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Histological based sub-chronic toxicity testing of target heavy metals in fresh water; Using the Histo-morphometry of Kidney of *Coptodon Zilli*

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Abstract

This histology based toxicological study is aimed to advance the histopathological characteristics of fish kidney exposed to some known crude oil spill heavy metals. Based on literature review on the environmental crude oil spill burdens of Niger Delta region of Nigeria, the following target chemicals (TC) were selected for the study: Cadmium (Cd), Chromium (Cr), Copper (Cu), Nickel (Ni) and Lead (Pb). The experimental type is a sub-chronicity fish exposure testing for twenty-eight (28) days duration. The experimental set up involves twenty-two pieces (22) of twenty-liter (20l) tanks of four (4) replicate tanks per target chemical (TC) containing ten (10) juvenile study fish per tank in a semi-static tank testing system. TC exposure concentrations above maximum allowable toxicant concentration (MATC) for fresh water were used for the study. Quality histologically results showed alteration which were consistent with heavy metal exposure studies. Semi-quantitative assessment showed significant difference between control and experimental kidney organ histopathological indices. The study further validates MATC fresh water guideline use for toxicity certification in Cr, Cd and Ni exposure.

Keywords: Histology; Toxicity; Toxicology; Sub-Chronic; Heavy Metals and Fish Kidney

1. Introduction

This study is aimed to simulate the ecological effect of exposure to some known heavy metals from crude oil spill. It is a laboratory based sub-chronic toxicity testing of heavy metal treated fresh water using the kidney of a bio-indicator fish species. Toxicity tests are used to expose test organisms to a medium—water, sediment, or soil—and evaluate the effects of contamination on behavioral, physiological, biochemical and histological end points. The goal of toxicity assessment is to identify adverse effects of a substance. Adverse effects depend on two main factors (Ottoboni, 1991);

- Dosage: has to do with duration and concentration of exposure. Both large single exposures (acute) and continuous small exposures (chronic) are studied.
- Route of exposure: The routes of exposure are Ingestion (oral), inhalation or skin absorption (dermal).
- Other factors are species, age, sex, health, environment and individual characteristics.

Based on the measured end points, toxicity testing divided into Acute, Sub-Chronic and Chronic toxicity testing (USEPA, 1994:

- Acute toxicity tests: are short-term tests that measure the effects of exposure to relatively high concentrations of chemicals. The measurement endpoint generally reflects the extent of lethality (USEPA, 1994).

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- Sub-Chronic Toxicity Testing: A toxicity test designed to investigate possible adverse effect occurring as a result of continuous or repeated exposure of several groups of experimental animals to a series of concentration of the test chemical for part (no exceeding 10%) of their life span (ECETOC. 1986)
- Chronic toxicity tests, on the other hand, generally are longer-term tests that measure the effects of exposure to relatively lower, less toxic concentrations. For a chronic toxicity test, the measurement endpoint concerns a sublethal effect (e.g., reproduction, growth) or both lethality and sub- lethal effect (USEPA, 1994). Results can be analyzed in several ways. One is simply by a direct comparison between percent effects occurring in organisms exposed to site media and those exposed to uncontaminated media.
- Other approaches to analysis determine the effect concentration on fifty percent (50%) of the sample (EC50) and the lowest observed effective concentration (LOEC), or the no observed effect concentration (NOEC). The effect, "E" in the aforementioned acronyms can be any of the following end points: mortality, morbidity, inhibition of growth and/ or reproduction.

Heavy metal contamination of aquatic ecosystem has attracted great attention of researchers over the last few decades (Farombi et al., 2007). Metal in the aquatic environment are bioaccumulated by organisms either passively from water or by facilitated uptake. Excess metal concentration in an organism must be actively excreted, compartmentalized in cells or tissues, or metabolically immobilized. Some metal escape all these actions causing toxic and other adverse effects (Chapman et al., 1996; Rand et al., 1985). Some heavy metals, Chromium (Cr), cadmium (Cd), copper (Cu), lead (Pb), nickel (Ni) and zinc (Zn), are found in the list of organic and inorganic hazardous pollutants which is prepared by the United States Environmental Protection Agency (USEPA) (Akar and Tunali, 2005). However, it is important to note that most essential metals may be toxic when present in concentrations that are in excess of the guideline levels (Agbozu et al., 2007). According to Roberts (1989 and 2001) Cu, Pb, mercury (Hg), Zn, Cr, manganese (Mn) and iron (Fe) are the most common cause of metal poisoning. Jarup (2003) documented that the main threat to human health from heavy metals are associated with exposure to Pb, Cd, Hg, and arsenic (As) (arsenic is a metalloid but is usually classified as a heavy metal).

Gerhardt (2004) established that bioindicators are organisms or communities of organisms which reactions are observed representatively to evaluate a situation, giving clues for the condition of the whole ecosystem; Gerhardt also indicate that bioindicators are species reacting to anthropogenical effects on the environment, concluding that a biological indicator would be: a species or group of species that readily reflects the abiotic or biotic state of an environment, represents the impact of environmental change on a habitat, community or ecosystem or is indicative of the diversity of a subset of taxa or the whole diversity within an area. In this sense, the primary role of ecological indicators is to measure the response of the ecosystem to anthropogenic disturbances (Niemi and McDonald, 2004).

Kidneys are vital organs in the maintenance of pH and volume of body fluids as well as erythropoiesis (Iqbal et al., 2004). In fish, they maintain a water and electrolyte balance in order to provide a stable internal environment. Due to these properties, the kidneys are also an outstanding indicator of possible pollution and environmental stress in the vicinity of the fish (Hinton and Lauren, 1990). Fish kidney nephrons consist of collecting ducts, proximal and distal tubules, and renal corpuscle. Moreover, the interstitial stroma of fish kidney includes hematopoietic tissue, which contains hematopoietic stem cells (HSC), blood cells, renal progenitors, and other mesenchymal cell types (Motamedi, et al., 2020). The kidney of mammals cannot form new nephrons to replace the lost ones when it is damaged, and it can repair only the proximal tubules and glomeruli partially through bone marrow cells (Motamedi et al., 2019) or intrarenal mesenchymal cells (Lin et al., 2005; Osafune et al., 2006). However, various fish species, including rainbow trout, tilapia, catfish, zebrafish, tomcod, and the Hormuz killifish Aphaniops hormuzensis are known to regenerate their kidneys ((Motamedi, et al., 2020). Fish kidneys are considered as an important vital organ to use as a biomarker in determining the health of fish in the freshwater ecosystem (Bernet et al., 1999). The tissues and organs which are in contact with the foreign compounds have the potential to suffer the most and cause a damage when exposed to higher concentrations of chemical pollutants (Timbrell, 1991).

Histology is the study of the structure of normal tissue while histopathology is the study of the structure of abnormal, diseased tissue. According to Hinton and Lauren (1990) histological alterations are the overall result of unfavorable physiological and biochemical alterations in an organism. They may be used to foresee the effects of alterations on processes or activities such as predator avoidance, growth, reproduction, and population stability that occur at higher levels of biological organization (Meyers and Hendricks, 1985). Negative effects as a result of toxicity generally begin at the cellular or tissue level long before the obvious signs such as behavioral changes, loss of appetite, discoloration, or death occur. This is where histology could be a useful tool in toxicity studies. Histopathology was introduced in studies where more detailed information was needed for further description or screening for early preneoplastic changes (Wester et al., 2002). Histopathological characteristics of specific organs express condition and represent time

integrated endogenous and exogenous impacts on the organism stemming from alterations at lower levels of biological organization (Hinton and Lauren, 1990; Swee et at., 1996).

Direct exposures to various toxic pollutants may result in abnormalities and pathological alterations in fish (Bernet et al., 1999). Therefore, to determine and simulate the effects of some target heavy metals exposed to *Coptodon Zilli*i, a histology-based fish health assessment protocol was used to evaluate the bio-indicator fish, using the kidney as a histologic biomarker

2. Materials and method

2.1. Study Location

The fish toxicity testing was done in a Wet laboratory of the Department of Aquaculture and Fisheries Management, Faculty of Agriculture, University of Benin, Nigeria. The department undertakes high level human resource development through research, training and technology transfer to ensure sustainable development of forest resources as well as the environmental and social impact on these resources.

2.2. Study Specie

2.2.1. Fish selection

EROCIPS (Emergency Response to coastal Oil, Chemical and Inert Pollution from Shipping), (2006), "Protocol for Selection of Sentinel Species" was basically used to select the appropriate sentinel specie for this study. The resident fish species, *Coptodon Zilli*i because it is a widely studied fish species (Harvey et al., 1999; Pietrapiana et al., 2002; Budzinski et al., 2004; EROCIPS, 2006; Marigo´mez et al., 2006; Martı´nez-Go´mez et al., 2006; Joly-Turquin et al., 2009), and it is a resident of the Niger Delta region of Nigeria. This region is crude oil production hub of Nigeria, with the highest level of crude oil spills and other crude oil related environmental crimes.

Figure 1 Picture of *C. zillii*

2.2.2. Fish Biodata

The chosen bio-indicator fish specie for this study is Tilapia zillii, now known as *Coptodon Zilli*i. The fish specie is a resident of the Niger Delta region of Nigeria. *Coptodon Zilli*i has a maximum length of 40cm (SL) and a maximum published weight of 300 grams with a total of 13 to 16 Dorsal spines (GISD, 2019). The non-breeding colouration of *C. zillii* is dark olive on top and light olive to yellow-brown on the sides, often with an iridescent blue sheen. Lips are bright green and the chest is pinkish. Six to seven dark vertical bars cross two horizontal stripes on the body and caudal peduncle. Fins are olivaceous, covered in yellow spots with the dorsal and anal fins displaying an outline of a thin orange band. Caudal fin often grey with pale interstices with dots covering the entire fin. Adults display a black spot outlined in yellow. *C. zillii* from 2 to 14cm (SL) have an entirely yellow to grey caudal fin with no dots, developing a greyish caudal fin with dots with increasing size. Spawning coloration is shiny dark green on top and sides, red and black on the throat and belly, and obvious vertical bands on the sides. Heads turn dark blue to black with blue-green spots. Eggs are green to olive green, sticky, 1-2 mm in diameter; relatively smaller than eggs of other cichlids (FishBase, 2008; GISD, 2019).

2.2.3. Fish Source

The fish for toxicity testing was acquired from Nigeria Institution for Oceanography and Maritime Research (NIORMR) in Sapele Delta State, Nigeria. The Institute is involved in farmers' field test and incubation validated research results and technologies. The test fish was positively identified by a taxonomist from NIOMR, to be the right species for the study. The test fish used was disease-free and appear healthy, behave normally, feed well, and had low mortality in cultures, during holding, and in test control. (USEPA, 2002).

2.2.4. Test Fish Guidelines

Juveniles of *Coptodon Zilli*i were chosen for the study in tandem with ISO,1994 recommendation for chronic toxicity study (ISO, 1994; CEPA, 1999). Young organisms are often more sensitive to toxicants than (are) adults. For this reason, the use of early life stages, such as juvenile is required for all tests (ISO, 1994; CEPA, 1999). Fish were approximately the same age and were gotten from the same source. Since age may affect the results of the tests, this would enhance the value and comparability of the data if the same species in the same life stages were used throughout a monitoring program at a given facility (ISO, 1994; CEPA, 1999).

2.3. Study Design

2.3.1. Study Guideline

International Standardization Organization (ISO) test guideline standard, ISO 10229:1994 – "Chronic Toxicity Test of Fish Water quality", was used for the laboratory study (ISO, 1994). The standard specified the method for the determination of the long-term toxicity of substances (pure chemicals, mixtures, wastewater etc.). This Standard allows for the use of a semi-static method. The endpoint response was a measure of the morphological changes of test fish exposed to a test substance for a period of 14 to 28 days. The standard permits to adapt this method for use with a wide variety of freshwater, marine and brackish water fish with appropriate modifications in test conditions (temperature, food, fish marking technique).

2.3.2. Experimental Set-up

Sub-Chronic Toxicity studies were done in Department of Aquaculture and Fisheries Management, Faculty of Agriculture, University of Benin. The experimental duration was 28 days. Twenty two pieces (22) of twenty liters (20L) tanks were acquired for the experiment. Four replicate tanks per Target Chemical (TC) were labeled and used for the group treated. Hence, five TC of Copper, Cadmium, Chromium, Lead and Nickel, a total of Twenty (20) tanks were set up for the experiment.

Figure 2 Experimental set-up in the Wet-laboratory of the Department of Agriculture University of Benin

2.3.3. Test Chemical/Concentration

Target Chemicals (TC), Cadmium (Cd0, Chromium (Cr), Copper (Cu), Nickel (Ni) and Lead (Pb) were chosen because of their cardiogenic quotient and presence in crude oil. A standard stock solution of 100mg/L of the TC (Cd, Cr, Cu, Ni and Pb) was prepared from analytical grade metallic salts of Cadmium Chloride (CdCl2), Lead II Nitrate (Pb(NO3)2), Nickel II Sulphate (NiSO4.(H2O)6), Copper Sulphate (CuSO4) and Potassium Dichromate (K2Cr2O7). Stock solution was prepared with de-ionized water in I-L volumetric. The maximum acceptable toxicant concentration (MATC) for estuarine fresh water was set as the concentration of stock solutions for the laboratory testing (Gheorghe et al., 2017). Two concentrations below and above the estimated MATC were used for the testing (Gheorghe et al., 2017).

Table 1 The results of TC doses applied for the fish toxicity study

Target Chemicals (TC)	MATC (mg/L)	FED: Dose above MATC (mg/L)		FED: Dose below MATC (mg/L)	
		C1	C2	C3	C4
Cadmium (Cd)	0.00001	0.1	0.01	0.0001	0.0001
Chromium (Cr)	0.001	10.0	1.0	0.01	0.001
Copper (Cu)	0.002	20.0	2.0	0.02	0.002
Nickel (Ni)	0.025	25.00	2.5	0.05	0.025
Lead (Pb)	0.001	10.0	1.0	0.01	0.001

Key: TC = Target Chemicals; FED = Fish Exposure Dose; C1, C2, C3 and, C4 = Dose Concentrations of Heavy metal applied per Fish Tank; MATA = CCME, 2001

2.3.4. Test Chamber

Twenty (20) liters of chemically inert vessels (plastic Tanks) were used for the study. Each tanked was stocked with 25 fish, a stock capacity that was enough to allow for proper growth and maintenance of dissolved oxygen concentration. This is in compliance with the guideline loading rate criteria (OECD, 2013). The test chambers was randomly positioned in the test area and shielded from unwanted disturbance. The test was carried out without adjustment of pH. Nevertheless, where there is evidence of marked changed in the pH of the Tank water after addition of the test substance, the test would be repeated, adjusting the pH of the stock solution to the tank water before addition of the test substance. The PH adjustment will be made (preferably with HC1 or Na0H) in such a way that the stock solution concentration is not changed to any significant extent and that no chemical reaction or physical precipitation of the test substance is caused (ISO, 1994; CEPA, 2004; Gheorghe et al., 2017).

2.3.5. Test Solution Conditions

The test solution is fresh water, which was used to simulated fresh surface water. Water temperature was maintained between 20^0C to 25^0C (ISO, 1994; CEPA, 2004). The temperature of the test solutions was measured by placing a thermometer directly into the test solutions. Temperature was recorded continuously in at least one vessel during the duration of each. DO concentration was maintained at not less than 60% of the maximum air saturation value throughout the test. DO and pH was checked at the beginning of the test and daily, throughout the test period. Light quality was set at ambient laboratory illumination. Photoperiod was set at a minimum of ration of 12 hours light to 12hours dark, with a light intensity maintained at 10 to 20 µE/m2/s. Feeding was at least once daily, the quantity of food being kept constant and related to the initial fish weight, at least 2% body weight (ISO, 1994; CEPA, 2004).

2.3.6. Validity of Test

- For the conditions of validity, ISO (1994) and OECD (2013) conditions for the validity of test were adopted for this study:
- The mortality in the controls should not exceed 10% at the end of test.
- The dissolved oxygen concentration should be at least 60% of the air saturation value throughout the test
- In semi-static procedures, aeration can be used, provided it does not lead to a significant loss of test substance
- There should be evidence that the concentration of the substance being tested has been satisfactorily maintained (it should be at least 80% of the nominal concentration) over the test period. The results should be based on measured concentration if the deviation from the nominal concentration is greater than 20%

2.4. Histological Evaluation

At the end of the experiment, 5 fishs from each tank was sacrificed and the target organ of gills were excised and preserved in 10% buffered formalin solution and sent to the laboratory for tissue preparation. Tissue preparation was done in accordance to Drury and Wallington (1980). The following histological evaluations were made:

2.4.1. Qualitative Analysis

A Light microscopy (Olympus BH2) was used to identify and interpret (histological description) tissue slides and micrograph specimens at 40X, X100 and X400 magnification. The percentage prevalence of tissue histopathology was noted. Histological findings in the prepared slides from Fish Exposed Dosage (FED) of TCs and control were noted.

2.4.2. Semi-quantitative analysis

A standard semi-quantitative histological assessment according to the modified protocol of Bernet et al. (1999) modified by Van Dyk et al. (2009) was done to quantify the histopathological alterations observed in the gill of histology slides. This assessment is useful because it can be applied to any organ as well as allowing for standardized quantification of results. This assessment allows for the comparison of observed histopathologic lesions between the different Fish Exposure Dosage (FED) of TCs and controls (USEPA, 1994).

3. Results

3.1. Qualitative Histological Assessment

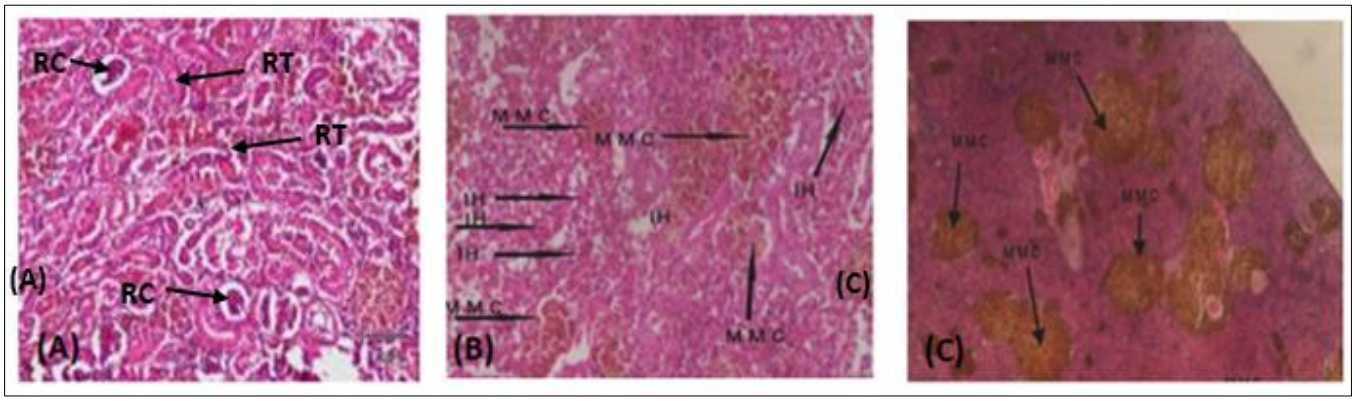

Figure 3 Photomicrographs (H&E:400X). (A) Normal Kidney show Renal Corpuscles (RC) and Renal Tubules (RT) (B): Showing multi focal Melano Macrophage Centres (MMC) and diffuse Interstitial Haemorhage (IH) (C): Showing Multi-focal MMC with marked structural alteration – hardening with atrophy of renal corpuscles and renal tubules

3.2. Semi-Quantitative Histopathological Assessment

Below (Table 2) is the kidney histopathologic organ index (I_{org}) result collected with a score chart quantified by Bernet et al. (1999) protocol (modified by Van Dyk et al., (2009a), showed that: Liver I_{org} of Cd1-Cd3, Cu3, Ni3, Pb2 and Pb3 were above control; Kidney I_{org} of Cd1, Cd2, Cr1, Cr2, Cu3, Ni3, Pb2 and Pb3 were above control. Gills I_{org} of Cd1, Cd2, Cr1-Cr3, Cu3, Ni3, Pb2 and Pb3 were above control. Mean fish indices result showed that Cd1, Cd2, Cr1, Cr2, Cu3, Ni3, Pb2 and Pb3 were above the control values

Table 2 The mean Organ and fish Indices of cultured fish lesions

TC	FEC	Kidney Mean I_{org}
Con		1.0
Cd	Cd1	13.4
	Cd2	8.6
	Cd3	0.6
	Cd4	0.2
Cr	Cr1	11.0
	Cr2	10.4
	Cr3	0.4
	Cr4	0.4
Cu	Cu1	Nil
	Cu2	Nil
	Cu3	4.6
	Cu4	1.0
Ni	Ni1	Nil
	Ni2	Nil
	Ni3	2.6
	Ni4	0.4
Pb	Pb1	Nil
	Pb2	9.6
	Pb3	1.2
	Pb4	0.2

Key: Cd1, Cd2…Cr1, Cr2… etc = Concentrations of Heavy metal applied per Fish Tank; Conc = Concentration: red = values above the control values; FEC = Fish Exposure Concentration; Iorg = Organ Index; Nil – Fish in the test chamber did not survive the duration of the experiment

Table 3 Kidney Paired Sample T Test of the Fish Toxicity Study, comparing the mean Organ Indices (Iorg) of fish from the different FED tanks with the control fish

		Paired Differences					t	Df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval				
					Lower	Upper			
Cd	Cont - Cd1	-12.800	6.140	2.746	-20.424	-5.176	-4.661	4	.010
	Cont - Cd2	-8.000	2.915	1.304	-11.620	-4.380	-6.136	4	.004
	Cont - Cd3	0.000	1.000	.447	-1.242	1.242	0.000	4	1.000
	Cont - Cd3	.400	1.140	.510	-1.016	1.816	.784	4	.477
Cr	Cont - Cr1	-10.400	7.021	3.140	-19.118	-1.682	-3.312	4	.030
	Cont - Cr2	-9.800	4.658	2.083	-15.584	-4.016	-4.704	4	.009
	Cont - Cr3	.200	.837	.374	-.839	1.239	.535	4	.621

	Cont - Cr4	.200	1.304	.583	-1.419	1.819	.343	4	.749
Cu	Cont - Cu1	.600	.894	.400	-.511	1.711	1.500	4	.208
	Cont - Cu2	.600	.894	.400	-.511	1.711	1.500	4	.208
	Cont - Cu3	-4.000	2.345	1.049	-6.912	-1.088	-3.814	4	.019
	Cont - Cu4	-.400	1.140	.510	-1.816	1.016	-.784	4	.477
Ni	Cont - Ni1	.600	.894	.400	-.511	1.711	1.500	4	.208
	Cont - Ni2	.600	.894	.400	-.511	1.711	1.500	4	.208
	Cont - Ni3	-2.000	1.871	.840	-4.323	.323	-2.390	4	.075
	Cont - Ni4	.200	1.304	.583	-1.419	1.819	.343	4	.749
Pb	Cont - Pb1	.600	.894	.400	-.511	1.711	1.500	4	.208
	Cont - Pb2	-9.000	5.657	2.530	-16.024	-1.976	-3.558	4	.024
	Cont - Pb3	-.600	.547	.245	-1.280	.080	-2.449	4	.070
	Cont - Pb4	.400	1.140	.510	-1.016	1.816	.784	4	.477

Key: TC = Toxicant Conc,; FEC = Fish Exposure Conc. NA = Not Application (Fish group all died); RED = values above the control values

4. Discussion

The kidney histolological alterations include: Diffuse Interstitial Oedema; generalized vascular rupture with Interstitial Haemorrhage (IH); Melano-Macrophage Centre; Vacuolation; Diffuse Coagulative Necrosis; Renal Tubular Hypertrophy; and Necrotic Foci. This study findings was consistent with histopathological alterations found in fish exposed to various contaminants which include: tubular epithelium and glomerulus (Teh et al.,1997); dilation of Bowman's space (Jiraungkoorskul et al., 2002); tubular necrosis, desquamation and vacuolation of tubular epithelial cells (Ortiz et al., 2003); degeneration of glomeruli and increased in intratubular hematopoietic tissue (Iqbal et al., 2004); and degeneration of glomerulus, hypertrophied cells and narrowing of the tubular lumen (Cengiz, 2006).

The semi-quantitative analysis showed that the TC concentrations above MATC had worse kidney organ histopathologic indices (Iorg) than control. This validates the guideline MATC for fresh water aquatic life (ECETOC,2002) which proposes deleterious ecological exposure effect beyond the allowable environmental concentration of the TC. Though concentrations of Cu3, Cu4 & Pb3 caused hazardous effect even at concentrations lower that the MATC. This might not be unrelated to the hazardous quotient of the metals, but nevertheless needs further investigation. Furthermore, there was significant difference ($P<0.005$) for the kidney histopathologic organ indices (I_{org}) between the control and TC concentrations of Cd1, Cd2, Cu3 and Pb2.This further validates the MATC guideline for fresh water aquatic life (ECETOC,2002). Cu3, which is a Cu concentration below MATC, showed hazardous exposure effect which was inconsistent with MATC guideline. This might be due Cu toxic affinity for kidney and or Hazardous Quotient in fresh water bodies. Nevertheless, this fact needs further investigation

5. Conclusion

This study was toxicologically relevant. The study was able to demonstrate that heavy metal toxicity can be simulated in a laboratory setting. It has further proven that the target chemicals concentration above the guideline levels or regulatory standards of MATC were hazardous to the bio-indicator fish. Though there is need to further investigate the pharmacokinetics behind the hazardous exposure effects caused by the concentrations of Cu and Pb that were below MATC guideline, which was inconsistent with MATC regulatory provision. The study has also proven that histopathologic organ indexing is an explorable environmental tool for histological based toxicity certification

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of ethical approval

Ethical approval was obtained.

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