

Assessing the Bioaccumulative Impact of Four Heavy Metals on the Endocrine System of *Tilapia rendalli* Fish Species in the Kafue River

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Authors' contributions

This work was carried out in collaboration between both authors. Author JN designed the study and wrote the protocol. Author BK carried out the sampling and experiment. Authors JN and BK analyzed data. Authors JN and BK did a literature search and wrote the paper. Both authors read and approved the final manuscript.

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ABSTRACT

Some heavy metals (HMs) are of biological importance in animal life while others are important trace elements for plant growth and in higher animals. Despite various uses, their biotoxic effects arise once accumulation levels in animal bodies go beyond maximum permissible limits. Heavy Metal contamination is an environmental problem worldwide. This study aimed at assessing the bioaccumulative impact of Cd, Cu, Ni and Pb on the endocrine system of *Tilapia rendalli* fish in Kafue River, Zambia. Water samples collected in replicate, were stored at 8°C in polypropylene bottles. Samples were filtered at room temperature for analysis. Blood, gills, liver and muscles were extracted per fish sample collected from upper, middle and lower site of the river; KUP, ITT and SH respectively. Gills, livers and muscles were cleaned, oven dried at 110°C, weighed and digested using 55% nitric acid and 70% perchloric acid (ANALAR) at about 200 – 250°C on a hot plate to a transparent solution after disappearance of initial brown fumes. Solutions were cooled and diluted

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using distilled water. The samples were assayed for the metals using FAAS. Blood samples were thawed at room temperature, centrifuged to collect supernatant serum. Serum was assayed for Estradiol (E2) and Testosterone (T) using ELISA method. Hormone levels varied significantly among fish samples. This difference was related to variations of HM levels in fish tissues from respective sites. Highest mean levels of T (13.58 ± 2.8 ng/mL) and E2 (774.33 ± 66.98 pg/mL) were measured in SH while lowest levels of T (5.78 ± 0.69 ng/mL) and E2 (63.75 ± 45.39 pg/mL) were measured in ITT. The study showed that low levels of Cd, Cu, and Ni in SH correlated significantly to high hormone levels, while high levels of Cu in livers and muscles; 496.73 ± 184.96 mg/kg and 43.68 ± 18.32 mg/kg respectively recorded in ITT, correlated negatively to low hormone levels in fish. A positive correlation between hormone levels and Pb concentrations in tissues was observed. Therefore, HM bioaccumulation may affect expression levels of sex hormones in fish as shown in this study. Since the levels of HM were high in internal organs than in muscle (flesh), fish of Kafue River may be safe for consumption, but may pose a health risk if consumed together with internal organs.

Keywords: Bioaccumulation; heavy metals (HMs); endocrine system; estradiol; testosterone; *Tilapia rendalli*.

1. INTRODUCTION

Some heavy metals (copper, iron and zinc among others) are of biological importance in animal life while others are important trace elements for plant growth and in higher animals. Despite the various uses of HMs, [1] argued that their biotoxic effects come into play once accumulation levels in water, plants and animal bodies go beyond maximum permissible limits. Heavy metal contamination is a major problem of the environment today, especially for growing medium sized cities in developing countries mainly due to uncontrolled pollution levels argued [2-4]. These, eventually bioaccumulate in organisms' bodies.

1.1 Environmental Effects of Heavy Metal Pollution

Heavy metal pollution threatens the health and life of many aquatic/non-aquatic organisms such as birds, fish, humans among others, by way of the food chain. The author [5], reported that the most unfortunate thing is that this kind of pollution is covert, long term and irreversible while [6] suggested that it threatens the health and human life by way of the food chain as man is a major consumer along the trophic levels.

Heavy metals like mercury (Hg), lead (Pb), cadmium (Cd) are toxic even at low concentrations and may affect deoxyribonucleic acid (DNA) and enzymatic processes [7]. These interfere with bio reactions of biomolecules in an organism. However, other essential HMs like zinc (Zn) and copper (Cu), are strongly regulated by

metabolic processes, as they are important constituents of enzymes and other compounds, argued [7]. The poisoning and toxicity in animals occur frequently through exchange and co-ordination mechanisms. When ingested, the HMs combine with the body's biomolecules like water, proteins, enzymes to form very stable biotoxic compounds in the body, thereby inactivating the biomolecules. This affects the functionality of biomolecules in the body, resulting in many associated disorders like gastrointestinal tract (GI), reproductive inefficiency, diarrhea, stomatitis, tremor, haemoglobinuria among others, reported [8].

Currently, most research documented suggests that the development of underground deep mining, waste water disposals from city centres, cosmetic activities, industries and agricultural run-offs, all promote the rapid increase of environmental heavy metal pollution in the aquatic waters, reported [7,9,6]. In the case of Zambia, many studies such as [9-11] have revealed that HM pollution is quite common in the Kafue River, and the predominant elements being Zn, Cu, Cd, Pb and Ni. However, actual data of HM concentration levels in Kafue River is quite scanty and a recent study on assessing HM accumulation values in lake sediments and fish (*Oreochromis niloticus* and *Serranochromis thumbergi*) and crayfish in Lake Itezhi-Tezhi and Lake Kariba [9] revealed the following values for the respective HMs as shown in Table 1-1. Note that only part of the data is shown in this table.

Currently, heavy metal pollution is among the major areas of concern in environmental

research today. In view of this problem, monitoring and prevention of this case has taken centre stage so as to reduce a great deal of its adverse effects. A related study [12], reported that monitoring techniques such as bioaccumulation, biochemical alterations, morphological and behaviour observations, population and community level approaches and modelling are used in modern research for assessment of heavy metal pollution in aquatic life as well as human beings.

1.2 Bioaccumulation

Bioaccumulation is the increase in concentration of a chemical in a biological organism over a period of time compared to the chemical's concentration in the environment. It occurs when an organism absorbs a toxic substance at a rate greater than the rate at which the substance is lost [12] and this threatens the health of human life as man is a major consumer along the trophic levels [6]. The authors [13] also opined that deleterious effects of HMs do not normally manifest themselves immediately after the toxin enters the organism; but usually become apparent only after a few years. However, [14], opined that sometimes, despite a relatively shorter exposure period, the amount of a metal deposited in an organism may be considerably high due to different feeding and elimination mechanisms. This observation coincides with [15]'s opinion that in some cases, the level of a metal in the tissues of one organism does not elicit any toxic effects at all, but may constitute a danger to its predators. The authors [14] investigated levels of Zn, Cu, Cd, As and Pb in kidneys, liver, gills and heart of African catfish (*Clarias gariepinus*), and the study showed high levels of Zn, Cu and Pb low levels of As and Cd in all organs. The study further showed that Cu, As and Pb were highly accumulated in kidneys and livers while concentrations of Cd and Zn were mostly high in gills and liver, as indicated in the comparison sketch;

Heart:- Zn >Cu> Pb >As>Cd; **Gill:-** Zn>Cu>Pb>Cd >As; **Kidney:-** Zn >Cu>Pb >As >Cd; **Liver:-** Zn >Cu>Pb >As >Cd. This information indicated the order of concentration of metals in the organs as:-

Arsenite: Kidneys >Liver >gills> heart; **Lead:-** liver >kidneys >gills >heart; **Zinc:** gills >liver >kidney >heart; **Copper:** kidneys >liver >gills >heart & **Cadmium:-** liver >gills >kidneys >heart.

1.3 Biochemical Mechanisms of Heavy Metal Toxicity

A related study [16], reported that Pb toxicity is inclusive of inhibition of cellular enzymes, binding to sulphhydryl groups or dissociation of biologically active metal ions from metalloenzymes. The HMs combine with the body 's biomolecules such as proteins and enzymes to form strong and stable chemical bonds argued [8]. This way, a metal ion in the body's metalloenzyme is conveniently replaced by another metal ion of a similar size. Thus Cd²⁺ can replace Zn²⁺ in some dehydrogenating enzymes, leading to cadmium toxicity. In this process of inhibition, the structure of a protein molecule can be converted to a bio-inactive form, and in the case of an enzyme, it may completely be denatured. The equations below show their reactions during bond formation with sulphhydryl groups (-SH) of cysteine and sulphur atoms of methionine (-SCH₃) reported [17].

1.4 Metallothionein (MT)'s Protective Mechanisms in Fish Bodies

MT is a polypeptide and may be referred to be a polyfunctional group as it has many sulphhydryl groups for effective metal binding during transportation and detoxification processes in the body. This protein is synthesised in fish body due to the presence of exogenous substances such as heavy metals. MT is mainly composed of the amino acids; cysteine, glycine and serine, but devoid of any aromatic amino acids.

Table 1-1. Mean (±SD) values (mg/kg dry-wt) of HMs in fish liver and muscle of *O. niloticus*.[†]

Tissue	BW	BL	K	Cu	Cd	Pb	Ni
Liver ITT	1 100±409	32.5±3.8	3.2±0.4	1345±930	0.80±0.73	0.19±0.15	0.52±0.28
Liver LK	849±4.2	29.3±4.2	3.2±0.9	587±320	1.21±0.88	0.50±0.24	0.52±0.56
Muscle ITT				3±1	0.003±0.005	0.12±0.25	0.38±0.29
Muscle LK				2±1	0.002±0.006	0.04±0.06	0.82±0.51

Where: BW= Body Weight, K= Coefficient condition, BL= Body Length, ITT= Itezhi-Tezhi and LK = Lake Kariba

[†] Adopted and modified from Table 2 of Nakayama et al., 2010

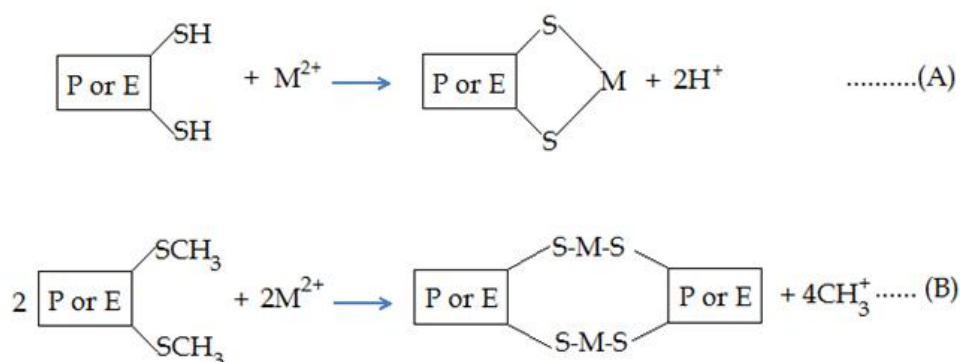


Fig. 1.1. Biochemical mechanisms of heavy metal toxicity

Equation A shows intramolecular bonding, B shows intermolecular bonding, P = protein, E = enzyme and M = metal; adopted from [17]

The authors, [7] opined that elements like Cd, Cu or mercury (Hg) have greater affinity for ligands than zinc, and will tend to displace it (Zn) at MT binding sites. These, further expressed that toxic metals inhibit cellular enzyme activity in that physiological metals (Cu, Zn or Ca) are displaced by xenobiotic ones like Cd, Pb, Ni and Hg. This way, MT proteins, bind and detoxify the metal ions as they have high affinities for heavy metals; thereby concentrate and regulate these metals in the liver. The author, [18] also opined that binding of MT with both physiological and xenobiotic metals in an organism protects an organism from toxic effects of those metals that would occur if they became bound to proteins or enzymes with important functions in metabolic or enzymatic processes. Interestingly, [19] also suggested that Cu and Zn, even though are essential cofactors in biochemical and many other physiological processes in organisms, any increased concentrations of these, may result in deleterious effects in the organism such as impairment of growth and reproduction.

1.5 Sex Steroid Hormone Levels in Fish

The data in the Table 1-2 was obtained from a study on the effect of heavy metals on the reproductive system of *cyprinid fish* (common carp) from a polluted Kor river, Fars province in Iran. The results of the study showed that no significant differences were detected in concentrations of Testosterone (T) among female fish from the three sampling zones ($P > 0.05$) while a significant difference in T concentration between male and female sexes was observed from the lower sampling zones reported [20].

Another similar study by [21], was carried out to investigate hormonal disruptors in Nile Tilapia fish species, exposed to pesticides. The group 1 of female Nile tilapia was treated as a control while the other four groups were treated with different pesticides of varying concentrations. For the male tilapia, only three groups were involved, group 1 was the control while the other were treated with two different pesticides and separately for 21 days. The study bioaccumulation of pesticides in both male and female fish tissues. The study further showed that the level of E2 and P concentration in females were significantly decreased in all treatments, while T levels in male fish was decreased significantly when compared to the control groups. Table 1-3 shows the results of the study.

In view of these findings from many articles, which indicating depressed steroid levels arising from reproductive impairment, [22] among others, this study focused on assessing the bioaccumulative impact of Cd, Cu, Ni and Pb on the endocrine system of the red-breasted breams (*Tilapia rendalli*) in the Kafue River, Zambia. The study determined the levels of the sex hormones (E2 and T) and correlated these to HM concentrations in the fish.

2. MATERIALS AND METHODS

2.1 Materials and Reagents

Reagents used were 70% concentrated perchloric acid, 65% concentrated nitric acid (all ANALAR grade), Testosterone (KGE010) and Estradiol (KGE014) ELISA Parameter Assay kits from R and D Systems while materials included a

microplate reader machine, 1.50 mL microcentrifuge tubes (eppendorf), 15 mL (falcon) tubes, 500 mL polypropylene bottles, polyethylene/polypropylene buckets, polyethylene plastic bags, 5 mL heparinized syringes and 23G needles, blotting paper, storage cups, gill net, vacutainers (red tops and green tops) tube racks, gloves, stainless steel knives and a grinder.

2.2 Study Design

This was a Cross - Sectional Study involving *Tilapia rendalli* fish species.

2.3 Study Area and Field Sampling

The sampling sites were; upper Kafue River (KUP), in Chililabombwe, upper lake Itezhi-Tezhi (ITT) and Shimungalu (SH), in Mazabuka along the Kafue River. These are shown in Fig. 2-1. Within this area, Kafue River receives and drains effluents from different sources like Kitwe City Council drainage system, Mopani copper mining, Konkola copper mining, city centre washings (rainy season), run offs from Zambia sugar plantation agricultural fields, among others. These, the study assumed could be the source of HM pollution in the river, thereby affecting

growth, reproductive systems of fishes, among other effects in aquatic organisms.

2.4 Sample Size Calculation

Sample size was calculated using data in Table 1-2 for a similar study that was done to determine the difference in sex hormone levels for fish caught from different sample zones along Kor river [20]. The mean values and standard errors for both hormones (E2 and T) were used to calculate the standard deviations using the formula:

$$S.E. = s.d. / \sqrt{n}$$

where **S.E.** = standard error; **s.d** = standard deviation and **n** = sample size, s.d.1 and s.d.2 for E2 were 18.4 and 16.7 respectively. Using Stata software version 12;

2.4.1 Sample size 1 (n_1)

Using E2 mean concentration values for upper and lower sample zones (13.4±2.13) and (5.72±1.93) respectively, $n = 75$; $s.d_1 = 18.4$ and $s.d_2 = 16.7$, confidence level of 80 % , $p = 0.20$; a value of n_1 , for a difference of two means, was calculated and found to be 83.

Table 1-2. Plasma concentrations of sex steroid hormones in exposed fish from different zones¹

Heavy Metal (HM)	HM Conc. in fish from sample zones of Kor River (mean±s.e) mg/mL		
	D.dam (upper)	Amir (middle)	Korbal (lower)
Cadmium (Cd)	0.44±0.14	4.51±0.94	1.73±0.48
Lead (Pb)	2.83±0.80	7.49±1.85	4.68±0.92
Mercury (Hg)	0.006±0.001	0.036±0.012	0.011±0.01
Arsenic (As)	0.80±0.17	1.80±0.19	1.10±0.76
Hormones	Plasma Concentrations of Sex Hormones in Fish (mean±s.e) ng/mL		
17β-Estradiol (E2)	13.40±2.13	1.93±0.65	5.72±1.93
Progesterone (P)	0.09±0.01	0.77±0.015	0.25±0.05
Testosterone (T)	1.74±0.32	0.44±0.22	0.91±0.15

Part of data for hormone levels of cyprinid fish exposed to heavy metal pollution from Kor river, s.e. = standard error

Table 1-3. Hormonal levels in control and exposed fish to pesticides in Sinaloa, Mexico²

Hormone	Range of hormonal concentrations (ng/mL) at 205 nm and 270 nm	
	Exposed group	Control Group
17β-Estradiol (E2)	0.1 – 0.5	0.6 – 0.9
Progesterone (P)	0.5 – 1.8	1.6 – 2.2
Testosterone (T)	4.0 – 6.8	9.0 – 12.0

Note that hormone levels for both groups (control and exposed) were assayed at both wavelengths

¹ Adopted from Ebrahimi and Taherianfard (2011).

² Adopted from Hormonal disruptions in fish exposed to pesticides, Reyes et al., (2014).

Fish Sampling Sites for Heavy Metals

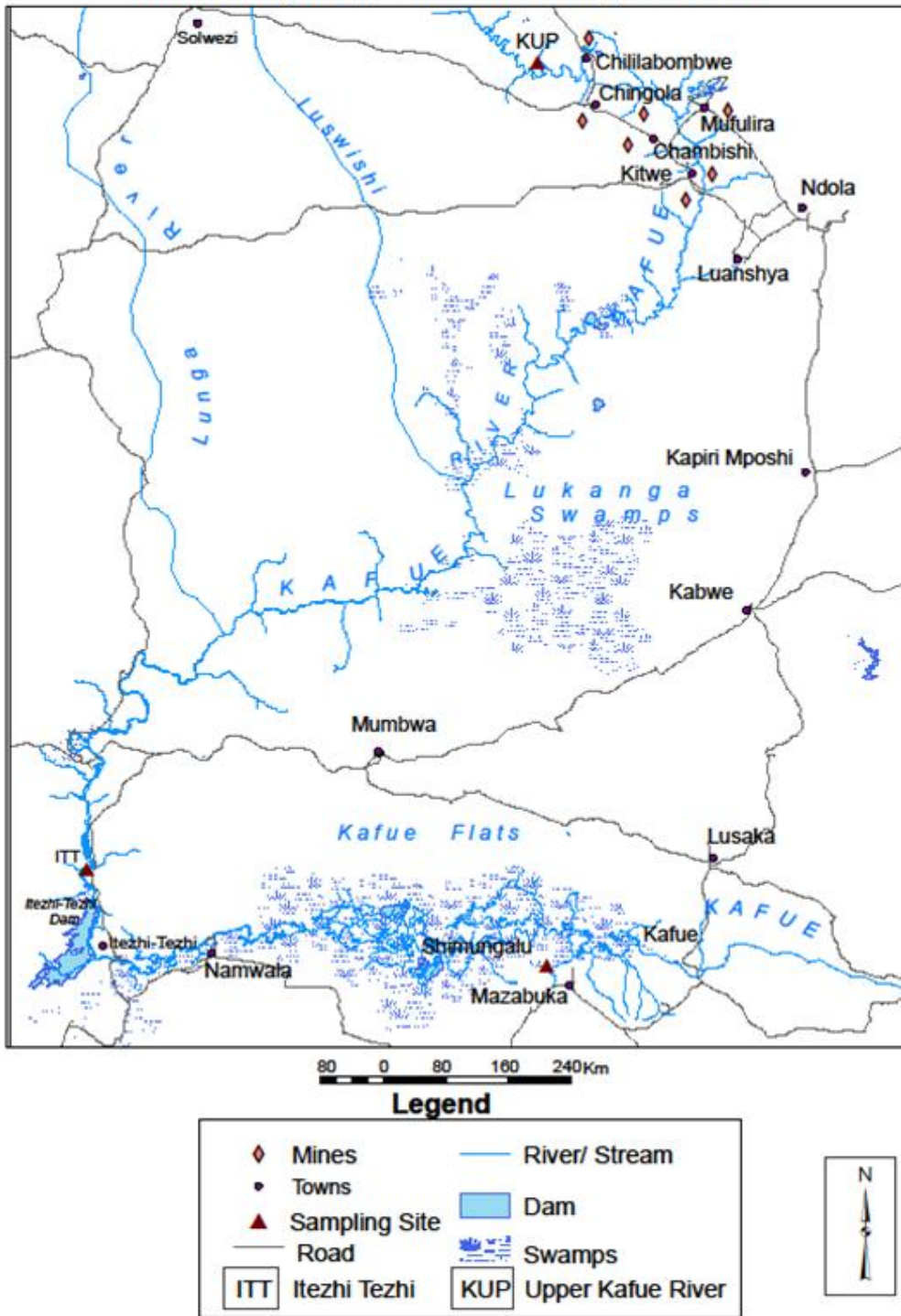


Fig. 2-1. Map showing sampling sites: KUP = site 1, ITT = site 2, Shimungalu (SH) = site 3
 Source: Geography and Environmental Studies Department; Cartographic unit, UNZA

2.4.2 Sample size 2 (n_2)

Using T mean concentration values for the upper and lower sample zones (1.74 ± 0.32) and (0.91 ± 0.15) respectively and $n = 75$; $s.d_1 = 2.77$; $s.d_2 = 1.30$, confidence level of 80%, $p = 0.20$; the value of n_2 , for a difference of two means was calculated and found to be 107. Therefore, sample size 2 (107 samples) was used for this study since it was inclusive of the smaller sample size 83. However, 14 samples were spoiled and 93 samples were used in the study.

2.5 Inclusion Criteria and Exclusion Criteria

The inclusion and exclusion criteria was based on fish species and the mature age range of the fish species. The study only focused on red – breasted breams (*Tilapia rendalli*) species while other fish species were excluded. The sample population was the *T. rendalli* of a mature age group while the target population was all fish species in the Kafue River.

2.6 Ethical Consideration

The permission to carry out this study was sought from the University of Zambia Biomedical Research Ethics Committee (UNZABREC). We also sought permission and guidance from Chilanga Fisheries Department, Ministry of Agriculture and livestock.

2.7 Methodology of Study

2.7.1 Study sites

This study was carried out between March and April (Autumn season), 2015. The Fig. 2-1 is a map showing three study sites along the river: Upper Kafue River (KUP) site 1, upper lake Itezhi-Tezhi (ITT) site 2 and Shimungalu fishing camp (SH) site 3, in Mazabuka.

2.8 Sample Collection and Preparation

2.8.1 Fish samples

A total of 107 mature healthy *T. rendalli* (T.r.), body length range 156 – 214 cm and weighing 156 – 210 g per fish were sampled from the sites as follows: 35 from KUP, 37 from ITT and 35 from Shimungalu (SH) fishing camp, near Mazabuka along the Kafue River. Fishing was

done with help from fishermen and research officers from Chilanga Fisheries department. For this purpose, a gill net with floaters at the top and sinkers at the bottom was used. Sex identification was done by observing the genital papilla; males have two openings in the papilla, whereas females have three. The sex was further verified by checking on the gonads; males have elongated gonads while females have slightly shorter and thicker gonads. The weight and length of each fish was measured, recorded and the fish was sacrificed by bisecting it over the ventrilia to the head using a clean knife (precleaned in distilled water) and blood collected. The gills, liver and muscle piece (cut from the inside of the flesh) were removed from each fish and identification codes put on cleaned storage polypropylene cups and containers before putting them in ice – cooled boxes. The fish was allowed to freeze in deep freezers before transporting them to the laboratory.

2.8.2 Blood samples

About 2 – 4 mL of blood samples were collected using a 23G needle and a 5 mL syringe, into a labeled red - top vacutainer after puncturing the caudal vasculature vein of the fish using a pre-cleaned stainless steel knife. Care was taken to avoid contamination of blood with body fluids. The blood samples were kept in ice-cooled racks in cooler boxes, and then frozen before transporting them to the University of Zambia, School of Veterinary Medicine, Department of Biomedical sciences for continued freezing before assay. The samples were centrifuged at $2016 \times g$ (3000 rpm) for 10 minutes to separate serum from blood. For E2, serum was removed after centrifugation, pretreated, and then assayed for E2 concentration following the protocol in R and D Systems KGE014 Parameter Estradiol assay kit. For T, serum was removed and prepared for assay following the protocol in R and D Systems KGE010 Parameter Testosterone assay kit.

2.9 Determination of Optical Densities (OD)

The OD for each sample was determined using a microplate reader set at 450 nm. For correction of readings obtained at 450 nm, the microplate reader was again set at 492 nm, (i.e. 492 nm was chosen as a closest value to a prescribed wavelength of 540 nm in the kits). The other suggested wavelength was 570 nm. OD's

measured at 492 nm were subtracted from OD's measured at 450 nm to get the corrected OD's. Using corrected OD's for the standard concentrations of E2 and T shown in Table 3-2, respective standard curves were generated and a four parameter logistic analysis in Graphpad prism version 6.01 was performed to interpolate unknown concentrations of samples using their OD's from a standard curve.

2.10 Tissue Digestion

Varying sizes of the muscle, liver and gills of each fish sample were rinsed in distilled water thoroughly and then dried to constant weight at about 110°C. Dried gills, livers and muscles were ground separately to powder, and then packed in precleaned polypropylene containers and polyethylene plastics in the oven. Before digestion, each sample was weighed, mass recorded and then transferred quantitatively to the digestion flask. Sample digestion was carried out according to the methods by [23]. About 10 mL of nitric acid (55%) and 5 mL of perchloric acid (70%) (both ANALAR grade) were added to each digestion flask. The flasks were shaken gently and put on the hot plate to digest tissues at about 200-250°C until a clear transparent solution was observed in the flask. Initially, dark, brown fumes were observed, followed by dense white fumes which disappeared later. The disappearance of dense white fumes indicated completion. This coincided with the appearance of a transparent solution in the flask in about 30 to 50 minutes. More drops of nitric acid, followed by perchloric acid were added into the digestion flask until a clear transparent solution was observed. About 8 to 10 mL of solution remaining in the flask were cooled and diluted to 20 mL with distilled water in the 25 mL capacity measuring test-tubes. This was done by transferring quantitatively the sample solution from the digestion flask into the measuring test-tubes. The prepared sample solutions were transferred into precleaned 100 mL polypropylene bottles for storage. Metal concentrations were then determined using the Varian 220 Atomic Absorption Spectrophotometer within a week of preparation. Concentrations of the metals in the samples were determined after instrument calibration using respective working standards (Merck Germany) (2 ppm, 4 ppm, 8 ppm for Cu and Ni; 1 ppm, 2 ppm, 3 ppm for Cd; while 5 ppm, 10 ppm and 15 ppm for Pb) prepared from 1000 mg/L stock solutions of the respective metals. Table 2-1 shows the settings for determination of each metal concentration in the sample. Determined mean concentrations for

Cd, Cu, Ni and Pb in water and tissue samples are shown in Tables 3-1 and 3-5 respectively.

2.11 Statistical Analysis

Unpaired t-test was carried out to test for any significant differences in variable means between any two sites. This was followed by one-way analysis of variance (ANOVA), to test for any statistically significant variance in heavy metal concentrations and hormones in among the three sites. All data were checked beforehand for normality. The data which were not normally distributed were transformed using Grubb's test. ANOVA was followed by testing for Pearson's correlation coefficient between dependent variables (E2 & T) and independent variables (HM concentrations in tissues). This was followed by linear regression (univariate and multivariate) analysis to determine the strength of association (correlation) between the outcome variable and the independent variables. All statistical calculations were carried out using Stata version 13 for windows and Confidence Interval set at 95% ($P \leq .05$).

3. RESULTS AND DISCUSSION

3.1 Results

In this study, 93 samples (46 females and 47 males) from an original sample size of 107 were used. Fourteen were spoiled, representing 87% of the original size. The mean weights of fish from the sites, KUP, ITT and SH were (154.79±11.58, 209.15±18.80 and 182.59±7.76) g respectively, while mean lengths were (185.25±4.36, 210.85±6.11 and 191.91±3.03) mm respectively. Mean concentrations of the HMs (mg/L) in water along the Kafue River at KUP, ITT and SH are given in the Table 3-1. Only Cd was detected at all sites with mean values (0.031, 0.021 and 0.004) for KUP, ITT and SH respectively as shown in Table 3-1.

3.1.1 Cadmium (Cd) concentration in tissues

The highest Cd level of 7.41±1.45 mg/kg in livers was recorded from SH, followed by KUP with 5.43±0.80 mg/kg and a lowest level of 3.96±0.86 mg/kg dry weight (DW), from ITT. Similarly, Cd concentrations in gills showed a decreasing order of 4.96±0.43 mg/kg, 4.27±0.24 mg/kg and 4.16±0.22 mg/kg for SH, ITT and KUP respectively, representing no statistically significant difference in both tissues ($P > .05$).

Cadmium showed lowest concentration levels in muscles and a wider range among the three tissues. ANOVA test results showed a statistically significant difference for Cd_muscle among the three sites ($P = .036$) as shown in Table 3-8. Lowest concentration recorded in muscles was 0.21 ± 0.09 mg/kg from KUP while the highest was 1.03 ± 0.30 mg/kg from ITT as shown in Table 3-5.

3.1.2 Copper (Cu) concentration in tissues

The highest level of the essential element Cu, measured in the liver was 830.98 ± 178.58 mg/kg from KUP while ITT recorded 496.73 ± 184.96 mg/kg and SH recorded a lowest value of 69.09 ± 26.77 mg/kg. This represented a statistically significant variance of ($P = .011$) among the sites. The highest concentration of Cu measured in gills was 10.38 ± 1.12 mg/kg from KUP, followed 8.00 ± 1.67 mg/kg from SH while the lowest value 4.67 ± 1.25 mg/kg was recorded from ITT, representing a statistically significant variance ($P = .017$) as shown in Table 3-8. In muscles, highest Cu level measured 127.19 ± 61.34 mg/kg from SH, followed by 43.68 ± 18.32 mg/kg from ITT while Cu levels were below detection in KUP as shown in Table 3-5.

3.1.3 Nickel (Ni) concentration in tissues

Among the four heavy metals; (Cd, Cu, Ni and Pb) investigated, Ni was the only one

not detected in muscles from all the sites. Its highest concentration of 8.94 ± 0.73 mg/kg, was detected in gills from KUP, followed by 7.36 ± 0.77 mg/kg from SH and a lowest level of 7.35 ± 0.95 mg/kg was recorded from ITT. In livers; a highest Ni concentration of 2.61 ± 0.79 mg/kg was recorded from ITT, followed by 0.58 ± 0.31 mg/kg, from SH and a lowest level of 0.14 ± 0.06 mg/kg was measured from KUP, representing a wide range of 2.47 (2.61 – 0.14) and a statistically significant variation of ($P = 0.0098$) as shown in the Tables 3-5 and 3-8 respectively.

3.1.4 Lead (Pb) concentration in tissues

The highest concentration of Pb measured was 59.14 ± 6.67 mg/kg in gills from SH, followed by 28.97 ± 1.77 mg/kg from ITT and a lowest level of 28.60 ± 1.75 mg/kg was measured in gills from KUP, representing a great statistically significant variance of ($P < .0001$) among the sites. High levels of Pb were also recorded in the liver; SH recorded highest level of 43.63 ± 11.12 mg/kg, followed by 40.74 ± 7.00 mg/kg for KUP and 11.21 ± 3.21 mg/kg was recorded for ITT. This represented a statistically significant variance ($P = .0030$) among the sites. Interestingly, a similar decreasing order of Pb concentrations among the three tissues from the sites (SH > KUP > ITT) was observed, although no statistically significant variation was observed in muscles.

Table 2-1. FAAS settings for HM determination

Heavy metal	Wavelength (nm)	Lamp type	Oxidant	Detection limit
Cadmium (Cd)	228.8	Cd HC lamp	Acetylene gas	0.002
Copper (Cu)	324.8	Cu HC lamp	Acetylene gas	0.03
Nickel (Ni)	232.0	Ni HC lamp	Acetylene gas	0.002
Lead (Pb)	217.0	Pb HC lamp	Acetylene gas	0.01

FAAS instrumental settings and the detection limits for determination of the heavy metals, HC = hollow cathode

Table 3-1. Heavy metal concentrations in water samples for the sites

Heavy metal	Sampling sites				
	KUP (upper)	ITT (middle)	SH (lower)	MPL	Source
Cadmium (Cd)	0.031	0.021	0.004	0.005	WHO (2004)
Copper (Cu)	ND	ND	ND	1.0	WHO (2004)
Nickel (Ni)	ND	ND	ND	0.02	WHO (2004)
Lead (Pb)	ND	ND	ND	0.005	WHO (2004)

Heavy metal levels in water at all three sites. It shows that heavy metal concentrations in water are below maximum permissible limits set by WHO / FAO, except for Cd which was detected slightly above MPL at sites KUP and ITT respectively. The concentrations of Cd at KUP and ITT are within the permissible range. This suggests that the water from the sites is safe for drinking. ND = not detected; MPL = maximum permissible limit

Table 3-2. Optical Densities (OD's) for E2 and T standards

E2 pg/mL	OD at 450 nm	OD at 492 nm	Diff. OD	Aver. OD	Corr-d OD	T ng/mL	OD at 450 nm	OD at 492 nm	Diff. OD	Aver. OD	Corr-d OD
NSB	0.065	0.051	0.014			NSB	0.053	0.050	0.003		
	0.066	0.052	0.014	0.014			0.052	0.049	0.003	0.003	
	0.467	0.131	0.336				1.692	0.371	1.321		
0 (Bo)	0.444	0.124	0.320	0.328	0.314	0 (Bo)	1.698	0.384	1.314	1.32	1.31
	0.334	0.101	0.233				1.489	0.359	1.130		
12.3	0.337	0.110	0.227	0.230	0.216	0.041	1.358	0.315	1.043	1.09	1.08
	0.352	0.115	0.237				1.443	0.326	1.117		
37	0.352	0.110	0.242	0.240	0.226	0.123	1.383	0.317	1.066	1.09	1.09
	0.344	0.101	0.243				1.274	0.291	0.983		
111	0.334	0.103	0.231	0.237	0.223	0.37	1.247	0.288	0.959	0.971	0.97
	0.267	0.098	0.169				0.763	0.200	0.563		
333	0.263	0.088	0.175	0.172	0.158	1.11	0.806	0.205	0.601	0.58	0.58
	0.169	0.076	0.093				0.457	0.140	0.317		
1000	0.172	0.082	0.09	0.092	0.078	3.33	0.466	0.137	0.329	0.32	0.32
	0.134	0.078	0.056				0.223	0.084	0.139		
3000	0.127	0.066	0.061	0.059	0.045	10	0.217	0.082	0.135	0.14	0.13

Shows the Optical Densities for E2 and T standard solutions. Aver. = Average, Differ. = Difference, Corr-d OD = Corrected optical density

Table 3-3. Optical densities for estradiol levels in female *Tilapia rendalli*

Site	KUP			ITT			SH				
Sample ID	OD at 450 nm	OD at 492nm	Corr. OD	Sample ID	OD at 450nm	OD at 492nm	Corr. OD	Sample ID	OD at 450nm	OD at 492nm	Corr. OD
KUP T.r03	0.281	0.111	0.170	ITT T.r03	0.433	0.129	0.304	SH T.r07	0.120	0.058	0.062
KUP T.r04	0.186	0.070	0.116	ITT T.r10	0.443	0.124	0.319	SH T.r29	0.240	0.079	0.161
KUP T.r05	0.287	0.096	0.191	ITT T.r12	0.332	0.099	0.233	SH T.r30	0.233	0.084	0.149
KUP T.r06	0.281	0.087	0.194	ITT T.r21	0.372	0.109	0.263	SH T.r31	0.329	0.115	0.214
KUP T.r07	0.128	0.064	0.064	ITT T.r25	0.404	0.119	0.285	SH T.r32	0.177	0.072	0.105
KUP T.r08	0.182	0.074	0.108	ITT T.r26	0.255	0.088	0.167	SH T.r33	0.187	0.089	0.098
KUP T.r11	0.166	0.075	0.091	ITT T.r27	0.434	0.132	0.302	SH T.r35	0.237	0.097	0.140
KUP T.r12	0.212	0.081	0.131	ITT T.r29	0.379	0.114	0.265	SH T.r39	0.191	0.075	0.115

Site	KUP			ITT			SH				
Sample ID	OD at 450 nm	OD at 492nm	Corr. OD	Sample ID	OD at 450nm	OD at 492nm	Corr. OD	Sample ID	OD at 450nm	OD at 492nm	Corr. OD
KUP T.r14	0.238	0.09	0.148	ITT T.r41	0.396	0.113	0.283	SH T.r41	0.237	0.112	0.125
KUP T.r18	0.295	0.093	0.202	ITT T.r42	0.365	0.104	0.261	SH T.r42	0.245	0.087	0.158
KUP T.r19	0.287	0.093	0.194	ITT T.r46	0.373	0.107	0.266	SH T.r43	0.194	0.091	0.103
KUP T.r21	0.298	0.099	0.199	ITT T.r50	0.302	0.098	0.204	SH T.r44	0.198	0.085	0.113
KUP T.r26	0.198	0.085	0.113	ITT T.r51	0.198	0.078	0.120	SH T.r45	0.174	0.077	0.097
KUP T.r27	0.245	0.087	0.158					SH T.r46	0.208	0.084	0.124
KUP T.r36	0.290	0.094	0.196					SH T.r48	0.209	0.077	0.132
KUP T.r37	0.206	0.078	0.128					SH T.r50	0.244	0.083	0.161

Optical Densities for E2 levels in unknown female *T. rendalli* samples from all sites. The lowest OD of 0.062 was recorded from SH while a highest OD of 0.304 was from ITT. Corr. OD = corrected optical density

Table 3-4. Optical densities for testosterone in male *Tilapia rendalli*

Site	KUP			ITT			SH				
Sample ID	OD at 450 nm	OD at 492 nm	Corr. OD	Sample ID	OD at 450 nm	OD at 492 nm	Corr. OD	Sample ID	OD at 450 nm	OD at 492 nm	Corr. OD
KUP T.r09	0.886	0.226	0.660	ITT T.r02	0.916	0.228	0.688	SH T.r13	0.775	0.284	0.491
KUP T.r10	1.093	0.279	0.814	ITT T.r15	1.189	0.281	0.908	SH T.r14	0.351	0.132	0.219
KUP T.r13	0.867	0.247	0.620	ITT T.r16	1.155	0.265	0.890	SH T.r16	1.245	0.285	0.960
KUP T.r23	0.663	0.170	0.493	ITT T.r20	0.972	0.231	0.741	SH T.r18	1.080	0.256	0.824
KUP T.r29	0.837	0.204	0.633	ITT T.r23	1.302	0.308	0.994	SH T.r21	0.557	0.160	0.397
KUP T.r30	1.040	0.247	0.793	ITT T.r24	1.306	0.294	1.012	SH T.r22	1.116	0.268	0.848
KUP T.r32	1.049	0.267	0.782	ITT T.r28	0.920	0.228	0.692	SH T.r23	0.754	0.197	0.557
KUP T.r33	1.186	0.281	0.905	ITT T.r30	1.023	0.238	0.785	SH T.r24	1.135	0.264	0.871
KUP T.r38	1.080	0.254	0.826	ITT T.r31	1.111	0.265	0.846	SH T.r25	0.496	0.140	0.356
KUP T.r43	0.895	0.215	0.680	ITT T.r32	1.167	0.296	0.871	SH T.r26	1.143	0.269	0.874
KUP T.r44	1.149	0.269	0.880	ITT T.r33	1.309	0.296	1.013	SH T.r27	0.927	0.226	0.701
				ITT T.r34	0.887	0.216	0.671	SH T.r34	0.941	0.242	0.699
				ITT T.r35	0.979	0.225	0.754	SH T.r36	1.022	0.270	0.752
				ITT T.r36	0.811	0.206	0.605	SH T.r37	0.389	0.130	0.259
				ITT T.r37	0.911	0.225	0.686	SH T.r38	0.572	0.159	0.413
				ITT T.r38	1.364	0.324	1.040	SH T.r40	0.672	0.168	0.504

Site	KUP			ITT			SH				
Sample ID	OD at 450 nm	OD at 492 nm	Corr. OD	Sample ID	OD at 450 nm	OD at 492 nm	Corr. OD	Sample ID	OD at 450 nm	OD at 492 nm	Corr. OD
				ITT T.r39	1.353	0.316	1.037				
				ITT T.r43	0.911	0.212	0.699				
				ITT T.r47	1.401	0.317	1.084				
				ITT T.r48	1.186	0.274	0.912				

Optical Densities for T levels in unknown male *T. rendalli* samples from all sites. The lowest OD of 0.219 was from SH and a highest OD of 1.084 was from ITT (1.084). Note that OD is inversely related to hormone concentrations in blood

Table 3-5. Mean HM concentrations in tissues and hormonal levels in blood

Tissue	H M	KUP		ITT		SH		MPL	C F	Source(s)
		Mean	S.E	Mean	S.E	Mean	S.E			
Gills	Cd	4.16	0.22	4.27	0.24	4.96 ^x	0.43	1		WHO/FAO(1993)
	Cu ^a	10.38 ^x	1.12	4.67	1.25	8.00	1.67	30		WHO/FAO(2008)
	Ni	8.94 ^x	0.73	7.35	0.95	7.36	0.77	10		WHO/FAO(1993)
	Pb ^a	28.60	1.75	28.97	1.77	59.14 ^x	6.67	2		WHO/FAO(1993)
Liver	Cd	5.43	0.80	3.96	0.86	7.41 ^x	1.45	1	0.80±0.73	
	Cu ^a	830.98 ^x	178.58	496.73	184.96	69.09	26.77	30	1345±930	Nakayama et al.,2010
	Ni ^a	0.14	0.06	2.61 ^x	0.79	0.58	0.31	10	0.52±0.28	
	Pb ^a	40.74	7.00	11.21	3.21	43.63 ^x	11.12	2	0.19±0.15	
Muscle	Cd ^a	0.21	0.09	1.03 ^x	0.30	0.36	0.17	1	0.003±0.005	
	Cu	N.D.		43.68	18.32	127.19 ^x	61.34	30	3.0±1.0	Nakayama et al.,2010
	NI	N.D.		N.D.		N.D.		10	0.38±0.29	
	Pb	1.61	0.33	1.40	0.34	1.76 ^x	0.44	2	0.12±0.25	
Hormone		KUP	S.E	ITT	S.E	SH	S.E		Normal levels	
E2 ^a (pg/mL)		608.8	77.64	63.75	45.39	774.33 ^x	66.98		600 - 900	Reyes J. G. G. et al., 2014
T ^a (ng/mL)		7.54	0.76	5.78	0.69	13.58 ^x	2.80		9.0 - 12.0	

This shows concentrations of heavy metals in tissues (gills, livers and muscles) of *Tilapia rendalli* from all the sites KUP, ITT and SH. Where S.E = standard error, N D = not detected, small letter "a" = significant variation among sites while "x" indicates highest concentration value among the sites, MPL = maximum permissible limits and CF = comparable figures, from sources indicated in the table

3.1.5 Hormone levels in blood serum

3.1.5.1 17 β – Estradiol (E2) pg/mL

For this study, SH recorded a highest level of E2 (774.33±66.98 pg/ mL), followed by 608.80±77.64 pg/mL from KUP while ITT recorded a lowest level of 63.75 ± 45.39 pg/mL. A t-test comparison of means results between KUP and SH showed no statistically significant difference in the means (P = .059), but statistically significant differences in means were observed between KUP and ITT (P<.0001); and ITT and SH (P <.0001) respectively as shown in Table 3-7. ANOVA test results also revealed a statistically significant variance for E2 levels among the sites (P < .0001), as shown in Table 3-8.

3.1.5.2 Testosterone (T) ng/mL

Similarly, a highest levels of 13.58±2.80 ng/mL was recorded for SH; followed by 7.54±0.75 ng/mL, from KUP; while a lowest level of 5.78±0.69 ng/mL, was measured from ITT.

ANOVA test results for these hormones revealed great statistically significant variances among the sites, with p values of P < .0001 and P = .0049 respectively as indicated in Table 3-8.

3.1.6 Summary of mean HM concentrations in tissues and hormone levels in blood

The graphical representation of mean heavy metal concentrations in gills, liver and muscle are shown in the Fig. 3-1.

3.1.7 Bioaccumulation factors

Bioaccumulation factors (BAFs) for the metals Cd, Cu, Ni and Pb in gills, livers and muscles are shown in Table 3-6. The accumulation levels were significantly different, except for Cd in gills and Ni and Pb in muscles. Ni levels in muscles from all sites were below detection (ND) as shown in Table 3-5. Bioaccumulation factors (BAF's) were calculated using the formula as shown:

$$BAF = \frac{\text{concentration of HM in tissue mgkg}^{-1}}{\text{concentration of HM in water mgL}^{-1}}$$

In a case where the HM was not detected in water, the detection limit for the respective metal was used.

3.1.8 Statistical analysis of data

The results for statistical analysis of data using t-test and ANOVA are represented in the Tables 3-7 and 3-8 respectively.

ANOVA test results for Cd_livers and Cd_gills showed that no statistically significant variance was detected among the sites (P > .05). However, a statistically significant difference in Cd concentrations in muscles among the three sites was determined (P = .036) as shown in Table 3-8. Analysis of variance test for Cu revealed statistically significant differences in mean concentrations of Cu in gills and livers (P = .017 and P = .011) respectively, while in muscles, no statistically significant variance was detected (P > 0.05) as shown in Table 3-8. ANOVA test results for Ni revealed that statistically significant variance in mean concentrations was only detected in livers among sites (P = .0098) while ANOVA test results for Pb revealed statistically significant variances in mean concentrations of the metal in gills and livers (P < .001 and P = .0030) respectively as shown in Table 3-8.

The Tables 3-9 and 3-10 show results univariate and multivariate analysis of E2 and T levels in blood with HM levels in tissues. The analysis was done at 95% confidence interval to show the strength of association between the independent and dependent variables.

The Tables 3-11 and 3-12 show determined correlation values between the hormones (E2 & T) and HM levels in tissues of the fish. Correlation values closer to 1 or -1 showed high positive or negative correlations between the variables.

3.2 Discussion

3.2.1 Bioaccumulation of heavy metals in fish

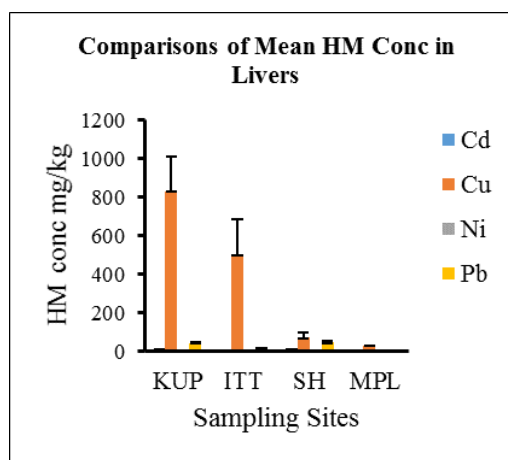
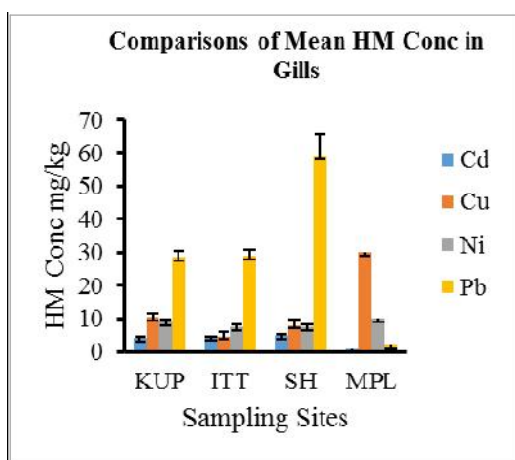
Fish tissues like gills, kidneys, livers, intestinal tissues and muscles are good biomarkers of heavy metal bioaccumulation. As observed by earlier authors like [7], results from this study have shown that essential metals like copper (Cu), zinc (Zn) and Iron (Fe) are accumulated mainly in the liver while lead (Pb) is mainly accumulated in gills [24]. This study chose gills, liver and muscles to assess the bioaccumulative impact of Cd, Cu, Ni and Pb on expression levels of Estradiol and Testosterone. Results indicated that levels of Cu and Ni were highest in the liver.

This observation is consistent with earlier studies [25,26]. The liver plays a key part in metabolism of vertebrate animals, as it is the site not only of metal bioaccumulation, but also their biotransformation, detoxification and enhanced elimination [7]. Previously, [9] also reported high levels of copper in the liver (1345±930 mg/kg) and low levels in the muscles (3±1 mg/kg) of *O. niloticus* from ITT. However, this study revealed lower levels of Cu in liver (496.73±184.96) and high levels in muscles (43.68±18.32 mg/kg). The low levels could be explained by reduced mining activities and improved pollution control measures such construction of Pollution Control Dams (PCD) by mining companies upstream. Despite this, recorded levels of Cu in livers from all sites (830.98±178.58, 496.73±184.96 & 69.09±26.77) mg/kg and in muscles; 43.68±18.32 mg/kg and 127.19±61.34 mg/kg from ITT and SH respectively, all are still higher than a MPL of 30 mg/kg set by WHO/FAO. The accumulation of Cu in the liver is linked to its role in metabolism like binding MT proteins, enzymatic and other biochemical and physiological processes. But at somewhat, higher concentrations it causes persistent toxicity

to aquatic life. Exposure of fishes to sub lethal concentrations of Cu leads to low cardiac activity and reduced heart rate [27]. Even though the muscle (flesh) is not an active tissue in accumulating heavy metals, the present study considered it important to analyze HM accumulation in this tissue because is the most consumed portion of fish by humans. Usually, concentrations of metals in muscles reflect concentrations of metals in the waters where the fish lives; whereas concentration in liver represents storage of metals argued [28]. This study also observed that in the liver, high levels of non-essential metals (Cd, Ni and Pb) (3.96±0.86, 2.61±0.79, 11.21±3.21) respectively were recorded. This confirms the observed high bioaccumulation rates of the tissues for the metals (Table 3-6). These findings are consistent with [24]. According to these authors, the observation for Cd could be explained by its ability to displace essential metals associated with MT in hepatic tissues. The accumulation pattern of Cd and Ni has been reported to fluctuate between the liver and the gills, with Pb showing similar accumulation patterns to Cd.

HM	KUP	ITT	SH	MPL
Cd	4.16	4.27	5	1
Cu	10.38	4.67	8	30
Ni	8.94	7.35	7.4	10
Pb	28.6	28.97	59	2

HM	KUP	ITT	SH	MPL
Cd	5.43	3.96	7.41	1
Cu	830.98	496.73	69.09	30
Ni	0.14	2.61	0.58	10
Pb	40.74	11.21	43.63	2



HM	KUP	ITT	SH	MPL
Cd	0.21	1.03	0.36	1
Cu	0	43.68	127.19	30
Ni	0	0	0	10
Pb	1.61	1.4	1.76	2

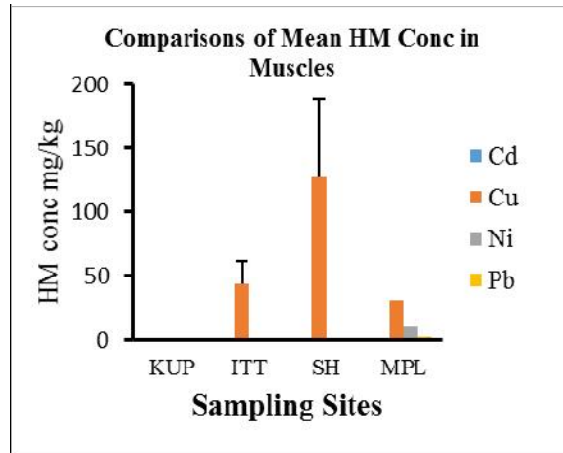


Fig. 3-1. Bar Graphs for comparisons of mean HM conc. in gills, livers and muscles
 Gills recorded high levels of Cd & Pb; Livers recorded high levels of Cd, Cu & Pb while muscles recorded high levels of Cu compared to maximum permissible limits

The Fig. 3-2 shows the variations of Estradiol and Testosterone levels in the fish among the sites.

	KUP	ITT	SH	R C L		KUP	ITT	SH	R C L
E2	608.8	63.75	774.33	600-900	T	7.54	5.78	13.58	9.0-12.0
Source	This study			Reyes et al., 2014	Source	This study			Reyes et al., 2014

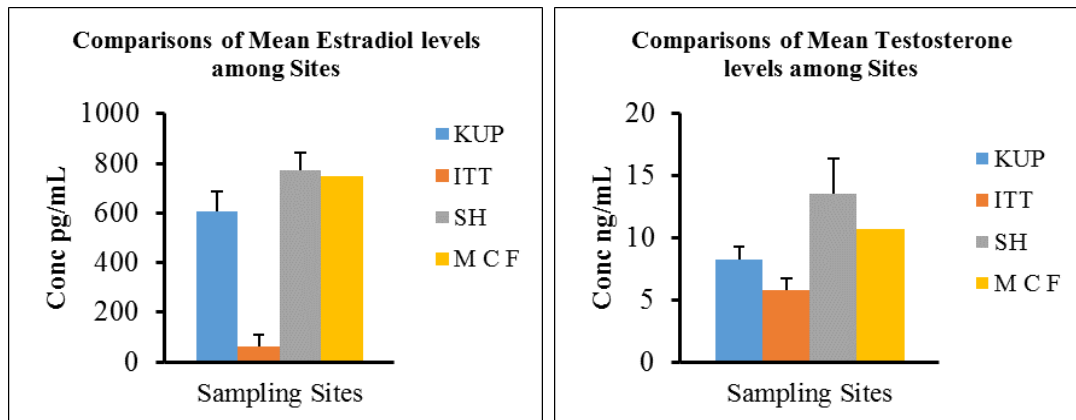


Fig. 3-2. Bar graphs for Estradiol and Testosterone levels in blood of the fish
 Where RCL = Range of Comparable Levels, MCF = Mean Comparable Figure (750 & 10.5) for E2 and T respectively. Low levels of E2 and T were recorded at ITT and high levels of E2 and T recorded at SH

3.2.2 Effects of heavy metal concentrations on steroid hormone levels

3.2.2.1 Cadmium (Cd)

This study has demonstrated that E2 and T levels in blood of *T. rendalli* showed a strong correlation with HM levels in tissues (Table 3-9 and Table 3-10). A multivariate regression analysis revealed a strong correlation between

E2 levels in blood and HM concentrations (Cd_gills, Ni_liver and Pb_gills, Pb_liver and Pb_muscle) in tissues. The strength of association (R^2) between E2 and HM concentrations was found to be 0.703 (70.3%) and $P = .029$ while that between T and HM concentrations (Cd_muscle, Cu_liver and Cu_muscle) in tissues was found to be 0.696 (69.6 %) and $P = .018$. Therefore 70.3% of the changes in E2 levels may be attributed to

changes in Cd, Ni and Pb concentrations in tissues while 69.6% of the changes in T concentration may be attributed to changes in Cd and Cu concentrations in fish tissues (See Tables 3-9 and 3-10). These findings are similar to observations by [29-33] which suggest that cadmium ions (Cd^{2+}) compete with other bivalent ions such as zinc ions (Zn^{2+}), calcium (Ca^{2+}), magnesium ions (Mg^{2+}) for binding to proteins of sperm cells, thereby affecting numerous reproductive processes in fish such as sexual maturation, spermatogenesis, fertilization success and development of embryonic and post embryonic stages, where they operate as cofactors. This could be arresting fish reproduction, thereby affecting its population at large. These findings are consistent with [34]'s observation that disturbances on the reproductive function in field - caught fish have been inferred from changes in the endocrine and reproductive function biomarkers, such as altered sex steroid hormones, typical gonadotropin and vitellogenin concentrations in circulation. Furthermore, these results provide evidence that the HMs (Cd, Ni Pb and Cu) are potential

endocrine disruptors (causes either over expression or suppress expression). These authors suggested that Cd and Pb effects on reproductive impairment seem to be biphasic. Cd at low concentrations has stimulatory effects while at high concentrations exhibits its inhibitory effects. These findings outlined complement [35]'s observation that Cd exposure increased glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression in the testis of rats as GAPDH is a potent co activator of androgen receptor-mediated transcription. Furthermore, up-regulation was probably a compensatory response to reduced testosterone concentrations. This finding is interesting since GAPDH has been proposed to have an important role in the regulation of apoptosis as well as sperm motility [35]. The stimulatory and inhibitory effects of Cd were also reported by [42] that fish from contaminated sites have been shown to be partially feminized, as evidenced by the presence of vitellogenin (Vg) in male Mediterranean killifish (*Aphanius fasciatus*), providing more evidence of endocrine disrupting properties of the HMs under study.

Table 3-6. Bioaccumulation Factors (BAF's) for HM in tissues at each site

Tissue	H M	S1 (KUP)		S2 (ITT)		S3 (SH)	
			BAF		BAF		BAF
Gills	Cd	4.16/0.031	134	4.27/0.021	203	4.96/0.004	1,240
	Cu	10.38/0.03	346	4.67/0.03	156	8.00/0.03	267
	Ni	8.94/0.002	4,470	7.35/0.002	3,675	7.36/0.002	3,680
	Pb	28.60/0.01	2,860	28.97/0.01	2,897	59.14/0.01	5,914
Livers	Cd	5.43/0.031	175	3.96/0.021	189	7.41/0.004	1,853
	Cu	830.98/0.03	27,699	496.73/0.03	16, 558	69.09/0.03	2, 303
	Ni	0.14/0.002	70	2.61/0.002	1,305	0.58/0.002	290
	Pb	40.74/0.01	4 074	11.21/0.01	1, 121	43.63/0.01	4,363
Muscles	Cd	0.21/0.031	6.8	1.03/0.021	49	0.36/0.004	90
	Cu	N.D.	1	43.68/0.03	1,456	127.19/0.03	4,240
	Ni	N.D.	1	N.D.	1	N.D.	1
	Pb	1.61/0.01	161	1.40/0.01	140	1.76/0.01	176

These BAFs confirm that highest bioaccumulation rates of Cd were in livers followed by gills and lowest muscles; while highest bioaccumulation rates of Cu were in livers, muscles and lowest in gills. Highest bioaccumulation rates of Ni were in gills, livers and lowest in muscles. Similarly, highest bioaccumulated rates of Pb were in gills, livers and lowest in muscles

Table 3-7. t-t results for comparison of means for variables with P < 0.1 (95% CI)

s/n	Variable (v)	Sites	Obs	Df	T value	P value
1	Cd_gill (v4)	KUP	27	56	-1.577	0.060
		SH	31			
2	Cd_gill (v4)	ITT	32	61	-1.409	0.082
		SH	31			
3	Cd_gill (v4)	KUP	28	58	3.348	0.0007
		ITT	32			

s/n	Variable (v)	Sites	Obs	Df	T value	P value
4	Cu_gill (v5)	ITT	32	61	-1.602	0.057
		SH	31			
5	Pb_gill (v7)	KUP	27	56	-4.161	0.0001
		SH	31			
6	Pb_gill (v7)	ITT	33	62	-4.492	< 0.0001
		SH	31			
7	Cd_liver (v8)	ITT	14	23	-2.152	0.021
		SH	11			
8	Cu_liver (v9)	KUP	9	17	4.450	0.0002
		SH	10			
9	Cu_liver (v9)	ITT	13	21	2.005	0.029
		SH	10			
10	Ni_liver (v10)	KUP	9	20	-2.583	0.0088
		ITT	13			
11	Ni_liver (v10)	KUP	9	17	2.157	0.021
		SH	10			
12	Ni_liver (v10)	ITT	13	21	-1.330	0.101
		SH	10			
13	Pb_liver (v11)	KUP	9	21	4.307	0.0002
		ITT	14			
14	Pb_liver (v11)	ITT	14	22	-3.220	0.0020
		SH	10			
15	Cd_muscle (v12)	KUP	9	20	-2.231	0.019
		ITT	13			
16	Cd_muscle (v12)	ITT	13	21	1.818	0.042
		SH	10			
17	Cu_muscle (v13)	KUP	9	21	-1.896	0.036
		ITT	14			
18	Cu_muscle (v13)	KUP	9	17	-1.961	0.033
		SH	10			
19	Estradiol (v16)	KUP	15	25	5.674	< 0.0001
		ITT	12			
20	Estradiol (v16)	KUP	15	28	-1.614	0.059
		SH	15			
21	Estradiol (v16)	ITT	12	25	-8.326	< 0.0001
		SH	15			
22	Testosterone (v17)	KUP	10	28	1.573	0.064
		ITT	20			
23	Testosterone (v17)	KUP	10	23	-1.723	0.049
		SH	15			
24	Testosterone (v17)	ITT	20	33	-3.067	0.0021
		SH	15			

T-test comparison of means results for variables: v4 (KUP vs SH), v4 (ITT vs SH), v5 (ITT vs SH), v16 (KUP vs SH) and v17 (KUP vs SH) are not statistically significant. Differences in means of E2 and T for KUP and SH, not statistically significant indicate similar exposure conditions. T and P values are inversely related

Table 3-8. One – way ANOVA results with P values less than 0.05 (P < .05)

s/n	Variable	Source	Df	F value	P value
1	Cu_gill (v5)	Between groups	2	4.26	.017
		Within groups	88		
2	Pb_gill (v7)	Between groups	2	17.67	< .001
		Within groups	88		

s/n	Variable	Source	Df	F value	P value
3	Cu_liver (v9)	Between groups	2	5.24	.011
		Within groups	29		
4	Ni_liver (v10)	Between groups	2	5.44	.0098
		Within groups	29		
5	Pb_liver (v11)	Between groups	2	7.09	.0030
		Within groups	30		
6	Cd_muscle (v12)	Between groups	2	3.73	.036
		Within groups	29		
7	Estradiol (v16)	Between groups	2	28.18	< .0001
		Within groups	39		
8	Testosterone (v17)	Between groups	2	6.04	.0049
		Within groups	42		

Analysis of Variance results and the inverse proportionality of F and P values. The higher F is, the smaller a P value will be and more significant the observation

Table 3-9. Univariate and multivariate regression analysis of E2 and HM Conc. in tissues

Univariate analysis of E2 & HM Conc. in tissues				Multivariate analysis of E2 & HM Conc. in tissues			
Variable	R ² (%)	F	P value	Variables	R ² (%)	F	P value
Cd_gill (v4)	4.5	1.8	0.187	V16 v4 v7	70.3	4.26	0.0290
Cu_gill (v5)	4.1	1.62	0.211	v10 v11 v15			
Pb_gill (v7)	11.9	5.26	0.027	V16 v4 v7	68.3	5.35	0.0144
Cd_liver (v8)	22.8	4.42	0.0528	v10 v11			
Ni_liver (v10)	14.9	2.44	0.14				
Pb_liver (v11)	44.9	11.4	0.005				
Pb_muscle (v15)	36.6	9.22	0.008				

F is directly proportional to strength of alternative hypothesis (H₁), P value shows probability or percentage of error and R² = strength of association between dependent and independent variables. The R² = 70.3 %, P = 0.0290 showed the strength of association between E2 and the variables v4, v7, v10, v11 and v15. Indicating that 70.3 % of variations in E2 levels could be attributed to variations in Cd_gills, Pb_gills, Ni_liver, Pb_liver and Pb_muscle

Table 3-10. Univariate and multivariate regression analysis of T and HM Conc. in tissues

Univariate regression analysis of T and HM Conc. in Tissues				Multivariate Regression Analysis of T and HM Conc. in Tissues			
Variable	R ² (%)	F	P value	Variables	R ² (%)	F	P value
Pb_gill (v7)	7.6	3.46	0.0698	V17 v7 v9 v12 v13	69.8	4.05	0.0519
Cu_liver (v9)	7.8	1.1	0.312	V17 v9 v12 v13	69.6	6.09	0.0184
Cd_muscle (v12)	14.6	2.05	0.178	V17 v12 v13	68.6	9.85	0.0054
Cu_muscle (v13)	65.7	21.05	0.001	V17 v9 v13	66.1	9.77	0.0045

F is directly proportional to strength of alternative hypothesis (H₁), P value shows probability or percentage of error and R² = strength of association between dependent and independent variables. The R² = 69.6 %, P = 0.0184 showed the strength of association between T and the variables v9, v12 and v13, indicating that 69.6 % of the variations in T levels could be attributed to variations in Cu_liver, Cd_muscle and Cu_muscle

Table 3-11. Correlation values for E2 levels against HM Conc. in tissues

Variable	Cd_gill	Ni_gill	Cu_liver	Ni_liver	Cd_muscle
Negative correlation value	-0.1023	-0.2055	-0.3476	-0.3219	-0.2796
Variable	Cu_gill	Pb_gill	Pb_liver	Cu_muscle	Pb_muscle
Positive correlation value	0.3032	0.3176	0.4964	0.3956	0.4032

A correlation value closer to 1 or -1 shows a stronger positive/negative relationship between estradiol and heavy metal concentration in the respective tissue

Table 3-12. Correlation values for T levels against HM Conc. in tissues

Variable	Cu_gill	Ni_gill	Cu_liver	Ni_liver	Cd_muscle	Pb_muscle
Negative Corr. Value	-0.4019	-0.2288	-0.3779	-0.3229	-0.4819	-0.3981
Variable	Pb_gill	Pb_liver	Cu_muscle			
Positive Corr. Value	0.6030	0.1236	0.8259			

A correlation value closer to 1 or -1 shows a stronger positive/negative relationship between testosterone and heavy metal concentration in the respective tissue

3.2.2.2 Copper (Cu)

Copper levels in the liver exceeded MPL of 30 mg/kg [36] while in gills it was below MPL. The levels of copper in the liver from all sites as well as muscles of the fish from SH are quite higher than MPL. This correlated with calculated bioaccumulation factors (BAFs) of the metal, which were also higher at the respective sites (Table 3-6). Higher Cu levels observed at KUP could be attributed from local sources and proximity to natural copper deposits and tailings which may have been washed by acidic rainy water runoffs. The station is also subjected to more human activities such as bathing, washing of clothes, which could have an effect on pH of the water due to high basic chemicals in soap used. This could be impairing fish in terms of reproduction, respiratory distress due to gill impairment, biochemical and physiological processes of fish. These observations of Cu toxicity at higher concentrations are consistent with statistical results which suggest high strength of association between the HMs and hormones levels in fish as outlined in section 6.3.1. Even though Cu is an essential element, it manifests its disruptive effects at higher concentrations as reported by [37] and [19]. Furthermore, these observations are in unison with [6] who also suggested that Cu is an important element and poisonous to numerous biological systems when in higher concentrations in an organism. These effects result in biochemical and physiological changes in fish blood and can be an indicator of the physiological state of fish argued [38]. The recorded levels of Cu in liver at KUP and ITT are quite higher compared to the MPL of 30 mg/kg set by [36]. These high levels have shown a negative correlation with E2 and T levels at the sites (Tables 3-9, 3-10, 3-11 & 3-12) complementing findings observed by other authors outlined above on its toxicity.

3.2.2.3 Nickel (Ni)

This study observed increased levels of Ni in livers from ITT compared to the level 0.52 ± 0.28 mg/kg reported by [9] for the same site. The

increase in Ni concentration in ITT could be due to heavy acidic rainy water runoffs through industrial wastes, municipal/city wastes from densely populated towns of the Copperbelt (e.g. Ndola, Kitwe, and Chingola). Heavy acidic rainy water runoffs through soils and rocky deposits could have led to such increases. The high levels of Ni recorded in SH in the gills could be explained by increased levels of waste runoffs from agricultural/plantation fields near the river; especially Zambia Sugar plantation fields which are in close proximity to the river and are heavily treated with insecticides rich in these heavy metals [2] and [39]. This is consistent with the observation that fish are known to accumulate Ni in different tissues when exposed to elevated levels in their environment [40] and [41]. Even though concentrations of Ni in the tissues from all sites were below MPL of 10 mg/kg set by [36]. Univariate and multivariate regression analysis results show strong, statistically significant negative correlations (-69) and (-34) between Ni and E2, suggesting that E2 being a more sensitive biomarker than Testosterone could be impaired. This observation by [42] corroborates with the findings reported here of a correlation between high levels of Ni concentrations against low E2 levels recorded in ITT. In aquatic ecosystems, Ni toxicity has been shown to vary significantly with organism species, pH and water hardness reported [43]. Furthermore, [44] and [45] also reported adverse effects of Ni such as reduced numbers of spermatozoa and reduced expression levels of testicular enzymes such as steroid 3β – hydroxysteroid dehydrogenase in nickel treated rats.

3.2.2.4 Lead (Pb)

Lead (Pb) in the environment arises from both natural and anthropogenic sources. Exposure to lead can occur through drinking water, food, air, soil, dust from old paint containing lead, wine bottle wraps, mirror coatings, batteries, old paints and tiles amongst others and that infants are more susceptible to the endangering effects of exposure to lead reported [46]. Municipal or city water runoffs, during rainy season are other sources of lead pollution in lakes, rivers and

underground water. Results of the present study show a decreasing order of Pb levels as SH > KUP > ITT for the sites. This coincides with the decreasing order of E2 levels (SH > KUP > ITT) for the sites as (774.33±66.98, 608.80±77.64; 63.75±45.39) pg/mL. Similarly, T levels also decreased in the order of SH > KUP > ITT (13.58±2.80; 7.54±0.75; 5.78±0.69) ng/mL respectively, indicating a positive correlation between hormone levels and HM concentrations in the fish's body. Univariate and multivariate regression analysis of E2 and Pb levels in tissues showed a statistically significant positive correlation (P = .027), while analysis of T and Pb concentrations in tissues showed no significant correlation (P =.070) as shown in Table 3-9 and Table 3-10 respectively. Furthermore, statistical results of correlation values (Table 3-11) for E2 levels and T levels (Table 3-12) against HM concentrations in tissues gave more evidence positive correlation between hormone levels and HM concentrations in tissues as well as the biphasic effects of Pb on hormone levels in fish.

The observed positive correlation of hormones with Pb concentrations in this study are consistent with findings of [47,48], (Chaube, Mishra et al. 2010) & [20], who suggested that effects of Lead exposure on fish are biphasic, with inhibitory effects after low-level exposure and stimulatory effects after high-level exposure. This may explain the recorded high levels of E2 and T from sites, positively correlating to high levels of Pb as shown in Table 3-5. These findings are also consistent with [49], who observed that elevated LH levels and increased size of gonads among other effects in high Pb dose treated fish groups (24- and 49-mg/kg) were associated with high Pb concentrations, compared to decreased LH levels, small gonad sizes from groups treated with low Pb dose (8 mg/kg). This provides more evidence to suggest that the body's response to lower exposure doses may be governed by a different mechanism (inhibitory effects) than in the case of higher exposure doses (stimulatory effects). They naturally react with a diverse array of environmental antigens and may confer an undefined degree of natural immunity to fish due to prolonged periods of exposure to pollutants. These observations could be similar to findings of this study. Due to chronic exposure of *T. rendalli* to high levels of Pb in the Kafue River particularly in SH, fish could have developed a defense mechanism against negative effects of lead on reproduction, resulting in observed increased levels of E2 and T in the exposed fish.

These findings are also consistent with Mager's opinion in [50] that several lines of evidence seem to indicate that fish are able to acclimatize, at least to some extent, to Pb during chronic exposures, henceforth develop some defense mechanisms which copy with its excess. Therefore, findings from this study suggest that environmental Pb may be a potential endocrine disruptor, affecting ovarian steroidogenesis, gametogenesis, and ovulation, which may lead to adverse impact on fish reproduction and decreased population density of fish in the river.

4. CONCLUSION

The negative effects of HM exposure are manifested by decreased E2 and T levels in fish. Heavy metals displace essential metals like Ca, Cu and Zn from proteins, enzymes and haemocyanin, where they are indispensable for the proper functioning of metabolic processes, thereby eliciting toxicity. These HMs elicit other adverse effects such as behavioral change, haemoglobinuria and reproductive dysfunctions among other effects. The study further showed that aquatic life in rivers and lakes open to human anthropogenic activities is exposed to heavy metal pollution and that fish particularly, bioaccumulate heavy metals in its tissues over a long period of time. The study observed that bioaccumulation levels of Cd, Cu, Ni and Pb in some tissues of fish exceeded MPL set by WHO and FAO (Table 3-5). There is great bioaccumulation of HMs especially in gills and livers of the fish, while muscles showed low bioaccumulation rates. The study also showed that bioaccumulation of heavy metals may be affecting the reproductive system and other biochemical and physiological processes in bodies of fish and that environmental Cd, Cu, Ni and Pb could be potential endocrine disruptors in the Kafue River.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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