# Bioaccumulation and toxicity of cadmium, chromium and lead to live fish food, the tubificid worms*(Tubifex spp.)*

A thesis submitted to the Department of Fisheries, University of Dhaka

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# Declaration by Student

I hereby declare that the dissertation entitled "Bioaccumulation and toxicity of cadmium, chromium and lead to live fish food, the tubificid worms(*Tubifex spp.*)" submitted to the Department of Fisheries, University of Dhaka for the degree of Master of Science (MS) is based on self-investigation carried out under the supervision of Mosammat Salma Akter, Assistant Professor, Department of Fisheries, University of Dhaka, Dhaka- 1000, Bangladesh.

I also declare that this or any part of this work has not been submitted for any other degree anywhere. All sources of knowledge used have been duly acknowledged.

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# Certificate

We certify that the research work embodied in this thesis entitled "Bioaccumulation and toxicity of cadmium, chromium and lead to live fish food, the tubificid worms(*Tubifex spp.*)" submitted by Md. Abedur Rahman, examination roll number: 807, session: 2015-2016, registration number: 2011- 812- 762 has been carried out under our supervision.

This is further to certify that it is an original work and suitable for the partial fulfillment of the degree of Master of Science (MS) in Fisheries from the Department of Fisheries, University of Dhaka.

We wish him every success in his life.

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The author,

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# Abstract

Tubificid worms, a popular live fish food are commonly found in the sediment-water interface in heavy metal contaminated habitats. These are also widely used as a test organism for evaluating aquatic environmental health. However, the toxic potential of metals can hinder the potential of these worms as safe live fish food as well as pose threat to other organisms through bioaccumulation in successive trophic levels. In the present study, acute toxicity and bioaccumulation of three commonly occurred heavy metals in polluted water, namely, cadmium ($Cd^{2+}$), chromium ($Cr^{6+}$) and lead ($Pb^{2+}$) were evaluated in *Tubifex spp*. The worms were exposed to various concentrations of these three metals in water only static acute toxicity tests for each metal separately. The concentration-mortality (%) data were analyzed through graphical analysis and Probit analysis to estimate the 96 h median lethal concentration ($LC_{50}$). The worms were subjected to sub-lethal concentration for 96 h and periodic accumulated metal were measured using Atomic Absorption Spectrophotometer. The worms showed various behavioral responses including increased movement, decreased clumping tendency and mucus secretion with increasing metal concentrations and exposure duration. Posterior part of the body was most affected resulting in random loss of hind parts and tail bifurcation at high concentrations. $Cd^{2+}$ was found to be the most toxic followed by $Cr^{6+}$ and $Pb^{2+}$ with $LC_{50}$values 0.0762, 1.4995, 1.8799µM respectively. In contrast, order of bioaccumulation (ppm) was $Pb^{2+} > Cd^{2+} > Cr^{6+}$. The accumulation increased up to 72 h and decreased afterwards which supports the responses and mortality trend observed. The current study confirms that metal toxicity to *Tubifex spp.*varies among different regions as reported in other studies and should be evaluated accordingly. This study found both the linear model from Probit analysis and 4 parameter sigmoid model are suitable ($R^2$>0.95) to predict the toxicity endpoints in *Tubifex spp.* The findings of this study can provide crucial information to establish water and sediment quality guidelines, and also can provide toxicological understanding essential in the mass production of *Tubifex spp.* as live fish food under polluted environment.

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# List of Abbreviations

| | | |
|---|---|---|
| µM | : | Micro molar |
| µS | : | Micro Simmens |
| AAS | : | Atomic Absorption Spectrometer |
| ASTM | : | American Society for Testing Materials |
| ATSDR | : | Agency for Toxic Substances and Disease Registry |
| BCSIR | : | Bangladesh Council of Scientific and Industrial Research |
| DO | : | Dissolved oxygen |
| DoF | : | Department of Fisheries |
| dw | : | Dry weight |
| $EC_{50}$ | : | Median effective concentration |
| $ED_{50}$ | : | Median effective dose |
| et al. | : | *et* alia(L), and others |
| etc | : | et cetra |
| FAO | : | Food and Agriculture Organization |
| GDP | : | Gross domestic product |
| i. e | : | That is |
| $LC_{50}$ | : | Median lethal concentration |
| $LD_{50}$ | : | Median lethal dose |
| MTLP | : | Metallotheonine like protein |
| OECD | : | Organization for Economic Cooperation and Development |
| pH | : | Puissance of hydrogen |
| ppm | : | Parts per million |
| SD | : | Standard deviation |
| SPSS | : | Scientific Package for Social Sciences |
| TDS | : | Total dissolved solid |
| USEPA | : | United States Environmental Protection Association |
| ww | : | Wet weight |

# Chapter 1 – Introduction

## 1.1 Background

The world's population is increasing day by day. The increasing population needs more protein as food. People obtain about 25% of their animal protein from fish and shellfish globally (Bahnasawy et al., 2009). The demand for food fish has increased over last 60 years worldwide (FAO, 2016).In 2014, about 87% (146.3 million ton) of estimated world fish production was used for direct human consumption (FAO, 2016). Apparent per capita fish consumption in the world increased from an average of 9.9 kg in the 1960s to 20.1 kg in 2014 (FAO, 2016).Only capture fisheries can't meet the demand of food fish. Moreover it leads to overfishing. To meet the demand of food fish and to save the fishes from being overfished, aquaculture has emerged as one of the most promising industries in the world. It also contributes to a considerable growth potential to improve human dietary standards by providing protein rich food and diversifying rural production and aquaculture potential (Gupta and Dhawan, 2013). In the last five decades global fish production through aquaculture has grown steadily from 55.7 in 2009 to 73.8 in 2016 (FAO, 2016). In 2014 total world aquaculture production was 167 million tons of which 44% came from aquaculture (FAO, 2016).

Bangladesh is one of the most important inland fishing nations where fish occupies a position in people's regular diet historically. The country's fisheries sector contributes 3.69% to the national GDP and 60% of animal protein (DoF, 2016). Bangladesh is ranked 4th in the world in inland culture fisheries (FAO, 2016). The fish production was 36,84,245 Metric Tons in 2015 (DoF, 2016). However, the fish production from open water bodies is decreasing day by day in the country (DoF, 2016).Along with the fishing mortality other anthropogenic cause including aquatic pollution is responsible for decreased fish production from rivers and estuaries (Ahmed et al., 2015). Various organic and inorganic pollutants including the heavy metals have destroyed the fish habitats (Ahmed et al., 2015). There has been studies on the pollution status of several rivers (Ahmed et al., 2015). The sediment compartment is vital for fish health. Contaminants are mainly associated to the fine

particulate material (both organic and mineral) in the sediments (Burton, Jr., 2002) and they can act as reservoir and source of contamination to the water column and aquatic biota. This fact can complicate the assessment of the relationship between contaminants exposure and toxicity. In an attempt to better understand the relationships between environmental chemical concentration and toxicity, an approach has been proposed based on body residue of the chemical in the exposed organisms and the toxic effects. So, monitoring the environmental health of sediment water interface using locally available organisms is necessary. Sediment toxicity test is useful to determine the toxic effects of metals to organisms that lives within the sediment water interface.

In Bangladesh, in contrast to the decreasing capture fisheries production, the population of the country has increased from 70.88 million in 1974 To 162.66 million in 2016 (Bangladesh Population (2017) - Worldometers, 2017)which has increased the demand for food fish production. Under the circumstances, the closed water or pond/tank aquaculture is increasing day by day due to meet the demand of the people (e-Jahan et al., 2010).The increased pressure to increase the production aquaculture is heading towards intensification (Zaki et al., 2012). For intensive aquaculture, fish require nutrient rich food for their better growth, efficient breeding and survival. Farmers are shifting gradually from no feed, through the use of farm-made feeds, to factory-made feeds (Zaki et al., 2012). The success of intensive and semi-intensive fish culture depends on a large extent to the application of suitable feeds. Different feeds are used in the fish farms and hatcheries in Bangladesh. A reliable and adequate supply of good quality seed of the desired species is essential to sustain the aquaculture industry, which in turn depends on the successful production and rearing of fish larvae. Besides formulated feeds, live foods are used in the hatcheries. Live food increases the growth and survival of juvenile catfishes and crustaceans, and helps in the raising of ornamental fishes (Proulx and de la Noüe, 1985). Supply of safe and healthy live food can play crucial role in the success of intensive aquaculture.

Use of live food in aquaculture depends mainly on their availability, ease of use, preference by the cultured species and price. Shrimp larvae are fed with *Artemia* as live food. Cost of *Artemia* is very high. *Tubificid* worms (Oligochaeta, Tubificidae), play the most important role in the rearing process of catfish larvae and carnivorous fish larvae. High food value (5,575 cal·g-1 on a dry weight basis) of tubificid worms makes them one of the best quality

live feed used in intensive aquaculture (Olaf, 1982). These worms have already been tested in commercial fish culture in the former Union of the Soviet Socialist Republic (Lietz, 1987). They are now being used across the world, including Bangladesh, as feed for ornamental aquarium fishes and for catfishes.

The tubificid worms grow in sewerage drains and canals where organic load is very high. In addition, it is hazardous due to the unhealthy conditions prevailing in the natural habitats. They can be mixed with heavy metals in the natural environment where tubificid worms are grown. The metals can be bioaccumulated in the tissues of tubificid worms. They can be toxic to the worms, even kill a whole population of the worms. Moreover, when fishes consume these metal accumulated worms, these metals also pass to the fish's body. This is called bio magnification. The metals cause various problems to the fish like endocrine disruption, brain damage, reproductive failure etc.

## 1.2 Tubificid worms

Tubificid worms are cosmopolitan genus of tubificid annelids that inhabits the sediments of lakes, rivers and occasionally sewer lines. At least 13 species of *Tubifex* have been identified, with the exact number not certain, as the species are not easily distinguishable from each other.

Tubificid worms are hermaphroditic: each individual has both male (testes) and female (ovaries) organs in the same animals. These minute reproductive organs are attached to the ventral side of the body wall in the coelomic cavity. In mature specimens, the reproductive organs are clearly found on the ventral side of the body. Although the tubificid worms are hermaphrodites, the male and female organs become mature at different times; thus self-fertilization is avoided, and cross-fertilization is encouraged. Two mature Tubificid worms undergo copulation by joining their ventral and anterior surfaces together with their anterior ends pointing opposite directions. Thus, the spermathecal openings of each worm is nearer to the male apertures of another worm. The pennial setae of one worm penetrate into the tissues of other worm and thus the conjugants are held together. At this stage, the sperm of one worm is passed into the spermathecae of the other worm. After copulation, they separate and begin to produce egg cases containing eggs, called cocoons. The cocoon is formed

around the clitellum as a soft, box-like structure into which the ova and the sperm are deposited. Soon, the Tubificid worm withdraws its body from the egg case by its backward wriggling movements.

### 1.2.1 Tubificid species

The genus includes the following species

1. *Tubifex blanchardi* (Vejdovský, 1891)
2. *Tubifex costatus*
3. *Tubifex ignotus* (Stolc, 1886)
4. *Tubifex kryptus* (Bülow, 1957)
5. *Tubifex longipenis* (Brinkhurst, 1965)
6. *Tubifex montanus* (Kowalewski, 1919)
7. *Tubifex nerthus* (Michaelsen, 1908)
8. *Tubifex newaensis* (Michaelsen, 1903)
9. *Tubifex newfei*
10. *Tubifex pescei* (Dumnicka 1981)
11. *Tubifex pomoricus* (Timm, 1978)
12. *Tubifex smirnowi* (Lastockin, 1927)
13. *Tubifex tubifex* (Mueller, 1774)

### 1.2.2 Tubificid worms as live fish food

Tubificid worms are often used as a live food for fish, especially tropical fish and certain other freshwater species. The tubificid worms have high food value (5,575 cal·g-1 on a dry weight basis), that's why tubificid worms are one of the best quality live feed used in intensive aquaculture (Olaf, 1982) They have been a popular food for the aquarium trade almost since its inception, and gathering them from open sewers for this purpose was quite common until recently. Most are now commercially obtained from the effluent of fish hatcheries, or from professional worm farms.

### 1.3 Heavy metals

Heavy metals are natural components of the earth's crust and they can enter the water and food cycles through a variety of chemical and geochemical processes (Tinsley, 1979).The term heavy metal is a general collective term which applies to group of metals and metalloids with atomic density greater than 4g/cm$^3$ or 5 times or greater than water (Duruibe et al., 2007), they are also known as trace elements because they occur in minute concentrations in biological systems. There are over 50 elements that can be classified as heavy metals, among them 17 are considered to be both very toxic and accessible. Toxicity level depends on the type of metal, its biological role and the type of organisms that are exposed to it.

#### 1.3.1 Cadmium (Cd)

Cadmium is an industrial and environmental pollutant that affects adversely a number of organs in humans. Cadmium is a metal from group II B that has an atomic weight of 112.41; the ionic form of cadmium ($Cd^{2+}$) is usually combined with ionic forms of oxygen (cadmium oxide, $CdO_2$), chlorine (cadmium chloride, $CdCl_2$), or sulfur (cadmium sulfate, $CdSO_4$). There are estimates that 30,000 tons of cadmium are released into the environment each year, with an estimated 4000–13,000 tons coming from human activities(ATSDR, 2012). Natural as well as anthropogenic sources of cadmium, which include industrial emissions and the application of fertilizer and sewage sludge to farm land, increased cadmium environmental levels (ATSDR, 2003b). It has been established that, although cadmium occurs in the aquatic organism and marine environment only in trace concentrations, the salinity can affect the speciation of this metal, and bioaccumulation is affected both by temperature and salinity(Ray, 1986).Cadmium has oxidation state of +2 and forms a number of inorganic compounds such as sulphates, chlorides and acetates most of which are water soluble. Ingestion of Cd can rapidly cause feelings of nausea, vomiting, abdominal cramp and headache, as well as diarrhoea and shock. Itai-itai disease in Japan was identified among people living in Cadmium-polluted areas where rice was irrigated. Target organs include liver, placenta, kidneys, lungs, brain and bones(Satarug et al., 2002).

### 1.3.2 Chromium (Cr)

Chromium has density of 7.2g/cm$^3$ and is the 21st most abundant element in Earth's crust with an average concentration of 100 ppm (van Rensburg et al., 2001). Chromium compounds are found in the environment, due to erosion of Cr -containing rocks, animals, plants, soil and can be a liquid, solid or gas. Cr can exist in valences of +3and +6 with oxidation state in Cr (III) being stable and give series of chromic compounds, like oxides ($Cr_2O_{3)}$, chlorides ($CrCl_3$) and sulphates ($Cr_2(SO_4)_3$) (Castro-González and Méndez-Armenta, 2008). Cr is used in metal alloys such as stainless steel, protective coatings of metal (electroplating), magnetic tapes, and pigments for paints, cement, paper, rubber and its soluble form is used in wood preservatives as well as additive in water to prevent corrosion in industrial and other cooling system (Hingston et al., 2001). Hexavalent Cr is very toxic and mutagenic when inhaled and is a known human carcinogen. Breathing high levels of the element in this form can cause irritation to the lining of the nose and breathing problems such as asthma, cough, shortness of breath, or long term exposure can cause damage to liver, kidney circulatory and nerve tissues, as well as skin irritation (Dayan and Paine, 2001). Cr is particularly dangerous as it can accumulate in many organisms, sometimes as much as 4 000 times above the level of the surrounding environment in aquatic algae (Laws, 2000).

### 1.3.3 Lead (Pb)

Lead has a density of 11.3g/cm$^3$ atomic number 82 and is obtained from its sulphide mineral galena, carbonate cerussite, and sulphate anglesite. The ores are frequently found in combination with other recoverable metals such as Cu, Zn and Cd. Lead exists in various oxidation states (O, I, II and IV), which are of environmental importance with oxidation +2, the form in which most Pb is bio-accumulated by aquatic organisms. Lead was placed position 2 on the Agency for Toxic Substances and Disease Registry's (ATSDR, 2007) top 20 list of most dangerous heavy metals and it accounts for most of the cases of pediatric heavy metal poisoning (ATSDR, 2007).Lead has been used in pipe making, drains and soldering materials as well as battery manufacture, plumbing, ammunition, fuel additives, paint pigments and pesticides (ATSDR, 2007).

Lead has been of particular concern due to its toxicity and ability to bioaccumulation aquatic ecosystems, as well as persistence in the natural environment. Lead is known to accumulate

in fish tissues such as bones, gills, liver, kidneys and scales, while gaseous exchange across the gills to the blood stream is reported to be the major uptake mechanism (Oronsaye et al., 2010). Some effects of Pb poisoning include deficiency in cognitive function due to destruction of the central nervous system, abdominal pain and discomfort, formation of weak bones as Pb replaces calcium and causes anaemia due to reduction of enzymes concerned with synthesis of red blood cells (Jarup and M, 2003).

Lead also leads to decreased fertility, causes cancer and other minor effects like vomiting, nausea, and headache (Jarup and M, 2003). Exposure to high Pb levels can severely damage the brain and kidneys, cause miscarriage in pregnant women, damage the organs responsible for sperm production in men and it may ultimately cause death (ATSDR, 2007). Since fish have ability to bioaccumulate metals for a long time, the level of metal ions at a particular time may not give accurate information on concentration at that particular time.

### 1.3.4 Environmental fate of metals and Bioaccumulation

Depending upon the concentration of the metal, the metal may exert beneficial or harmful effects on plant, animal and human life (Förstner, 1981). Some of these metals are toxic to living organisms even at low concentrations, whereas others are biologically essential and become toxic at relatively high concentrations. When ingested in excess amounts heavy metals combine with body's biomolecules, like proteins and enzymes to form stable biotoxic compounds, thereby mutilating their structures and hindering them from the bioreactions of their functions (Duruibe et al., 2007).

### 1.3.5 Heavy metal contamination of aquatic systems

In the last decades, contamination of aquatic systems by heavy metals has become a global problem. Heavy metals may enter aquatic systems from different natural and anthropogenic (human activities) sources. It includes industrial or domestic wastewater, application of pesticides and inorganic fertilizers, storm runoff, leaching from landfills, shipping and harbour activities, geological weathering of the earth crust and atmospheric deposition etc. The pollution of aquatic environment by heavy metals affects aquatic biota posse's considerable environmental risks and concerns. Compared with other types of aquatic

pollution, heavy metal pollutants less visible but its effects on the ecosystem and humans are intensive and very extensive due to their toxicity and their ability to accumulate in the biota (Shanmugam et al., 2007). The soluble forms are thought to be more dangerous because it is easily transported and more readily available to plants and animals

Bangladesh has a number of waterbodies. Everyday a huge amount of untreated domestic and industrial wastes is being discharged into open water bodies and its adjacent lands. The wastes carry heavy metals to the waterbodies. Fresh waterbodies like rivers and canals are mainly affected by anthropogenic pollution. Different studies shows heavy metal pollution in the waterbodies of Bangladesh.

Table 1. 1Concentrations of Heavy Metals water and sediment in various rivers in Bangladesh.Values presented as mean ±SD

| Name of the river | Heavy metal | Concentration of heavy metal | | Source |
|---|---|---|---|---|
| | | Water (µg/L) | Sediment (mg/kg dw) | |
| **Dhaleswari river** | Pb | 50.05±19.28 | 64.22±3.80 | (Ahmed et al., 2009) |
| | Cd | 6.49±0.87 | 3.23±0.61 | |
| | Cr | 441.34±42.48 | 117.56±19.57 | |
| **Korotoa river** | Cr | 83±27 | 118±50 | (Islam et al., 2015b) |
| | Pb | 35±19 | 63±16 | |
| | Cd | 11±8 | 1.5±0.77 | |
| **Shitalakkha river** | Cd | 7.12-10.11 | 1.71-2.17 | (Ahmed et al., 2010) |
| | Cr | 192.18-234.32 | 60.09-91.02 | |
| | Pb | 41.24-63.5 | 1.71-2.17 | |
| **Buriganga river** | Cr | 56.5-92.1 | 2039-2471 | (Islam et al., 2014) |
| | Cd | 5.0-5.9 | 26-29 | |
| | Pb | 2.9-8.1 | 1300-1802 | |

### 1.3.6 Fate of heavy metals in aquatic system and Bioaccumulation

In natural aquatic ecosystems, metals occur in low concentrations. They cannot be degraded, they are deposited, assimilated or incorporated in water, sediment and aquatic animals and thus, cause heavy metal pollution in water bodies (Abdel-Baki et al., 2011). Metals entering the aquatic ecosystem can be deposited in aquatic organisms through the effects of bio-concentration, bioaccumulation via the food chain process and become toxic when accumulation reaches a substantially high level. Heavy metals are dangerous because they tend to bioaccumulate. Bioaccumulation means an increase in concentration of a pollutant from the environment to the first organisms in a food chain. It means how pollutants enter a food chain of an organism. These chemicals accumulate in the tissues of aquatic organisms at concentrations many times higher than concentrations in water and may be biomagnified in the food chain to levels that cause physiological impairment at higher tropic levels and in human consumers (Simons and Raposo, 2009). In aquatic organisms, at the higher level of the aquatic food chain, substantial amounts of metals may accumulate in their soft and hard tissues. Pollutants enter into aquatic organisms through a number of routes: via skin, gills, oral consumption of water, food and non-food particles. Once absorbed, pollutants are transported in the blood stream to either a storage point (i.e. bone) or to the liver for transformation and/or storage. The bioaccumulation of heavy metals in living organisms and biomagnifications describes the processes and pathways of pollutants from one trophic level to another. Heavy metals can enter the food web through direct consumption of water or organisms taken as food (zooplankton, phytoplankton, and faunal of the bottom) or by uptake through the gills and skin and be potentially accumulated in edible fish in aquatic ecosystem.

In Bangladesh, aquatic organisms are accumulated with heavy metals due to pollution. Different studies shows the accumulation of heavy metals in the fishes, crustaceans, shellfishes etc.

Table 1. 2Heavy metal accumulation in fishes, crustaceans and shellfishes in Bangladesh. (Values presented as mean ±SD)

| Species | River | Heavy metal | Amount ( mg/kg ww) | Source |
|---|---|---|---|---|
| ***C. punctatus*** | Turag | Cr | 1.9-2.9 | (Islam et al., 2015a) |
| | | Cd | 0.008-0.013 | |
| | | Pb | 0.49-0.72 | |
| ***M. rosenbergii*** | Buriganga | Cr | 1.59±0.93 | (Ahmed et al., 2015) |
| | | Cd | 1.51±0.04 | |
| | | Pb | 0.51±0.01 | |
| ***L. marginalis*** | Meghna | Cr | 4.24±0.17 | (Ahmed et al., 2011) |
| | | Cd | 1.09±1.21 | |
| | | Pb | 10.06±0.09 | |
| ***H. fossilis*** | Buriganga | Cr | 4.01±0.04 | (Begum et al., 2013) |
| | | Cd | 1.85±0.13 | |
| | | Pb | 9.07±0.28 | |

### 1.3.7 Heavy metal toxicity

Toxicity is the degree to which a substance can damage an organism. Toxicity can refer to the effect on a whole organism, such as an animal, bacterium, or plant, as well as the effect on a substructure of the organism, such as a cell (cytotoxicity) or an organ such as the liver (hepatotoxicity). By extension, the word may be metaphorically used to describe toxic effects on larger and more complex groups, such as the family unit or society at large. Sometimes the word is more or less synonymous with poisoning in everyday usage. In biological systems, heavy metals have been reported to affect cellular organelles and components such as cell membrane, mitochondrial, lysosome, endoplasmic reticulum,

nuclei, and some enzymes involved in metabolism, detoxification, and damage repair (Wang and Shi, 2001). Several studies revealed that toxicity and carcinogenicity of metals such as cadmium, chromium and lead are very high (Omole et al., 2006; Patlolla et al., 2009; Tchounwou et al., 2001)

### 1.3.8 Toxicity Estimation

Acute toxicity describes the adverse effects of a substance that result either from a single exposure or from multiple exposures in a short period of time (usually less than 24 hours). To be described as acute toxicity, the adverse effects should occur within 14 days of the administration of the substance. Acute toxicity is distinguished from chronic toxicity, which describes the adverse health effects from repeated exposures, often at lower levels, to a substance over a longer time period (months or years).

It is widely considered unethical to use humans as test subjects for acute (or chronic) toxicity research. However, some information can be gained from investigating accidental human exposures (e.g., factory accidents). Otherwise, most acute toxicity data comes from animal testing or, more recently, in vitro testing methods and inference from data on similar substances.

Toxicity can be measured by measuring $LC_{50}$, $LD_{50}$, $EC_{50}$, $ED_{50}$ etc.$LC_{50}$ is the lethal concentration required to kill 50% of the population is killed in a given period of time. On the other hand, $LD_{50}$ is defined as the lethal dose at which 50% of the population is killed in a given period of time.The $EC_{50}$ is the concentration of a drug that gives half-maximal response.$ED_{50}$ is the "median effective dose". The dose that produces a quintal effect (all or nothing) in 50% of the population that takes it (median referring to the 50% population base). It is also sometimes abbreviated as the $ED_{50}$, meaning "effective dose, for 50% of people receiving the drug".

Bioassay tests have been used to establish the toxicity levels of compounds for aquatic organisms. Many types of bioassays are available and tests can be conducted in the laboratory or in the field and monitored manually or automatically.The Organization for Economic Cooperation and Development (OECD) has suggested a set of minimum data

needed to assess effects of chemicals in the environment. There are different types of bioassays for testing toxicity. Some of them are chemical bioassay, microbial bioassay, fish bioassay, plant and algal bioassay etc. Fishes, shellfishes, crustaceans etc can be tested under fish bioassay types.

### 1.4 Heavy metal toxicity and tubificid worms in literature

All aquatic invertebrates including tubificid worms accumulate trace metals in their tissues, whether or not these metals are essential to metabolism (Eisler, 1981). In addition, the tubificid worms are significant part of the detritus food chains. They feed on sediments which involves the intake of large amounts of substrate (Wang† and Matisoff, 1997). Tubificid worms have been used as a test organism for sediment bioassays (Wiederholm et al., 1987) and to assess the acute toxicity of various metals and organic compounds (Brković-Popović and Popović, 1977).

A Quality assurance or quality control program for any sediment bioassay should include methods to evaluate the sensitivity and quality of test-organisms. (Hoffman et al., 2003) have suggested that all toxicity sediment bioassays should include positive controls conducted with reference toxicants in the absence of sediment to provide insight into changes in organism sensitivity that may result from acclimation, disease, loading density or handling stress. Acute toxicity tests with reference substances also provide relevant information about the health of test organisms in culture, as well as data to compare the sensitivity of organisms from different laboratories (Rand, 1985). Routine acute toxicity tests using reference toxicants are recommended in several guidelines (ASTM, 1997; OECD, 2006) as acceptability requirements for chronic bioassays. Thus, when measuring the toxicity of sediment-associated contaminants with freshwater invertebrates ASTM (2005) recommends performing periodically 96-h water only reference toxicity tests to assess the sensitivity of culture organisms.

Due to their toxicity and accumulation in biota, determination of toxicity and the levels of heavy metals in tubificid worms have received attention in different countries in the region and around the world. Some of the important documented contributions relevant to the present study are as follows:

Redeker, (2004), studied the compartmentalization of cadmium and zinc in the oligochaete *Tubifex tubifex*. They followed subcellular distribution over time and measured levels of metallothionein-like proteins. They found the whole body tissue concentrations of cadmium was 0.0218µmol/g and zinc was 0.34 µmol/g. they also found that Cadmium accumulates in the metabolically available pool over time but is at the same time detoxified and/or stored but Zinc in the two pools differs from the distribution of cadmium. The majority of zinc was found in the metabolically available fraction.

Gillis et al., (2004) studied uptake and depuration of cadmium, nickel and lead in laboratory exposed *Tubifex tubifex* and corresponding changes in the concentration of a metallothionein like protein. *Tubifex tubifex* were exposed to sediment spiked with just Cd (3.66 mmol/g). They found that Cadmium uptake and induction of metallothionein-like protein (MTLP) were rapid. Metallothionein-like protein (8.7 6 1.8 nmol/g) and Cd (60.8 6 11.0 mmol/g) reached maximum concentrations after 96 h and four weeks, respectively.

Steen Redeker et al., (2007), studied accumulation and toxicity of Cadmium in the aquatic oligocheate *Tubifex tubifex* in a kinetic modeling approach. They analyzed toxicity of cadmium by determining the lethal exposure concentration associated with a mortality of 50% (LC50) at different time points and critical body concentrations (CBC) associated with 50% mortality were calculated by combining the model-predicted pharmacokinetic parameters and the measured LC50 values. They found the predicted mean CBC (0.32 µmol/g wet weight ±0.02) in good agreement with the experimentally obtained CBC for cadmium found in *T. tubifex* (0.37 µmol/g wet weight ±0.07) and it appeared to be independent of exposure time and exposure concentration. Thus a pharmacokinetic modeling approach provides a tool to link metal exposure to availability, accumulation, and toxicity under variable exposure scenarios taking into account the kinetics of the processes.

Dhara et al., (2014), studied acute toxicity of cadmium to benthic oligochaete worm, *Branchiura sowerbyi* and juvenile catfish, *Clarias batrachus*. They found the 96 h median lethal concentrations (with 95 % confidence limit) of cadmium for *B. sowerbyi* and juvenile catfish *C. Batrachus* were 15.98 (10.78–20.82) and 29.39 (23.70–33.42) mg/l respectively.

Rathore and Khangarot, (2002) studied the effects of temperature on the sensitivity of *Tubifex tubifex* to selected heavy metal ions. Metals used in this study were cadmium, chromium, cobalt, copper, iron, lead, manganese, mercury, nickel, and zinc. They studied

acute toxicity of these heavy metals at 15, 20, 25, and 30℃. They determined percentage mortality, relative toxicity, and $EC_{50}$ values and their 95% confidence limits from 24 to 96 h at varying temperatures. The EC50 values (mg/liter) of metal ions at 15℃ were $Hg^{2+}$, 0.034; $Cu^{2+}$, 0.340; $Cr^{6+}$, 1.846; $Zn^{2+}$, 10.99; $Ni^{2+}$, 25.10; $Cd^{2+}$, 56; $Fe^{3+}$, 86.09; $Co^{2+}$, 239.39; $Pb^{2+}$, 456.76; and $Mn^{2+}$, 164.55. At 30℃ the values were $Hg^{2+}$, 0.014; $Cu^{2+}$, 0.031; $Cr^{6+}$, 0.872; $Zn^{2+}$, 3.37; $Ni^{2+}$, 18; $Cd^{2+}$, 28.55; $Fe^{3+}$, 71.26; $Co^{2+}$, 95.35; $Pb^{2+}$, 165.22; and $Mn^{2+}$, 239.39. The results indicate that the acute toxicity of cadmium, chromium, cobalt, copper, lead, mercury, nickel, and zinc increases with temperature increase. The toxicity of manganese was not influenced by temperature, and temperature had little effect on iron toxicity. The study indicates that seasonal temperature changes are an important variable in determining the amount of heavy metals that may be safely released from metal industries and other similar sources into the aquatic environment.

Méndez-Fernández et al., (2013) studied toxicity and critical body residues of Cd, Cu and Cr in the aquatic oligochaete *Tubifex tubifex* based on lethal (LBR) and sublethal (CBR) effects. They estimated $LC_{50}$, $EC_{50}$, $LBR_{50}$ and $CBR_{50}$ for each metal by means of data on survival and on several sub-lethal variables measured in short-term (4 days), water-only exposures and in long-term, chronic (14 and 28 days) exposures using metal-spiked sediment. They estimated $LC_{50}$ values for Cd, Cu, Cr on tubificid worms were 11.75, 0.79, 697.87 µmol/L respectively and $CBR_{50}$ values for Cd, Cu, Cr on tubificid worms were 60.4, 6.76, 0.50 µmol/g dw respectively. They found $LBR_{50}$ and $CBR_{50}$ were 3–6 times higher in sediment than in water-only exposure to Cd and about 2–11 times higher for Cu, depending on the measured endpoint; for Cr these parameters varied only by a factor of 1.2. They found in the metal-spiked sediments, 28 d $CBR_{50}$ values for autotomy, reproduction and growth ranged 6.76–29.54 µmol g-1 dw for Cd, 3.88–6.23 µmol g-1 dw for Cu and 0.65 µmol g-1 dw for Cr.

Trojner et al., (2014) studied bioaccumulation and purification of cadmium on *Tubifex tubifex*. They studied the comparision of accumulation of cadmium (Cd) in *Tubifex* worms (*Tubifex tubifex*) during exposure to different doses of cadmium (0.9 and 2.5 mg/kg) in bottom sediments and in water (0.9 and 2.5 mg/l). They examined the elimination of Cd from these invertebrates by prior exposure also. They analyzed Cd concentration in water, sediments and worms by Atomic Absorption Spectroscopy (AAS) method. They found that

*Tubifex* worms are more sensitive to Cd concentration in water (Concentration factors from 16 to 60) than bottom sediments (CF from 0.44 to 0.77). They also found *Tubifex tubifex* rapidly reduced Cd concentration (from 28.5 to 0.13 mg/kg) in 3 days after exposure cessation.

**1.5 Rationale**

Cadmium (Cd), chromium (Cr) and lead (Pb) are the most deleterious and used extensively in industries. As stated in the above sections these metals discharged from various industries in Bangladesh are carried through discharge channels towards the rivers. These metals readily bio-accumulate in invertebrate organisms such as tubificid worms (Bouche *et al.*, 2000), which pose a risk to the organisms high up in the food chain; especially the non-predatory fish form is toxic. Although the toxicity of different heavy metals on tubificid worms have been assessed worldwide, but such studies yet not done in Bangladesh. Previous literature also showed that toxicity varies depending on culture/habitat condition and other environmental factors. Moreover, the toxicity endpoints of metals to tubificids in existing literature are variable. If it is studied in Bangladesh, it would help comparing results from other parts of the world and in Bangladesh. Bioaccumulation of different heavy metals has been studied on different phytoplanktons and zooplanktons and also on tubificid worms using spiked sediment bioassay. But it has never conducted using water only exposures. However, bioaccumulation studies also yet not done in Bangladesh. Despite the potential transfer of metals through the use of contaminated tubificid worms as fish food. The growth of tubificid worms as live fish food rises the necessity of assessment of potential bioaccumulation and transformation through food chain. Therefore, assessment of metal bioaccumulation in tubificid worms is required before using as live food to fishes in Bangladesh. So, metal toxicity and bioaccumulation on tubificid worms under local environmental condition can be useful information for environmental monitoring and risk assessment of using tubificid worms as live fish food in economically and nutritionally crucial aquaculture sector.

## 1.6 Objectives

The overall objective of the present study was to estimate the toxicity and bioaccumulation of Cd, Cr and Pb to tubificid worms.

The specific objectives of the present study was to-

i. evaluate the behavioral response of tubificid worms to various concentrations of $Cd^{2+}$, $Cr^{6+}$ and $Pb^{2+}$;
ii. observe the morphological characteristics of tubificid worms exposed to $Cd^{2+}$, $Cr^{6+}$ and $Pb^{2+}$;
iii. estimate the median lethal concentration ($LC_{50}$) of $Cd^{2+}$, $Cr^{6+}$ and $Pb^{2+}$on tubificid worms;
iv. estimate the bioaccumulation of $Cd^{2+}$, $Cr^{6+}$ and $Pb^{2+}$ in tubificid worms.

# Chapter 2 - Materials and Methods

## 2.1 Experimental organism

Tubificid worms were the experimental organism for this study. The organisms were identified based on morphological characteristics upto genus.

Kingdom: Animalia

Phylum: Annelida

Class: Clitellata

Order: Oligochaeta

Family: Naididae

Subfamily: Tubificinae

Genus: *Tubifex* (Lamarck, 1816)

Figure 2.1A tubificid worm (*Tubifex spp.*)

### 2.1.2 Collection of tubificid worms

The tubificid worms were bought from Katabon Aquarium fish market, Dhaka, Bangladesh. The worms were collected from pre-contacted person to ensure that the worms were not collected from any highly polluted place. Immediately after collection the worms were brought to the laboratory in a plastic bag with sufficient water. The worms were investigated under light microscope to identify the upto the genus and to ensure the absence of other worms except from *Tubifex spp.*

### 2.1.3 Identification of tubificid worms (*Tubifex spp.*)

Tubificid worms were identified based on morphometric characteristics under light microscope measuring body color, segments, number, size and shape of setae, number and position of gonad according to (Brinkhurst, 1984).

### 2.1.4 Acclimatization

The tubificid worms were gently washed in a plastic box with tap water and kept in 1L plastic box with running water flow in the laboratory for 24 hours for acclimatization. During the acclimatization the worms were kept under continuous water flow to ensure wash out of the wastes and excretion of the worms. Water flow also provided required oxygen for the tubificid worms. Moreover, running water flow helped in detoxification of the worms from any possible significant pre-accumulated metals. No feed were provided during this period.

Figure 2.2Acclimatization of tubificid (*Tubifexspp.*) worms with water flow

**2.2Place of experiments**

Acute toxicity tests were done on the Aquatic laboratory of Department of Fisheries, University of Dhaka.

Test for bioaccumulation of heavy metals were doneat Zoology section laboratory, Biological Research Division,Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka, Bangladesh.

Heavy metal concentrations were measured in the Soil and Environment Section laboratories, Biological Research Division, Bangladesh Council of Scientific and Industrial Research (BCSIR), Dhaka, Bangladesh.

**2.3 Water quality estimation**

Water temperature, DO, pH, conductivity and total dissolved substance (TDS) were measured daily during both acute toxicity tests and bioaccumulation tests. DO meter (HANNA), pH meter (HANNA) and conductivity meter (HANNA) were used in water quality estimation.

**2.4 Acute toxicity test**

Water only exposure tests were done for estimation of the median lethal concentration ($LC_{50}$) of Cadmium ($Cd^{2+}$), Chromium ($Cr^{6+}$) and Lead ($Pb^{2+}$). Each tests were conducted for 96 hours. Several concentrations were selected on the basis of previous literature and trial exposures.

**2.4.1 Preparation of stock solutions**

Chromium's stock solution was prepared from $K_2Cr_2O_7$ (MERCK, India). Molar weight of $K_2Cr_2O_7$ is 294.19 g/mol. The prepared stock solution was 5000 µM for acute toxicity tests. And for bioaccumulation tests, 1000 µM stock solution wereprepared. From the stock solutions, a dilution series was prepared. Distilled water was used to prepare stock solution.

Cadmium's stock solution was prepared from $CdSO_4$ (SIGMA ALDRICH, India). Molar weight of $CdSO_4$ is 208.47 g/mol. The prepared stock solution was 1000 µMfor acute toxicity tests. And for bioaccumulation tests, 500 µM stock solution wereprepared. From

this stock solution, a dilution series was prepared. Distilled water was used to prepare stock solution.

Lead's stock solution was prepared from $Pb(NO_3)_2$ (SIGMA ALDRICH, India). Molar weight of $Pb(NO_3)_2$ is 331.21 g/mol. The prepared stock solution was 20000 µMfor acute toxicity tests. And for bioaccumulation tests, 10000 µM stock solution wereprepared. From this stock solution, a dilution series was prepared. Distilled water was used to prepare stock solution.

### 2.4.2 Determination of Exposure Concentrations

Trial exposure concentrations (APPENDIX-I) of $Cd^{2+}$, $Cr^{6+}$, $Pb^{2+}$ were determined by reviewing previous literature. After observing the condition of the worms in the trial exposures, final exposure concentrations were calculated as presented in the Table 2.1.

**Table 2. 1Exposure concentrations of $Cd^{2+}$, $Cr^{6+}$, $Pb^{2+}$ (µM) used in the water only toxicity tests determined by primary trails based on literature.**

| Name of the metal | Concentration of metals (µM) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Cd | 0.00 | 0.02 | 0.04 | 0.06 | 0.08 | 0.10 | 0.20 | 0.30 |
| Cr | 0.00 | 0.20 | 0.40 | 0.80 | 1.60 | 3.20 | 6.40 | 10.00 |
| Pb | 0.00 | 0.50 | 1.00 | 1.50 | 2.00 | 3.00 | 4.00 | 5.00 |

### 2.4.3 Exposure to chemical

Healthy and similar sized worms were carefully selected from the pre-acclimatized worm stock through visual observation. The worms were considered healthy when it was of dark red colour in appearance and moving spontaneously in the water. The selected worms were placed in a petri dish with distilled water. 25 worms were picked using a dropper and kept in 250 mL glass beaker with small volume of distilled water (Figure). Care was taken to minimize the transfer of water from petri dish to beaker. Each beaker with 25 worms was then filled with distilled water and required volume of stock solution of chemical for each exposure concentration (Figure). The final volume of solution for each worm was 10 mL as

used by Mendez *etal,.*2013. A control group was maintained along with the exposure concentrations where worms were kept in distilled water only. 2 replicates were maintained for each exposure concentration and control group.

Plate 2. 1Experimental setup for acute toxicity tests

### 2.4.4 Mortality estimation

Mortality of the worms was observed by both naked eye observation and on compound electronic microscope. Mortality was observed and recorded (APPENDIX II) regularly in every 8 hours during the exposure. A worm was considered to be dead when there was no response in 10 s after a slight disturbance with a bar(Rand, 1985). Dead worms were taken away from the beakers using dropper.

### 2.4.5 Median Lethal Concentration ($LC_{50}$) analysis

The 96 h Median Lethal Concentration ($LC_{50}$) was calculated from the 96 h cumulative mortality (%) using two different methods, i.e., through graphical analysis and Probit analysis.

#### 2.4.5.1 Graphical analysis

The exposure concentration and mortality (%) data were plotted using the graphical software SIGMAPLOT 10. The curve was tested with different fit models to find the best fit. The 4

parameter sigmoid curve was found to be the best fit model with the data. The best fit models were determined by the respective $R^2$ values. The 4 parameter sigmoid curve yielded equation (1) for each of the metals which was used to estimate the $LC_{50}$ of each metal.

$$f = y_0 + \frac{a}{1+e^{\frac{-(x-x_0)}{b}}} \quad \text{.....(1)}$$

Where, $f$= Mortality (%)

$x$ = concentration of the metal

$a$ = parameter 1

$b$ = parameter 2

$x_0$= parameter 3

$y_0$= parameter 4

The descriptions of parameters and fit equation are presented in APPENDIX-III.

**2.4.5.2 Probit analysis**

The exposure concentration and mortality (%) data were input into the PROBIT ANALYSIS software (Dr.Alpha Raj.M, MVSc, PhD Assistant Professor, Veterinary Pharamcology & Toxicology, SVVU, India. alpharajm@gmail.com) to obtain the various $LC_n$ values along with their plots and goodness of fit coefficients. The following steps were used in the calculation of LC in this method.

1. Converting doses to log (10) doses (x)
2. Converting mortality to proportions
3. The proportions are corrected for control mortality if it is more than 10% using Schneider-Orelli's (1947) formula:

$$\text{Corrected mortality, P} = \frac{\%\ \text{Responded} - \%\ \text{Responded in Control}}{100 - \text{Responded}\ \%\ \text{in Control}} \times 100$$

4. Converting corrected proportions (p) to empirical probits (y).
   A dose response curve is drawn using the $\log_{10}$ doses (x) and empirical probits (y) and the regression equation is derived (y=5+(x-μ)/s). .Empirical probits less than 1 and more than 7 are ignored as they have little and no significance in the estimation of LC (Hayes, 2014).
5. From the equation of the curve and log10 doses, the expected probits (Yi) are derived
6. From the expected probits (Yi), expected mortality proportion followed by expected no.of animals are derived
7. The original mortality (Observed) and derived mortality (Expected) are used to calculate the Chi-Square test with (No. of log doses used -2) degrees of freedom. If the Chi-square test is non-significant, it indicates good curve fitting.
8. Z value is derived using the formula,Z=1/(v2p)e(-1/2(Yi-5)^2)
   Where, Yi = Expected probits
9. The weighting coefficents (W) are derived using the formula,W=Z^(2 )/PQ,
   Where, P = Expected proportion and Q=(1-P)
10. The weighted coefficients were used to calculate the standard error,SE= s/v?nW
    Where, s = Standard deviation (1/slope)
    n= number of animals in each group
    W= Weighting coefficient
11. Working probits (Yw) are derived from the regression equation,Yw = Yi-(P/Z)-p/Z
    Where, Y = Expectedl probits;
    P = Expected Proportion;
    p = Observed proportion.
12. The LD or LC values are derived from the curve drawn using working probits and log doses. Antilog of the dose corresponding to respective probit value.
13. 12. 95% Fiducial confidence limits are calculated using the formula

    Fiducial Limits =Antilog (Log10 Dose ± 1.96 (SE)

### 2.4. 6 Observation of behavioral response and physical conditions

Behavioral response and physical condition of the worms was observed by both naked eye observation and on microscope. Mucous secretion, movement of the worm, clumping tendency etc behavioral response of the tubificid worms were observed at all the concentrations at different time period. Autotomy, degeneration of the hind part of the body of the worms etc were seen in different concentrations. The worms were taken on a slide and physical conditions of the worms were observed on microscope.

### 2.5 Bioaccumulation of metals in tubificid worms

Water only toxicity test was followed to determine bioaccumulation of Cd, Cr and Pb on the tubificid worms. Metal concentrations were tested at regarded as endpoints of acute toxicity tests at 24, 48, 72 and 96 h exposure. One-fourth of $LC_{50}$ values were taken as the exposure concentration for bioaccumulation tests were followed by (Méndez-Fernández et al., 2013)

### 2.5.1 Exposure set-up

Healthy and pre-acclimatized worms were exposed to a sub-lethal concentration based on the estimated $LC_{50}$ concentration ($1/4^{th}$ of $LC_{50}$) along with a control group in distilled water in 2L glass beakers. Each beaker consists of 200 worms. 10 ml solution was given for each worm as the acute toxicity test. Worms were collected after 24, 48, 72 and 96 hours of exposure.

### 2.5.2 Preparation of samples

After each exposure, the samples were filtered at sieve. The water was totally drained. Fresh weight of the samples were taken using electronic balance. More than 1g samples were taken in each plastic sample bags and freeze at -20℃ until the samples to be digested for metal analysis.

Plate 2.2Tubificid worm (*Tubifexspp.*) samples collected after exposure for bioaccumulation studies

### 2.5.3 Digestion of the samples

About 1g wet sample was taken in 50 ml beakers. 5 ml $HNO_3$ were added to each sample. Each beaker was covered by cap. The samples were kept on hot plate at 60℃ for 2 hours. Then the caps were removed and the solution of samples kept on hot plate for another 1hour. When the solution was almost dried, they were taken off from hot chamber and cooled. Then volume of solution was change into 25 ml with distilled water and filtered. The filtered solution was kept in a plastic sample bottle for analysis on AAS.

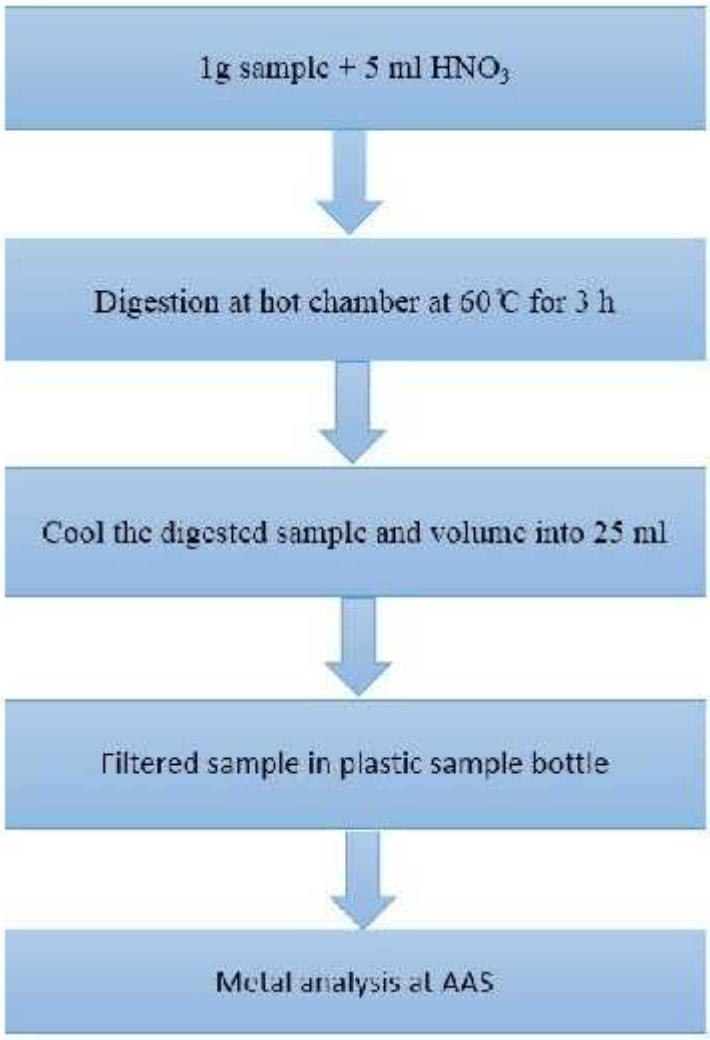


Figure 2.3The overall procedures of metal analysis in the sample using AAS.

Plate 2.3Samples on hot chamber for digestion

Plate 2.4digested samples to be analyzed on AAS

### 2.5.4 Estimation of metal content in the tubificid worms

Flame atomic absorption spectrophotometer is very common technique for detecting metals and metalloids in environmental samples. It is very reliable and simple to use. The technique is based on the principle of ground state metals absorbing light at specific wave length. Metal ions in a solution are converted to atomic state by means of a flame. Light of the appropriate wave length is supplied and the amount of light absorbed can be measured against a standard curve. The technique makes use of absorption spectrometry to assess the concentration of an analyte in a sample. It requires a standard with known analyte content to establish the relation between the measured and the analyte concentrations and relies on Beer Lambert's law (Skoog *et al*., 2005; Christian, 2005).

Plate 2.5AAS machine at Soil and Environmental Laboratory at BCSIR

The sample is converted into atomic vapours by a process known as atomization. The precision and accuracy of this method depends on the atomization step and therefore a good choice of the atomization method is required. The two types of atomizers are continuous and discreet atomizers. In continuous atomizers the sample is fed into the atomizer continuously at a constant rate giving a spectral signal which is constant with time. Atomization methods that are of continuous type are flame, inductively coupled argon plasma and direct current argon plasma. With the discrete atomizers, a measured quantity of a sample is introduced as a plug of liquid or solid. The spectral signal in this case rises to a maximum and then decreases to zero. An electro thermal atomizer is one of the discretetypes. The atoms then absorb radiations of characteristic wavelengths from an external source. The atoms of lead, nickel, copper, iron, cadmium and chromium, absorb radiations of wavelengths of 217.0 nm, 232.0 nm, 324.8nm, 248.3 nm 228.8nm and 357.9nm, respectively from an external source which is usually a hollow cathode lamp. This technique has been widely employed for elemental analysis in a number of matrices such as soils, water, nuts, wine and wine products (Narin et al., 2000).

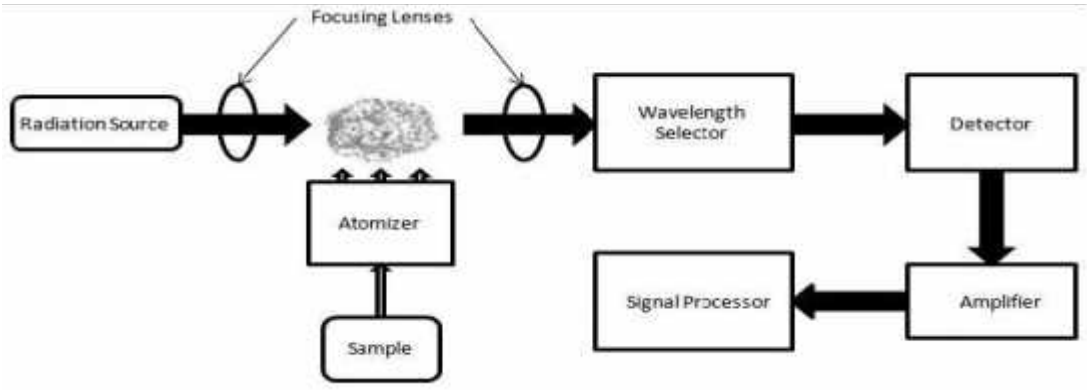


Figure 2.4Schematic diagram of AAS equipment

Figure shows a schematic diagram for the components of AAS. The two sources of radiation are continuous source which makes use of deuterium and mercury lamps and a hollow lamp which consists of an anode made of either tungsten wire or wink and a hollow cathode made of either the element of interest or its own salt. Flame atomization method consists mainly of a fuel and oxidant. Their temperatures are determined by flow rate and ratio of oxidant and fuel while the electro thermal atomizer is basically made of carbon rods. The free atoms are vaporized from the carbon atomizer into the optical light path to a monochromator which

presents a monochromatic radiation to the detector. The radiations from the monochromators are received by detectors which converts them to electrical signals. Some commonly used detectors are photocells and photo multiplier tubes.

a. Radiation source (Hollow cathode lamp)

This is the source of analytical light line for the element of interest and gives a constant and intense beam of that analytical line.

b. Atomiser (Flame)

The atomiser will destroy any analyte ions and break complexes to create atoms of the element of interest.

c. Wavelength selector (Monochromator)

A wavelength selector isolates analytical line photons passing through the flame and remove scattered light of the other wavelength from the flame. This only impinges a narrow line on the photomultiplier tube.

d. Detector (Photomultiplier tube (PMT))

It determines the intensity of the analytical line exiting the monochromator. The PMT is the most commonly used detector for AAS.

### 2.5.5 Sample analysis

Analysis of the heavy metal content of the samples was performed with a flame atomic absorption spectrophotometer (Model Shimadzu AA-7000) using acetylene gas as fuel and air as an oxidizer. Digested samples were aspirated into the fuel-rich air acetylene flame and the metal concentrations were determined from the calibration curves obtained from standard solutions. Each determination was based on the average values of three replicate samples.

### 2.5.6 Analytical technique

Trace elements relate to the very small amounts of the analyte found in the sample which required special instrumental techniques to be determined. Not long ago, trace levels were around µg/g levels, nowadays concentration levels are ranging from µg/g to ng/g or lower. On the other hand, one element at a high concentration in a sample can be considered as a trace in another. The analytical technique used to determine heavy metal levels in all samples was thermoelement Solar S4 Atomic Absorption Spectroscopy (International Equipment Trading Ltd, USA). It is a standard laboratory analytical tool for metal analysis and is based on the absorption of electromagnetic radiation by atoms. The absorption wavelengths and detections limits for the heavy metals were 217.0 nm and 0.001ppmfor Pb, 228.8 nm and 0.002 ppm for Cd , 324.7nm and 0.02 ppm for Cu and 232.0 nm and 0.01 ppm for Ni.

The key feature is the production of free, ground state atoms from the sample, whichpass through the light beam from the hallow cathode lamb. For many conditions the absorption of radiation follows Beer's law:

$$A = abc$$

Where, $A$ is the absorbance, $a$ is the absorptivity, $b$ is the bath-length of absorption and $c$ is the concentration of the absorbing species.

Beer's law shows a relation between absorption and concentration of analyte, so calibration of the instrument is needed.

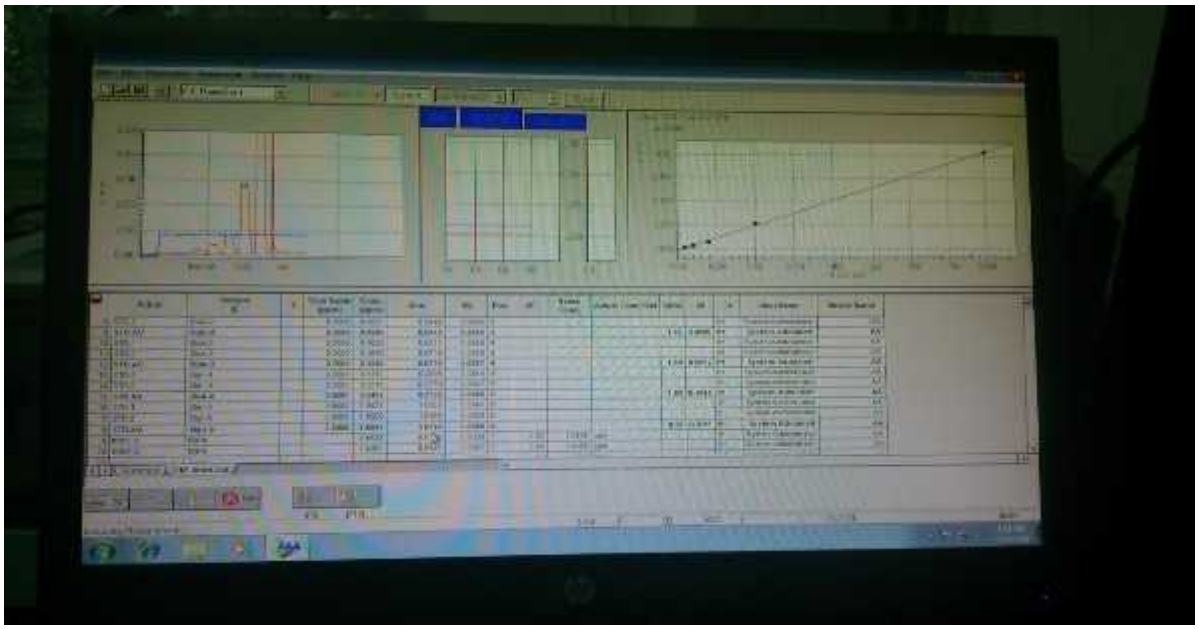

Figure 2.5: standard curve from standard solution

### 2.5.7 Calibration of instrument

Calibration requires the establishment of a relationship between signal response and known set of standards. The standards in atomic absorption spectrometry refer to the production of a series of aqueous solutions of varying concentrations (working standards) of the analyte of interest. By measuring the signals for a series of working solutions of known concentrations it is possible to construct a suitable graph. Then, by presenting a solution of unknown concentration to the instrument, a signal is obtained which can be interpreted from the graph, thereby determining the concentrations of the element in the unknown.

The actual concentration of each metal was calculated using the formula:

Actual concentration of metal in sample = (mg/g)R × dilution factor

Where:

(mg/g)R = AAS Reading of digest- blank reading

Dilution Factor = Volume of digest used / Weight of digested sample

**Table 2. 2 The AAS operating conditions**

| Operating parameters | Pb | Cd | Ni | Cr | Fe | Cu | Na |
|---|---|---|---|---|---|---|---|

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Wavelength (nm) | 283.2 | 228.9 | 232.2 | 357.9 | 248.3 | 324.8 | 589.6 |
| Flame type | Air Acetylene | | | | | | |
| Oxidant flow rate (l/min) | 1.5 | | | | | | |
| Sensitivity (ppm) | 0.11 | 0.011 | 0.066 | 0.055 | 0.055 | 0.011 | 0.015 |
| Detection limit (ppm) | 0.02 | 0.0006 | 0.008 | 0.005 | 0.05 | 0.008 | .0053 |
| Lamp current (mA) | 6 | 3 | 5 | 5 | 3 | 3 | |

**2.6 Precautionary measures**

All glasswares were soaked in 10% $HNO_3$ and distilled water before each exposure. Mask and hand gloves were worn during preparation of stock solution. All the chemicals handled carefully that they couldn't harm other animal or organisms.

**2.7 Data analysis**

The mortality data were analyzed using both SIGMAPLOT 10, SYSTAT, Inc and CALCULATION OF $LC_{50}$ USING PROBIT ANALYSIS, (Dr.Alpha Raj.M, MVSc, PhD Assistant Professor, Veterinary Pharamcology & Toxicology, SVVU, India. alpharajm@gmail.com). The dose response data were analyzed using IBM SPSS 21, (SPSS, USA). Two way ANOVA followed by homogeneity test, LSD and Tukey HSD Post Hoc Tests were done to analyze the mortality data obtained from acute toxicity tests using IBM SPSS 21,(SPSS, USA).

# Chapter 3 – Results

## 3.1 Physico-chemical properties of the exposure medium

The physico-chemical properties of the exposure medium (temperature, dissolved oxygen (DO), pH, conductivity, total dissolved substance (TDS)) did not vary during the experimental period as presented in Table 3.1

Table 3. 1Physico-chemical properties of the exposure medium (mean±SD)

| Physico-chemical properties | $Cd^{2+}$ | $Cr^{6+}$ | $Pb^{2+}$ |
|---|---|---|---|
| DO (mg/L) | 6.67±0.01 | 6.13±0.02 | 6.35±0.15 |
| pH | 6.72±0.02 | 6.71±0.01 | 6.74±0.01 |
| Temperature (℃) | 24.63±0.21 | 24.70±0.20 | 24.5±0.53 |
| Conductivity (µS) | 75.5±0.26 | 75.87±0.32 | 75.43±0.15 |
| TDS (ppm) | 48.3±0.17 | 48.67±0.23 | 48.27±0.11 |

## 3.2 Behavioral responses of the worms to metal exposure

Tubificid worms exposed to different concentrations of $Cd^{2+}$, $Cr^{6+}$ and $Pb^{2+}$ showed variable behavioral responses compared to the control groups. Change in degree of movement, clumping tendency and mucous secretion were most commonly observed responses. In control group, the worms were found to be clumped to each other in the form of a colony

(figure 3.1). In contrast, the organisms became more active and the tail movement frequency increased in higher exposure concentrations and longer exposure durations. The continuous increased movement resulted in reduced clumping tendency and the organisms were found separated as individuals without any colony (figure 3.2). The worms were found to secrete mucus like white semi-liquid substances (figure 3.3) at higher exposure concentrations and longer exposure durations to all there metals. The behavioral responses and their intensity were different at different exposure concentrations and durations. There were differences in the degree and time of appearance of various responses observed in different metals. However, there was no clear pattern in the behavioral responses to distinguish any metal specific responses.

Figure 3. 1Strong clumping tendency of tubificid worms (*Tubifexspp.*) in control group (no metal added).

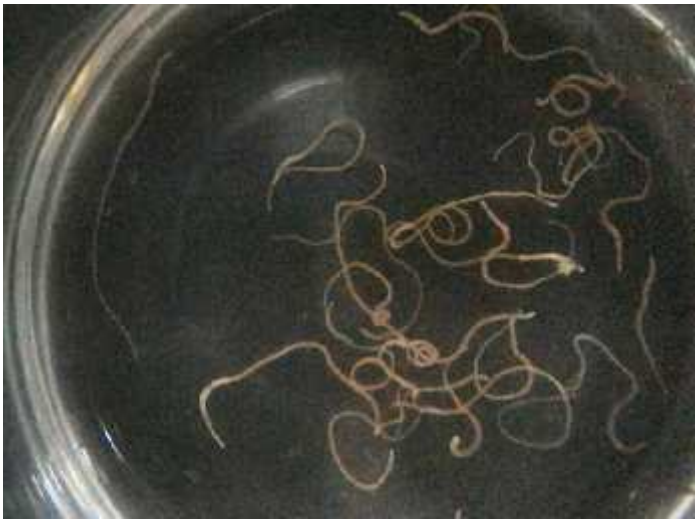

Figure 3. 2Absence of clumping tendency of tubificid worms (*Tubifexspp.*) at 3.20 µM of $Cr^{6+}$ during the 96 h acute toxicity test

Figure 3. 3Mucous secreted from tubificid worm (*Tubifexspp.*) exposed at 0.06 µM of $Cd^{2+}$ seen under light microscope (40X)

### 3.2.1 Responses to $Cd^{2+}$

Tubificid worms showed abnormalities in movement, clumping tendency and mucous secretion in response to different concentrations of $Cd^{2+}$. The degree of responses increased or decreased in an exposure concentration and duration dependent manner (table 3.2).

Worms in the control group and the lowest exposure concentrations (0.02 µM of $Cd^{2+}$) were in healthy condition because there were regular movement, strong clumping tendency and mucous secretion was absent during the 96h exposure. In contrast, with increasing concentration and exposure duration the worms became more active and showed increased movement, while the clumping tendency showed the opposite trend (table 3.2). However, at high concentrations (>0.06µM) the worms showed higher frequent within 24 h. After longer exposure durations (>72 h) the worms continued to show increased abnormal activity as separation from colony even at lower concentrations before they died.

With increasing movement of the, worms the individuals started to secrete mucus appeared as white semi-liquid substance around the body. There was no noticeable mucus secretion below 0.06 µM $Cd^{2+}$ at all durations. Heavy mass of white mucus was noticed around the individuals/colony with the shutdown of movement and the observation revealed the organisms dead. Within 72 h of exposure at 0.040 µM and higher concentrations of $Cd^{2+}$, worms showed mild mucous secretion in both individually and in colonies. However, at higher concentrations (>0.20 µM $Cd^{2+}$) the occurrence of mucus was noticeable within the first 24 h and in 72 h, heavy mass of white mucus were secreted by the worms that the worms could hardly be seen..

At the control and lower concentrations (<0.06 µM $Cd^{2+}$) strong clumping tendency of the worms were seen at all exposure durations. However, the clumping tendency decreased with increasing exposure duration and concentrations of $Cd^{2+}$. After longer exposure durations (>72 h), at the concentrations (>0.06 µM $Cd^{2+}$) the worms showed reduced clumping behavior when 1 or 2 individuals were often seen separated from the colony. At higher concentrations (>0.20 µM $Cd^{2+}$) clumping behavior of the worms could hardly be seen and the worms were found to be freely distributed on the surface as individual organism.

Table 3. 2Behavioral responses of tubificid worms exposed to different concentrations (µM) of $Cd^{2+}$ in 96 h water only acute toxicity test

| Concentration (µM) | 24h | | | 48h | | | 72h | | | 96h | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | CT | MS | M | CT | MS | M | CT | MS | M | CT | MS |
| 0.00 | + | +++ | - | + | +++ | - | + | +++ | - | + | +++ | - |
| 0.02 | + | +++ | - | + | +++ | - | + | +++ | - | + | +++ | - |
| 0.04 | + | +++ | - | + | +++ | - | + | ++ | + | ++ | ++ | + |
| 0.06 | + | +++ | + | + | ++ | ++ | ++ | ++ | ++ | ++ | + | ++ |
| 0.08 | ++ | ++ | + | ++ | ++ | ++ | ++ | + | ++ | ++ | + | ++ |
| 0.10 | ++ | ++ | + | ++ | ++ | ++ | ++ | + | ++ | ++ | + | ++ |
| 0.20 | ++ | ++ | + | ++ | + | ++ | ++ | + | ++ | ++ | + | +++ |
| 0.30 | +++ | + | +++ | +++ | - | +++ | - | - | - | - | - | - |

(M= Movement, MS= Mucous Secretion, CT= Clumping Tendency, + = Mild. ++ = Moderate, +++ = Strong, - = None)

### 3.2.2 Responses to $Cr^{6+}$

Tubificid worms showed abnormalities in movement, clumping tendency and mucous secretion in response to different concentrations of $Cr^{6+}$. The degree of responses increased or decreased in an exposure concentration and duration dependent manner (table 3.3).

Worms in control group and the lowest exposure concentrations (0.20 µM of $Cr^{6+}$) were in healthy condition because there were regular movement, strong clumping tendency and mucous secretion was absent during the 96h exposure. In contrast, with increasing concentration and exposure duration the worms became more active and showed increased

movement, while the clumping tendency showed the opposite trend (table 3.3). However, at high concentrations (>0.80 µM) the worms showed higher movement frequency within 24 h. After longer exposure durations (>72 h) the worms continued to show increased abnormal activity as separation from colony even at lower concentrations (< 1.60 µM) before they died.

Table 3. 3Behavioral responses of tubificid worms exposed to different concentrations (µM) of $Cr^{6+}$ in 96 h water only acute toxicity test

| Concentration (µM) | 24 h | | | 48 h | | | 72 h | | | 96 h | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | CT | MS | M | CT | MS | M | CT | MS | M | CT | MS |
| 0.00 | + | +++ | - | + | +++ | - | + | +++ | - | + | +++ | - |
| 0.20 | + | +++ | - | + | +++ | - | + | +++ | - | + | +++ | - |
| 0.40 | + | +++ | - | + | +++ | - | + | +++ | - | + | +++ | + |
| 0.80 | + | +++ | + | ++ | +++ | + | ++ | +++ | ++ | ++ | ++ | ++ |
| 1.60 | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | +++ |
| 3.20 | ++ | ++ | ++ | ++ | + | ++ | +++ | + | +++ | + | - | +++ |
| 6.40 | ++ | + | ++ | +++ | - | +++ | - | - | - | - | - | - |
| 10.00 | +++ | + | ++ | - | - | - | - | - | - | - | - | - |

(M= Movement, MS= Mucous Secretion, CT= Clumping Tendency, + = Mild. ++ = Moderate, +++ = Strong, - = None)

With increasing movement of the, worms the individuals started to secrete mucus appeared as white semi-liquid substance around the body. There was no noticeable mucus secretion below 0.80 µM$Cr^{6+}$ at all durations. Heavy mass of white mucus was noticed around the

individuals/colony with the shutdown of movement and the observation revealed the organisms dead. Within 72 h of exposure at 1.60µM and higher concentrations of $Cr^{6+}$, worms showed moderate mucous secretion in both individually and in colonies. However, higher concentrations (>1.60 µM$Cr^{6+}$), the occurrence of mucus was noticeable within the first 24 h and in 72 h, heavy mass of white mucus were secreted by the worms that the worms could hardly be seen.

At the control and lower concentrations (<0.80 µM$Cr^{6+}$) strong clumping tendency of the worms were seen at all exposure durations. However, the clumping tendency decreased with increasing exposure duration and concentrations of $Cr^{6+}$. After longer exposure durations (>72 h), at the concentrations (>0.80 µM$Cr^{6+}$) the worms showed reduced clumping behavior when 1 or 2 individuals were often seen separated from the colony. At higher concentrations (>3.20 µM$Cr^{6+}$) clumping behavior of the worms could hardly be seen and the worms were found to be freely distributed on the surface as individual organism.

### 3.2.3 Responses to $Pb^{2+}$

Tubificid worms showed abnormalities in movement, clumping tendency and mucous secretion in response to different concentrations of $Pb^{2+}$. The degree of responses increased or decreased in an exposure concentration and duration dependent manner (table 3.4).

Worms in control group and the lowest exposure concentrations (0.50 µM of $Pb^{2+}$) were in healthy condition because there were regular movement, strong clumping tendency and mucous secretion was absent during the 96h exposure. In contrast, with increasing concentration and exposure duration the worms became more active and showed increased movement, while the clamping tendency showed the opposite trend (table 3.3). However, at high concentrations (>2.00 µM of $Pb^{2+}$) the worms showed higher movement frequency within 24 h. After longer exposure durations (>72 h) the worms continued to show increased abnormal activity as separation from the colony even at lower concentrations before they died.

With increasing movement of the, worms the individuals started to secrete mucus appeared as white semi-liquid substance around the body. There was no noticeable mucus secretion

below 1.50µM $Pb^{2+}$ at all durations. Heavy mass of white mucus was noticed around the individuals/colony with the shutdown of movement and the observation revealed the organisms dead. Within 48 h of exposure at 1.50 µM and higher concentrations of $Pb^{2+}$, worms showed mild mucous secretion in both individually and in colonies which got higher after 72 h of exposure to higher concentrations (>3.00 µM of $Pb^{2+}$). However, higher concentrations (>4.00 µM of $Pb^{2+}$), the occurrence of mucus was noticeable within the first 24 h and in 72 h, heavy mass of white mucus were secreted by the worms that the worms could hardly be seen. At the control and lower concentrations (<2.00 µM of $Pb^{2+}$) strong clumping tendency of the worms were seen at all exposure durations. Clumping tendency decreased with increasing exposure duration and concentrations of $Pb^{2+}$. However, longer exposure durations (>72 h), at higher concentrations (>3.00 µM of $Pb^{2+}$) the worms showed reduced clumping behavior as 1 or 2 individuals were often seen separated from the colony. At higher concentrations (>4.00 µM of $Pb^{2+}$) clumping behavior of the worms could hardly be seen and the worms were found to be freely distributed on the surface as individual organism.

Table 3. 4Behavioral responses of tubificid worms (*Tubifexspp.*) exposed to different concentrations (µM) of $Pb^{2+}$ in 96 h water only acute toxicity test

| Concentration (µM) | 24h | | | 48h | | | 72h | | | 96h | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | M | CT | MS | M | CT | MS | M | CT | MS | M | CT | MS |
| 0.00 | + | +++ | - | + | +++ | - | + | +++ | - | + | +++ | - |
| 0.50 | + | +++ | - | + | +++ | - | + | +++ | - | + | +++ | - |
| 1.00 | + | +++ | - | + | +++ | - | + | +++ | - | ++ | +++ | + |
| 1.50 | + | +++ | - | + | +++ | + | + | +++ | + | ++ | ++ | ++ |
| 2.00 | + | ++ | + | + | ++ | + | ++ | ++ | + | ++ | ++ | ++ |
| 3.00 | + | ++ | + | + | ++ | + | ++ | ++ | ++ | ++ | + | +++ |
| 4.00 | ++ | ++ | ++ | +++ | + | +++ | +++ | - | +++ | - | - | - |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **5.00** | +++ | + | +++ | +++ | - | +++ | - | - | - | - | - | - |

(M= Movement, MS= Mucous Secretion, CT= Clumping Tendency, + = Mild. ++ = Moderate, +++ = Strong, - = None)

### 3.3 Morphological responses of the worms

The tubificid worms without any chemical exposure in the control group were found healthy in appearance with no damage done at the body, especially with regular shape of tail and without loss of any organ (Figure 3.4). On the other hand, worms exposed to different concentrations of $Cd^{2+}$, $Cr^{6+}$ and $Pb^{2+}$ showed various morphological alterations including organ loss (figure 3.5), autotomy (figure 3.6) and tail bifurcation (figure 3.7) as observed under compound light microscope at 40X. The degree of alteration or physical damage varied at different concentrations. Autotomy of the hind part of the body was common for all threemetals. The morphological alterations of worms didn't show any metal specificity during the experimental duration.

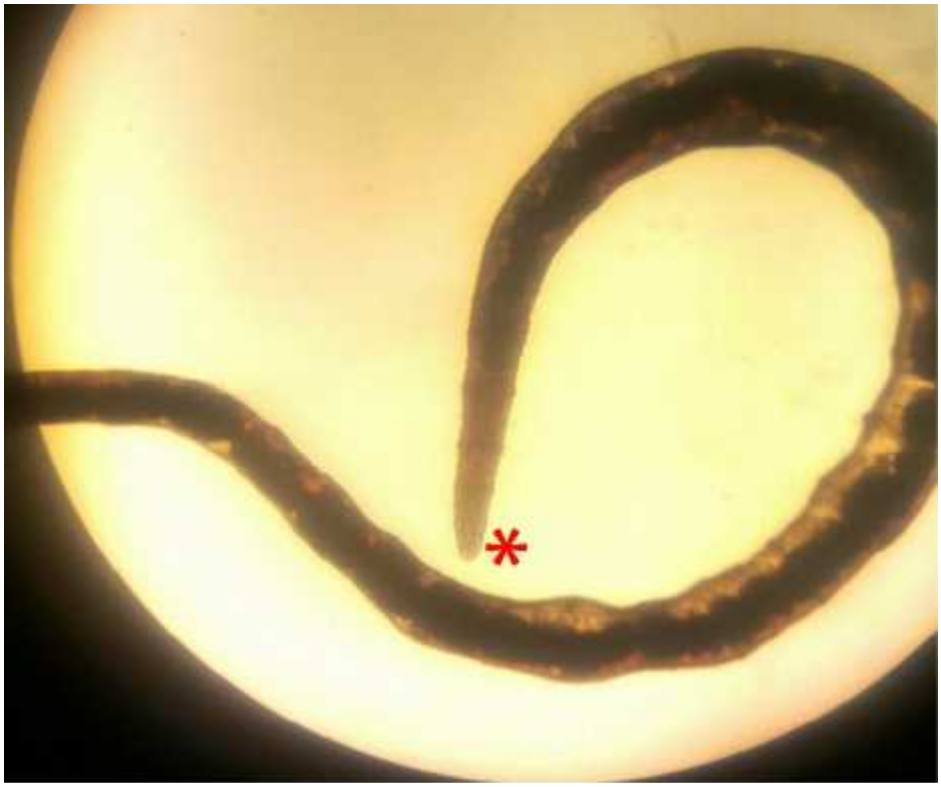

Figure 3. 4A healthy (no damage done at the body, especially with regular shape of tail and without loss of any organ) tubificid worm (*Tubifexspp*.) in control group (no metal added) were observed under light microscope (40X); '*' sign indicates the tail part of tubificid worm is at normal condition

Figure 3. 5Organ loss at the hind part of the body (pointed) of tubificid worm (*Tubifexspp*.) at 48 h exposure to 3.00 µM $Pb^{2+}$ seen under light microscope (40X)

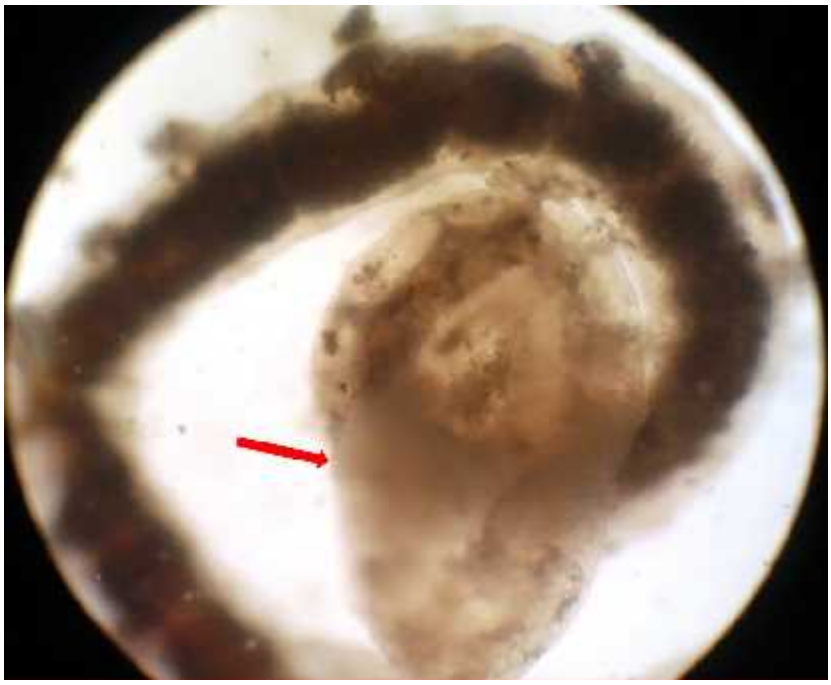

Figure 3. 6Autotomy at the hind part of the body (pointed) of tubificid worm (*Tubifexspp*.) at 72 h exposure to 0.06 µM $Cd^{2+}$ seen under light microscope (40X)

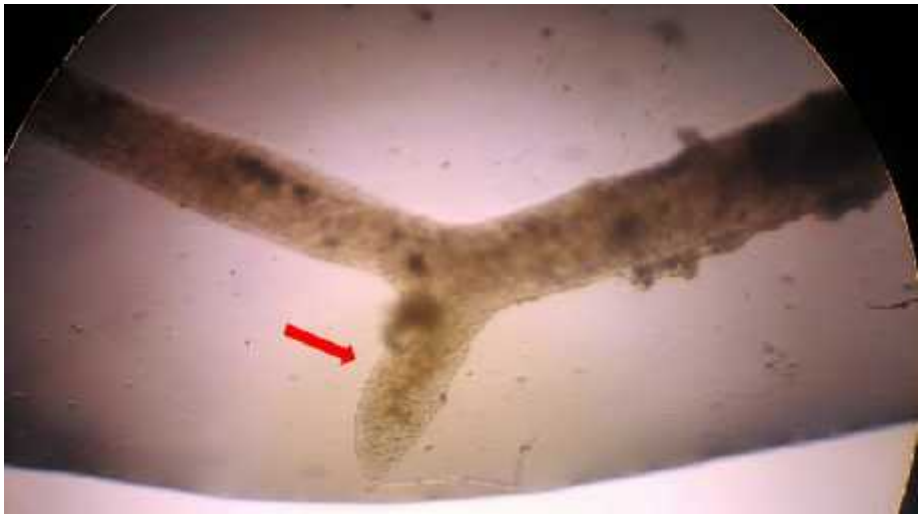

Figure 3. 7Tail bifurcation (deviation at the tail) of tubificid worm (*Tubifexspp*.) at 48 h exposure to 3.2 µM $Cr^{6+}$ (pointed) seen under light microscope (40X)

### 3.3.1 Responses to $Cd^{2+}$

Worms were in healthy condition in the control groups (figure 3.4). In contrast, worms suffered from morphological abnormalities after $Cd^{2+}$ exposure. Autotomy at the hind part of the body of the tubificid worms were the major morphological response to exposure to $Cd^{2+}$ concentrations. However, autotomy at the hind part of the body was first observed at 0.06 µM of $Cd^{2+}$ (figure 3.6). The rate of autotomy at the hind part of the body increased with increasing concentration of $Cd^{2+}$ and exposure duration. The degree of autotomy increased till the worm died.

Degeneration at the body was another morphological response to exposure to $Cd^{2+}$ concentrations. It was first observed at 48 h exposure to 0.08 µM of $Cd^{2+}$. It followed the duration and concentration dependent manner. However, it was more likely to appear at the higher concentrations (>0.10 µM $Cd^{2+}$). Therefore, it results in the death of the worm.

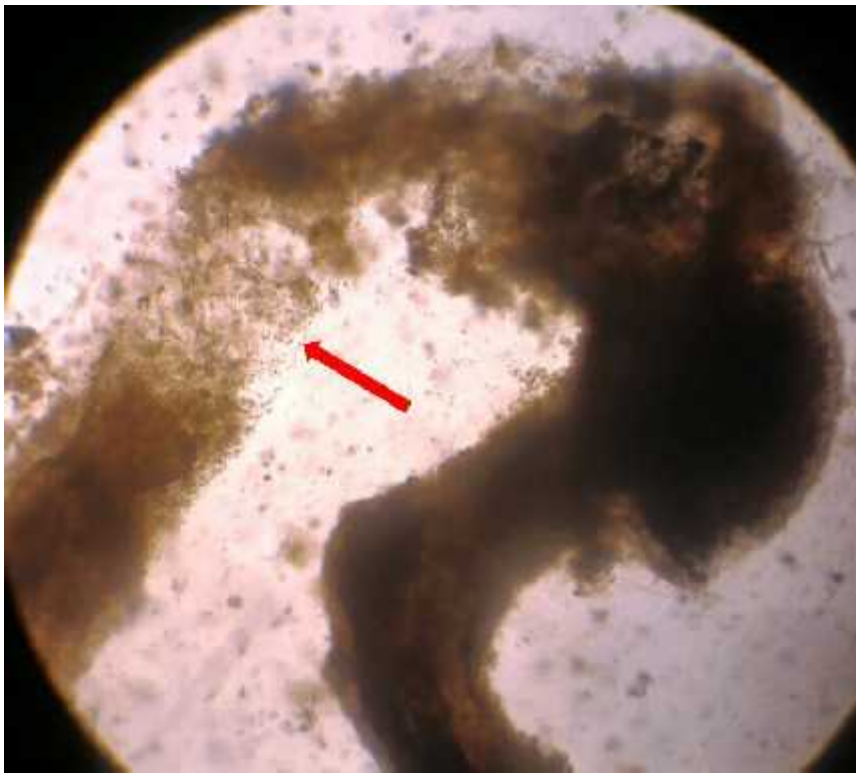

Figure 3. 8Degeneration at the body of tubificid worm (*Tubifexspp*.) at 48 h exposure to 0.20 µM $Cd^{2+}$ seen under light microscope (40X)

### 3.3.2 Response to $Cr^{6+}$

Tubificid worms suffered from morphological abnormalities at exposure to different concentrations of $Cr^{6+}$. The worms were in healthy condition in the control groups (figure 3.4). Autotomy at the hind part of the body were observed at almostall the concentrations (>0.8 µM $Cr^{6+}$). However, autotomy rate increased in a duration and concentration dependent trend. Increasing in both concentration and duration, autotomy increased. Autotomy continued till the worms are dead. At higher concentrations (>6.4 µM $Cr^{6+}$)autotomy at the hind part was severe that the body parts at the hind part of the body were degenerated (Figure 3.8).

Tail bifurcation (deviation at the tail), a unique morphological response of the tubificid worms observed on exposure to $Cr^{6+}$ concentrations. Tail bifurcation was observed only at 1.6 and 3.2 µM $Cr^{6+}$ (Figure 3.9) concentrations after 48 h exposure. More worms exhibited tail bifurcation at 3.2 µM $Cr^{6+}$ than at 1.6 µM $Cr^{6+}$ concentrations. It was not observed before 48 h exposure to these concentrations.

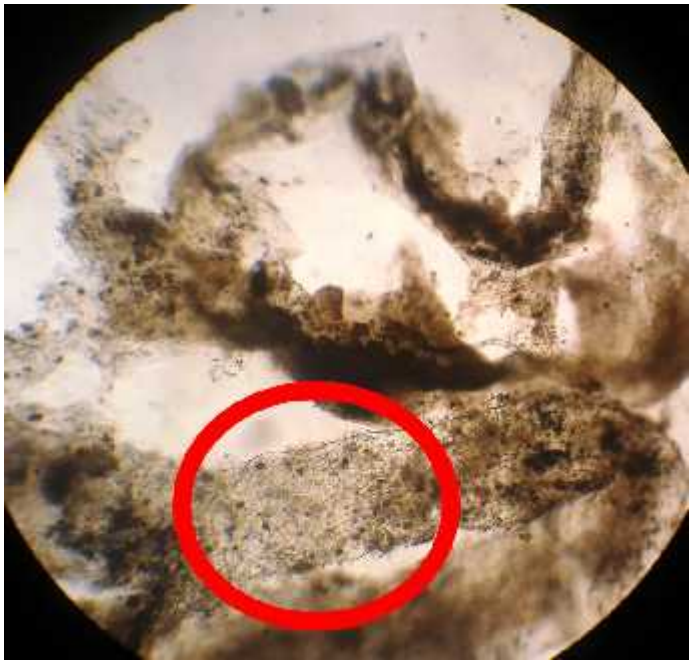

Figure 3. 9Degeneration at the hind part of the body (marked) of tubificid worm (*Tubifexspp*.) at 24 h exposure to 6.4 µM $Cr^{6+}$ seen under light microscope (40X)

### 3.3.3 Response to $Pb^{2+}$

Worms were in healthy condition in the control groups (figure 3.4) and in lower concentrations (<1.50 µM $Pb^{2+}$). In contrast, tubificid worms suffered from physical abnormalities at higher concentrations of $Pb^{2+}$. Autotomy at the hind part of the body were first observed at 1.50 µM $Pb^{2+}$ concentration. However it was present in later all the concentrations of $Pb^{2+}$. Autotomy rate increased in a duration and concentration dependent trend. Therefore, autotomy increased at the higher concentration and also at higher exposure durations. However, autotomy continued till the worm was dead. At higher concentrations (>3.00 µM $Pb^{2+}$) autotomy at the hind part was intense that the body parts at the hind part of the body of the worms were broken (Figure 3.8). After breakdown of hind part of the body, the tubificid worms died in less than 6 h.

## 3.4 Acute toxicity tests

Water only acute toxicity tests were conducted to estimate median lethal concentration ($LC_{50}$) of $Cd^{2+}$, $Cr^{6+}$ and $Pb^{2+}$ to tubificid worms. The cumulative mortality (%) increased in a duration and concentration dependent manner. The significant difference in mortality were evaluated based on the two-way ANOVA outputs (APPENDIX-V).

### 3.4.1 Mortality of worms exposed to $Cd^{2+}$

Worms in control group were in healthy condition and no mortality were observed at control group and at the lowest concentration (0.02 µM $Cd^{2+}$). First mortality of the worms occurred at 0.04 µM $Cd^{2+}$ after 24 h of exposure. Mortality (%) increased with increasing duration of exposure and concentration. 100% mortality occurred at the highest two concentrations (0.02 and 0.03) µM $Cd^{2+}$. Mortality (%) at different exposure durations with$Cd^{2+}$ were significantly different ($P<0.05$) as revealed by two-way ANOVA. Mortality (%) at different concentrations of $Cd^{2+}$ at all the exposure durations were also significantly different($P<0.05$) accept 0.00 and 0.04 µM. Mortality (%) at all the concentrations of $Cd^{2+}$ were homogenous

(P<0.05) accept (0.04 to 0.06 µM) $Cd^{2+}$. Mortality (%) to different concentrations of $Cd^{2+}$ at different exposure durations are presented at Figure 3.9.

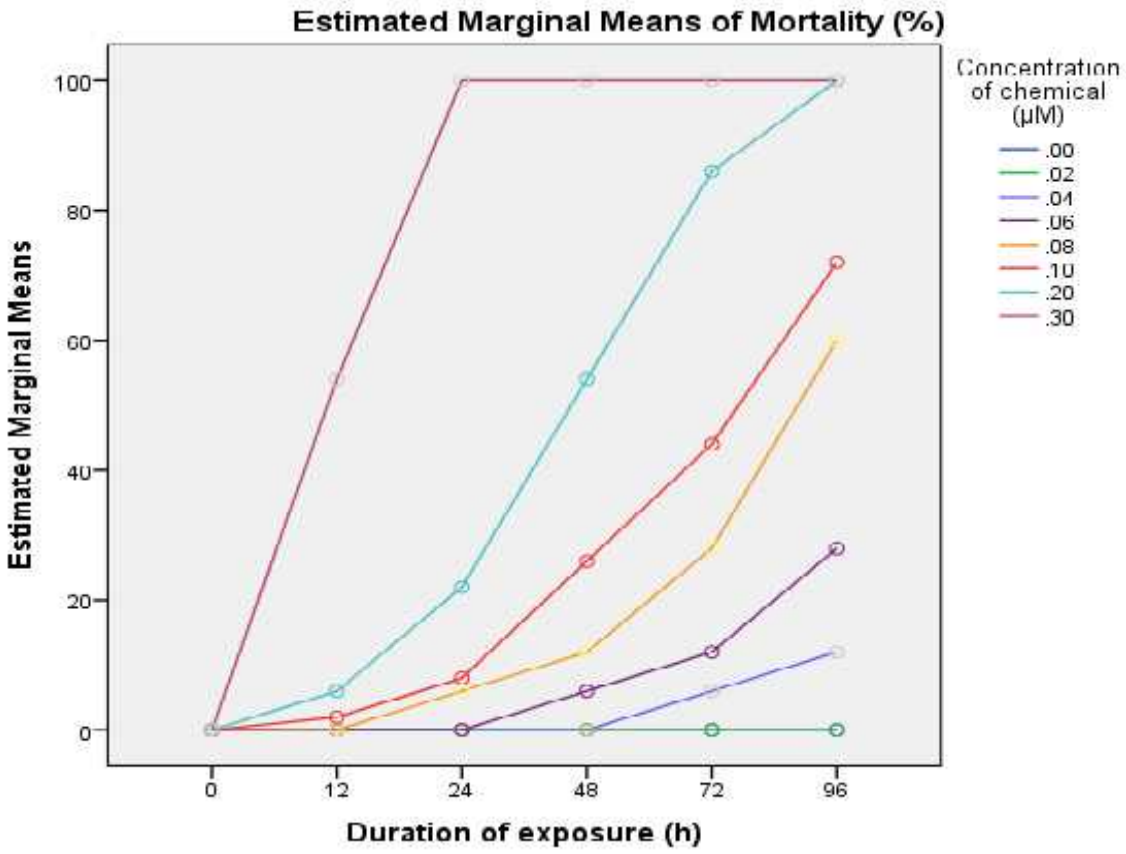


Figure 3. 10Mortality (%) of tubificid worms (*Tubifexspp*.) exposed to different concentrations of $Cd^{2+}$ at different exposure durations (h)

### 3.4.2 Mortality of worms exposed to $Cr^{6+}$

Control worms were in healthy condition and no mortality were observed at control and at the lowest concentration (0.20 µM $Cr^{6+}$). First mortality of the worms occurred at (0.40 µM $Cr^{6+}$). However, mortality increased with increasing exposure duration and concentration of $Cr^{6+}$. 100% mortality occurred at the highest two concentrations (6.40 and 10.00 µM $Cr^{6+}$). All exposure durations of $Cr^{6+}$ were significantly different (P<0.05) and homogenous accept 12 h exposure. All the concentrations of $Cr^{6+}$ were significantly different (P<0.05) accept (0.00 to 0.40) µM $Cr^{6+}$. Mortality (%) at all the concentrations of $Cr^{6+}$ were homogenous (P<0.05) accept (0.04 to 0.06) µM $Cr^{6+}$. Mortality (%) to different concentrations of $Cr^{6+}$ at different exposure durations are presented at Figure 3.10.

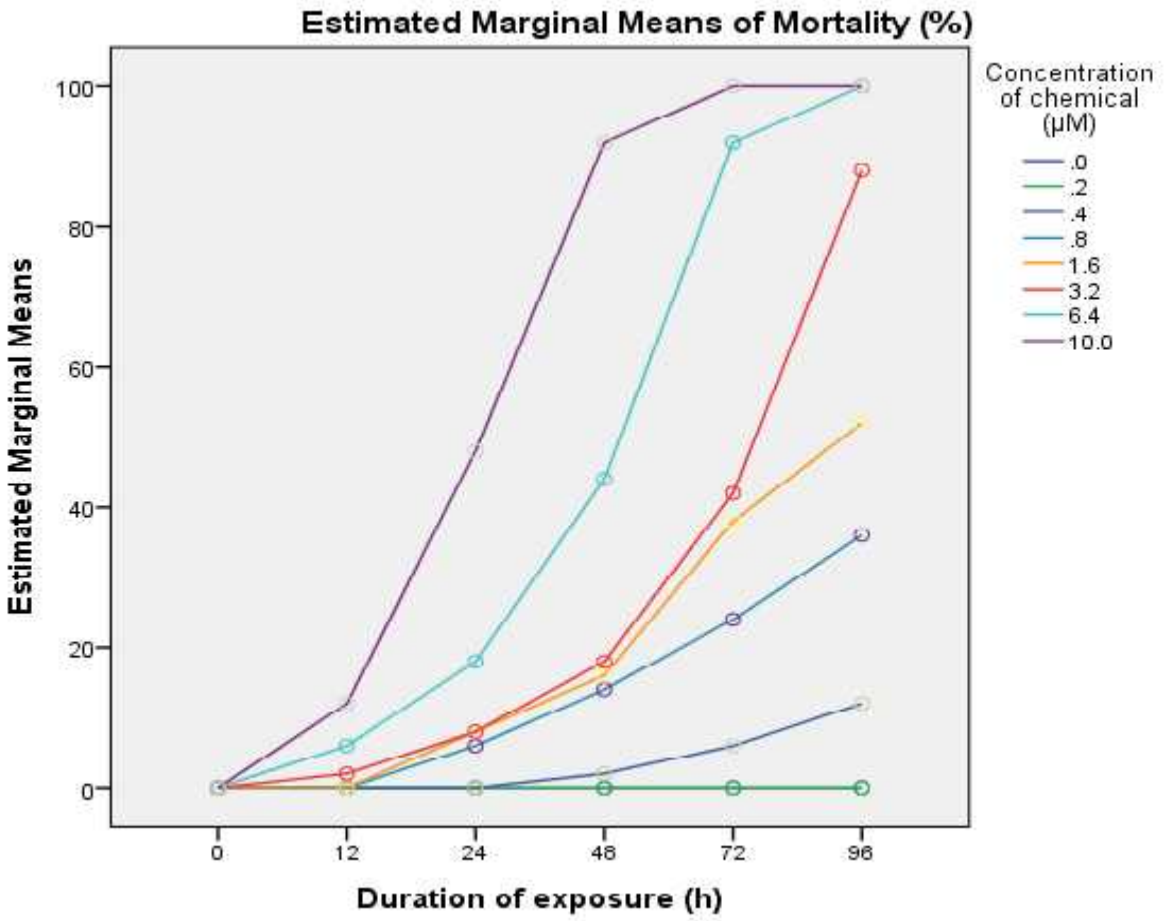


Figure 3. 11Mortality (%) of tubificid worms (*Tubifexspp*.) exposed to different concentrations of $Cr^{6+}$ at different exposure durations (h)

### 3.4.3 Mortality estimation for $Pb^{2+}$

No mortality were observed at control and at the lowest concentration (0.05 µM $Pb^{2+}$). First mortality of the worms occurred at (1.00 µM $Pb^{2+}$). However, mortality increased with increasing duration of exposure and concentration. 100% mortality occurred at the highest two concentrations (4.00 and 5.00 µM $Pb^{2+}$). All exposure durations of $Pb^{2+}$ were significantly different ($P<0.05$) accept 12 h exposure. Mortality (%) to different concentrations of $Pb^{2+}$ at all exposure durations were homogenous ($P<0.05$) accept 12 h exposure. All the concentrations of $Pb^{2+}$ were significantly different ($P<0.05$) accept (0.00 to 1.00) µM $Pb^{2+}$. Mortality (%) at all concentrations of $Pb^{2+}$ were homogenous ($P<0.05$) accept (0.00 to 1.00) µM $Pb^{2+}$. Mortality (%) to different concentrations of $Pb^{2+}$ at different exposure durations are presented at Figure 3.11.

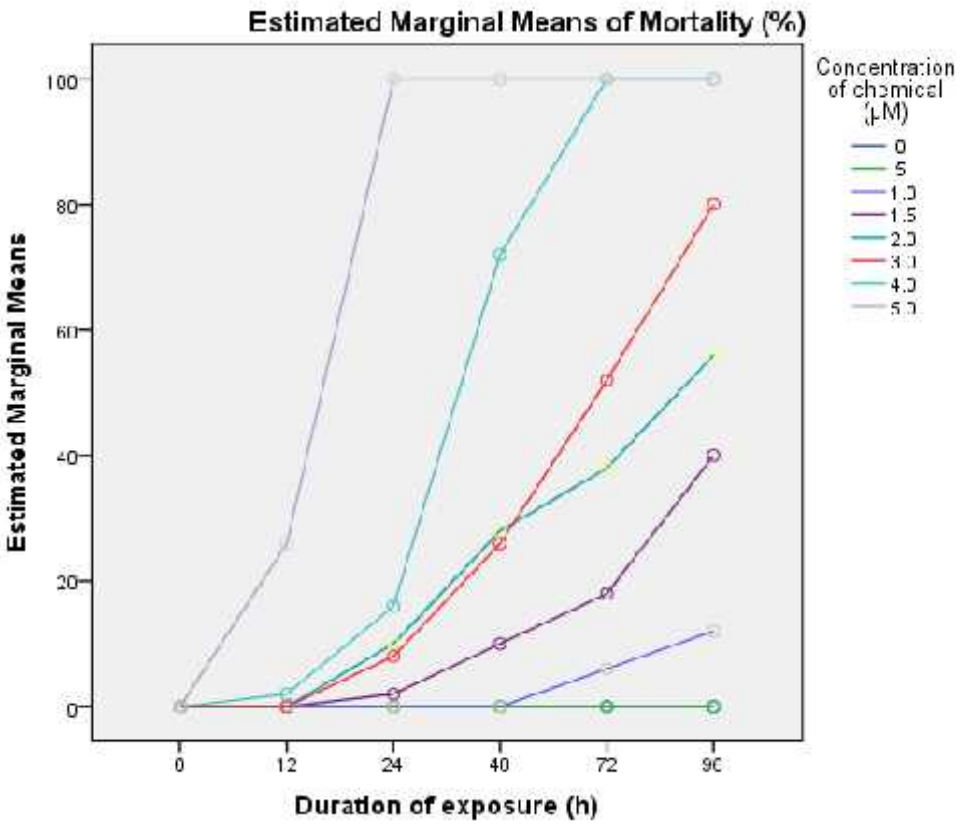


Figure 3. 12Mortality (%) of tubificid worms (*Tubifexspp*.) exposed to different concentrations of $Pb^{2+}$ at different exposure durations (h)

### 3.4.4 Median lethal concentration ($LC_{50}$) estimation

The 4 parameter sigmoid curve obtained from the concentration and mortality (%) data for each metal is presented in the figure 3.13 to 3.15. $LC_{50}$ values estimated by the graphical and probit analysis methods along with their best fitted model and goodness of fit are presented in table 3.5 and table 3.6 respectively (detailed statistical output are presented in APPENDX VI and VII).

$LC_{50}$ values obtained from using both the methods wear nearly equal. From the calculated values, $LC_{50}$ values of three metals to tubificid worms were in the order of $Pb^{2+} > Cr^{6+} > Cd^{2+}$. The metal have lower $LC_{50}$ value, is more toxic to the organism than which have higher $LC_{50}$. $Cd^{2+}$ was 19.67 times more toxic than $Cr^{6+}$ and 24.67 times more toxic than $Pb^{2+}$, while $Cr^{6+}$ was 1.25 times more toxic than $Pb^{2+}$.

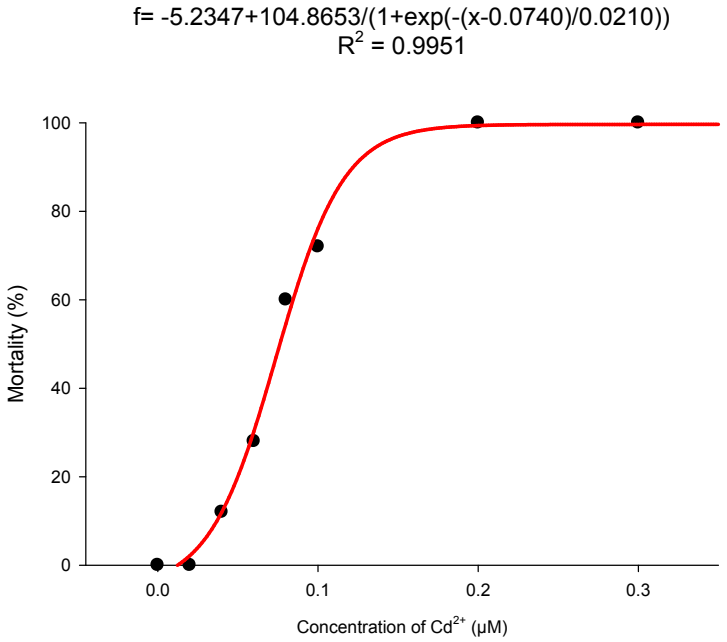


Figure 3. 13The 4 parameter sigmoid curve obtained from 96 h cumulative mortality (%) of tubificid worms (*Tubifexspp*.) exposed to different Concentrations (µM) of $Cd^{2+}$

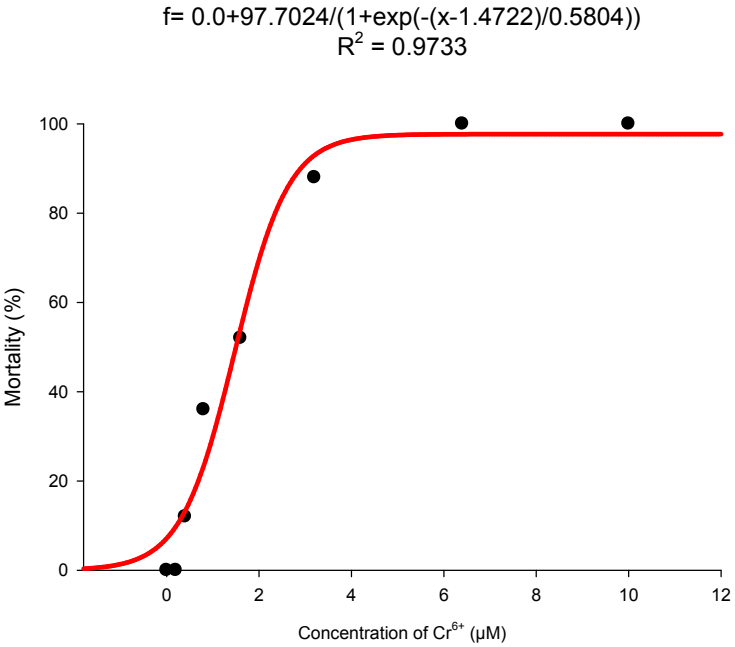


Figure 3. 14The 4 parameter sigmoid curve obtained from 96 h cumulative mortality (%) of tubificid worms (*Tubifexspp*.) exposed to different Concentrations (µM) of $Cr^{6+}$

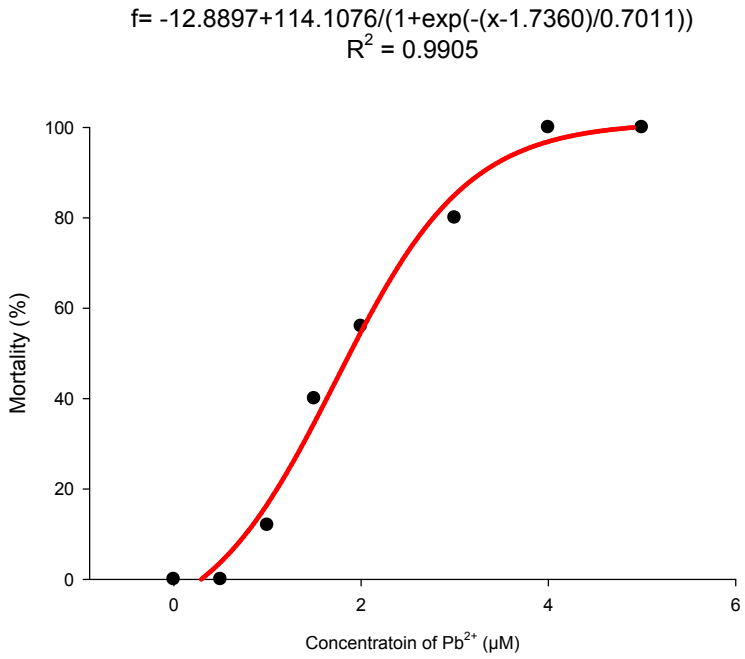


Figure 3. 15The 4 parameter sigmoid curve obtained from 96 h cumulative mortality (%) of tubificid worms (*Tubifexspp*.) exposed to different Concentrations (µM) of $Pb^{2+}$

Table 3. 5The 96 h $LC_{50}$values (µM) of $Cd^{2+}$, $Cr^{6+}$ and $Pb^{2+}$ to tubificid worms (*Tubifexspp*.) estimated by graphical analysis using SIGMAPLOT 10

| Metal | The best fitted model | $R^2$ | $LC_{50}$ (µM) |
|---|---|---|---|
| **$Cd^{2+}$** | f = -5.2347+104.8653/(1+exp(-(x-0.0740)/0.0210)) | 0.9951 | 0.0762 |
| **$Cr^{6+}$** | f = 0.0+97.7024/(1+exp(-(x-1.4722)/0.5804)) | 0.9733 | 1.4995 |
| **$Pb^{2+}$** | f = -12.8897+114.1076/(1+exp(-(x-1.7360)/0.7011)) | 0.9905 | 1.8799 |

Table 3. 6The 96 h $LC_{50}$ values (µM) of $Cd^{2+}$, $Cr^{6+}$ and $Pb^{2+}$to tubificid worms (*Tubifex spp.*) estimated by graphical analysis using CALCULATION OF $LC_{50}$ USING PROBIT ANALYSIS

| Metal | Function | $R^2$ | $LC_{50}$ (µM) | SD | SE | 95% confidence Level | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower | Upper |
| $Cd^{2+}$ | y= 4.5984x + 10.192 | 0.9794 | 0.074 | 0.217 | 0.045 | 0.061 | 0.091 |
| $Cr^{6+}$ | y= 2.4777x + 4.7901 | 0.9697 | 1.220 | 0.404 | 0.082 | 0.842 | 1.767 |
| $Pb^{2+}$ | y= 3.6561x + 4.0835 | 0.9973 | 1.839 | 0.240 | 0.047 | 1.490 | 2.269 |

### 3.5 Bioaccumulation of $Cd^{2+}$, $Cr^{6+}$ and $Pb^{2+}$ in tubificid worms (*Tubifex spp.*)

Bioaccumulation tests were carried out to measure the amount of $Cd^{2+}$, $Cr^{6+}$ and $Pb^{2+}$ bioaccumulated on the body of the tubificid worms exposed to a sub-lethal concentration ($1/4^{th}$ of the $LC_{50}$) of the metal. Exposure concentrations and periodic accumulation of $Cd^{2+}$, $Cr^{6+}$ and $Pb^{2+}$ in the whole body of tubificid worms obtained from the AAS are presented in the table 3.7 and figure 3.15 to figure 3.17.

Highest amount of accumulation was found for $Pb^{2+}$ at 72 h exposure duration. The accumulation of $Pb^{2+}$ and $Cd^{2+}$ increased till 48 h exposure then it gradually decreased. However, lowest amount of accumulation found for $Cr^{6+}$. Accumulation of $Cr^{6+}$ continuously decreased with the increase of exposure duration.

Table 3. 7Periodic accumulation of $Cd^{2+}$, $Cr^{6+}$ and $Pb^{2+}$ in the whole body of tubificid worms (*Tubifexspp.*) obtained from AAS

| Exposure duration (h) | $Cd^{2+}$ (ppm) | $Cr^{6+}$ (ppm) | $Pb^{2+}$ (ppm) |
|---|---|---|---|
| **24** | 1.7796 | 1.5917 | 71.0488 |
| **48** | 3.7241 | 1.2998 | 80.4973 |
| **72** | 5.9948 | 1.1674 | 133.8470 |
| **96** | 4.6925 | 0.9556 | 128.4771 |
| **Exposure concentration** | 0.002 | 0.020 | 0.100 |

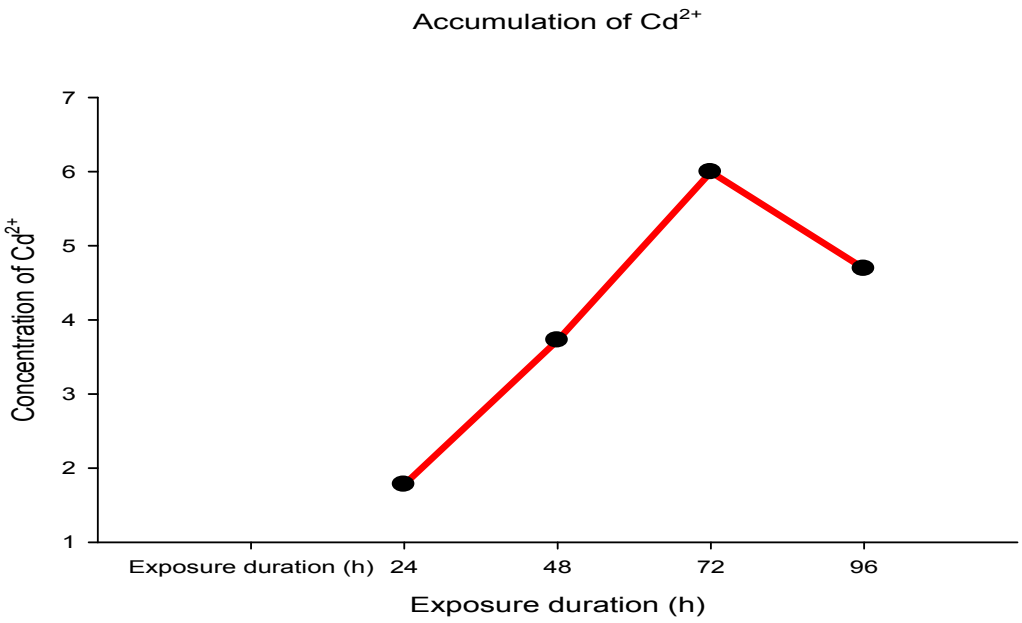


Figure 3. 16Accumulation of $Cd^{2+}$ (ppm) in the whole body of tubificid worms (*Tubifexspp.*) exposed to 0.002 ppm of $Cd^{2+}$ (ppm) upto 96 h.

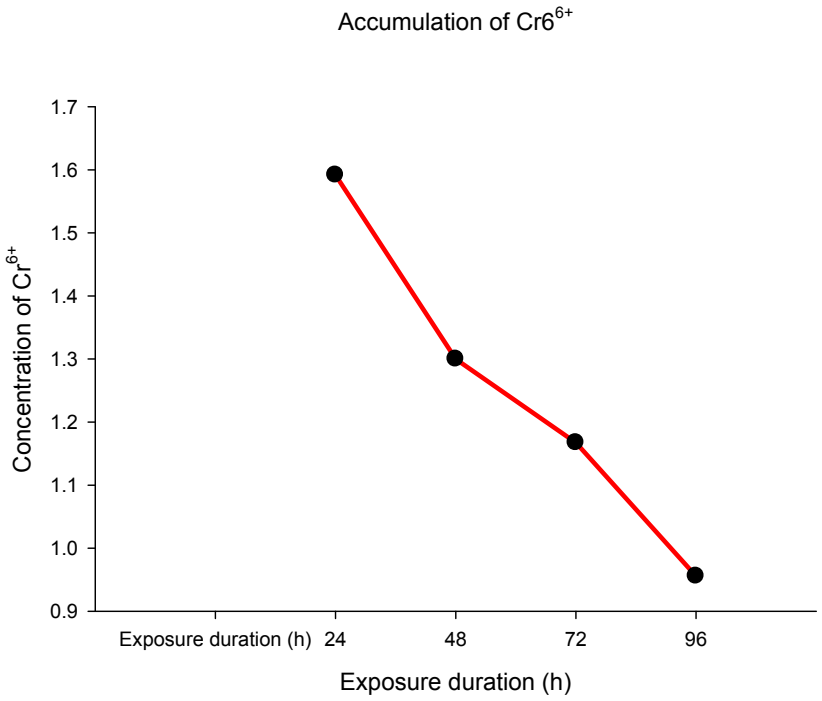


Figure 3. 17Accumulation of $Cr^{6+}$ (ppm) in the whole body of tubificid worms (*Tubifex spp*.) exposed to 0.020 ppm of $Cr^{6+}$ (ppm) upto 96 h.

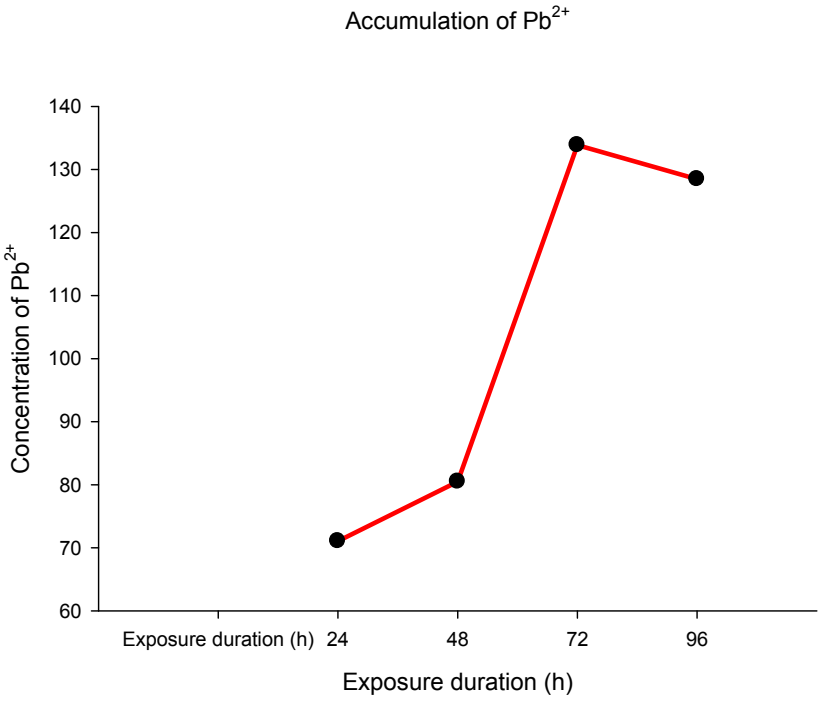


Figure 3. 18Accumulation of $Pb^{2+}$ (ppm) in the whole body of tubificid worms (*Tubifexspp*.) exposed to 0.100 ppm of $Pb^{2+}$ (ppm) upto 96 h.

# Chapter 4 - Discussion

The accumulation rate and consequent toxicity of metals varies with temperature , pH, amount dissolved solids, hardness (Hamelink et al., 1994; Sorensen, Elsa, 1991) conductivity, amount of organic matter present in the medium, etc (Sparling, 2016). Toxicity decreases with the increase of pH and/or hardness (Brković-Popović and Popović, 1977). Temperature is also an important factor in the short-term acute toxicity tests(Rathore and Khangarot, 2002).

The physico-chemical parameters of the exposure medium did not varied in a great extent. Water temperature fluctuations were very little (24.60-25.0) ˚C. Dissolved oxygen (DO) were within the normal range (6.12-6.94) mg/L. pH ranging from 6.64-6.74. So, the environmental parameters did not vary among the treatments groups throughout the experimental period. The present study were conducted in water only experiment using distilled water to avoid the presence of any organic matter or other compounds which can impact the partitioning of metals ions between test solution and organisms. During the experiment, no feed were given to avoid fluctuations caused by the metabolic waste of the worms.So, only the exposure concentration and duration were expected to be the only sources of variation between or within treatment groups.

In the present study, short-term (96 h) water only acute toxicity tests were done to estimate median lethal concentration ($LC_{50}$) of $Cd^{2+}$, $Cr^{6+}$ and $Pb^{2+}$ to tubificid worms (*Tubifexspp*.). Test with aquatic oligochaetes, absence of sediment is already a source of stress. It reduces realism in exposure conditions and also has clear limitations for the selection of sub-lethal endpoints, such as growth and reproduction.However, in mortality analysis, water-only exposures are still useful to conduct toxicity comparison of different chemicals to a particular species (Rodriguez and Reynoldson., 2011).

There has been a wide array of methods used to calculate the $LC_{50}$ from the mortality data of acute toxicity test including graphical, Spearman-Karber,Trimmed Spearman-Karber, or Probit Method(USEPA, 2002). Being commonly noticed. In this study, the use of graphical analysis and Probit analysis provided the opportunity to test the suitability of methods to obtain toxicity endpoints. The $R^2$ (>0.95) in all the cases suggests both the methods suitable to obtain a robust dose-response model for *Tubifex spp.* Moreover, the nearness of $LC_{50}$ values obtained from both the methods confirm and establish the confidence on the methods for toxicity studies.

The $LC_{50}$ values of the metals suggest that $Cd^{2+}$ was the most toxic among the three followed by $Cr^{6+}$ and $Pb^{2}$. Studies by other researchers also reported that $Cd^{2+}$ is more toxic than Pb (Fargasova 1994) and Cr (Méndez-Fernández et al., 2013). Rathore and Khangarot (2002) also reported the similar findings. The highest potential for toxicity of Cd is probably due to structure and denticity of the ligands which determine the pre-organization of the complex(Remelli et al., 2016).

The $LC_{50}$ estimated in the present study were considerably different from the values reported in literatures. In previous studies by other authors, the $LC_{50}$ values for the same metal ion were also varied over a long range. This variation were due to variation in species (Bræk et al., 1976) and other experimental conditions including temperature, pH, etc.Moreover, the mortality results not necessarily from a single key mechanism but also from the lineage of a suite of physiological processes (Meador et al., 2011), which may vary from metal to metal making the toxic potential vary among metals and their speciation.

Although the parent compound contributing the metal ions varied in different studies, we can still compare them because the cations are responsible for toxicity, anions hardly affect in toxicity (Parker et al., 2001). The free-ion-activity model (FIAM) formulated by Morel (1983) indicates the free-metal ion activity of cations, which reflects the reactivity of the metal and its bioavailability and toxicity.

Since various studies reported the toxicity endpoints in different units, prior to compare with other authors all the $LC_{50}$ values were preferably transformed from µM to mg/L unit. The 96 h $LC_{50}$ of $Cd^{2+}$ (0.008 mg/L) obtained in the present study is lower than reported by Bouché et al., (2000) (0.030 mg/L) and Chapman et al., (1982) (0.32 mg/L) who used a test medium of low hardness (5.3 mg/L CaCO3) although it is generally accepted that increased water hardness reduces the toxicity of Cu to freshwater organisms (Sorensen, Elsa, 1991). This variation might be due to the species and/or temperature variation. However, $LC_{50}$ of $Cd^{2+}$ estimated in the present study (0.0762µM ) is many folds lower than the finding (11.75 µM ) of Méndez-Fernández et al., (2013), might because of the cultured *T. tubifex* species. Other 96 h $LC_{50}$ of $Cd^{2+}$found in the literature also validated a great variability and ranged from 0.4 to 47.5 mg/L (Fargasova, 1994; Khangarot, 1991; Reynoldson et al., 1996), where experimental conditions were different from each other. The 96 h $LC_{50}$of $Cr^{6+}$(0.078 mg/L) in this study is lower than 2.42 mg/L reported by Rathore and Khangarot, (2002) and 4.89-7.22 mg/L by Maestre et al., (2009), both used laboratory cultured *T. tubifex* same population for all bioassays. The $LC_{50}$of $Pb^{2+}$at 96 h 0.389 mg/L, which is obtained from the present study is also lower than 165-239 mg/L(Rathore and Khangarot, 2002).

It is evident from the above discussion that in the present study, the metals were found to be more toxic with lower $LC_{50}$values than reported by other studies. Apart from the various physico-chemical variabilities the origin of the worms can also affect toxicity. Reynoldson et al., (1996) reported that the 96 h $LC_{50}$ of Cd on *Tubifex* harvested in Canada was two to eight times higher than that on *tubifex* which werecollected from Spain.Several authors also found that worms collected from a polluted area or an area with a long-term history of exposure to a metal were more tolerant when they were further exposed (Bryan et al., 1985; Pesch and Hoffman, 1982; Reinecke et al., 1999). Therefore,toxicity may vary due to factors such as test water quality characteristics (such as hardness, alkalinity, pH, temperature), metal salt used, test-organism source, different populations strains (Reynoldson et al., 1996; Sturmbauer et al., 1999) and other biological factors (such as species life stages, age, or sensitivity) that may alter the toxicity of the chemical and also the physiological condition of the organisms (Burton et al., 1996; Rathore and Khangarot, 2003). All these factors makes it very difficult to compare $LC_{50}$ values of different studies (Bouché et al., 2000).

Different behavioral abnormalities of the worms were seen in response to exposure to the metal concentrations in the present study. Mucous secretion is one of the behavioral abnormalities found in the present study is supported by (Bouché et al., 2000). However, mucus secretion may be an adaptive response of the worms that is related to the physiological resistance phase. Generally tubificid worms clump together to form a colony and remains in the colony. However, when they are at colony, their movement is limited. The present study reveals that in response to metal concentrations, movement of tubificid worms gets higher and clumping tendency weakens. Therefore, at the higher concentrations and higher exposure durations, the worms separates as individual worm with a rapid movement. The same trend was reported by (Dhara et al., 2014) for another aquatic oligochaete *B. sowerbyii*.

Some morphological abnormality (such as autotomy, organ loss, breakdown at hind part of the body etc) occurs when tubificid worms are exposed to a toxicant. In the present study, the worms undergo some morphological abnormalities. Among them autotomy was common for all three metals. However, autotomy frequency in the exposed worms has been shown to be related to the metal levels the worms are exposed (Meller et al., 1998). Autotomy has been shown to be anearly response indicator of toxicity for *T. tubifex*. It is supported by other author's findings of using autotomy as a useful toolfor monitoring exposure and effects of metal contamination inaquatic oligochaetes (Lagauzère et al., 2009; Meller et al., 1998; Méndez-Fernández et al., 2013).

The sub-lethal toxic effects are often expressed in the form of behavioral and morphological alterations. Tail bifurcation and loss or shrink of hind part was observed in the present study. Generally tubificid worms lives in a colony form with upside down and their hind part of the body moves highly. Therefore, hind part of the gets in contact with the metal concentrations more than anterior. This can be resulted more damage at the hind part of the body.

Tail bifurcation of the tubificid worms were reported by Méndez-Fernández et al., (2013) to the response of Cu exposure. However, tail bifurcation found for $Cr^{6+}$ exposure in the present study only at two concentrations. The presence of tail bifurcation in this study cannot be correlated with metal exposure as it was recorded vary randomly in different

treatments groups. However,it is an interesting observation given that the existence of malformations and/or abnormalities after metal exposure,which is poorly documented for oligochaetes(Méndez-Fernández et al., 2013). Lagauzère et al., (2009) have reported malformations in the prostomium (e.g., formation of two heads) and epidermal growths in oligochaetes exposed to uranium, although also in that case malformations, that could not be statistically analyzed for the small sample size. On the other hand, autotomy was a mechanismto detoxify metals and organic complexes in aquatic oligochaetes as proposed by several authors (Lagauzère et al., 2009; Paris-Palacios et al., 2010; Vidal and Horne, 2003).

The morphological and behavioral responses and other toxic effects are the consequences of metal uptake by organisms due to the lipophilic property of metal ions (Viarengo, 1985). The continuous uptake of metals by organism results in accumulation of the ions in their tissue. The accumulation process continues depending on the partitioning coefficients between organism and its surrounding environment (Meylan et al., 1999). However, the organisms were reported to respond by reducing metal uptake by various means or losing accumulated metal through excretion process (Luoma, 1983). This makes the study of bioaccumulation important in the study of toxicity. However, after bioaccumulation of metal, toxicity happens (Lagauzère et al., 2009) and the metal might be biomagnified to higher trophic level.In this study, $Pb^{2+}$ accumulation was highest among the three metals followed by $Cr^{6+}$and $Cd^{2+}$. Excessive mucous secretion found in $Cr^{6+}$ exposed organisms may be a cause to lower accumulation of $Cr^{6+}$. The production of a mucus/metal complex has been interpreted as a barrier to metal uptake in tubificids exposed to Pb and Zn (Whitley, 1968).Therefore, this may be a cause to decrease of $Cd^{2+}$ accumulation after 72 h exposure to tubificid worms. Gillis et al., (2004) reported metallotheonein like protein (MTLP) formulated in the body of tubificid worms in exposure to Cd which increases in amount in with the increase of exposure duration. Metals can be removed from the body by the broken parts at the posterior end which may also cause decrease in the accumulation of the metals. Moreover, reduced accumulation of the metals at longer exposure durations can be related to mortality. In this study, the accumulation pattern of metals over time are well in agreement with the sigmoid curves of mortality over time. Therefore, it is evident from the study that the metals caused the various chronic and acute toxic impacts on *Tubifex spp.*

The findings of the present study can be useful in understanding the toxicological impacts of these metals in *Tubifex spp.* and the potential impacts to other organisms as well throughout the world. Since the toxicity varies with region as stated above, the findings from this study can also provide crucial information in formulating the pollution control guidelines compatible under the conditions of Bangladesh.

Tubificid worms have been used as live feed for fishes because of its high food value (Olaf, 1982). There has been many approaches to increase the production of these worms through culture. However, the quality guidelines for the culture conditions to ensure the environmental and public health safety are still unknown. The findings of the present study suggest that Cd, Cr and Pb are accumulated in the tissue of *Tubifex spp.* which are toxic to these organism above the certain concentration which can be predicted from the respective $LC_{50}$s of the metals. The metals can be transferred to fish and subsequently reaching human through food chain. The toxicity assessment of metals to tubificid worms would help us to develop the guidelines for culture medium of the worms as the demand for these worms are increasing as live fish food.

# Chapter 5 - Conclusion and Recommendations

## 5.1 Conclusion

The study evaluated the toxicity and bioaccumulation of cadmium, chromium and lead to tubificid worms. Cd was the most toxic among the three metals followed by Cr and Pb respectively. The $LC_{50}$s of $Cd^{2+}$, $Cr^{6+}$ and $Pb^{2+}$ to tubificid worms *(Tubifex spp.)* were lower than many studies in other regions of the world. So, the toxicity of metals to tubificid worms *(Tubifex spp.)* varies depending on the local conditions and origin of the organism. The toxicity of these metals were the result of bioaccumulation of the respective cations in their tissues which can cause accumulation and subsequent toxic effects in fish if used as live fish food.

## 5.2 Recommendations

Toxicological information is very important for assessing the environmental health and food safety. But for proper understanding the assessment under variable conditions are necessary. Since the metal uptake is competitive in the presence of multiple cations, studies should be done to evaluate the joint toxicities of metals on these organisms under variable environmental conditions. In addition, the findings of bioaccumulation in the present study also arose the question of long term exposure experiment with the worms. Since tubificid worms are popular live fish food the trophic transfer of the metals from worms accumulated with heavy metals are required. Therefore, the study findings recommends to establish guidelines for using the tubificid worms *(Tubifix spp.)* as fish foo

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# Appendices

## Appendix I

Trial exposures concentrations (µM) of $Cd^{2+}$, $Cr^{6+}$ and $Pb^{2+}$ for 96 h acute toxicity tests

| Metal | Trial 1 | Trial 2 | Trial 3 |
|---|---|---|---|
| $Cd^{2+}$ | 0.00 | 0.00 | 0.00 |
| | 0.20 | 0.01 | 0.005 |
| | 0.40 | 0.02 | 0.01 |
| | 0.80 | 0.04 | 0.02 |
| | 1.60 | 0.08 | 0.04 |
| | 3.20 | 0.16 | 0.06 |
| | 6.40 | 0.32 | 0.08 |
| | 10.00 | 0.64 | 0.10 |
| | 15.00 | 1.28 | 0.20 |
| | 20.00 | 2.56 | 0.30 |
| $Cr^{6+}$ | 0.00 | 0.00 | 0.00 |
| | 25.00 | 0.50 | 0.10 |
| | 50.00 | 1.00 | 0.20 |
| | 75.00 | 2.00 | 0.40 |
| | 100.00 | 4.00 | 0.80 |
| | 200.00 | 8.00 | 1.60 |
| | 300.00 | 12.00 | 3.20 |
| | 400.00 | 16.00 | 6.40 |

| | | | |
|---|---|---|---|
| | 600.00 | 20.00 | 10.00 |
| | 800.00 | 24.00 | 15.00 |
| $Pb^{2+}$ | 0.00 | 0.00 | 0.00 |
| | 50.00 | 5.00 | 0.50 |
| | 100.00 | 10.00 | 1.00 |
| | 150.00 | 15.00 | 2.00 |
| | 200.00 | 20.00 | 3.00 |
| | 300.00 | 25.00 | 4.00 |
| | 500.00 | 30.00 | 5.00 |
| | 800.00 | 40.00 | 6.00 |
| | 1000.00 | 50.00 | 8.00 |
| | 1200.00 | 100.00 | 10.00 |

**Appendix II**

Mortality (%) of tubificid (*tubifex*spp.) worms at different concentrations of $Cd^{2+}$, $Cr^{6+}$ and $Pb^{2+}$ at different exposure durations in the final exposure.

| Metal | Treatment no | Concentration of chemical (µM/L) | Exposure duration (hours) | | | | |
|---|---|---|---|---|---|---|---|
| | | | Cumulative mortality (%) | | | | |
| | | | 12 h | 24 h | 48 h | 72 h | 96 h |
| $Cr^{6+}$ | Control | 0.00 | 0 | 0 | 0 | 0 | 0 |
| | 1 | 0.20 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0.40 | 0 | 0 | 2 | 6 | 12 |
| | 3 | 0.80 | 0 | 6 | 14 | 24 | 36 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | 4 | 1.60 | 0 | 8 | 16 | 38 | 52 |
| | 5 | 3.20 | 2 | 8 | 18 | 42 | 88 |
| | 6 | 6.40 | 6 | 18 | 44 | 92 | 100 |
| | 7 | 10.00 | 12 | 48 | 92 | 100 | 100 |
| $Cd^{2+}$ | Control | 0.00 | 0 | 0 | 0 | 0 | 0 |
| | 1 | 0.02 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 0.04 | 0 | 0 | 0 | 6 | 12 |
| | 3 | 0.06 | 0 | 0 | 6 | 12 | 28 |
| | 4 | 0.08 | 0 | 6 | 12 | 28 | 60 |
| | 5 | 0.10 | 2 | 8 | 26 | 44 | 72 |
| | 6 | 0.20 | 6 | 22 | 54 | 86 | 100 |
| | 7 | 0.30 | 54 | 100 | 100 | 100 | 100 |
| $Pb^{2+}$ | Control | 0.00 | 0 | 0 | 0 | 0 | 0 |
| | 1 | 0.50 | 0 | 0 | 0 | 0 | 0 |
| | 2 | 1.00 | 0 | 0 | 0 | 6 | 12 |
| | 3 | 1.50 | 0 | 2 | 10 | 18 | 40 |
| | 4 | 2.00 | 0 | 10 | 28 | 38 | 56 |
| | 5 | 3.00 | 0 | 8 | 26 | 52 | 80 |
| | 6 | 4.00 | 2 | 16 | 72 | 100 | 100 |
| | 7 | 5.00 | 26 | 100 | 100 | 100 | 100 |

## Appendix III

**Fit Equation Description of 4 Parameter Sigmoidal curve**

***Variables: x and y***

reciprocal_y = 1/abs(y)
reciprocal_ysquare = 1/y^2
[Parameters]
a = max(y)-min(y) "Auto {{previous: 104.865}}
b = xwtr(x,y-min(y),.5)/4 "Auto {{previous: 0.0209542}}
x0 = x50(x,y-min(y),.5) "Auto {{previous: 0.0740099}}
y0 = min(y) "Auto {{previous: -5.23467}}
[Equation]
f= y0+a/(1+exp(-(x-x0)/b))
fit f to y
"fit f to y with weight reciprocal_y
"fit f to y with weight reciprocal_ysquare

## Appendix IV

**Statistical outputs of Analysis of 4 Parameter Sigmoidal curve fitting analysis**

**Equation: Sigmoidal, Sigmoid, 4 Parameter**

f= y0+a/(1+exp(-(x-x0)/b))

**Analysis of $LC_{50}$ of $Cd^{2+}$ using graphic analysis:**

| R | Rsqr | Adj Rsqr | Standard Error of Estimate |
|---|---|---|---|
| 0.9975 | 0.9951 | 0.9914 | 3.9088 |

| | Coefficient | Std. Error | t | P | VIF |
|---|---|---|---|---|---|
| a | 104.8653 | 5.6725 | 18.4867 | <0.0001 | 6.4663< |
| b | 0.0210 | 0.0031 | 6.7573 | 0.0025 | 2.5560 |
| x0 | 0.0740 | 0.0033 | 22.5191 | <0.0001 | 2.9124 |
| y0 | -5.2347 | 4.5954 | -1.1391 | 0.3182 | 11.0570< |

**Analysis of Variance:**

Uncorrected for the mean of the observations:

| | DF | SS | MS |
|---|---|---|---|
| Regression | 4 | 29650.8845 | 7412.7211 |
| Residual | 4 | 61.1155 | 15.2789 |
| Total | 8 | 29712.0000 | 3714.0000 |

Corrected for the mean of the observations:

| | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Regression | 3 | 12352.8845 | 4117.6282 | 269.4983 | <0.0001 |

| | | | |
|---|---|---|---|
| Residual | 4 | 61.1155 | 15.2789 |
| Total | 7 | 12414.0000 | 1773.4286 |

**Statistical Tests:**

**PRESS** 318.7954

**Durbin-Watson Statistic** 3.2658 Failed

**Normality Test** Passed (P = 0.9662)

K-S Statistic = 0.1662 Significance Level = 0.9662

**Constant Variance Test** Passed (P = 0.8849)

**Power of performed test with alpha = 0.0500: 1.0000**

**Regression Diagnostics:**

| Row | Std. Res. | Stud. Res. | Stud. Del. Res. |
|---|---|---|---|
| 1 | 0.5769 | 0.9801 | 0.9737 |
| 2 | -0.5548 | -0.6856 | -0.6320 |
| 3 | -0.0116 | -0.0149 | -0.0129 |
| 4 | -0.5871 | -0.8445 | -0.8068 |
| 5 | 1.3708 | 1.8363 | 4.0132< |
| 6 | -1.0492 | -1.8084 | -3.6669< |
| 7 | 0.1600 | 0.2221 | 0.1935 |
| 8 | 0.0950 | 0.1356 | 0.1177 |

**95% Confidence:**

| Row | Predicted | 95% Conf-L | 95% Conf-U | 95% Pred-L | 95% Pred-U |
|---|---|---|---|---|---|
| 1 | -2.2548 | -11.0285 | 6.5188 | -16.2103 | 11.7007 |
| 2 | 2.1688 | -4.2064 | 8.5439 | -10.4178 | 14.7553 |
| 3 | 12.0454 | 5.2786 | 18.8122 | -0.7440 | 24.8348 |
| 4 | 30.2949 | 22.4941 | 38.0956 | 16.9296 | 43.6602 |
| 5 | 54.6417 | 47.4208 | 61.8626 | 41.6063 | 67.6770 |
| 6 | 76.1010 | 67.2616 | 84.9405 | 62.1041 | 90.0980 |
| 7 | 99.3746 | 91.8487 | 106.9005 | 86.1679 | 112.5813 |
| 8 | 99.6285 | 91.8863 | 107.3706 | 86.2973 | 112.9597 |

**Analysis of $LC_{50}$ of $Cr^{6+}$ using graphic analysis:**

**Equation: Sigmoidal, Sigmoid, 4 Parameter**

f= y0+a/(1+exp(-(x-x0)/b))

| R | Rsqr | Adj Rsqr | Standard Error of Estimate |
|---|---|---|---|
| 0.9865 | 0.9733 | 0.9532 | 9.3488 |

| | Coefficient | Std. Error | t | P | VIF |
|---|---|---|---|---|---|
| a | 97.7024(NAN) | (+inf) | <0.0001 | 0.0000 | |
| b | 0.5804(NAN) | (+inf) | <0.0001 | 0.0000 | |
| x0 | 1.4722(NAN) | (+inf) | <0.0001 | 0.0000 | |
| y0 | 0.0000(+inf) | 0.0000 | 1.0000 | 0.0000 | |

**Analysis of Variance:**

Uncorrected for the mean of the observations:

| | DF | SS | MS |
|---|---|---|---|
| Regression | 4 | 31538.4014 | 7884.6003 |
| Residual | 4 | 349.5986 | 87.3997 |
| Total | 8 | 31888.0000 | 3986.0000 |

Corrected for the mean of the observations:

| | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Regression | 3 | 12720.4014 | 4240.1338 | 48.5143 | 0.0013 |
| Residual | 4 | 349.5986 | 87.3997 | | |
| Total | 7 | 13070.0000 | 1867.1429 | | |

**Statistical Tests:**

**PRESS** (NAN)

**Durbin-Watson Statistic** 1.5893 Passed

**Normality Test** Passed (P = 0.6579)

K-S Statistic = 0.2450 Significance Level = 0.6579

**Constant Variance Test** Passed (P = 0.3533)

**Power of performed test with alpha = 0.0500: 0.9999**

**Regression Diagnostics:**

| Row | Std. Res. | Stud. Res. | Stud. Del. Res. |
|---|---|---|---|
| 1 | -0.7663(+inf)< | (+inf)< | |
| 2 | -1.0499(+inf)< | (+inf)< | |
| 3 | -0.1395(+inf)< | (+inf)< | |

| | | |
|---|---|---|
| 4 | 1.3532(+inf)< | (+inf)< |
| 5 | -0.2360(+inf)< | (+inf)< |
| 6 | -0.5312(+inf)< | (+inf)< |
| 7 | 0.2479(+inf)< | (+inf)< |
| 8 | 0.2458(+inf)< | (+inf)< |

**95% Confidence:**

| Row | Predicted | 95% Conf-L | 95% Conf-U | 95% Pred-L | 95% Pred-U |
|---|---|---|---|---|---|
| 1 | 7.1642(NAN) | (NAN) | (NAN) | (NAN) | |
| 2 | 9.8157(NAN) | (NAN) | (NAN) | (NAN) | |
| 3 | 13.3042(NAN) | (NAN) | (NAN) | (NAN) | |
| 4 | 23.3491(NAN) | (NAN) | (NAN) | (NAN) | |
| 5 | 54.2063(NAN) | (NAN) | (NAN) | (NAN) | |
| 6 | 92.9658(NAN) | (NAN) | (NAN) | (NAN) | |
| 7 | 97.6823(NAN) | (NAN) | (NAN) | (NAN) | |
| 8 | 97.7023(NAN) | (NAN) | (NAN) | (NAN) | |

**Analysis of $LC_{50}$ of $Pb^{2+}$using graphic analysis:**

**Equation: Sigmoidal, Sigmoid, 4 Parameter**
f= y0+a/(1+exp(-(x-x0)/b))

| R | Rsqr | Adj Rsqr | Standard Error of Estimate |
|---|---|---|---|
| 0.9952 | 0.9905 | 0.9834 | 5.4444 |

| | Coefficient | | Std. Error | t | P | VIF |
|---|---|---|---|---|---|---|
| a | 114.1076 | 14.1987 | 8.0365 | 0.0013 | 22.1969< | |
| b | 0.7011 | 0.1793 | 3.9111 | 0.0174 | 5.7253< | |
| x0 | 1.7360 | 0.1708 | 10.1625 | 0.0005 | 4.9837< | |
| y0 | -12.8897 | 10.8795 | -1.1848 | 0.3017 | 31.9458< | |

**Analysis of Variance:**

Uncorrected for the mean of the observations:

| | DF | SS | MS |
|---|---|---|---|
| Regression | 4 | 31161.4361 | 7790.3590 |
| Residual | 4 | 118.5639 | 29.6410 |
| Total | 8 | 31280.0000 | 3910.0000 |

Corrected for the mean of the observations:

| | DF | SS | MS | F | P |
|---|---|---|---|---|---|
| Regression | 3 | 12343.4361 | 4114.4787 | 138.8105 | 0.0002 |
| Residual | 4 | 118.5639 | 29.6410 | | |
| Total | 7 | 12462.0000 | 1780.2857 | | |

**Statistical Tests:**

**PRESS** 598.3973
**Durbin-Watson Statistic** 2.5129 Failed

**Normality Test** Passed (P = 0.8739)

K-S Statistic = 0.1984 Significance Level = 0.8739

**Constant Variance Test** Passed (P = 0.2897)
**Power of performed test with alpha = 0.0500: 1.0000**
**Regression Diagnostics:**

| Row | Std. Res. | Stud. Res. | Stud. Del. Res. |
|---|---|---|---|
| 1 | 0.7418 | 1.4533 | 1.8320 |
| 2 | -0.7018 | -0.8630 | -0.8285 |
| 3 | -0.8628 | -1.1208 | -1.1720 |
| 4 | 0.9820 | 1.2953 | 1.4723 |
| 5 | 0.2238 | 0.3121 | 0.2736 |
| 6 | -0.9314 | -1.4346 | -1.7831 |
| 7 | 0.5745 | 0.7254 | 0.6741 |
| 8 | -0.0262 | -0.0445 | -0.0386 |

**95% Confidence:**

| Row | Predicted | 95% Conf-L | 95% Conf-U | 95% Pred-L | 95% Pred-U |
|---|---|---|---|---|---|
| 1 | -4.0389 | -17.0372 | 8.9595 | -23.9750 | 15.8972 |
| 2 | 3.8206 | -4.9778 | 12.6190 | -13.6695 | 21.3107 |
| 3 | 16.6974 | 7.0489 | 26.3460 | -1.2354 | 34.6303 |
| 4 | 34.6534 | 24.7967 | 44.5101 | 16.6077 | 52.6991 |
| 5 | 54.7817 | 44.2456 | 65.3179 | 36.3561 | 73.2073 |
| 6 | 85.0708 | 73.5738 | 96.5679 | 66.0794 | 104.0623 |
| 7 | 96.8720 | 87.6447 | 106.0993 | 79.1622 | 114.5817 |
| 8 | 100.1429 | 87.9357 | 112.3501 | 80.7134 | 119.5724 |

## Appendix V

**ANOVA Table for Cd**

**Univariate Analysis of Variance**

**Between-Subjects Factors**

| | | N |
|---|---|---|
| Duration of exposure (h) | 0 | 16 |
| | 12 | 16 |
| | 24 | 16 |
| | 48 | 16 |
| | 72 | 16 |
| | 96 | 16 |
| Concentration of chemical (µM) | .00 | 12 |
| | .02 | 12 |
| | .04 | 12 |
| | .06 | 12 |
| | .08 | 12 |
| | .10 | 12 |
| | .20 | 12 |
| | .30 | 12 |

**Levene's Test of Equality of Error Variances[a]**

Dependent Variable: Mortality (%)

| F | df1 | df2 | Sig. |
|---|---|---|---|
| . | 47 | 48 | . |

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.[a]

a. Design: Intercept + Durationofexposureh + ConcentrationofchemicalµM + Durationofexposureh * ConcentrationofchemicalµM

Tests of Between-Subjects Effects

Dependent Variable: Mortality (%)

| Source | Type III Sum of Squares | df | Mean Square | F |
|---|---|---|---|---|
| Corrected Model | 109226.000[a] | 47 | 2323.957 | 74.966 |
| Intercept | 45414.000 | 1 | 45414.000 | 1464.968 |
| Durationofexposureh | 23612.000 | 5 | 4722.400 | 152.335 |
| ConcentrationofchemicalµM | 59492.667 | 7 | 8498.952 | 274.160 |
| Durationofexposureh * ConcentrationofchemicalµM | 26121.333 | 35 | 746.324 | 24.075 |
| Error | 1488.000 | 48 | 31.000 | |
| Total | 156128.000 | 96 | | |
| Corrected Total | 110714.000 | 95 | | |

**Tests of Between-Subjects Effects**

Dependent Variable: Mortality (%)

| Source | Sig. |
|---|---|
| Corrected Model | .000[a] |
| Intercept | .000 |
| Durationofexposureh | .000 |
| ConcentrationofchemicalµM | .000 |
| Durationofexposureh * ConcentrationofchemicalµM | .000 |
| Error | |
| Total | |
| Corrected Total | |

a. R Squared = .987 (Adjusted R Squared = .973)

**Estimated Marginal Means**

**Grand Mean**

Dependent Variable: Mortality (%)

| Mean | Std. Error | 95% Confidence Interval | |
|---|---|---|---|
| | | Lower Bound | Upper Bound |
| 21.750 | .568 | 20.607 | 22.893 |

**Post Hoc Tests**

**Duration of exposure (h)**

**Multiple Comparisons**

Dependent Variable: Mortality (%)

| | (I) Duration of exposure (h) | (J) Duration of exposure (h) | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| Tukey HSD | 0 | 12 | -7.75* | 1.969 | .003 | -13.59 | -1.91 |
| | | 24 | -17.00* | 1.969 | .000 | -22.84 | -11.16 |
| | | 48 | -24.75* | 1.969 | .000 | -30.59 | -18.91 |
| | | 72 | -34.50* | 1.969 | .000 | -40.34 | -28.66 |
| | | 96 | -46.50* | 1.969 | .000 | -52.34 | -40.66 |
| | 12 | 0 | 7.75* | 1.969 | .003 | 1.91 | 13.59 |
| | | 24 | -9.25* | 1.969 | .000 | -15.09 | -3.41 |
| | | 48 | -17.00* | 1.969 | .000 | -22.84 | -11.16 |
| | | 72 | -26.75* | 1.969 | .000 | -32.59 | -20.91 |
| | | 96 | -38.75* | 1.969 | .000 | -44.59 | -32.91 |
| | 24 | 0 | 17.00* | 1.969 | .000 | 11.16 | 22.84 |
| | | 12 | 9.25* | 1.969 | .000 | 3.41 | 15.09 |
| | | 48 | -7.75* | 1.969 | .003 | -13.59 | -1.91 |
| | | 72 | -17.50* | 1.969 | .000 | -23.34 | -11.66 |
| | | 96 | -29.50* | 1.969 | .000 | -35.34 | -23.66 |
| | 48 | 0 | 24.75* | 1.969 | .000 | 18.91 | 30.59 |
| | | 12 | 17.00* | 1.969 | .000 | 11.16 | 22.84 |
| | | 24 | 7.75* | 1.969 | .003 | 1.91 | 13.59 |
| | | 72 | -9.75* | 1.969 | .000 | -15.59 | -3.91 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | 96 | -21.75* | 1.969 | .000 | -27.59 | -15.91 |
| | | 0 | 34.50* | 1.969 | .000 | 28.66 | 40.34 |
| | | 12 | 26.75* | 1.969 | .000 | 20.91 | 32.59 |
| | 72 | 24 | 17.50* | 1.969 | .000 | 11.66 | 23.34 |
| | | 48 | 9.75* | 1.969 | .000 | 3.91 | 15.59 |
| | | 96 | -12.00* | 1.969 | .000 | -17.84 | -6.16 |
| | | 0 | 46.50* | 1.969 | .000 | 40.66 | 52.34 |
| | | 12 | 38.75* | 1.969 | .000 | 32.91 | 44.59 |
| | 96 | 24 | 29.50* | 1.969 | .000 | 23.66 | 35.34 |
| | | 48 | 21.75* | 1.969 | .000 | 15.91 | 27.59 |
| | | 72 | 12.00* | 1.969 | .000 | 6.16 | 17.84 |
| | | 12 | -7.75* | 1.969 | .000 | -11.71 | -3.79 |
| | | 24 | -17.00* | 1.969 | .000 | -20.96 | -13.04 |
| | 0 | 48 | -24.75* | 1.969 | .000 | -28.71 | -20.79 |
| | | 72 | -34.50* | 1.969 | .000 | -38.46 | -30.54 |
| | | 96 | -46.50* | 1.969 | .000 | -50.46 | -42.54 |
| | | 0 | 7.75* | 1.969 | .000 | 3.79 | 11.71 |
| LSD | | 24 | -9.25* | 1.969 | .000 | -13.21 | -5.29 |
| | 12 | 48 | -17.00* | 1.969 | .000 | -20.96 | -13.04 |
| | | 72 | -26.75* | 1.969 | .000 | -30.71 | -22.79 |
| | | 96 | -38.75* | 1.969 | .000 | -42.71 | -34.79 |
| | | 0 | 17.00* | 1.969 | .000 | 13.04 | 20.96 |
| | 24 | 12 | 9.25* | 1.969 | .000 | 5.29 | 13.21 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 48 | -7.75* | 1.969 | .000 | -11.71 | -3.79 |
| | 72 | -17.50* | 1.969 | .000 | -21.46 | -13.54 |
| | 96 | -29.50* | 1.969 | .000 | -33.46 | -25.54 |
| | 0 | 24.75* | 1.969 | .000 | 20.79 | 28.71 |
| | 12 | 17.00* | 1.969 | .000 | 13.04 | 20.96 |
| 48 | 24 | 7.75* | 1.969 | .000 | 3.79 | 11.71 |
| | 72 | -9.75* | 1.969 | .000 | -13.71 | -5.79 |
| | 96 | -21.75* | 1.969 | .000 | -25.71 | -17.79 |
| | 0 | 34.50* | 1.969 | .000 | 30.54 | 38.46 |
| | 12 | 26.75* | 1.969 | .000 | 22.79 | 30.71 |
| 72 | 24 | 17.50* | 1.969 | .000 | 13.54 | 21.46 |
| | 48 | 9.75* | 1.969 | .000 | 5.79 | 13.71 |
| | 96 | -12.00* | 1.969 | .000 | -15.96 | -8.04 |
| | 0 | 46.50* | 1.969 | .000 | 42.54 | 50.46 |
| | 12 | 38.75* | 1.969 | .000 | 34.79 | 42.71 |
| 96 | 24 | 29.50* | 1.969 | .000 | 25.54 | 33.46 |
| | 48 | 21.75* | 1.969 | .000 | 17.79 | 25.71 |
| | 72 | 12.00* | 1.969 | .000 | 8.04 | 15.96 |

Based on observed means.

The error term is Mean Square (Error) = 31.000.

*. The mean difference is significant at the .05 level.

**Homogeneous Subsets**

**Mortality (%)**

| | Duration of exposure (h) | N | Subset | | | |
|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 |
| Tukey HSD[a,b] | 0 | 16 | .00 | | | |
| | 12 | 16 | | 7.75 | | |
| | 24 | 16 | | | 17.00 | |
| | 48 | 16 | | | | 24.75 |
| | 72 | 16 | | | | |
| | 96 | 16 | | | | |
| | Sig. | | 1.000 | 1.000 | 1.000 | 1.000 |

**Mortality (%)**

| | Duration of exposure (h) | Subset | |
|---|---|---|---|
| | | 5 | 6 |
| Tukey HSD[a,b] | 0 | | |
| | 12 | | |
| | 24 | | |
| | 48 | | |
| | 72 | 34.50 | |
| | 96 | | 46.50 |
| | Sig. | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square (Error) = 31.000.

a. Uses Harmonic Mean Sample Size = 16.000.

b. Alpha = .05.

**Concentration of chemical (µM)**

**Multiple Comparisons**

Dependent Variable: Mortality (%)

| | (I) Concentration of chemical (µM) | (J) Concentration of chemical (µM) | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| Tukey HSD | .00 | .02 | .00 | 2.273 | 1.000 | -7.20 | 7.20 |
| | | .04 | -3.00 | 2.273 | .887 | -10.20 | 4.20 |
| | | .06 | -7.67* | 2.273 | .029 | -14.87 | -.47 |
| | | .08 | -17.67* | 2.273 | .000 | -24.87 | -10.47 |
| | | .10 | -25.33* | 2.273 | .000 | -32.53 | -18.13 |
| | | .20 | -44.67* | 2.273 | .000 | -51.87 | -37.47 |
| | | .30 | -75.67* | 2.273 | .000 | -82.87 | -68.47 |
| | .02 | .00 | .00 | 2.273 | 1.000 | -7.20 | 7.20 |
| | | .04 | -3.00 | 2.273 | .887 | -10.20 | 4.20 |
| | | .06 | -7.67* | 2.273 | .029 | -14.87 | -.47 |
| | | .08 | -17.67* | 2.273 | .000 | -24.87 | -10.47 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | .10 | -25.33* | 2.273 | .000 | -32.53 | -18.13 |
| | .20 | -44.67* | 2.273 | .000 | -51.87 | -37.47 |
| | .30 | -75.67* | 2.273 | .000 | -82.87 | -68.47 |
| | .00 | 3.00 | 2.273 | .887 | -4.20 | 10.20 |
| | .02 | 3.00 | 2.273 | .887 | -4.20 | 10.20 |
| | .06 | -4.67 | 2.273 | .459 | -11.87 | 2.53 |
| .04 | .08 | -14.67* | 2.273 | .000 | -21.87 | -7.47 |
| | .10 | -22.33* | 2.273 | .000 | -29.53 | -15.13 |
| | .20 | -41.67* | 2.273 | .000 | -48.87 | -34.47 |
| | .30 | -72.67* | 2.273 | .000 | -79.87 | -65.47 |
| | .00 | 7.67* | 2.273 | .029 | .47 | 14.87 |
| | .02 | 7.67* | 2.273 | .029 | .47 | 14.87 |
| | .04 | 4.67 | 2.273 | .459 | -2.53 | 11.87 |
| .06 | .08 | -10.00* | 2.273 | .001 | -17.20 | -2.80 |
| | .10 | -17.67* | 2.273 | .000 | -24.87 | -10.47 |
| | .20 | -37.00* | 2.273 | .000 | -44.20 | -29.80 |
| | .30 | -68.00* | 2.273 | .000 | -75.20 | -60.80 |
| | .00 | 17.67* | 2.273 | .000 | 10.47 | 24.87 |
| | .02 | 17.67* | 2.273 | .000 | 10.47 | 24.87 |
| | .04 | 14.67* | 2.273 | .000 | 7.47 | 21.87 |
| .08 | .06 | 10.00* | 2.273 | .001 | 2.80 | 17.20 |
| | .10 | -7.67* | 2.273 | .029 | -14.87 | -.47 |
| | .20 | -27.00* | 2.273 | .000 | -34.20 | -19.80 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | .30 | -58.00* | 2.273 | .000 | -65.20 | -50.80 |
| | .00 | 25.33* | 2.273 | .000 | 18.13 | 32.53 |
| | .02 | 25.33* | 2.273 | .000 | 18.13 | 32.53 |
| | .04 | 22.33* | 2.273 | .000 | 15.13 | 29.53 |
| .10 | .06 | 17.67* | 2.273 | .000 | 10.47 | 24.87 |
| | .08 | 7.67* | 2.273 | .029 | .47 | 14.87 |
| | .20 | -19.33* | 2.273 | .000 | -26.53 | -12.13 |
| | .30 | -50.33* | 2.273 | .000 | -57.53 | -43.13 |
| | .00 | 44.67* | 2.273 | .000 | 37.47 | 51.87 |
| | .02 | 44.67* | 2.273 | .000 | 37.47 | 51.87 |
| | .04 | 41.67* | 2.273 | .000 | 34.47 | 48.87 |
| .20 | .06 | 37.00* | 2.273 | .000 | 29.80 | 44.20 |
| | .08 | 27.00* | 2.273 | .000 | 19.80 | 34.20 |
| | .10 | 19.33* | 2.273 | .000 | 12.13 | 26.53 |
| | .30 | -31.00* | 2.273 | .000 | -38.20 | -23.80 |
| | .00 | 75.67* | 2.273 | .000 | 68.47 | 82.87 |
| | .02 | 75.67* | 2.273 | .000 | 68.47 | 82.87 |
| | .04 | 72.67* | 2.273 | .000 | 65.47 | 79.87 |
| .30 | .06 | 68.00* | 2.273 | .000 | 60.80 | 75.20 |
| | .08 | 58.00* | 2.273 | .000 | 50.80 | 65.20 |
| | .10 | 50.33* | 2.273 | .000 | 43.13 | 57.53 |
| | .20 | 31.00* | 2.273 | .000 | 23.80 | 38.20 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | .02 | .00 | 2.273 | 1.000 | -4.57 | 4.57 |
| | | .04 | -3.00 | 2.273 | .193 | -7.57 | 1.57 |
| | | .06 | -7.67* | 2.273 | .001 | -12.24 | -3.10 |
| | .00 | .08 | -17.67* | 2.273 | .000 | -22.24 | -13.10 |
| | | .10 | -25.33* | 2.273 | .000 | -29.90 | -20.76 |
| | | .20 | -44.67* | 2.273 | .000 | -49.24 | -40.10 |
| | | .30 | -75.67* | 2.273 | .000 | -80.24 | -71.10 |
| | | .00 | .00 | 2.273 | 1.000 | -4.57 | 4.57 |
| | | .04 | -3.00 | 2.273 | .193 | -7.57 | 1.57 |
| | | .06 | -7.67* | 2.273 | .001 | -12.24 | -3.10 |
| LSD | .02 | .08 | -17.67* | 2.273 | .000 | -22.24 | -13.10 |
| | | .10 | -25.33* | 2.273 | .000 | -29.90 | -20.76 |
| | | .20 | -44.67* | 2.273 | .000 | -49.24 | -40.10 |
| | | .30 | -75.67* | 2.273 | .000 | -80.24 | -71.10 |
| | | .00 | 3.00 | 2.273 | .193 | -1.57 | 7.57 |
| | | .02 | 3.00 | 2.273 | .193 | -1.57 | 7.57 |
| | | .06 | -4.67* | 2.273 | .046 | -9.24 | -.10 |
| | .04 | .08 | -14.67* | 2.273 | .000 | -19.24 | -10.10 |
| | | .10 | -22.33* | 2.273 | .000 | -26.90 | -17.76 |
| | | .20 | -41.67* | 2.273 | .000 | -46.24 | -37.10 |
| | | .30 | -72.67* | 2.273 | .000 | -77.24 | -68.10 |
| | .06 | .00 | 7.67* | 2.273 | .001 | 3.10 | 12.24 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | .02 | 7.67* | 2.273 | .001 | 3.10 | 12.24 |
| | .04 | 4.67* | 2.273 | .046 | .10 | 9.24 |
| | .08 | -10.00* | 2.273 | .000 | -14.57 | -5.43 |
| | .10 | -17.67* | 2.273 | .000 | -22.24 | -13.10 |
| | .20 | -37.00* | 2.273 | .000 | -41.57 | -32.43 |
| | .30 | -68.00* | 2.273 | .000 | -72.57 | -63.43 |
| | .00 | 17.67* | 2.273 | .000 | 13.10 | 22.24 |
| | .02 | 17.67* | 2.273 | .000 | 13.10 | 22.24 |
| | .04 | 14.67* | 2.273 | .000 | 10.10 | 19.24 |
| .08 | .06 | 10.00* | 2.273 | .000 | 5.43 | 14.57 |
| | .10 | -7.67* | 2.273 | .001 | -12.24 | -3.10 |
| | .20 | -27.00* | 2.273 | .000 | -31.57 | -22.43 |
| | .30 | -58.00* | 2.273 | .000 | -62.57 | -53.43 |
| | .00 | 25.33* | 2.273 | .000 | 20.76 | 29.90 |
| | .02 | 25.33* | 2.273 | .000 | 20.76 | 29.90 |
| | .04 | 22.33* | 2.273 | .000 | 17.76 | 26.90 |
| .10 | .06 | 17.67* | 2.273 | .000 | 13.10 | 22.24 |
| | .08 | 7.67* | 2.273 | .001 | 3.10 | 12.24 |
| | .20 | -19.33* | 2.273 | .000 | -23.90 | -14.76 |
| | .30 | -50.33* | 2.273 | .000 | -54.90 | -45.76 |
| | .00 | 44.67* | 2.273 | .000 | 40.10 | 49.24 |
| .20 | .02 | 44.67* | 2.273 | .000 | 40.10 | 49.24 |
| | .04 | 41.67* | 2.273 | .000 | 37.10 | 46.24 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | .06 | 37.00* | 2.273 | .000 | 32.43 | 41.57 |
| | .08 | 27.00* | 2.273 | .000 | 22.43 | 31.57 |
| | .10 | 19.33* | 2.273 | .000 | 14.76 | 23.90 |
| | .30 | -31.00* | 2.273 | .000 | -35.57 | -26.43 |
| | .00 | 75.67* | 2.273 | .000 | 71.10 | 80.24 |
| | .02 | 75.67* | 2.273 | .000 | 71.10 | 80.24 |
| | .04 | 72.67* | 2.273 | .000 | 68.10 | 77.24 |
| .30 | .06 | 68.00* | 2.273 | .000 | 63.43 | 72.57 |
| | .08 | 58.00* | 2.273 | .000 | 53.43 | 62.57 |
| | .10 | 50.33* | 2.273 | .000 | 45.76 | 54.90 |
| | .20 | 31.00* | 2.273 | .000 | 26.43 | 35.57 |

Based on observed means.

The error term is Mean Square (Error) = 31.000.

*. The mean difference is significant at the .05 level.

**Homogeneous Subsets**

**Mortality (%)**

| | Concentration of chemical (µM) | N | Subset | | | |
|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 |
| Tukey HSD[a,b] | .00 | 12 | .00 | | | |
| | .02 | 12 | .00 | | | |
| | .04 | 12 | 3.00 | 3.00 | | |
| | .06 | 12 | | 7.67 | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | .08 | 12 | | | 17.67 | |
| | .10 | 12 | | | | 25.33 |
| | .20 | 12 | | | | |
| | .30 | 12 | | | | |
| | Sig. | | .887 | .459 | 1.000 | 1.000 |

**Mortality (%)**

| | Concentration of chemical (µM) | Subset | |
|---|---|---|---|
| | | 5 | 6 |
| Tukey HSD[a,b] | .00 | | |
| | .02 | | |
| | .04 | | |
| | .06 | | |
| | .08 | | |
| | .10 | | |
| | .20 | 44.67 | |
| | .30 | | 75.67 |
| | Sig. | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square (Error) = 31.000.

a. Uses Harmonic Mean Sample Size = 12.000.

b. Alpha = .05.

**ANOVA Table for Cr**

**Univariate Analysis of Variance**

**Between-Subjects Factors**

| | | N |
|---|---|---|
| Duration of exposure (h) | 0 | 16 |
| | 12 | 16 |
| | 24 | 16 |
| | 48 | 16 |
| | 72 | 16 |
| | 96 | 16 |
| Concentration of chemical (µM) | .0 | 12 |
| | .2 | 12 |
| | .4 | 12 |
| | .8 | 12 |
| | 1.6 | 12 |
| | 3.2 | 12 |
| | 6.4 | 12 |
| | 10.0 | 12 |

**Levene's Test of Equality of Error Variances[a]**

Dependent Variable: Mortality (%)

| F | df1 | df2 | Sig. |
|---|---|---|---|
| . | 47 | 48 | . |

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.[a]

a. Design: Intercept + Durationofexposureh + ConcentrationofchemicalµM + Durationofexposureh * ConcentrationofchemicalµM

Tests of Between-Subjects Effects

Dependent Variable: Mortality (%)

| Source | Type III Sum of Squares | df | Mean Square | F |
|---|---|---|---|---|
| Corrected Model | 96312.000[a] | 47 | 2049.191 | 120.541 |
| Intercept | 40344.000 | 1 | 40344.000 | 2373.176 |
| Durationofexposureh | 30778.000 | 5 | 6155.600 | 362.094 |
| ConcentrationofchemicalµM | 38410.667 | 7 | 5487.238 | 322.779 |
| Durationofexposureh * ConcentrationofchemicalµM | 27123.333 | 35 | 774.952 | 45.585 |
| Error | 816.000 | 48 | 17.000 | |
| Total | 137472.000 | 96 | | |
| Corrected Total | 97128.000 | 95 | | |

**Tests of Between-Subjects Effects**

Dependent Variable: Mortality (%)

| Source | Sig. |
|---|---|
| Corrected Model | .000[a] |
| Intercept | .000 |
| Durationofexposureh | .000 |
| ConcentrationofchemicalμM | .000 |
| Durationofexposureh * ConcentrationofchemicalμM | .000 |
| Error | |
| Total | |
| Corrected Total | |

a. R Squared = .992 (Adjusted R Squared = .983)

**Estimated Marginal Means**

**Grand Mean**

Dependent Variable: Mortality (%)

| Mean | Std. Error | 95% Confidence Interval | |
|---|---|---|---|
| | | Lower Bound | Upper Bound |
| 20.500 | .421 | 19.654 | 21.346 |

**Post Hoc Tests**

**Duration of exposure (h)**

**Multiple Comparisons**

Dependent Variable: Mortality (%)

| | (I) Duration of exposure (h) | (J) Duration of exposure (h) | Mean Differenc | Std. Error | Sig. | 95% Confidence Interval |
|---|---|---|---|---|---|---|

| | | | e (I-J) | | | Lower Bound | Upper Bound |
|---|---|---|---|---|---|---|---|
| | | 12 | -2.50 | 1.458 | .529 | -6.83 | 1.83 |
| | | 24 | -11.00* | 1.458 | .000 | -15.33 | -6.67 |
| | 0 | 48 | -23.25* | 1.458 | .000 | -27.58 | -18.92 |
| | | 72 | -37.75* | 1.458 | .000 | -42.08 | -33.42 |
| | | 96 | -48.50* | 1.458 | .000 | -52.83 | -44.17 |
| | | 0 | 2.50 | 1.458 | .529 | -1.83 | 6.83 |
| | | 24 | -8.50* | 1.458 | .000 | -12.83 | -4.17 |
| | 12 | 48 | -20.75* | 1.458 | .000 | -25.08 | -16.42 |
| | | 72 | -35.25* | 1.458 | .000 | -39.58 | -30.92 |
| | | 96 | -46.00* | 1.458 | .000 | -50.33 | -41.67 |
| Tukey HSD | | 0 | 11.00* | 1.458 | .000 | 6.67 | 15.33 |
| | | 12 | 8.50* | 1.458 | .000 | 4.17 | 12.83 |
| | 24 | 48 | -12.25* | 1.458 | .000 | -16.58 | -7.92 |
| | | 72 | -26.75* | 1.458 | .000 | -31.08 | -22.42 |
| | | 96 | -37.50* | 1.458 | .000 | -41.83 | -33.17 |
| | | 0 | 23.25* | 1.458 | .000 | 18.92 | 27.58 |
| | | 12 | 20.75* | 1.458 | .000 | 16.42 | 25.08 |
| | 48 | 24 | 12.25* | 1.458 | .000 | 7.92 | 16.58 |
| | | 72 | -14.50* | 1.458 | .000 | -18.83 | -10.17 |
| | | 96 | -25.25* | 1.458 | .000 | -29.58 | -20.92 |
| | 72 | 0 | 37.75* | 1.458 | .000 | 33.42 | 42.08 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| LSD | 96 | 12 | 35.25* | 1.458 | .000 | 30.92 | 39.58 |
| | | 24 | 26.75* | 1.458 | .000 | 22.42 | 31.08 |
| | | 48 | 14.50* | 1.458 | .000 | 10.17 | 18.83 |
| | | 96 | -10.75* | 1.458 | .000 | -15.08 | -6.42 |
| | | 0 | 48.50* | 1.458 | .000 | 44.17 | 52.83 |
| | 0 | 12 | 46.00* | 1.458 | .000 | 41.67 | 50.33 |
| | | 24 | 37.50* | 1.458 | .000 | 33.17 | 41.83 |
| | | 48 | 25.25* | 1.458 | .000 | 20.92 | 29.58 |
| | | 72 | 10.75* | 1.458 | .000 | 6.42 | 15.08 |
| | | 12 | -2.50 | 1.458 | .093 | -5.43 | .43 |
| | | 24 | -11.00* | 1.458 | .000 | -13.93 | -8.07 |
| | | 48 | -23.25* | 1.458 | .000 | -26.18 | -20.32 |
| | | 72 | -37.75* | 1.458 | .000 | -40.68 | -34.82 |
| | | 96 | -48.50* | 1.458 | .000 | -51.43 | -45.57 |
| | 12 | 0 | 2.50 | 1.458 | .093 | -.43 | 5.43 |
| | | 24 | -8.50* | 1.458 | .000 | -11.43 | -5.57 |
| | | 48 | -20.75* | 1.458 | .000 | -23.68 | -17.82 |
| | | 72 | -35.25* | 1.458 | .000 | -38.18 | -32.32 |
| | | 96 | -46.00* | 1.458 | .000 | -48.93 | -43.07 |
| | | 0 | 11.00* | 1.458 | .000 | 8.07 | 13.93 |
| | 24 | 12 | 8.50* | 1.458 | .000 | 5.57 | 11.43 |
| | | 48 | -12.25* | 1.458 | .000 | -15.18 | -9.32 |
| | | 72 | -26.75* | 1.458 | .000 | -29.68 | -23.82 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 96 | -37.50* | 1.458 | .000 | -40.43 | -34.57 |
| | 0 | 23.25* | 1.458 | .000 | 20.32 | 26.18 |
| | 12 | 20.75* | 1.458 | .000 | 17.82 | 23.68 |
| 48 | 24 | 12.25* | 1.458 | .000 | 9.32 | 15.18 |
| | 72 | -14.50* | 1.458 | .000 | -17.43 | -11.57 |
| | 96 | -25.25* | 1.458 | .000 | -28.18 | -22.32 |
| | 0 | 37.75* | 1.458 | .000 | 34.82 | 40.68 |
| | 12 | 35.25* | 1.458 | .000 | 32.32 | 38.18 |
| 72 | 24 | 26.75* | 1.458 | .000 | 23.82 | 29.68 |
| | 48 | 14.50* | 1.458 | .000 | 11.57 | 17.43 |
| | 96 | -10.75* | 1.458 | .000 | -13.68 | -7.82 |
| | 0 | 48.50* | 1.458 | .000 | 45.57 | 51.43 |
| | 12 | 46.00* | 1.458 | .000 | 43.07 | 48.93 |
| 96 | 24 | 37.50* | 1.458 | .000 | 34.57 | 40.43 |
| | 48 | 25.25* | 1.458 | .000 | 22.32 | 28.18 |
| | 72 | 10.75* | 1.458 | .000 | 7.82 | 13.68 |

Based on observed means.

The error term is Mean Square (Error) = 17.000.

*. The mean difference is significant at the .05 level.

**Homogeneous Subsets**

**Mortality (%)**

| | Duration of exposure (h) | N | Subset | | | |
|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 |
| Tukey HSD[a,b] | 0 | 16 | .00 | | | |
| | 12 | 16 | 2.50 | | | |
| | 24 | 16 | | 11.00 | | |
| | 48 | 16 | | | 23.25 | |
| | 72 | 16 | | | | 37.75 |
| | 96 | 16 | | | | |
| | Sig. | | .529 | 1.000 | 1.000 | 1.000 |

**Mortality (%)**

| | Duration of exposure (h) | Subset |
|---|---|---|
| | | 5 |
| Tukey HSD[a,b] | 0 | |
| | 12 | |
| | 24 | |
| | 48 | |
| | 72 | |
| | 96 | 48.50 |
| | Sig. | 1.000 |

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square (Error) = 17.000.

a. Uses Harmonic Mean Sample Size = 16.000.

b. Alpha = .05.

**Concentration of chemical (µM)**

**Multiple Comparisons**

Dependent Variable: Mortality (%)

| | (I) Concentration of chemical (µM) | (J) Concentration of chemical (µM) | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| Tukey HSD | .0 | .2 | .00 | 1.683 | 1.000 | -5.33 | 5.33 |
| | | .4 | -3.33 | 1.683 | .505 | -8.67 | 2.00 |
| | | .8 | -13.33* | 1.683 | .000 | -18.67 | -8.00 |
| | | 1.6 | -19.00* | 1.683 | .000 | -24.33 | -13.67 |
| | | 3.2 | -26.33* | 1.683 | .000 | -31.67 | -21.00 |
| | | 6.4 | -43.33* | 1.683 | .000 | -48.67 | -38.00 |
| | | 10.0 | -58.67* | 1.683 | .000 | -64.00 | -53.33 |
| | .2 | .0 | .00 | 1.683 | 1.000 | -5.33 | 5.33 |
| | | .4 | -3.33 | 1.683 | .505 | -8.67 | 2.00 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | .8 | -13.33* | 1.683 | .000 | -18.67 | -8.00 |
| | 1.6 | -19.00* | 1.683 | .000 | -24.33 | -13.67 |
| | 3.2 | -26.33* | 1.683 | .000 | -31.67 | -21.00 |
| | 6.4 | -43.33* | 1.683 | .000 | -48.67 | -38.00 |
| | 10.0 | -58.67* | 1.683 | .000 | -64.00 | -53.33 |
| | .0 | 3.33 | 1.683 | .505 | -2.00 | 8.67 |
| | .2 | 3.33 | 1.683 | .505 | -2.00 | 8.67 |
| | .8 | -10.00* | 1.683 | .000 | -15.33 | -4.67 |
| .4 | 1.6 | -15.67* | 1.683 | .000 | -21.00 | -10.33 |
| | 3.2 | -23.00* | 1.683 | .000 | -28.33 | -17.67 |
| | 6.4 | -40.00* | 1.683 | .000 | -45.33 | -34.67 |
| | 10.0 | -55.33* | 1.683 | .000 | -60.67 | -50.00 |
| | .0 | 13.33* | 1.683 | .000 | 8.00 | 18.67 |
| | .2 | 13.33* | 1.683 | .000 | 8.00 | 18.67 |
| | .4 | 10.00* | 1.683 | .000 | 4.67 | 15.33 |
| .8 | 1.6 | -5.67* | 1.683 | .030 | -11.00 | -.33 |
| | 3.2 | -13.00* | 1.683 | .000 | -18.33 | -7.67 |
| | 6.4 | -30.00* | 1.683 | .000 | -35.33 | -24.67 |
| | 10.0 | -45.33* | 1.683 | .000 | -50.67 | -40.00 |
| | .0 | 19.00* | 1.683 | .000 | 13.67 | 24.33 |
| | .2 | 19.00* | 1.683 | .000 | 13.67 | 24.33 |
| 1.6 | | | | | | |
| | .4 | 15.67* | 1.683 | .000 | 10.33 | 21.00 |
| | .8 | 5.67* | 1.683 | .030 | .33 | 11.00 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 3.2 | -7.33* | 1.683 | .002 | -12.67 | -2.00 |
| | 6.4 | -24.33* | 1.683 | .000 | -29.67 | -19.00 |
| | 10.0 | -39.67* | 1.683 | .000 | -45.00 | -34.33 |
| | .0 | 26.33* | 1.683 | .000 | 21.00 | 31.67 |
| | .2 | 26.33* | 1.683 | .000 | 21.00 | 31.67 |
| | .4 | 23.00* | 1.683 | .000 | 17.67 | 28.33 |
| 3.2 | .8 | 13.00* | 1.683 | .000 | 7.67 | 18.33 |
| | 1.6 | 7.33* | 1.683 | .002 | 2.00 | 12.67 |
| | 6.4 | -17.00* | 1.683 | .000 | -22.33 | -11.67 |
| | 10.0 | -32.33* | 1.683 | .000 | -37.67 | -27.00 |
| | .0 | 43.33* | 1.683 | .000 | 38.00 | 48.67 |
| | .2 | 43.33* | 1.683 | .000 | 38.00 | 48.67 |
| | .4 | 40.00* | 1.683 | .000 | 34.67 | 45.33 |
| 6.4 | .8 | 30.00* | 1.683 | .000 | 24.67 | 35.33 |
| | 1.6 | 24.33* | 1.683 | .000 | 19.00 | 29.67 |
| | 3.2 | 17.00* | 1.683 | .000 | 11.67 | 22.33 |
| | 10.0 | -15.33* | 1.683 | .000 | -20.67 | -10.00 |
| | .0 | 58.67* | 1.683 | .000 | 53.33 | 64.00 |
| | .2 | 58.67* | 1.683 | .000 | 53.33 | 64.00 |
| | .4 | 55.33* | 1.683 | .000 | 50.00 | 60.67 |
| 10.0 | .8 | 45.33* | 1.683 | .000 | 40.00 | 50.67 |
| | 1.6 | 39.67* | 1.683 | .000 | 34.33 | 45.00 |
| | 3.2 | 32.33* | 1.683 | .000 | 27.00 | 37.67 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| LSD | .0 | 6.4 | 15.33* | 1.683 | .000 | 10.00 | 20.67 |
| | | .2 | .00 | 1.683 | 1.000 | -3.38 | 3.38 |
| | | .4 | -3.33 | 1.683 | .053 | -6.72 | .05 |
| | | .8 | -13.33* | 1.683 | .000 | -16.72 | -9.95 |
| | | 1.6 | -19.00* | 1.683 | .000 | -22.38 | -15.62 |
| | | 3.2 | -26.33* | 1.683 | .000 | -29.72 | -22.95 |
| | | 6.4 | -43.33* | 1.683 | .000 | -46.72 | -39.95 |
| | | 10.0 | -58.67* | 1.683 | .000 | -62.05 | -55.28 |
| | .2 | .0 | .00 | 1.683 | 1.000 | -3.38 | 3.38 |
| | | .4 | -3.33 | 1.683 | .053 | -6.72 | .05 |
| | | .8 | -13.33* | 1.683 | .000 | -16.72 | -9.95 |
| | | 1.6 | -19.00* | 1.683 | .000 | -22.38 | -15.62 |
| | | 3.2 | -26.33* | 1.683 | .000 | -29.72 | -22.95 |
| | | 6.4 | -43.33* | 1.683 | .000 | -46.72 | -39.95 |
| | | 10.0 | -58.67* | 1.683 | .000 | -62.05 | -55.28 |
| | .4 | .0 | 3.33 | 1.683 | .053 | -.05 | 6.72 |
| | | .2 | 3.33 | 1.683 | .053 | -.05 | 6.72 |
| | | .8 | -10.00* | 1.683 | .000 | -13.38 | -6.62 |
| | | 1.6 | -15.67* | 1.683 | .000 | -19.05 | -12.28 |
| | | 3.2 | -23.00* | 1.683 | .000 | -26.38 | -19.62 |
| | | 6.4 | -40.00* | 1.683 | .000 | -43.38 | -36.62 |
| | | 10.0 | -55.33* | 1.683 | .000 | -58.72 | -51.95 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | .0 | 13.33* | 1.683 | .000 | 9.95 | 16.72 |
| | .2 | 13.33* | 1.683 | .000 | 9.95 | 16.72 |
| | .4 | 10.00* | 1.683 | .000 | 6.62 | 13.38 |
| .8 | 1.6 | -5.67* | 1.683 | .002 | -9.05 | -2.28 |
| | 3.2 | -13.00* | 1.683 | .000 | -16.38 | -9.62 |
| | 6.4 | -30.00* | 1.683 | .000 | -33.38 | -26.62 |
| | 10.0 | -45.33* | 1.683 | .000 | -48.72 | -41.95 |
| | .0 | 19.00* | 1.683 | .000 | 15.62 | 22.38 |
| | .2 | 19.00* | 1.683 | .000 | 15.62 | 22.38 |
| | .4 | 15.67* | 1.683 | .000 | 12.28 | 19.05 |
| 1.6 | .8 | 5.67* | 1.683 | .002 | 2.28 | 9.05 |
| | 3.2 | -7.33* | 1.683 | .000 | -10.72 | -3.95 |
| | 6.4 | -24.33* | 1.683 | .000 | -27.72 | -20.95 |
| | 10.0 | -39.67* | 1.683 | .000 | -43.05 | -36.28 |
| | .0 | 26.33* | 1.683 | .000 | 22.95 | 29.72 |
| | .2 | 26.33* | 1.683 | .000 | 22.95 | 29.72 |
| | .4 | 23.00* | 1.683 | .000 | 19.62 | 26.38 |
| 3.2 | .8 | 13.00* | 1.683 | .000 | 9.62 | 16.38 |
| | 1.6 | 7.33* | 1.683 | .000 | 3.95 | 10.72 |
| | 6.4 | -17.00* | 1.683 | .000 | -20.38 | -13.62 |
| | 10.0 | -32.33* | 1.683 | .000 | -35.72 | -28.95 |
| | .0 | 43.33* | 1.683 | .000 | 39.95 | 46.72 |
| 6.4 | .2 | 43.33* | 1.683 | .000 | 39.95 | 46.72 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | .4 | 40.00* | 1.683 | .000 | 36.62 | 43.38 |
| | .8 | 30.00* | 1.683 | .000 | 26.62 | 33.38 |
| | 1.6 | 24.33* | 1.683 | .000 | 20.95 | 27.72 |
| | 3.2 | 17.00* | 1.683 | .000 | 13.62 | 20.38 |
| | 10.0 | -15.33* | 1.683 | .000 | -18.72 | -11.95 |
| | .0 | 58.67* | 1.683 | .000 | 55.28 | 62.05 |
| | .2 | 58.67* | 1.683 | .000 | 55.28 | 62.05 |
| | .4 | 55.33* | 1.683 | .000 | 51.95 | 58.72 |
| 10.0 | .8 | 45.33* | 1.683 | .000 | 41.95 | 48.72 |
| | 1.6 | 39.67* | 1.683 | .000 | 36.28 | 43.05 |
| | 3.2 | 32.33* | 1.683 | .000 | 28.95 | 35.72 |
| | 6.4 | 15.33* | 1.683 | .000 | 11.95 | 18.72 |

Based on observed means.

The error term is Mean Square (Error) = 17.000.

*. The mean difference is significant at the .05 level.

**Homogeneous Subsets**

**Mortality (%)**

| | Concentration of chemical (µM) | N | Subset | | | |
|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 |
| Tukey HSD[a,b] | .0 | 12 | .00 | | | |
| | .2 | 12 | .00 | | | |
| | .4 | 12 | 3.33 | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | .8 | 12 | | 13.33 | | |
| | 1.6 | 12 | | | 19.00 | |
| | 3.2 | 12 | | | | 26.33 |
| | 6.4 | 12 | | | | |
| | 10.0 | 12 | | | | |
| | Sig. | | .505 | 1.000 | 1.000 | 1.000 |

**Mortality (%)**

| | Concentration of chemical (µM) | Subset | |
|---|---|---|---|
| | | 5 | 6 |
| Tukey HSD[a,b] | .0 | | |
| | .2 | | |
| | .4 | | |
| | .8 | | |
| | 1.6 | | |
| | 3.2 | | |
| | 6.4 | 43.33 | |
| | 10.0 | | 58.67 |
| | Sig. | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square (Error) = 17.000.

a. Uses Harmonic Mean Sample Size = 12.000.

b. Alpha = .05.

**ANOVA Table for Pb**

**Univariate Analysis of Variance**

**Between-Subjects Factors**

| | | N |
|---|---|---|
| Duration of exposure (h) | 0 | 16 |
| | 12 | 16 |
| | 24 | 16 |
| | 48 | 16 |
| | 72 | 16 |
| | 96 | 16 |
| Concentration of chemical (µM) | .0 | 12 |
| | .5 | 12 |
| | 1.0 | 12 |
| | 1.5 | 12 |
| | 2.0 | 12 |
| | 3.0 | 12 |
| | 4.0 | 12 |
| | 5.0 | 12 |

**Levene's Test of Equality of Error Variances[a]**

Dependent Variable: Mortality (%)

| F | df1 | df2 | Sig. |
|---|---|---|---|
| . | 47 | 48 | . |

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.[a]

a. Design: Intercept + Durationofexposureh + ConcentrationofchemicalµM + Durationofexposureh * ConcentrationofchemicalµM

Tests of Between-Subjects Effects

Dependent Variable: Mortality (%)

| Source | Type III Sum of Squares | df | Mean Square | F |
|---|---|---|---|---|
| Corrected Model | 116671.833[a] | 47 | 2482.379 | 97.348 |
| Intercept | 50600.167 | 1 | 50600.167 | 1984.320 |
| Durationofexposureh | 30428.833 | 5 | 6085.767 | 238.658 |
| ConcentrationofchemicalµM | 54659.833 | 7 | 7808.548 | 306.218 |
| Durationofexposureh * ConcentrationofchemicalµM | 31583.167 | 35 | 902.376 | 35.387 |
| Error | 1224.000 | 48 | 25.500 | |
| Total | 168496.000 | 96 | | |
| Corrected Total | 117895.833 | 95 | | |

**Tests of Between-Subjects Effects**

Dependent Variable: Mortality (%)

| Source | Sig. |
|---|---|
| Corrected Model | .000[a] |
| Intercept | .000 |
| Durationofexposureh | .000 |

| | |
|---|---|
| ConcentrationofchemicalµM | .000 |
| Durationofexposureh * ConcentrationofchemicalµM | .000 |
| Error | |
| Total | |
| Corrected Total | |

a. R Squared = .990 (Adjusted R Squared = .979)

**Estimated Marginal Means**

**Grand Mean**

Dependent Variable: Mortality (%)

| Mean | Std. Error | 95% Confidence Interval | |
|---|---|---|---|
| | | Lower Bound | Upper Bound |
| 22.958 | .515 | 21.922 | 23.995 |

**Post Hoc Tests**

**Duration of exposure (h)**

**Multiple Comparisons**

Dependent Variable: Mortality (%)

| | (I) Duration of exposure (h) | (J) Duration of exposure (h) | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| Tukey HSD | 0 | 12 | -3.50 | 1.785 | .380 | -8.80 | 1.80 |
| | | 24 | -17.00* | 1.785 | .000 | -22.30 | -11.70 |
| | | 48 | -29.50* | 1.785 | .000 | -34.80 | -24.20 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 72 | -39.25* | 1.785 | .000 | -44.55 | -33.95 |
| | 96 | -48.50* | 1.785 | .000 | -53.80 | -43.20 |
| | 0 | 3.50 | 1.785 | .380 | -1.80 | 8.80 |
| | 24 | -13.50* | 1.785 | .000 | -18.80 | -8.20 |
| 12 | 48 | -26.00* | 1.785 | .000 | -31.30 | -20.70 |
| | 72 | -35.75* | 1.785 | .000 | -41.05 | -30.45 |
| | 96 | -45.00* | 1.785 | .000 | -50.30 | -39.70 |
| | 0 | 17.00* | 1.785 | .000 | 11.70 | 22.30 |
| | 12 | 13.50* | 1.785 | .000 | 8.20 | 18.80 |
| 24 | 48 | -12.50* | 1.785 | .000 | -17.80 | -7.20 |
| | 72 | -22.25* | 1.785 | .000 | -27.55 | -16.95 |
| | 96 | -31.50* | 1.785 | .000 | -36.80 | -26.20 |
| | 0 | 29.50* | 1.785 | .000 | 24.20 | 34.80 |
| | 12 | 26.00* | 1.785 | .000 | 20.70 | 31.30 |
| 48 | 24 | 12.50* | 1.785 | .000 | 7.20 | 17.80 |
| | 72 | -9.75* | 1.785 | .000 | -15.05 | -4.45 |
| | 96 | -19.00* | 1.785 | .000 | -24.30 | -13.70 |
| | 0 | 39.25* | 1.785 | .000 | 33.95 | 44.55 |
| | 12 | 35.75* | 1.785 | .000 | 30.45 | 41.05 |
| 72 | 24 | 22.25* | 1.785 | .000 | 16.95 | 27.55 |
| | 48 | 9.75* | 1.785 | .000 | 4.45 | 15.05 |
| | 96 | -9.25* | 1.785 | .000 | -14.55 | -3.95 |
| 96 | 0 | 48.50* | 1.785 | .000 | 43.20 | 53.80 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | 12 | 45.00* | 1.785 | .000 | 39.70 | 50.30 |
| | | 24 | 31.50* | 1.785 | .000 | 26.20 | 36.80 |
| | | 48 | 19.00* | 1.785 | .000 | 13.70 | 24.30 |
| | | 72 | 9.25* | 1.785 | .000 | 3.95 | 14.55 |
| | | 12 | -3.50 | 1.785 | .056 | -7.09 | .09 |
| | | 24 | -17.00* | 1.785 | .000 | -20.59 | -13.41 |
| | 0 | 48 | -29.50* | 1.785 | .000 | -33.09 | -25.91 |
| | | 72 | -39.25* | 1.785 | .000 | -42.84 | -35.66 |
| | | 96 | -48.50* | 1.785 | .000 | -52.09 | -44.91 |
| | | 0 | 3.50 | 1.785 | .056 | -.09 | 7.09 |
| | | 24 | -13.50* | 1.785 | .000 | -17.09 | -9.91 |
| | 12 | 48 | -26.00* | 1.785 | .000 | -29.59 | -22.41 |
| | | 72 | -35.75* | 1.785 | .000 | -39.34 | -32.16 |
| LSD | | 96 | -45.00* | 1.785 | .000 | -48.59 | -41.41 |
| | | 0 | 17.00* | 1.785 | .000 | 13.41 | 20.59 |
| | | 12 | 13.50* | 1.785 | .000 | 9.91 | 17.09 |
| | 24 | 48 | -12.50* | 1.785 | .000 | -16.09 | -8.91 |
| | | 72 | -22.25* | 1.785 | .000 | -25.84 | -18.66 |
| | | 96 | -31.50* | 1.785 | .000 | -35.09 | -27.91 |
| | | 0 | 29.50* | 1.785 | .000 | 25.91 | 33.09 |
| | | 12 | 26.00* | 1.785 | .000 | 22.41 | 29.59 |
| | 48 | 24 | 12.50* | 1.785 | .000 | 8.91 | 16.09 |
| | | 72 | -9.75* | 1.785 | .000 | -13.34 | -6.16 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 96 | -19.00* | 1.785 | .000 | -22.59 | -15.41 |
| | 0 | 39.25* | 1.785 | .000 | 35.66 | 42.84 |
| | 12 | 35.75* | 1.785 | .000 | 32.16 | 39.34 |
| 72 | 24 | 22.25* | 1.785 | .000 | 18.66 | 25.84 |
| | 48 | 9.75* | 1.785 | .000 | 6.16 | 13.34 |
| | 96 | -9.25* | 1.785 | .000 | -12.84 | -5.66 |
| | 0 | 48.50* | 1.785 | .000 | 44.91 | 52.09 |
| | 12 | 45.00* | 1.785 | .000 | 41.41 | 48.59 |
| 96 | 24 | 31.50* | 1.785 | .000 | 27.91 | 35.09 |
| | 48 | 19.00* | 1.785 | .000 | 15.41 | 22.59 |
| | 72 | 9.25* | 1.785 | .000 | 5.66 | 12.84 |

Based on observed means.

The error term is Mean Square (Error) = 25.500.

*. The mean difference is significant at the .05 level.

**Homogeneous Subsets**

**Mortality (%)**

| | Duration of exposure (h) | N | Subset | | | |
|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 |
| Tukey HSD[a,b] | 0 | 16 | .00 | | | |
| | 12 | 16 | 3.50 | | | |
| | 24 | 16 | | 17.00 | | |
| | 48 | 16 | | | 29.50 | |
| | 72 | 16 | | | | 39.25 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 96 | 16 | | | | |
| | Sig. | | .380 | 1.000 | 1.000 | 1.000 |

**Mortality (%)**

| | Duration of exposure (h) | Subset |
|---|---|---|
| | | 5 |
| Tukey HSD[a,b] | 0 | |
| | 12 | |
| | 24 | |
| | 48 | |
| | 72 | |
| | 96 | 48.50 |
| | Sig. | 1.000 |

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square (Error) = 25.500.

a. Uses Harmonic Mean Sample Size = 16.000.

b. Alpha = .05.

**Concentration of chemical (µM)**

**Multiple Comparisons**

Dependent Variable: Mortality (%)

| | (I) Concentration of chemical (µM) | (J) Concentration of chemical (µM) | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | .5 | .00 | 2.062 | 1.000 | -6.53 | 6.53 |
| | | 1.0 | -3.00 | 2.062 | .827 | -9.53 | 3.53 |
| | | 1.5 | -11.67* | 2.062 | .000 | -18.20 | -5.14 |
| | .0 | 2.0 | -22.00* | 2.062 | .000 | -28.53 | -15.47 |
| | | 3.0 | -27.67* | 2.062 | .000 | -34.20 | -21.14 |
| | | 4.0 | -48.33* | 2.062 | .000 | -54.86 | -41.80 |
| | | 5.0 | -71.00* | 2.062 | .000 | -77.53 | -64.47 |
| | | .0 | .00 | 2.062 | 1.000 | -6.53 | 6.53 |
| | | 1.0 | -3.00 | 2.062 | .827 | -9.53 | 3.53 |
| | | 1.5 | -11.67* | 2.062 | .000 | -18.20 | -5.14 |
| Tukey HSD | .5 | 2.0 | -22.00* | 2.062 | .000 | -28.53 | -15.47 |
| | | 3.0 | -27.67* | 2.062 | .000 | -34.20 | -21.14 |
| | | 4.0 | -48.33* | 2.062 | .000 | -54.86 | -41.80 |
| | | 5.0 | -71.00* | 2.062 | .000 | -77.53 | -64.47 |
| | | .0 | 3.00 | 2.062 | .827 | -3.53 | 9.53 |
| | | .5 | 3.00 | 2.062 | .827 | -3.53 | 9.53 |
| | | 1.5 | -8.67* | 2.062 | .003 | -15.20 | -2.14 |
| | 1.0 | 2.0 | -19.00* | 2.062 | .000 | -25.53 | -12.47 |
| | | 3.0 | -24.67* | 2.062 | .000 | -31.20 | -18.14 |
| | | 4.0 | -45.33* | 2.062 | .000 | -51.86 | -38.80 |
| | | 5.0 | -68.00* | 2.062 | .000 | -74.53 | -61.47 |
| | 1.5 | .0 | 11.67* | 2.062 | .000 | 5.14 | 18.20 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | .5 | 11.67* | 2.062 | .000 | 5.14 | 18.20 |
| | 1.0 | 8.67* | 2.062 | .003 | 2.14 | 15.20 |
| | 2.0 | -10.33* | 2.062 | .000 | -16.86 | -3.80 |
| | 3.0 | -16.00* | 2.062 | .000 | -22.53 | -9.47 |
| | 4.0 | -36.67* | 2.062 | .000 | -43.20 | -30.14 |
| | 5.0 | -59.33* | 2.062 | .000 | -65.86 | -52.80 |
| | .0 | 22.00* | 2.062 | .000 | 15.47 | 28.53 |
| | .5 | 22.00* | 2.062 | .000 | 15.47 | 28.53 |
| | 1.0 | 19.00* | 2.062 | .000 | 12.47 | 25.53 |
| 2.0 | 1.5 | 10.33* | 2.062 | .000 | 3.80 | 16.86 |
| | 3.0 | -5.67 | 2.062 | .133 | -12.20 | .86 |
| | 4.0 | -26.33* | 2.062 | .000 | -32.86 | -19.80 |
| | 5.0 | -49.00* | 2.062 | .000 | -55.53 | -42.47 |
| | .0 | 27.67* | 2.062 | .000 | 21.14 | 34.20 |
| | .5 | 27.67* | 2.062 | .000 | 21.14 | 34.20 |
| | 1.0 | 24.67* | 2.062 | .000 | 18.14 | 31.20 |
| 3.0 | 1.5 | 16.00* | 2.062 | .000 | 9.47 | 22.53 |
| | 2.0 | 5.67 | 2.062 | .133 | -.86 | 12.20 |
| | 4.0 | -20.67* | 2.062 | .000 | -27.20 | -14.14 |
| | 5.0 | -43.33* | 2.062 | .000 | -49.86 | -36.80 |
| | .0 | 48.33* | 2.062 | .000 | 41.80 | 54.86 |
| 4.0 | .5 | 48.33* | 2.062 | .000 | 41.80 | 54.86 |
| | 1.0 | 45.33* | 2.062 | .000 | 38.80 | 51.86 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1.5 | 36.67* | 2.062 | .000 | 30.14 | 43.20 |
| | | 2.0 | 26.33* | 2.062 | .000 | 19.80 | 32.86 |
| | | 3.0 | 20.67* | 2.062 | .000 | 14.14 | 27.20 |
| | | 5.0 | -22.67* | 2.062 | .000 | -29.20 | -16.14 |
| | | .0 | 71.00* | 2.062 | .000 | 64.47 | 77.53 |
| | | .5 | 71.00* | 2.062 | .000 | 64.47 | 77.53 |
| | | 1.0 | 68.00* | 2.062 | .000 | 61.47 | 74.53 |
| | 5.0 | 1.5 | 59.33* | 2.062 | .000 | 52.80 | 65.86 |
| | | 2.0 | 49.00* | 2.062 | .000 | 42.47 | 55.53 |
| | | 3.0 | 43.33* | 2.062 | .000 | 36.80 | 49.86 |
| | | 4.0 | 22.67* | 2.062 | .000 | 16.14 | 29.20 |
| | | .5 | .00 | 2.062 | 1.000 | -4.15 | 4.15 |
| | | 1.0 | -3.00 | 2.062 | .152 | -7.15 | 1.15 |
| | | 1.5 | -11.67* | 2.062 | .000 | -15.81 | -7.52 |
| | .0 | 2.0 | -22.00* | 2.062 | .000 | -26.15 | -17.85 |
| | | 3.0 | -27.67* | 2.062 | .000 | -31.81 | -23.52 |
| LSD | | 4.0 | -48.33* | 2.062 | .000 | -52.48 | -44.19 |
| | | 5.0 | -71.00* | 2.062 | .000 | -75.15 | -66.85 |
| | | .0 | .00 | 2.062 | 1.000 | -4.15 | 4.15 |
| | | 1.0 | -3.00 | 2.062 | .152 | -7.15 | 1.15 |
| | .5 | 1.5 | -11.67* | 2.062 | .000 | -15.81 | -7.52 |
| | | 2.0 | -22.00* | 2.062 | .000 | -26.15 | -17.85 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 3.0 | -27.67* | 2.062 | .000 | -31.81 | -23.52 |
| | 4.0 | -48.33* | 2.062 | .000 | -52.48 | -44.19 |
| | 5.0 | -71.00* | 2.062 | .000 | -75.15 | -66.85 |
| | .0 | 3.00 | 2.062 | .152 | -1.15 | 7.15 |
| | .5 | 3.00 | 2.062 | .152 | -1.15 | 7.15 |
| | 1.5 | -8.67* | 2.062 | .000 | -12.81 | -4.52 |
| 1.0 | 2.0 | -19.00* | 2.062 | .000 | -23.15 | -14.85 |
| | 3.0 | -24.67* | 2.062 | .000 | -28.81 | -20.52 |
| | 4.0 | -45.33* | 2.062 | .000 | -49.48 | -41.19 |
| | 5.0 | -68.00* | 2.062 | .000 | -72.15 | -63.85 |
| | .0 | 11.67* | 2.062 | .000 | 7.52 | 15.81 |
| | .5 | 11.67* | 2.062 | .000 | 7.52 | 15.81 |
| | 1.0 | 8.67* | 2.062 | .000 | 4.52 | 12.81 |
| 1.5 | 2.0 | -10.33* | 2.062 | .000 | -14.48 | -6.19 |
| | 3.0 | -16.00* | 2.062 | .000 | -20.15 | -11.85 |
| | 4.0 | -36.67* | 2.062 | .000 | -40.81 | -32.52 |
| | 5.0 | -59.33* | 2.062 | .000 | -63.48 | -55.19 |
| | .0 | 22.00* | 2.062 | .000 | 17.85 | 26.15 |
| | .5 | 22.00* | 2.062 | .000 | 17.85 | 26.15 |
| | 1.0 | 19.00* | 2.062 | .000 | 14.85 | 23.15 |
| 2.0 | 1.5 | 10.33* | 2.062 | .000 | 6.19 | 14.48 |
| | 3.0 | -5.67* | 2.062 | .008 | -9.81 | -1.52 |
| | 4.0 | -26.33* | 2.062 | .000 | -30.48 | -22.19 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 5.0 | -49.00* | 2.062 | .000 | -53.15 | -44.85 |
| | .0 | 27.67* | 2.062 | .000 | 23.52 | 31.81 |
| | .5 | 27.67* | 2.062 | .000 | 23.52 | 31.81 |
| | 1.0 | 24.67* | 2.062 | .000 | 20.52 | 28.81 |
| 3.0 | 1.5 | 16.00* | 2.062 | .000 | 11.85 | 20.15 |
| | 2.0 | 5.67* | 2.062 | .008 | 1.52 | 9.81 |
| | 4.0 | -20.67* | 2.062 | .000 | -24.81 | -16.52 |
| | 5.0 | -43.33* | 2.062 | .000 | -47.48 | -39.19 |
| | .0 | 48.33* | 2.062 | .000 | 44.19 | 52.48 |
| | .5 | 48.33* | 2.062 | .000 | 44.19 | 52.48 |
| | 1.0 | 45.33* | 2.062 | .000 | 41.19 | 49.48 |
| 4.0 | 1.5 | 36.67* | 2.062 | .000 | 32.52 | 40.81 |
| | 2.0 | 26.33* | 2.062 | .000 | 22.19 | 30.48 |
| | 3.0 | 20.67* | 2.062 | .000 | 16.52 | 24.81 |
| | 5.0 | -22.67* | 2.062 | .000 | -26.81 | -18.52 |
| | .0 | 71.00* | 2.062 | .000 | 66.85 | 75.15 |
| | .5 | 71.00* | 2.062 | .000 | 66.85 | 75.15 |
| | 1.0 | 68.00* | 2.062 | .000 | 63.85 | 72.15 |
| 5.0 | 1.5 | 59.33* | 2.062 | .000 | 55.19 | 63.48 |
| | 2.0 | 49.00* | 2.062 | .000 | 44.85 | 53.15 |
| | 3.0 | 43.33* | 2.062 | .000 | 39.19 | 47.48 |
| | 4.0 | 22.67* | 2.062 | .000 | 18.52 | 26.81 |

Based on observed means.

The error term is Mean Square (Error) = 25.500.

*. The mean difference is significant at the .05 level.

**Homogeneous Subsets**

**Mortality (%)**

| | Concentration of chemical (µM) | N | Subset | | | |
|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 |
| Tukey HSD[a,b] | .0 | 12 | .00 | | | |
| | .5 | 12 | .00 | | | |
| | 1.0 | 12 | 3.00 | | | |
| | 1.5 | 12 | | 11.67 | | |
| | 2.0 | 12 | | | 22.00 | |
| | 3.0 | 12 | | | 27.67 | |
| | 4.0 | 12 | | | | 48.33 |
| | 5.0 | 12 | | | | |
| | Sig. | | .827 | 1.000 | .133 | 1.000 |

**Mortality (%)**

| | Concentration of chemical (µM) | Subset |
|---|---|---|
| | | 5 |
| Tukey HSD[a,b] | .0 | |
| | .5 | |
| | 1.0 | |
| | 1.5 | |

| | | |
|---|---|---|
| | 2.0 | |
| | 3.0 | |
| | 4.0 | |
| | 5.0 | 71.00 |
| | Sig. | 1.000 |

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square (Error) = 25.500.

a. Uses Harmonic Mean Sample Size = 12.000.

b. Alpha = .05.

**Appendix VI**

Curve fitting properties obtained from Probit analysis

| Curve fitting | $Cd^{2+}$ | $Cr^{6+}$ | $Pb^{2+}$ |
|---|---|---|---|
| Slope | 4.598 | 2.478 | 4.163 |
| Intercept | 10.192 | 4.790 | 3.898 |
| SD (σ) | 0.217 | 0.404 | 0.240 |
| SE | 0.045 | 0.082 | 0.047 |
| $R^2$ | 0.979 | 0.970 | 0.990 |
| Chi-test (χ2) Sig | 0.795 | 0.554 | 0.733 |
| df | 2 | 2 | 2 |
| Chi-Test | NON-SIG | NON-SIG | NON-SIG |
| Fitting | GOOD FIT | GOOD FIT | GOOD FIT |

**Appendix VII**

Various LC values estimated by Probit analysis

| Metal | Endpoints | Value (µM) | 95% confidence limit | |
|---|---|---|---|---|
| | | | upper | lower |
| $Cd^{2+}$ | $LC_{10}$ | 0.039 | 0.032 | 0.048 |
| | $LC_{50}$ | 0.074 | 0.061 | 0.091 |
| | $LC_{90}$ | 0.141 | 0.115 | 0.173 |
| $Cr^{6+}$ | $LC_{10}$ | 0.369 | 0.254 | 0.534 |
| | $LC_{50}$ | 1.220 | 0.842 | 1.767 |
| | $LC_{90}$ | 4.037 | 2.786 | 5.849 |
| $Pb^{2+}$ | $LC_{10}$ | 0.904 | 0.733 | 1.115 |
| | $LC_{50}$ | 1.839 | 1.490 | 2.269 |
| | $LC_{90}$ | 3.741 | 3.032 | 4.615 |