casting coal mine as compared to animals from an unpolluted area. The heavy metal levels were determined using the Particle-Induced X-ray Emission (PIXE) technique, and the DNA damage was determined using the Comet assay. The findings showed that, regardless of the organ, *H. faber* specimens collected from the coal mining area showed elements in the order Fe > Cu > Al > Zn > Rb > Mn > Br. In comparison to the reference animals, the values of Comet assay parameters i.e., DNA damage index and DNA damage frequency were

significant in specimens collected from the polluted study area. It was inferred that the residues of coal mines are genotoxic to amphibians and may harm vegetation, soil, water and wild biodiversity [35].

In a study of 2015, Priyadarshani, Madhushani *et al.*, investigated the effects of heavy metal intoxication on the immune system of the Indian green frog specie, *Euphlyctishexadactylus* for the comparison of *BellanwilaAttidiya*, an urban wetland polluted with high heavy metal concentrations, to a reference site in Bolgoda, Sri Lanka. Copper, Zinc, Lead, and Cadmium accumulation in the gastrocnemius muscle and liver of the frog was significantly higher in the polluted site than the reference site, according to Atomic Absorption Spectrophotometer (AAS) analysis. Standard immunotoxicity tests were performed. The polluted site's frogs had significantly lower test parameters than their counterparts from the reference site. Frog phagocytes' in vitro exposure to Copper, Zinc, Lead, and Cadmium at concentrations of 10⁻²–10⁻¹⁰M showed immunomodulation, with low concentrations stimulating phagocytosis and high concentrations showing a trend toward immunosuppression [36].

The concentration levels of selected 14 heavy metals in the tissues of *Rana ridibunda*, a frog specie native to a small river in Macedonia, Northern Greece, was studied by Loumbourdis and Wray in 1998. Copper, cobalt, molybdenum, cadmium and chromium were significantly elevated in the liver, while aluminium, manganese, nickel, strontium, and barium were significantly higher in the carcass. Chromium, zinc, copper, molybdenum, manganese and aluminium were found in high concentrations in areas that were heavily polluted. The river can be considered as fairly to heavily polluted for other metals like lead, cobalt, cadmium, nickel and cesium. Fertilizers having heavy metals as trace elements, urban runoff, pesticides containing any of the heavy metal in their composition chemistry or various light industries along the river that discharge their effluents into this river appear to be the point sources of pollution in the river [37].

In a recent study of 2020, Zhelev, Arnaudova *et al.* observed the effects of toxicants on marsh frog *P. ridibundus* individuals who have lived in an anthropogenically polluted habitat for a long time are assessed, as well as the changes toxicants cause in anurans' organisms. In situ measurements of different morphophysiological and haematological parameters were done to determine the health status of sample frogs and then the bioaccumulation of Lead, Cadmium, Zinc, and Copper in their body tissues (liver and muscles) was analyzed. *P. ridibundus* individuals living in an area polluted with industrial waste were found to be anaemic, according to the findings. In comparison to samples from the chosen reference site, high levels of heavy metals and metalloids (Lead, Cadmium, Copper, Zinc, Arsenic, and Selenium) were found in the tissues of both sexes of frogs inhabiting the polluted site [38].

Another important study focused at the presence of heavy metal toxicants in soil and water samples and bioaccumulation in the heart, kidney and liver of two sample amphibian species, *Amietophrynus maculatus* and *Ptychadenapumilio*, at sites of Ibafo, Iwaya, Makoko 1, Makoko 2, and the University of Lagos in Lagos. The Perkin Elmer Analyst 200 Atomic Absorption Spectrophotometer was used to examine heavy metals. The overall mean concentrations in the water samples, had Zinc as having the highest bioaccumulation in both species and all locations. Manganese was the second in the descending list followed by Lead. Manganese, Zinc, and Lead were found in higher concentrations in the liver, while Nickel was found in higher concentrations in the heart in both anuran species. Both anuran species had significantly different Chromium concentrations in their livers. The study revealed the current state of biological and environmental health, with anurans serving as remarkably significant bio indicators and preliminary warning signs of potential environmental destruction [39].

The goal of another study carried out was to see how sublethal doses of cadmium (Cd) affected *Rhinella arenarum* specimens of liver, kidney, and blood. When comparing animals subjected a 0.5 mg/kg dose to controls, serum markers of hepatic injury and markers of renal dysfunction showed variation.

Histopathological images of animals with abnormal serum markers revealed changes in the liver and kidney. With the 5 mg/kg dose, the above-mentioned lesions were more visible. With the exception of the leukocyte formula, haematological values remained normal at the 0.5 mg/kg dose. Red and white blood cell counts, as well as hematocrit and haemoglobin values, were significantly lower in animals given 5 mg/kg. Red and white blood cells both had morphological abnormalities. The findings revealed that morphological and functional parameters in the kidney, liver, and blood must be examined in order to monitor amphibian populations exposed to chemical pollutants. [26].

The concentration of lead in the blood of a South American toad population of 152 samples was analyzed by Arrieta Apartin et al. in 2001 over the course of ten samplings conducted in the period of December 1996 and November 1999. The population under study lived in and around La Plata City, Argentina's largest industrial-urban setting in the province of Buenos Aires. The heavy metal was found in all of the tested samples, with an average concentration range of 1.99–4.66 mg/dL. Some basic data on Lead content in the soil in the sampling area point to the metal's human activity origin, possibly due to the widespread use of Lead-containing gasoline in the recent past. The reported findings could be the result of a long-term local air, soil and water pollution process [40].

In 2020, Santana EM and Schiesari LC studied Industrial pollution and stated that, it is a major global biodiversity threat, but has a poorly understood consequences for rainforest ecosystems. Due to their aquatic-terrestrial life cycle, amphibians are particularly vulnerable to pollutants released in natural surroundings. The physiological conditions of *Rhinella ornate* individuals, endemic to Atlantic Forest toad species and a global biodiversity hotspot for severe historical air, sea and soil contamination of Cubatão Industrial Complex in Brazil was observed. As expected, toads at lower distances from the source of pollution resulted in detox organs, liver and kidney, extension and compensatory immunologic function. Findings deduce that the potential evolution in the local resistance to contamination of toads in

populations nearby pollution sources was also discussed and chemical contaminants in the area will be exposed for more than 60 years [41].

In 2021, a research has sought to monitor water quality in Medjardin in Northeast Algeria to provide basic data regarding to the heavy metals levels in the water and in the spiny toad *Bufo spinosus* specie, a potential amphibian bio-sentinel. The atomic absorption spectrophotometer was used to measure temperature, dissolved oxygen, pH and biological oxygen demand from the water and heavy metals of toad skin. The toad skin and water concentrations of lead (Pb) were respectively 96 and 60 times higher than the standard values of reference, at all sites. Heavy metal presence levels in water and male toads, were in descending order.

This study focused on the environmental condition of the neighboring areas of the study sites, as a source of heavy metal pollution upstream of the Medjardín basin. The non-invasive skin removal procedure was also stated to be an ethically sound method for the assessment of heavy metal accretion in aquatic animals, such as toad, without destructing and losing the biodiversity of the species [42].

Smalling, in 2019, studied that over the last several decades, energy production has increased rapidly in the Williston Basin in the Central North Prairie Pothole region. The products from energy production sites contain high levels of heavy metals and metalloids (lead, selenium, strontium, antimony and vanadium), ammonia, radionuclides and organic volatile hydrocarbons but not chloride and sodium. Chlorine sensitivity and certain metals are particularly high in amphibians and potentially increased in saurium-contaminated wetlands. A collection of sediments and larval amphibians from wetlands of Montana and North Dakota was done for the determination of contamination related to metal levels in sediments and metal accumulation in tissue which were whether different in sediment or not. In wetland sediments, strontium and sodium levels occurred in oil and gas wastewater, while not being related to mercury but positively associated with saline contamination. Salt and vanadium had been associated with salt contamination in amphibian tissues. In comparisons with predatory salamanders, metal tissue levels in tadpoles

were higher; which suggested that frequent sediment contact could lead to greater intake of heavy metal loads. Though most of these metals are not directly related to energy growth, potential exposure additives or synergies, together with high brine chloride, can have important impacts on aquatic organisms [43].

A study focused on the impact of heavy metals on reproduction and development of amphibians of highlands of South Virginia, United States. Metal residues from two lands was analyzed: cropland and native herbal lands. Concentrations Cadmium and Nickel differed between soil uses in *Buffo cognatus* tissue. *Spea* species. tissues had no different concentrations of metals from one country to the next. Ba, collected from the same grassland beaches, indicating a distinctive habitat use, was higher in *Spea* species. than *Buffo cognatus*. There have been no correlations between concentrations of sediment and tissue. Land usage seems to have little effect on metal levels and is below the levels known to cause-effect in amphibians [44].

In another study, levels of minerals in the anuran amphibian *Dryophytesplicatus* (tadpoles) of El Chico, Hidalgo, Mexico were determined in water, tissue (intestinal, gills and liver) and sediment. Spectrochemical analysis produced the metal concentrations. The findings showed that the Cadmium concentration for water exceeded the Mexican Official Quality Standard maximum permitted concentration. Cadmium concentrations in sediments exceeded the regulations of the Environmental Protection Agency. Tadpoles had higher Zinc and Cadmium concentrations in the gill samples and higher Lead, Chromium and Nickel concentrations in the liver tissue. There was no highest concentration of any metal in water or sediment, whereas the five heavy metals (Nickel, Lead, Zinc, Chromium and Cadmium) had highest concentrations in tadpole tissues. The result declared that the survival of the species is threatened by high concentrations of heavy metals [45].

The focus of another significant research was the wetland area of the Merri Creek corridor of south-east Australia. In this study anurian species richness and

distribution were assessed with regard to water chemistry and metal content in sediments. A total of six species were found at 60 percent of studies. Six heavy metals' sediment concentrations were negatively correlated at all sites: Mercury, Nickel, Zinc, Cadmium, Copper and Lead. The ranges of all three of the most regularly seen species, *C. signifera*, *L. tasmaniensis*, and *L. ewingii*, were prominently adversely linked with the level of metal contamination in total at the different sites. The study is the first one to show that the wealth and distribution of metal contamination in the southern hemisphere is related to anuran species, adding small but growing data that pollution from heavy metals has contributed to a global decline in amphibian activity [46].

The samples of soils and amphibian host specimens from Agbada, Nigeria's Niger Delta oil flow station for heavy metal analysis and also for the regeneration of amphibian holminth endoparasites were collected monthly for 12 months, wet digestion was done and Heavy Metal Analysis was performed using Atomic Absorption Spectrophotometer. Using standard protocols, parasites were collected. Environmental and biological samples both accumulated different heavy metal concentrations. All metal concentrations were lower in environment samples than the target values while all metals were concentrated above the permissible limits in biological samples. In addition to the findings showed among others, that lung trematodes, *Haematoloechusexoterorchis* and *H. micrurus* correlated considerably positively with Copper in the soil samples and negatively with Lead, Cadmium, and Chromium in Soil, Pearson's coefficients for correlations were calculated for pairs of heavy metal concentrations in biological and environmental samples. The results reflect the conditions that enhance and impede the parasites' ability to develop a host species infection [47].

Jayawardena et al. worked on the study of the contamination of heavy metals, particularly amphibians, that may have negative effects on the wetland biota, as a result of serious immunotoxic effects in *Euphlyctishexadactylus* (Indian green frog) due to metal exposure (Cadmium, Chromium, Copper, Lead and Zinc). The

histopathological and biochemical effects of the metal toxicity were evaluated in the reference population of *E. hexadactylus* and a particular laboratory exposure group i.e (28 days of exposure to Cadmium, Chromium, Copper, Lead, and Zinc). A histopathological assessment was carried out for the semi-quantification of impaired tissue. The results were very consistent for the two exposure cases for biochemical and histopathological markers and provided validation to the hypothesis of heavy metals exposure. The present study confirms biochemical alterations linked to histopathology as potential biomarkers for the toxicity of heavy metals in amphibians [48].

The research involved Tadpoles from the *Zhenhaim Brown Frog* that were subjected to a combination of three factorial combinations, with three Cadmium exposure, for the evaluation of the combined toxic effects of Cadmium on tadpoles' growth, development, and physiology, during the developmental period, indicating remarkable Cadmium-induced toxicity of *R. zhenhaiensis* tadpoles, which affected almost all measured characteristics. Most measured variables were also impacted by pH and its interaction with Cadmium. The toxicity of Cadmium caused to tadpoles seems to be enhanced by high pH (but not low pH). The findings revealed that Cadmium exposure may interact with different pH levels, resulting in a variety of combination amphibian larvae toxicities [49].

CHAPTER 3

METHODOLOGY

Samples were collected from the agricultural fields of two study areas namely; Gujranwala and Sialkot Districts and the following methodology was adopted:

3.1: MATERIALS AND METHODS:

PLAN OF WORK

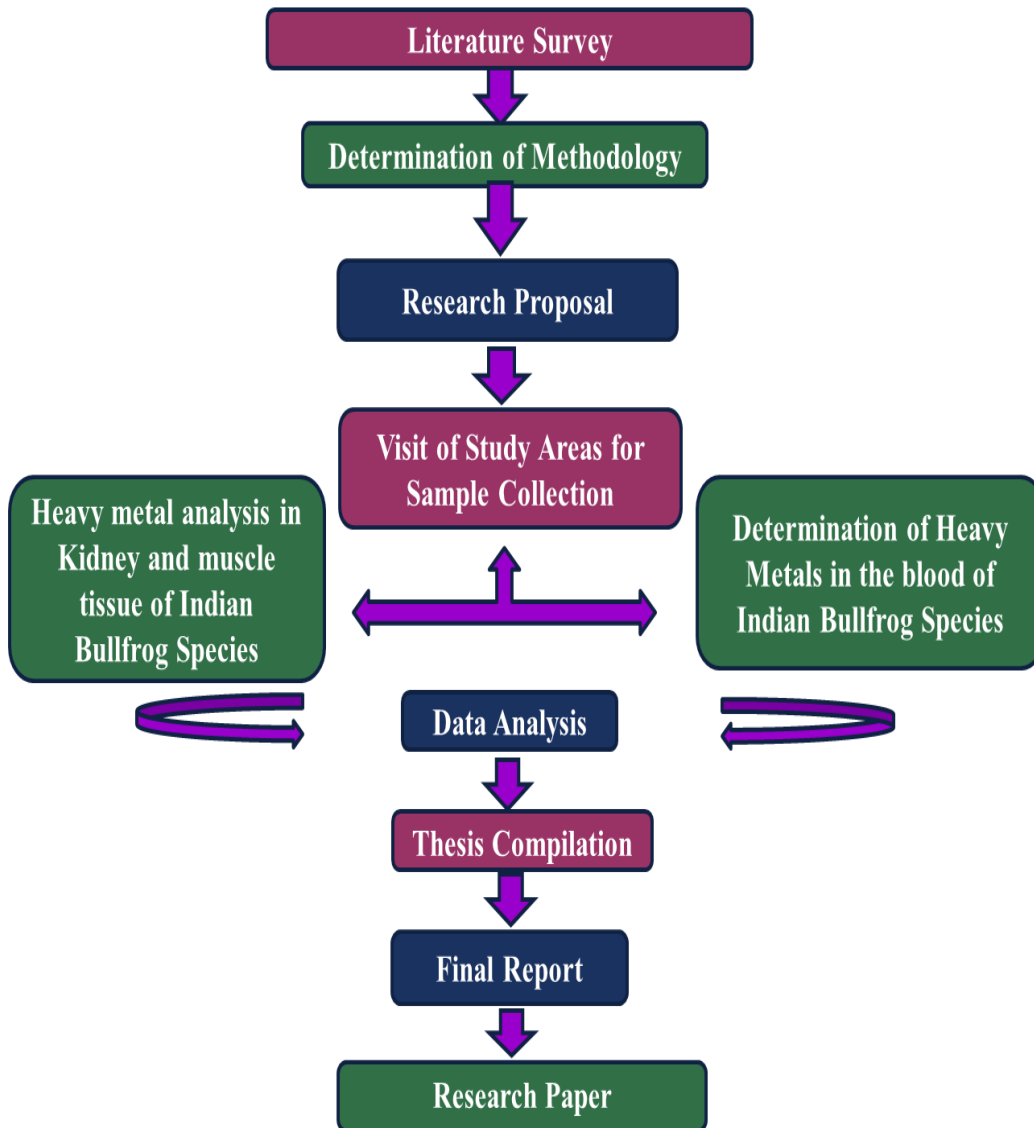


Figure 3.1: Flow sheet for the Plan of Work

Table 3.1: Materials and Reagents that were Required for Sample Collection and Analysis

Sr. No	Materials/Reagents Required	Quantity/Specification
1.	Plastic Jars	50
2.	Glass Vials	100
3.	Test Tubes With Covers	100
4.	Test Tube Stands	6
5.	Dissection Kits	2
6.	Gloves	50
7.	Cotton	1 Big Roll
8.	Formalin (10%)	1000 ml
9.	Volumetric Flasks	20
10.	Beakers	20
11.	Measuring Cylinders	20
12.	Hot Plate	Scilogex
13.	Chloroform	500 ml
14.	Weighing Scale	Benchmark
15.	Measuring Tape	1
16.	Atomic Absorption Spectrophotometer	210 A BUCK Scientific
17.	Sweeping Nets	2
18.	Plastic Bucket Fitted With Nylon Mesh	2
19.	Muffle Furnace	<i>WiseTherm</i> FHP Programmable Digital <i>Muffle Furnace</i>
20.	Ceramic Bowls	10
21.	Nitric Acid (Concentrated)	200 ml
22.	Perchloric Acid	50 ml
23.	Sulphuric Acid	50 ml
23.	Pipettes	5
24.	Vacutainers Containing EDTA (Purple Lid)	50
25.	Syringes	50 (3 cc)

Table 3.2: Collected Samples and their Identification Codes

Sr.no	Sample	City	Identification Codes
1.	Sample 1	Sialkot	S1
2.	Sample 2	Sialkot	S2
3.	Sample 3	Sialkot	S3
4.	Sample 4	Sialkot	S4
5.	Sample 5	Sialkot	S5
6.	Sample 6	Sialkot	S6
7.	Sample 7	Sialkot	S7
8.	Sample 8	Sialkot	S8
9.	Sample 9	Sialkot	S9
10.	Sample 10	Sialkot	S10
11.	Sample 11	Gujranwala	G1
12.	Sample 12	Gujranwala	G2
13.	Sample 13	Gujranwala	G3
14.	Sample 14	Gujranwala	G4
15.	Sample 15	Gujranwala	G5
16.	Sample 16	Gujranwala	G6
17.	Sample 17	Gujranwala	G7
18.	Sample 18	Gujranwala	G8
19.	Sample 19	Gujranwala	G9
20.	Sample 20	Gujranwala	G10
21.	Sample 21	Gujranwala	G11

3.2: SAMPLE COLLECTION:

Adult Indian Bullfrogs, *H. tigerinus*, were collected without sex discrimination from agricultural fields of Gujranwala and Sialkot districts, where small streams and water systems such as tubewells containing groundwater were present. They were caught using sweeping nets and then transferred to plastic buckets with aerated lids containing adequate amount of area's water to provide a nearly similar environment from which they were caught. The sample size of frogs collected was 10 from Sialkot and 11 from Gujranwala, respectively. They were caught during the post-monsoon season, when the heavy metals have leached down from the surface water and the heavy rainfall acts as a major dilution factor of contaminants [50].

3.3: SAMPLE PREPARATION

3.3.1: Blood Samples Collection and Digestion:

Blood of the frog was drawn by puncturing its heart using a 3cc syringe. Then it was transferred in the vacutainer containing EDTA as an anticoagulant agent. The vacutainer was then thoroughly shaken and kept in the refrigerator till further analysis.

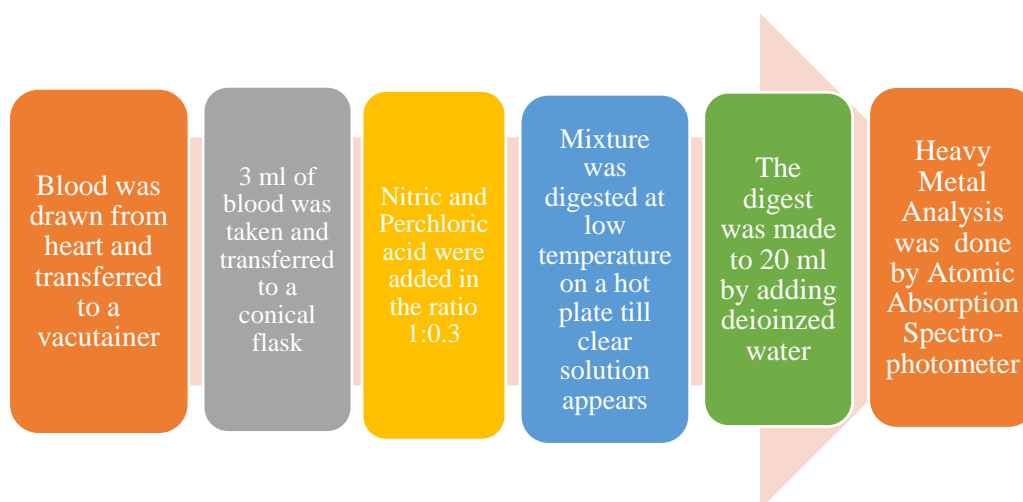


Figure 3.3.1: Flow sheet for Blood Sample Digestion

For preparing the sample for digestion, 3 ml of the blood was taken and transferred to a 100 ml conical flask. Nitric acid and perchloric acid were added in the ration 1:0.3. The conical flask was covered by an evaporating dish and the mixture was digested at low temperature with the aid of a hot plate, till a clear solution appeared. After that the digest was made up to 20 ml by adding deionized water and the sample solution was then analyzed with the help of Atomic Absorption Spectrophotometer for the presence of heavy metals [50, 51].

3.3.2: Organ Excision and Preservation:

The frogs were carefully pinned down on a wooden board after being anesthetized using chloroform and were dissected from their ventral side. The skin was carefully cut from the body of the frog, by making first a horizontal, followed by ventral and lateral incisions. After that the tissues were cut in order to fully expose the internal organs of the frog and the muscle tissue and kidney were excised from the frog's body. Both the muscle tissue and kidney samples were then preserved in 10% buffered formalin till further analysis [50].

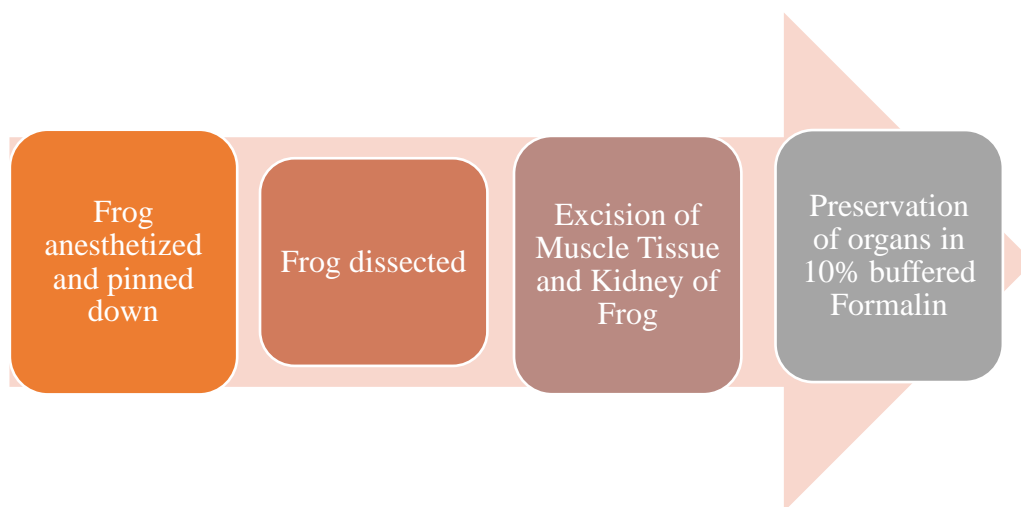


Figure 3.3.2: Flow sheet for Organ Excision and Preservation of Frogs

3.3.3: Organ Digestion:

Frogs' kidney and muscle tissue was burnt to ash at (600°C) for 2 hours in a

muffle furnace and then acid digested (1HNO₃: 1H₂SO₄). The digested samples were then filtered to remove any solid particles present and were later analyzed for the presence of heavy metals (Chromium, Cadmium, Cobalt, Nickel, Copper, Manganese and Zinc) using an Atomic Absorption Spectrophotometer (AAS) [12].

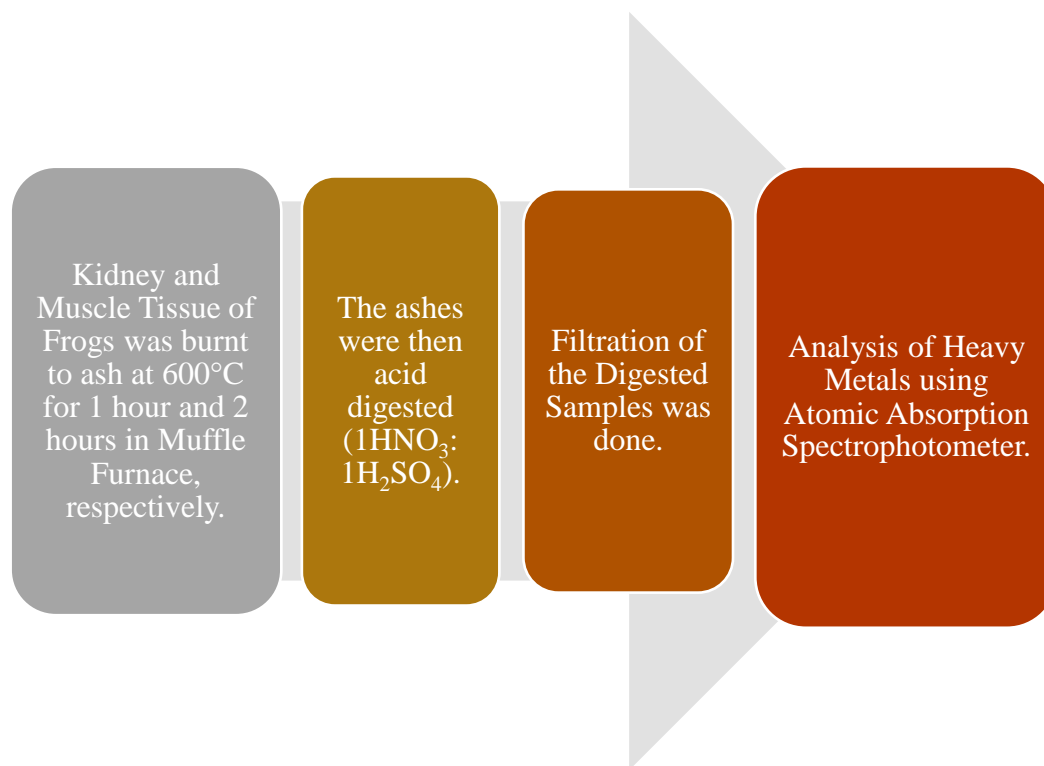


Figure 3.3.3: Flowsheet for Organ Digestion of Frogs

3.4: HEAVY METAL ANALYSIS

Concentration of heavy metals in soil samples were analyzed using Atomic Absorption Spectrophotometer. The prepared stock solutions were used in AAS to analyze the concentration of heavy metals, Chromium, Cadmium, Cobalt, Copper, Nickel and Manganese. Standard stock solutions of the salts were prepared, filtered and then stored in labeled vials. Particular emission spectrums for each metal were produced at various wavelengths. The concentrations of each heavy metal were obtained for all samples [52].

Table 3.3: Wavelengths of Heavy Metals

Heavy Metal	Wavelengths (nm)
Cadmium	228.8
Chromium	357.9
Nickel	232.0
Cobalt	240.7
Copper	324.8
Manganese	279.5
Zinc	213.9

3.5: DATA ANALYSIS:

The obtained data was represented in the form of graphs, made using Microsoft excel.

CHAPTER 4

RESULTS AND DISCUSSION

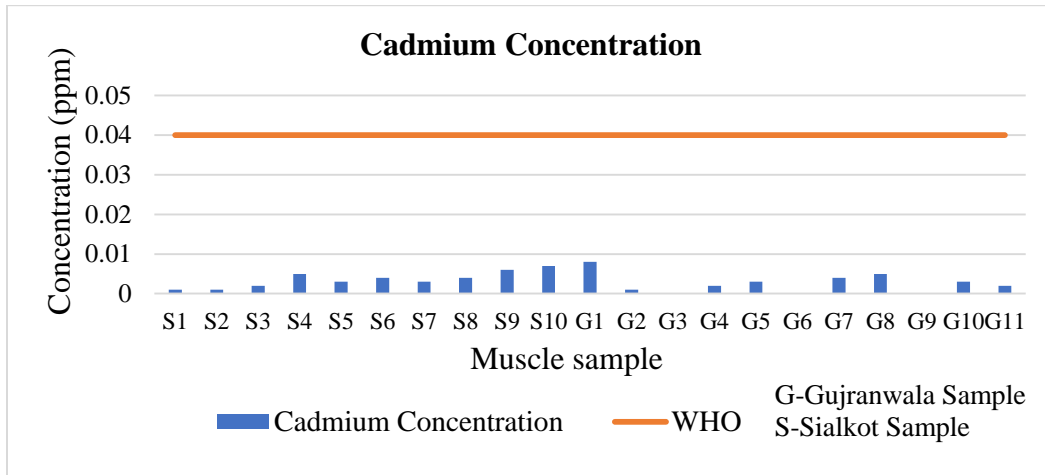
Samples of Indian Bullfrog were collected from the Gujranwala and Sialkot districts and the following heavy metal concentration results were obtained.

4.1: Concentration of Heavy Metals in Muscle Tissues:

Table 4.1: Concentration of Heavy Metals in Muscle Tissue Samples of Indian Bullfrog Species from Gujranwala and Sialkot Districts

Sr. No	Sample	Cd	Cr	Ni	Co	Cu	Mn	Zn
	WHO	0.04	0.07	0.2	0.05	2	0.5	5
1.	S1	0.001	0.000	0.000	0.003	0.000	0.000	0.001
2.	S2	0.001	0.004	0.003	0.001	0.002	0.007	0.005
3.	S3	0.002	0.001	0.004	0.000	0.003	0.008	0.004
4.	S4	0.005	0.002	0.000	0.004	0.000	0.001	0.003
5.	S5	0.003	0.008	0.005	0.005	0.004	0.005	0.001
6.	S6	0.004	0.004	0.006	0.001	0.005	0.001	0.002
7.	S7	0.003	0.001	0.000	0.002	0.001	0.003	0.004
8.	S8	0.004	0.002	0.004	0.000	0.002	0.000	0.005
9.	S9	0.006	0.000	0.004	0.003	0.001	0.004	0.006
10.	S10	0.007	0.003	0.003	0.004	0.000	0.005	0.004
11.	G1	0.008	0.004	0.001	0.001	0.003	0.007	0.001
12.	G2	0.001	0.001	0.002	0.002	0.004	0.008	0.002
13.	G3	0.000	0.002	0.001	0.000	0.006	0.000	0.003
14.	G4	0.002	0.000	0.001	0.005	0.009	0.009	0.004
15.	G5	0.003	0.003	0.000	0.001	0.000	0.001	0.001
16.	G6	0.000	0.001	0.001	0.002	0.001	0.002	0.002
17.	G7	0.004	0.000	0.002	0.000	0.002	0.001	0.001
18.	G8	0.005	0.002	0.001	0.003	0.004	0.003	0.002
19.	G9	0.000	0.000	0.001	0.004	0.005	0.004	0.003
20.	G10	0.003	0.001	0.001	0.000	0.000	0.001	0.004
21.	G11	0.002	0.002	0.000	0.003	0.003	0.003	0.005

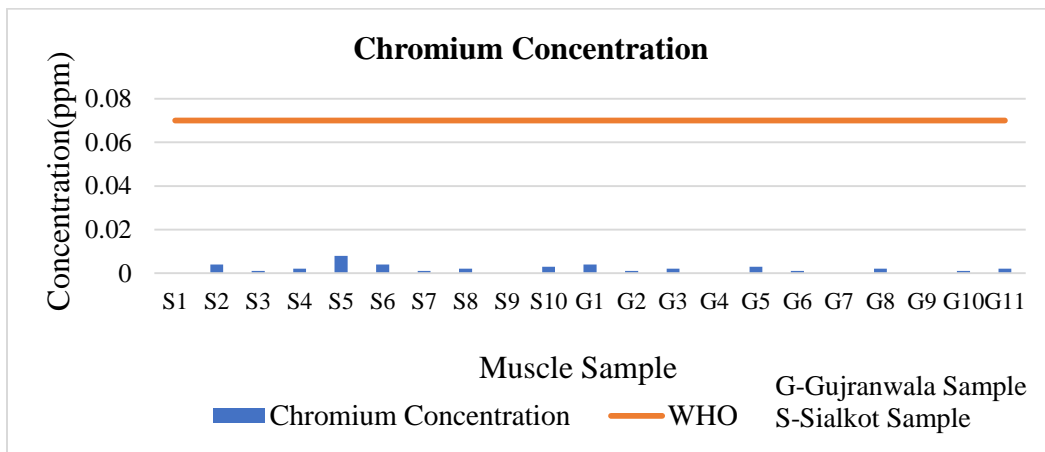
4.1.1: Cadmium Concentrations in Muscle Tissue Samples



Graph 4.1.1: Concentration of Cadmium in Muscle Sample

The graph shows that the concentration of Cadmium in Muscle Samples from Gujranwala and Sialkot, do not exceed the WHO permissible limit which is 0.04 ppm [53][54].

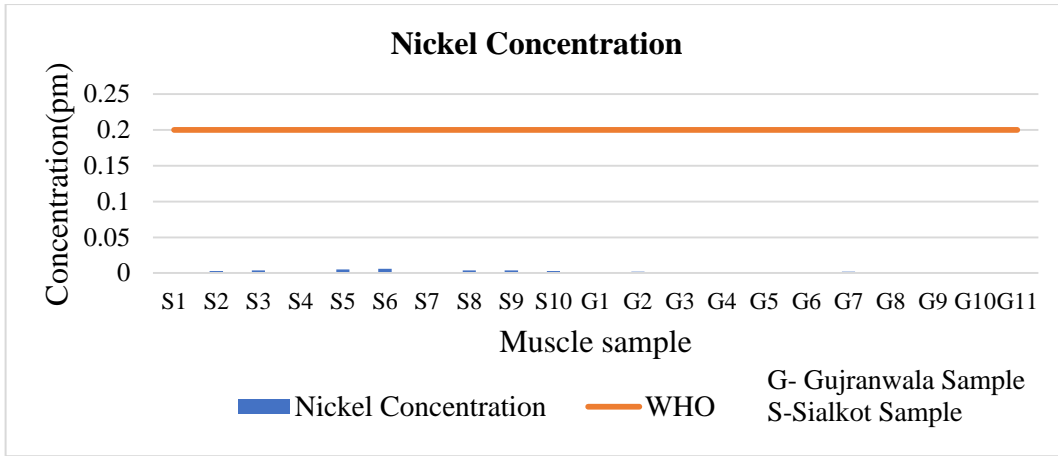
4.1.2: Chromium Concentrations in Muscle Tissue Samples



Graph 4.1.2: Concentration of Chromium in Muscle sample

The graph shows that the concentration of Chromium in Muscle Samples of Sialkot and Gujranwala is within the WHO permissible limit which is 0.07 ppm [53]. Maximum value of chromium was observed as 0.008 ppm in S5 sample[54].

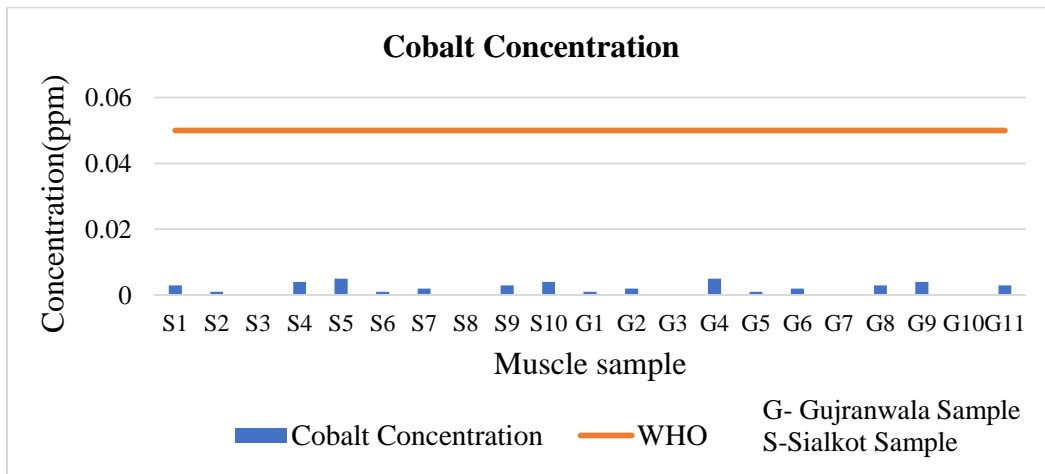
4.1.3: Nickel Concentrations in Muscle Tissue Samples



Graph 4.1.3: Concentration of Nickel in Muscle Sample

The graph shows the concentrations of nickel in muscle samples of frogs in Gujranwala and Sialkot were within the WHO permissible limit which is 0.2 ppm [53]. Not all samples contained nickel concentration so the levels were quite safer in muscle tissue. Samples from Gujranwala had almost negligible values for nickel concentrations [56].

4.1.4: Cobalt Concentrations in Muscle Tissue Samples

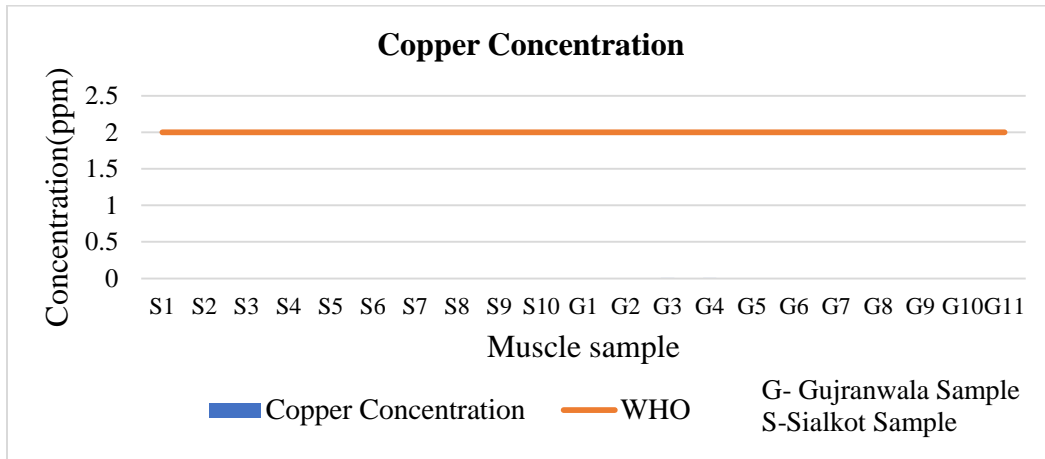


Graph 4.1.4: Concentration of Cobalt in Muscle Sample

This graph shows the concentration of Cobalt in the Muscle samples of frogs in Gujranwala and Sialkot did not cross the WHO permissible limit of 0.05 ppm [53].

On average considerable amounts were present in most samples. The maximum values were observed in the samples S5 and G4 that was 0.005 ppm [54].

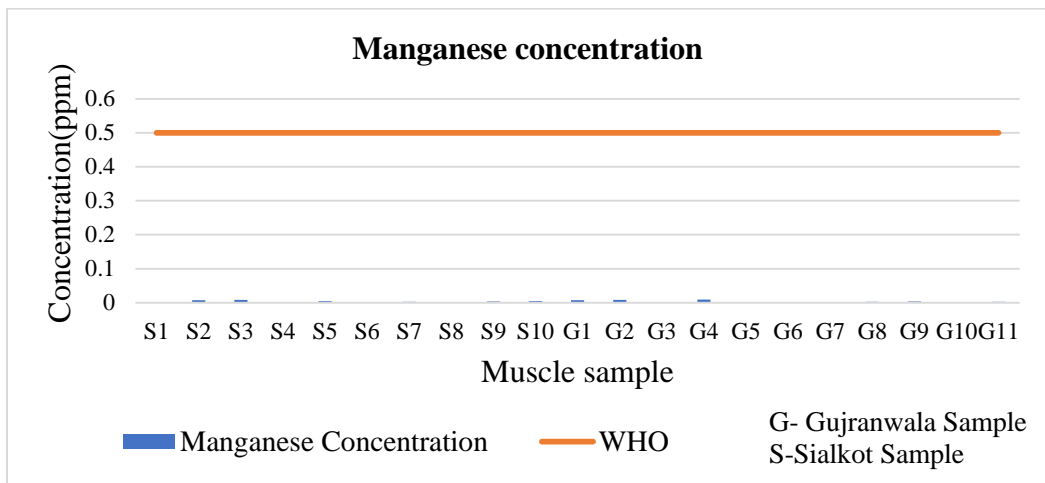
4.1.5: Copper Concentrations in Muscle Tissue Samples



Graph 4.1.5: Concentration of Copper in Muscle Samples

The graph shows the concentration of Copper in the Muscle Samples of frogs in Gujranwala and Sialkot. Concentration of copper is observed to be almost negligible in both study areas which is way less than the WHO permissible limit of 2 ppm [53]. In both study areas, Copper is present in very minute quantity which means that existence of amphibian species remains unaffected [55].

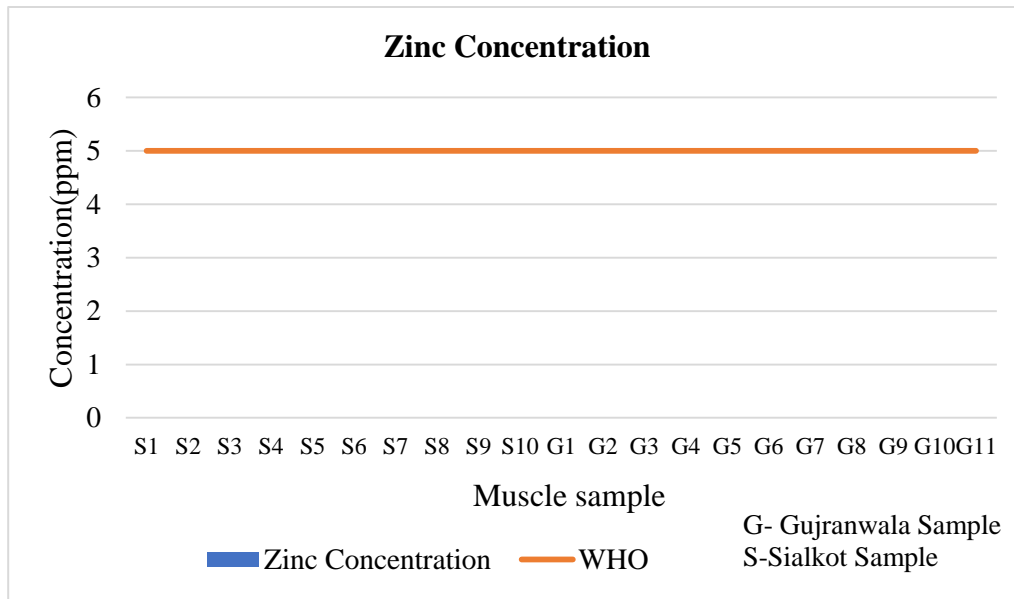
4.1.6: Manganese Concentrations in Muscle Tissue Samples



Graph 4.1.6: Concentration of Manganese in Muscle Samples

This graph shows the concentration of Manganese in the Muscle Samples of frogs in Gujranwala and Sialkot Districts. Manganese is present in minute quantities in all samples as compared to the WHO permissible limit that is 0.5 ppm [53]. The observed amounts of Mn amounts are required to regulate muscle contractions [54].

4.1.7: Zinc Concentrations in Muscle Tissue Samples



Graph 4.1.7: Concentration of Zinc in Muscle Samples

The graph shows the concentration of Zinc in the Muscle samples of frogs collected from Gujranwala and Sialkot districts. Zinc concentrations are observed to be negligible and did not exceed the WHO permissible limit of 5 ppm [53][54].

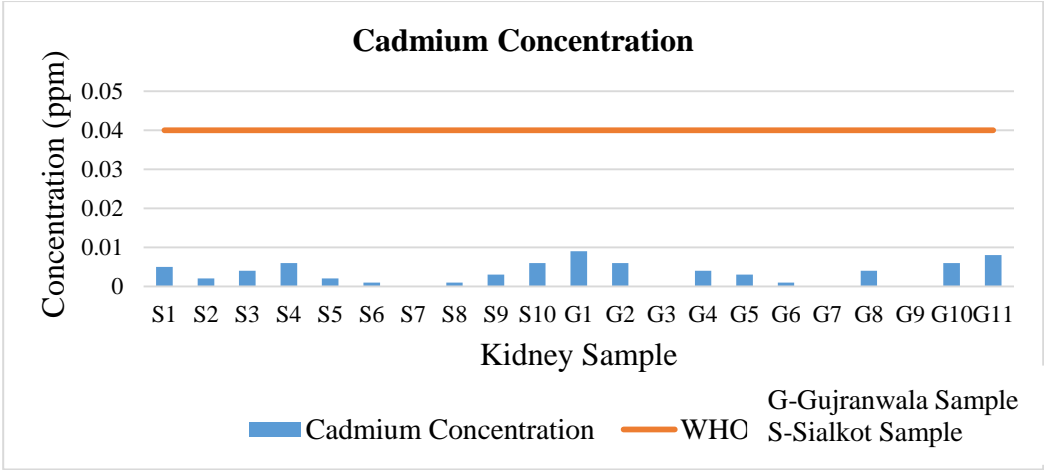
4.2: Concentration of Heavy Metal in Kidney Samples:

Table 4.2: Concentration of Heavy Metals in Kidney Samples of Indian Bullfrog Species from Gujranwala and Sialkot Districts

Sr. No	Sample	Cd	Cr	Ni	Co	Co	Mn	Zn
	WHO	0.04	0.07	0.2	0.05	2	0.5	5
1.	S1	0.005	0.000	0.000	0.003	0.004	0.001	0.031
2.	S2	0.002	0.001	0.000	0.001	0.005	0.005	0.029
3.	S3	0.004	0.002	0.001	0.004	0.000	0.002	0.035
4.	S4	0.006	0.004	0.001	0.005	0.001	0.003	0.040
5.	S5	0.002	0.005	0.000	0.000	0.002	0.004	0.045
6.	S6	0.001	0.001	0.002	0.001	0.003	0.000	0.051
7.	S7	0.000	0.003	0.000	0.001	0.000	0.009	0.062
8.	S8	0.001	0.004	0.003	0.001	0.004	0.001	0.019
9.	S9	0.003	0.001	0.001	0.002	0.005	0.002	0.018
10.	S10	0.006	0.002	0.001	0.001	0.006	0.003	0.042
11.	G1	0.009	0.000	0.000	0.001	0.001	0.004	0.045
12.	G2	0.006	0.008	0.005	0.002	0.005	0.000	0.040
13.	G3	0.000	0.009	0.000	0.003	0.003	0.001	0.038
14.	G4	0.004	0.010	0.008	0.001	0.002	0.003	0.040
15.	G5	0.003	0.000	0.000	0.001	0.000	0.004	0.035
16.	G6	0.001	0.008	0.005	0.001	0.001	0.005	0.011
17.	G7	0.000	0.004	0.004	0.004	0.001	0.001	0.072
18.	G8	0.004	0.003	0.000	0.001	0.000	0.002	0.033
19.	G9	0.000	0.006	0.001	0.003	0.002	0.003	0.044
20.	G10	0.006	0.005	0.000	0.004	0.000	0.001	0.055

21.	G11	0.008	0.002	0.003	0.000	0.003	0.000	0.001
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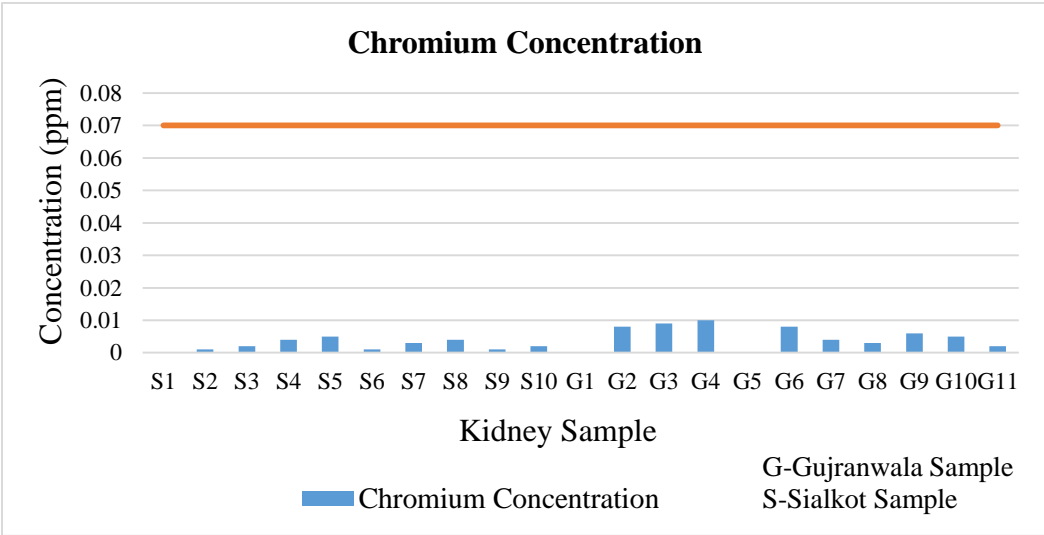
4.2.1: Cadmium Concentrations in Kidney Samples



Graph 4.2.1: Concentration of Cadmium in Kidney Samples

This graph shows the concentration of cadmium in kidney samples of Sialkot and Gujranwala districts that did not exceed the WHO permissible limit which is 0.04 ppm [53].

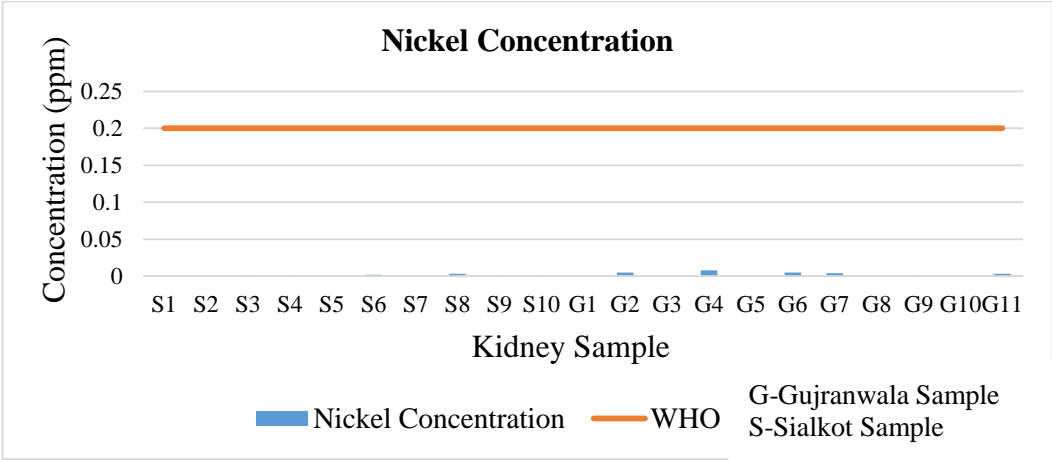
4.2.2: Chromium Concentrations in Kidney Samples



Graph 4.2.2: Concentration of Chromium in Kidney Samples

The graph shows the concentration of chromium in kidney samples of Sialkot and Gujranwala districts. Maximum concentration of chromium was observed in G4 which was 0.02 and did not exceed the WHO permissible limit which is 0.07 ppm [53].

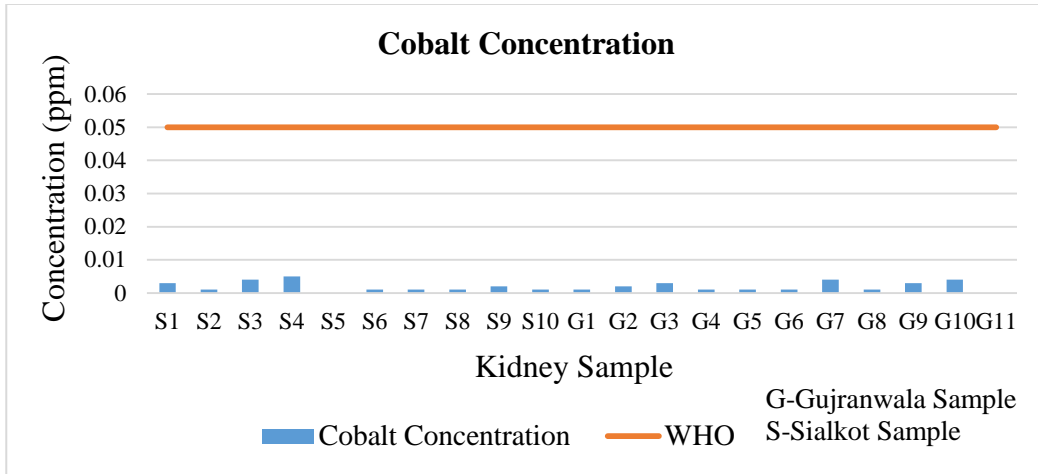
4.2.3: Nickel Concentrations in Kidney Samples



Graph 4.2.3: Concentration of Nickel in Kidney Samples

The graph shows the concentration of nickel in kidney samples from Gujranwala and Sialkot districts. Concentration of nickel in kidney samples of both areas did not exceed the WHO permissible limit which is 0.2 ppm [58]. Maximum concentration of nickel observed in the samples is 0.008 ppm which was for sample G4.

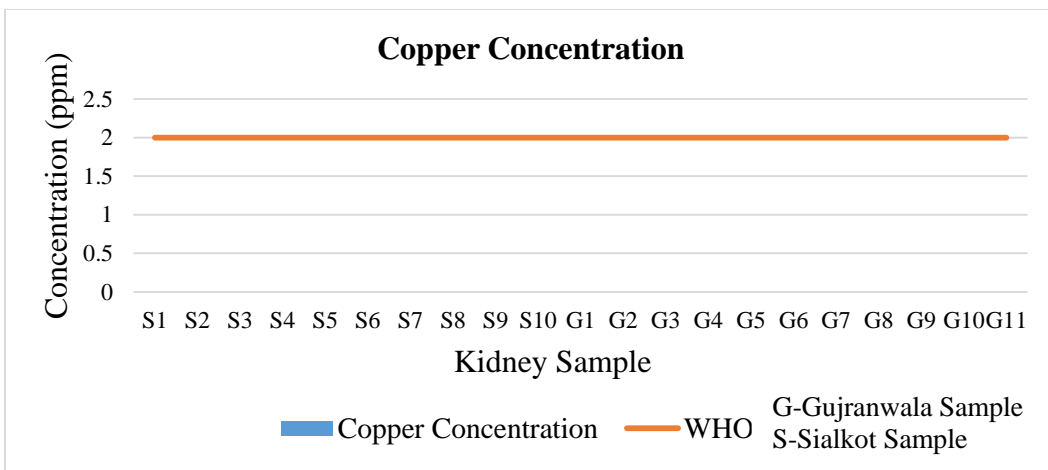
4.2.4: Cobalt Concentrations in Kidney Samples



Graph 4.2.4: Concentration of Cobalt in Kidney Samples

The graph shows the concentration of cobalt in kidney samples of Sialkot and Gujranwala districts. Maximum concentration observed is 0.005 ppm for sample S4 which is too less than the WHO permissible limit that is 0.05 ppm [58]. The concentration of cobalt in all the samples, collected from the selected study areas is within the allowable limits proposed by WHO.

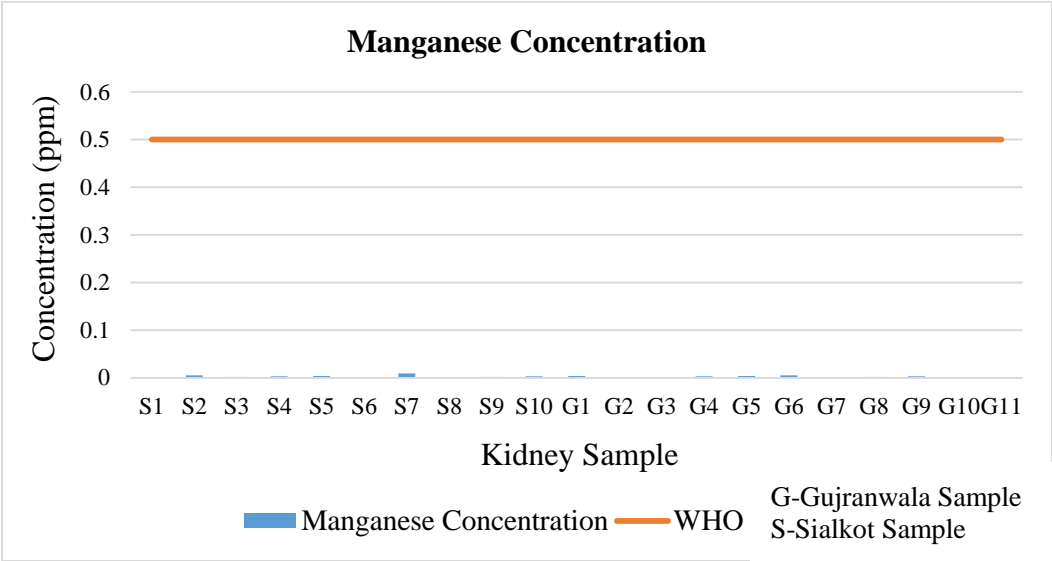
4.2. 5: Copper Concentrations in Kidney Samples



Graph 4.2.5: Concentration of Copper in Kidney Samples

This graph shows the concentration of copper in kidney samples of Sialkot and Gujranwala Districts. Maximum concentration of copper is 0.006 ppm which is observed in sample S10 that is much less than the WHO permissible limit of 2 ppm [60].

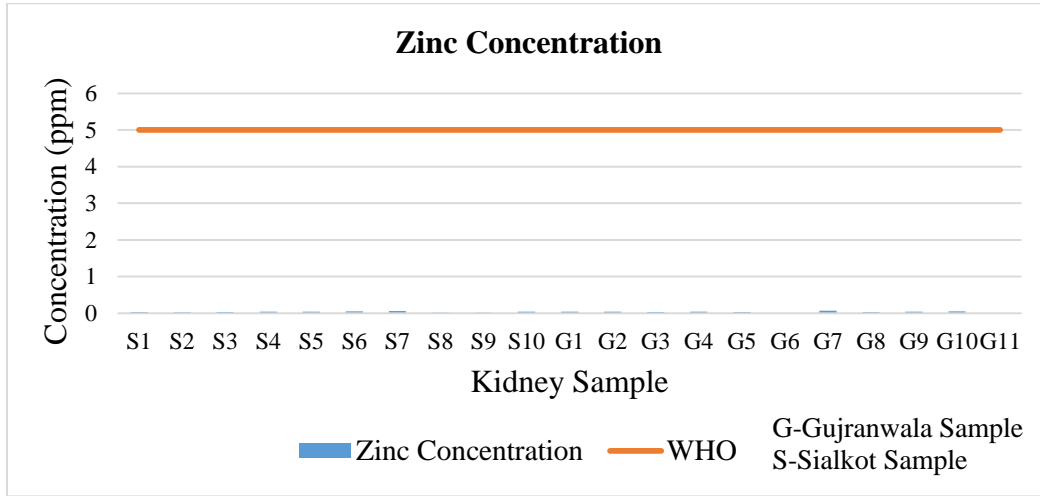
4.2.6: Manganese Concentrations in Kidney Samples



Graph 4.2.6: Concentration of Manganese in Kidney Samples

The graph shows the concentrations of manganese in kidney Samples of Sialkot and Gujranwala Districts. Maximum concentration of manganese is observed to be 0.005 ppm for the sample S2 and G6 which is less compared to the WHO permissible limit that is 0.5 ppm [58]. At this time, these activities are under control in the area hence, the values obtained are lesser.

4.2.7: Zinc Concentrations in Kidney Samples



Graph 4.2.7: Concentration of Zinc in Kidney Samples

The graph shows the concentration of zinc in kidney samples from Sialkot and Gujranwala Districts. Maximum concentration of zinc was observed to be 0.072 ppm in sample G7 which is less than the WHO permissible limit that is 5 ppm [60]. Zinc is present in very minute quantities [54].

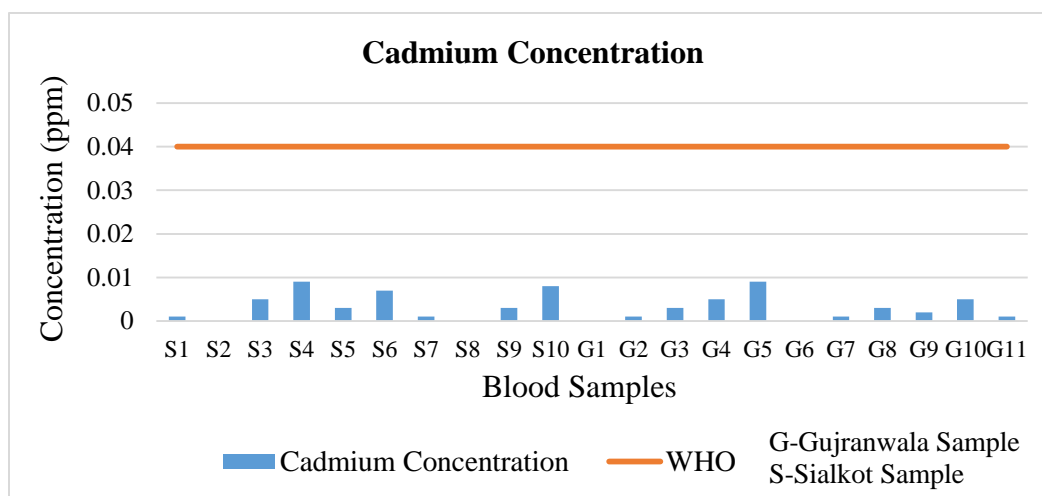
4.3: Concentration of Heavy Metals in Blood Samples:

Table 4.3: Concentration of Heavy Metal in Blood Samples of Indian Bullfrog Species from Gujranwala and Sialkot Districts

Sr. No	Sample	Cd	Cr	Ni	Co	Cu	Mn	Zn
	WHO	0.04	0.07	0.2	0.05	2	0.5	5
1.	S1	0.001	0.003	0.005	0.011	0.009	0.001	0.035
2.	S2	0.000	0.001	0.004	0.009	0.002	0.000	0.040
3.	S3	0.005	0.002	0.005	0.005	0.008	0.002	0.029
4.	S4	0.009	0.005	0.003	0.008	0.001	0.000	0.030
5.	S5	0.003	0.000	0.005	0.010	0.005	0.001	0.019
6.	S6	0.007	0.002	0.002	0.004	0.001	0.000	0.020

7.	S7	0.001	0.006	0.003	0.000	0.002	0.002	0.031
8.	S8	0.000	0.004	0.004	0.001	0.004	0.000	0.040
9.	S9	0.003	0.002	0.000	0.008	0.003	0.003	0.043
10.	S10	0.008	0.003	0.001	0.009	0.001	0.001	0.039
11.	G1	0.000	0.001	0.002	0.003	0.005	0.001	0.017
12.	G2	0.001	0.008	0.004	0.000	0.001	0.001	0.015
13.	G3	0.003	0.004	0.005	0.001	0.000	0.000	0.011
14.	G4	0.005	0.005	0.003	0.002	0.001	0.002	0.030
15.	G5	0.009	0.000	0.007	0.004	0.005	0.003	0.042
16.	G6	0.000	0.002	0.008	0.003	0.004	0.001	0.037
17.	G7	0.001	0.001	0.009	0.008	0.006	0.000	0.018
18.	G8	0.003	0.003	0.006	0.001	0.003	0.001	0.016
19.	G9	0.002	0.004	0.005	0.002	0.000	0.000	0.015
20.	G10	0.005	0.000	0.004	0.008	0.001	0.001	0.021
21.	G11	0.001	0.001	0.003	0.004	0.001	0.002	0.035

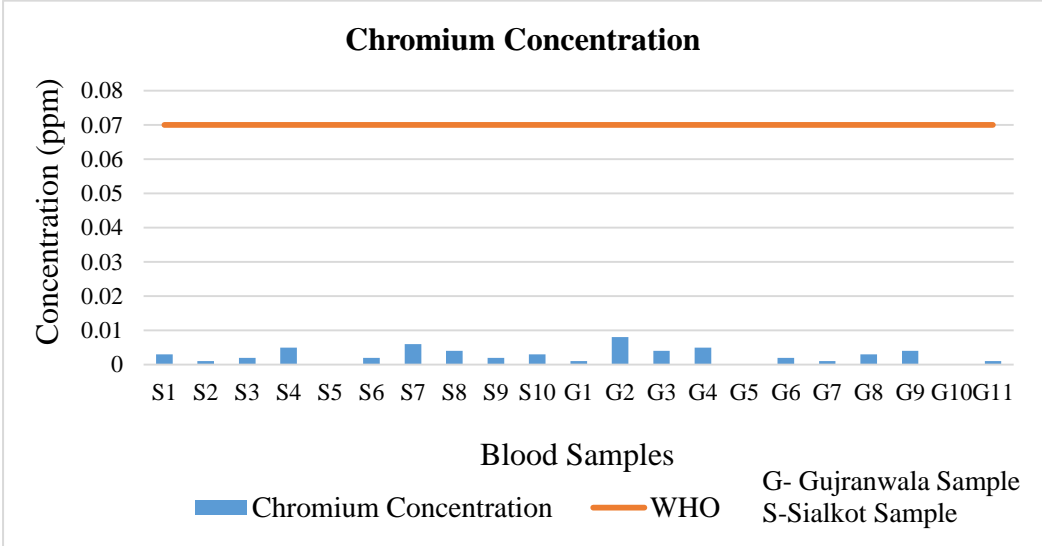
4.3.1: Cadmium Concentrations in Blood Samples



Graph 4.3.1: Concentration of Cadmium in Blood Samples

The graph shows that concentration of cadmium in blood samples of Gujranwala and Sialkot Districts did not exceed the WHO permissible limit of 0.04 ppm [53]. Maximum concentration is observed in samples G4 and S5. Further the cadmium contents in the area can be reduced by softening of hard wastewater.

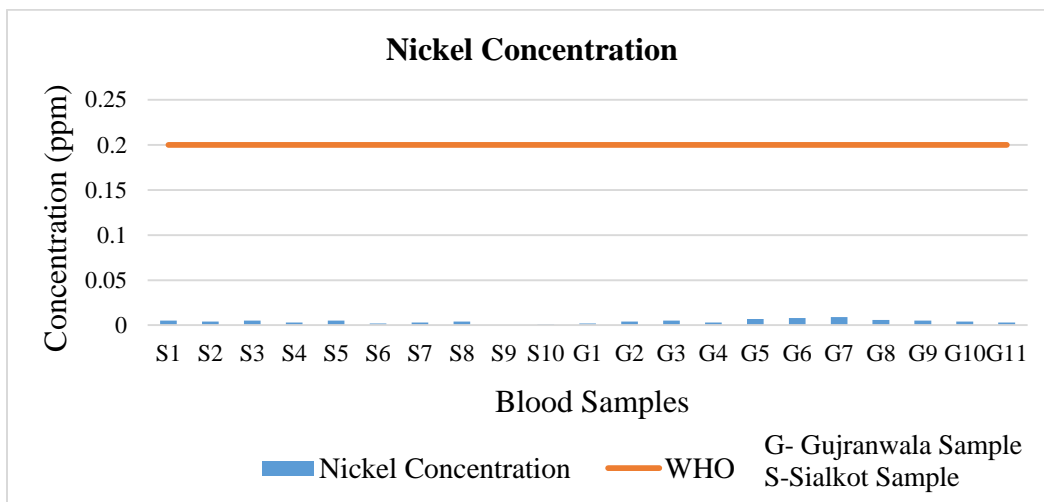
4.3.2: Chromium Concentrations in Blood Samples



Graph 4.3.2: Concentration of Chromium in Blood Samples

The graph shows the concentration of chromium in blood samples of frog species of Gujranwala and Sialkot Districts that did not exceed the WHO permissible limit which is 0.07 ppm [53]. Maximum value observed for the presence of chromium is 0.008 ppm in sample G2. Though the levels of chromium were under control in the study areas but treatment of this hard water is further recommended before directing it to the groundwater reserves and agricultural lands.

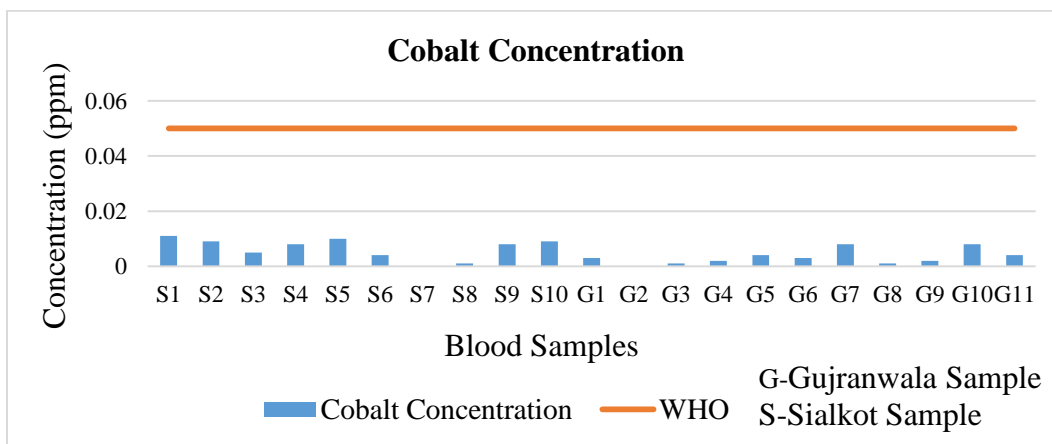
4.3.3: Nickel Concentrations in Blood Samples



Graph 4.3.3: Concentration of Nickel in Blood Samples

The graph shows the concentrations of nickel in blood samples of frogs from Gujranwala and Sialkot Districts. Maximum concentration of nickel observed in G7 is 0.009 ppm. Concentration of nickel of samples of both the districts did not exceed WHO permissible limit which is 0.2 ppm [53].

4.3.4: Cobalt Concentrations in Blood Samples

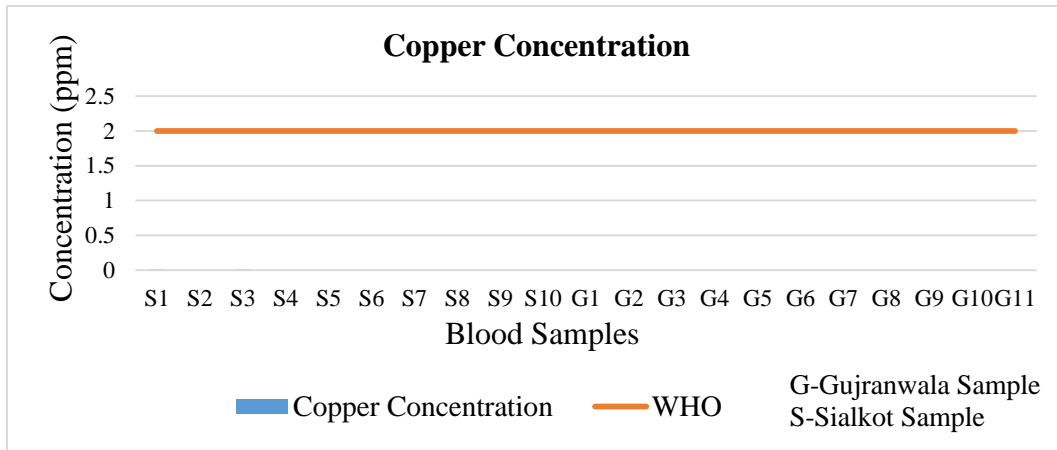


Graph 4.3.4: Concentration of Cobalt in Blood Samples

The graph shows the concentration of cobalt in the blood samples of frogs in Gujranwala and Sialkot Districts. Maximum concentration of cobalt is observed in S1 which is 0.011 ppm. Concentrations did not exceed the WHO permissible limit

which is 0.05 ppm but notable amounts are known to be absorbed in the blood samples [53]. Wastewater treatment is recommended for the areas [54].

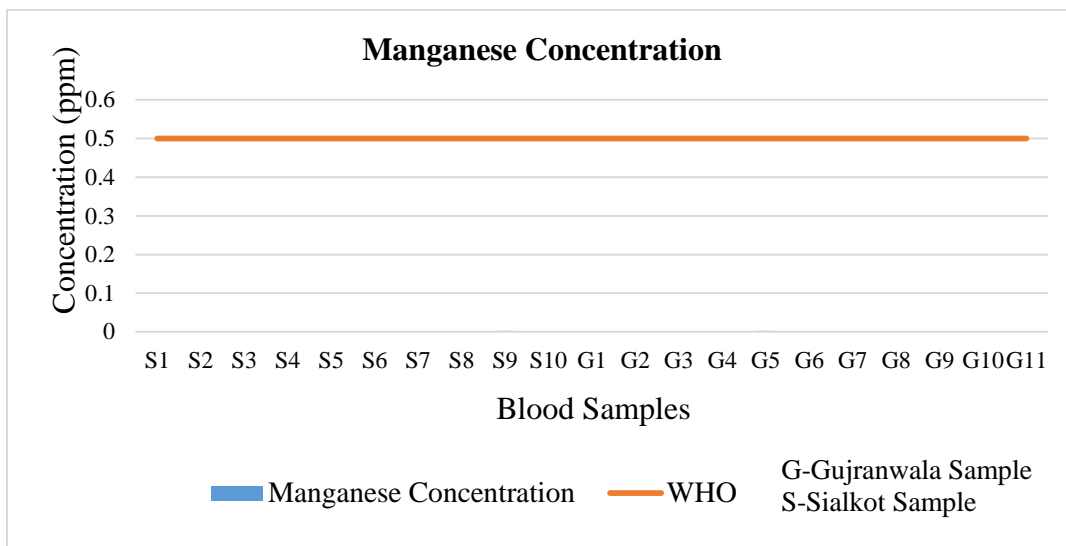
4.3.5: Copper Concentrations in Blood Samples



Graph 4.3.5: Concentration of Copper in Blood Samples

The graph shows the concentration of copper in the blood samples of frogs in Gujranwala and Sialkot Districts. Maximum concentration of copper is observed in S1 which is 0.009 ppm which is prominently lesser than the WHO permissible limit 2 ppm [53]. So, copper levels were in allowable limits by WHO.

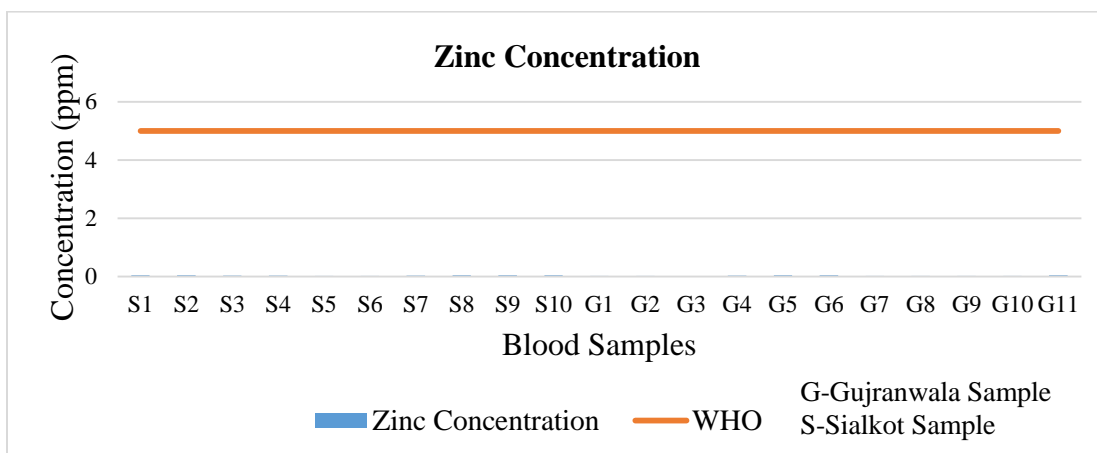
4.3.6: Manganese Concentrations in Blood Samples



Graph 4.3.6: Concentration of Manganese in Blood Samples

The graph shows the concentration of manganese in the blood samples of frogs in Gujranwala and Sialkot Districts. Maximum concentration of manganese is observed to be 0.003 ppm for the sample S9 and G5 which is less as compared to the WHO permissible limit that is 0.5 ppm [53].

4.3.7: Zinc Concentrations in Blood Samples



Graph 4.3.7: Concentration of Zinc in Blood Samples

The graph shows the concentration of zinc in the blood samples of frogs in Gujranwala and Sialkot Districts that does not exceed the WHO permissible limit 5 ppm in samples from both the study areas [53]. The maximum concentration was observed as 0.043 in S9 sample. Its concentrations are negligible because it is not absorbed by soil and rocks as effectively as other heavy metals [54].

Both the study areas are industrial areas because of untreated wastewater being dumped and solid waste being dumped on ground, results in the liquids from solid waste leach into the ground and sewage mixing with ground water. Since the frogs were collected from ponds containing ground water and tube wells near agricultural fields, therefore heavy metals are detected in the bodies of the frogs which must have absorbed in their bodies from the water. River and well water are primarily used for drinking and agriculture. It is had been studied that some concentrations of Heavy Metals are naturally present in ground water. The assessment reveals a rather normal situation in terms of heavy metal quality in Tap water suitable for drinking. For Drinking water, Cu, Mn, and Zn concentrations are assessed to be below WHO permissible values while the level of Chromium crosses the WHO standards by 21% in some areas of Pakistan. So, the results obtained depict that the concentrations of heavy metals in kidney, muscle tissue and blood of samples from both study areas are within the permissible limits overall and both the natural contents and contamination of groundwater due to industrial wastewater leaching contribute to this presence of heavy metals.

CONCLUSION

Indian Bullfrog specie samples were collected from Sialkot and Gujranwala distinctly. Twenty-one samples were analyzed; 10 from Sialkot and 11 from Gujranwala. Sialkot and Gujranwala study sites are industrial cities with major tanneries set up there. Wastewater pollution has impacted the biota of the area. In order to observe the pollution indicating capability of amphibians, standard procedures of dissection, organ preservation and organ digestion were performed. Atomic Absorption Spectrophotometer analysis were performed to obtain the results. The obtained values of heavy metals present in the water samples and the organs were then compared with the WHO Standards for heavy metals. The values of heavy metals [Cadmium, Chromium, Cobalt, Nickel, Zinc, Manganese and Copper] were well within the permissible limits of WHO, in the kidney, muscle and blood samples. Since, the frogs were caught from the Agricultural fields of the selected Areas, where ground water was used as a source of irrigation therefore, the Industrial effluent waste and sewage must have leached into the soil and contaminated the groundwater thus resulting in the occurrence of Heavy Metals in the Water and in the body of amphibian species. Some concentrations of heavy metals are also naturally present in the groundwater and drinking tap water. Thus, it was deduced that the water quality is above average in both of the study areas and it can be further improved by regular monitoring, with sensible use of fertilizers, enhanced sewage treatment practices and wastewater treatment and management over the years. This study has been important in depicting the amphibians as pollution indicators by analysis of major heavy metals concentration in samples taken from the study sites.

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ANNEXURES I



Figure. 1: Sample Collection



Figure. 2: Dissection of Frog



Figure. 3: Withdrawing of Blood Sample from heart



Figure. 4: Blood Sample Digestion on hot plate



Figure. 5: Water Sample Digestion



Figure. 6: Specimen of Kidney and Muscle in Muffle Furnace

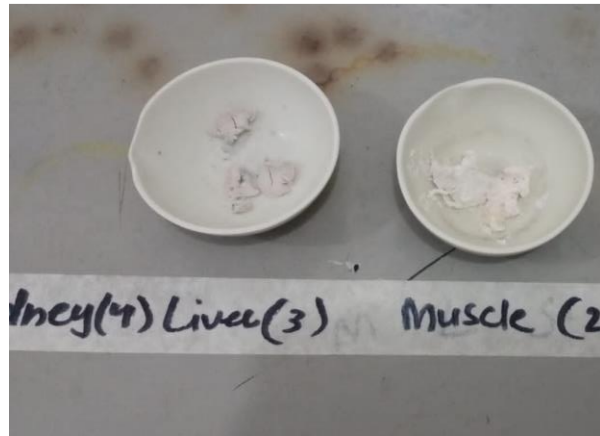


Figure. 7: Ashes of Specimen



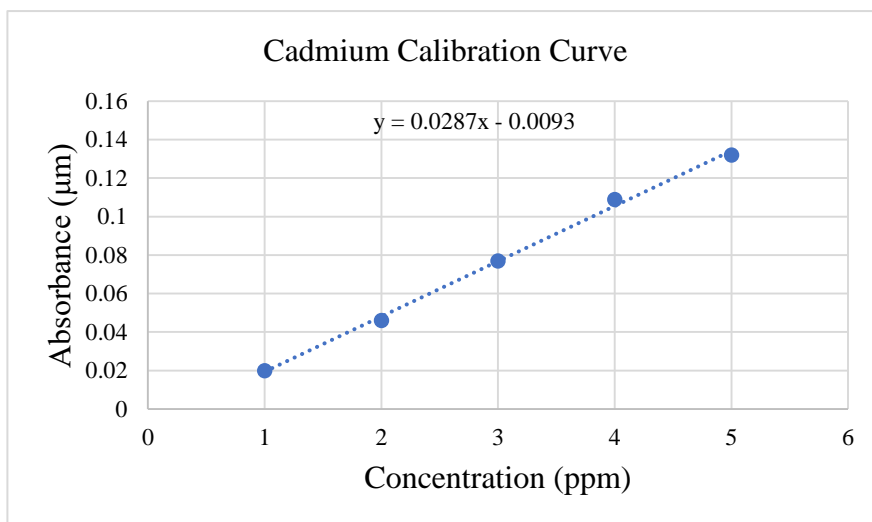
Figure. 8: Filtered Organ Samples

ANNEXURES II

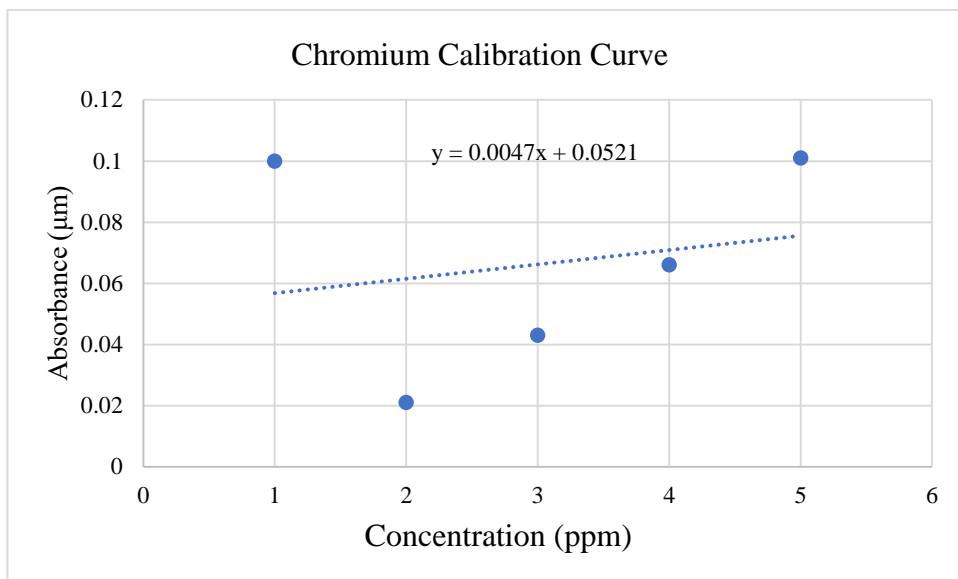
Table 1: Concentration of Heavy Metals in Stock Solutions

Sr. No	Sample	1 ppm	2 ppm	3 ppm	4 ppm	5 ppm
1.	Cadmium	0.02	0.046	0.077	0.109	0.132
2.	Chromium	0.10	0.021	0.043	0.066	0.101
3.	Nickel	0.005	0.011	0.017	0.023	0.029
4.	Cobalt	0.009	0.020	0.028	0.037	0.046
5.	Copper	0.001	0.003	0.045	0.060	0.075
6.	Manganese	0.003	0.007	0.011	0.015	0.018
7.	Zinc	0.061	0.125	0.190	0.261	0.289

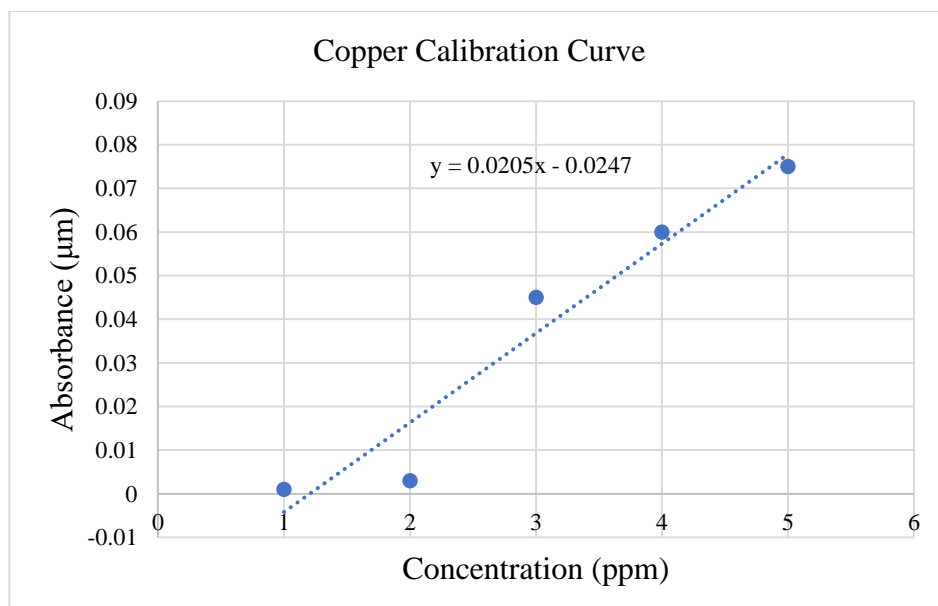
Calibration Curves of Stock Solutions:



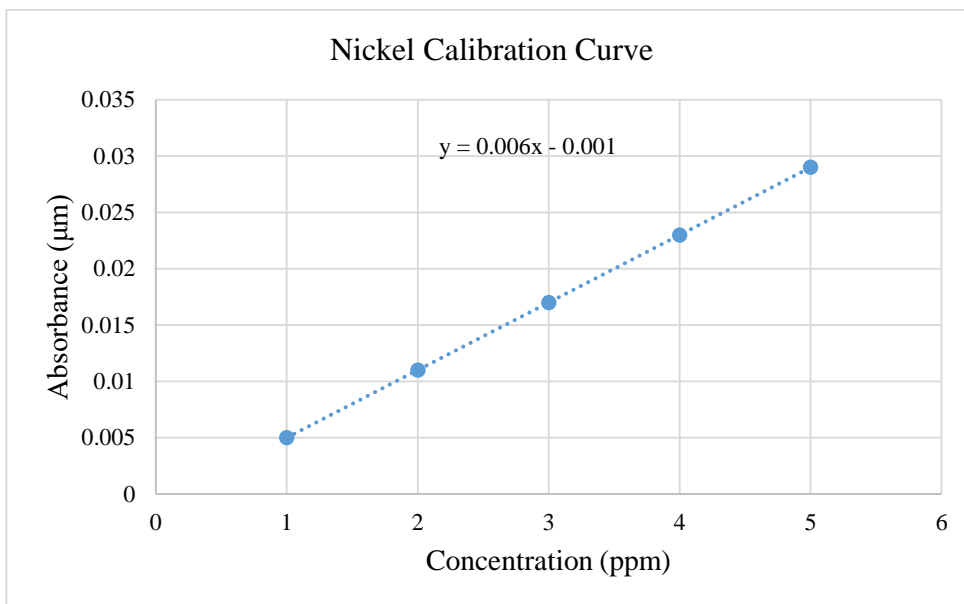
Graph 1: Calibration Curve of Stock Solution of Cadmium



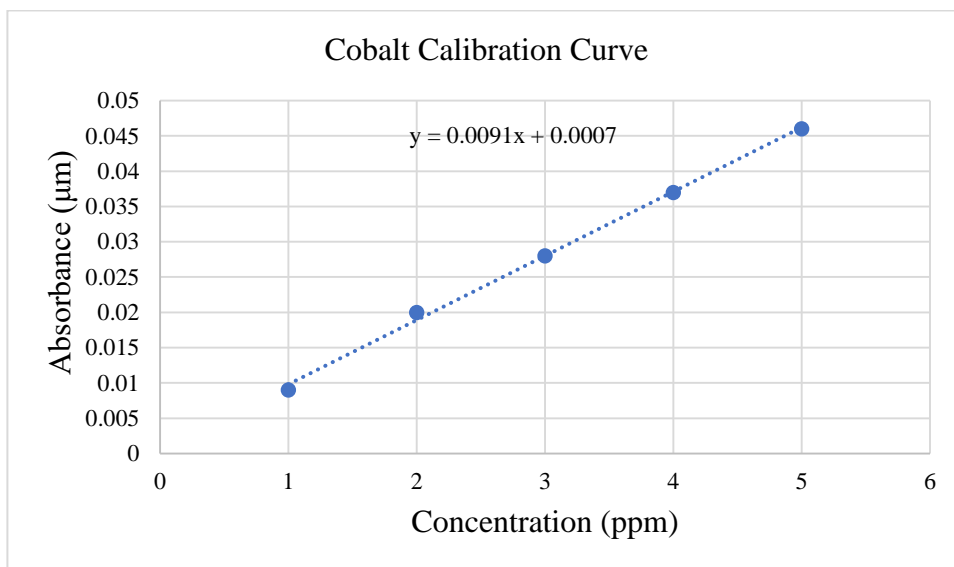
Graph 2: Calibration Curve of Stock Solution of Chromium



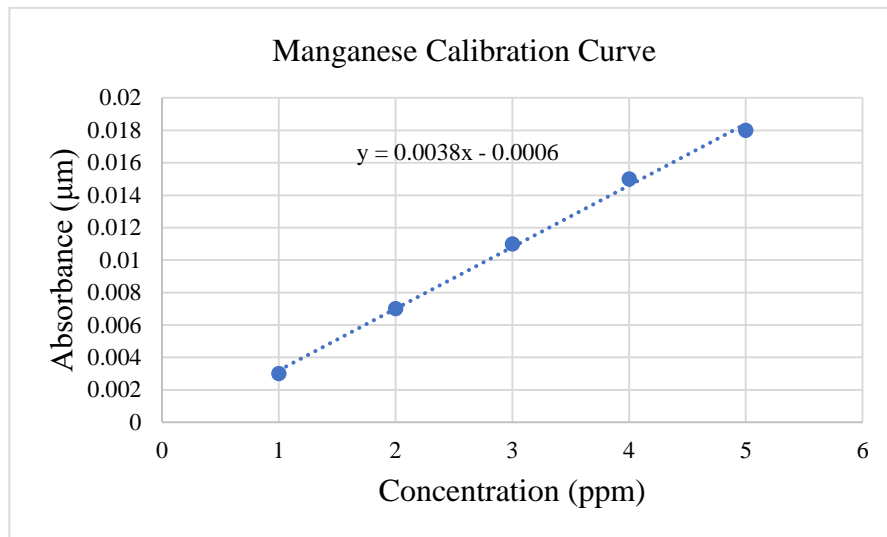
Graph 3: Calibration Curve of Stock Solution of Copper



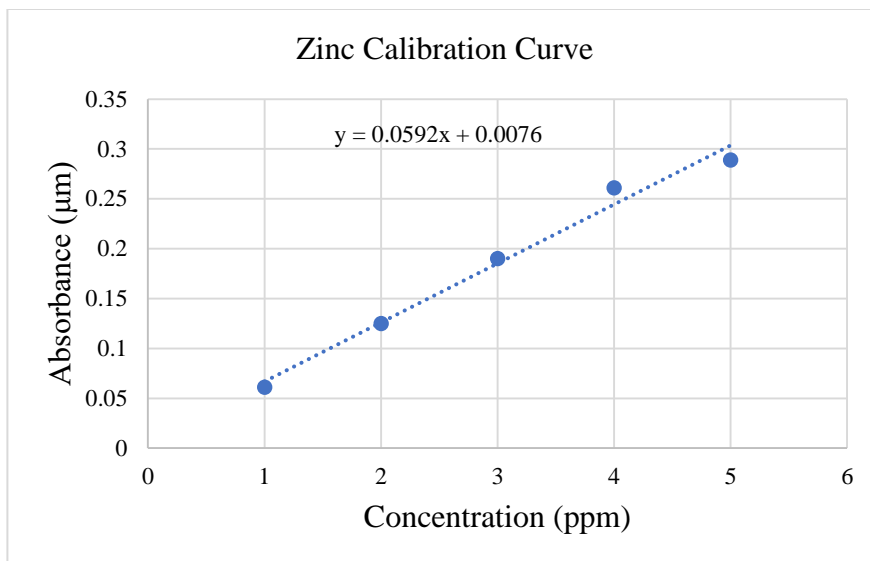
Graph 4: Calibration Curve of Stock Solution of Nickel



Graph 5: Calibration Curve of Stock Solution of Cobalt



Graph 6: Calibration curve of Stock Solution of Manganese



Graph 7: Calibration curve of Stock Solution of Zinc

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