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Cadmium Bio-Accumulation and the Associated Biomarkers in Edible Frog Species (*Hoplobatrachus Occipitalis)* in Ibadan, Oyo State, Nigeria

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ABSTRACT

The spate of natural emissions and anthropogenic activities has comparatively increased cadmium pollution in recent times. This has also increased the attendant hazardous implication on both the aquatic and terrestrial ecosystems. In this study, a total of 50 edible frog species (*Hoplobatrachus occipitalis*) sourced from the Ogunpa river in Ibadan, Oyo state were sampled. Atomic Absorption Spectrophotometry (AAS) was used for the evaluation of the blood, kidney and liver cadmium level. The frogs were grouped into Below Permissible Limit (BPL) and Above Permissible Limit (APL) groups using the FAO/WHO cadmium permissible level of 0.5mg/kg. 86% of the sampled frogs had blood cadmium level above the permissible limit while the liver and kidney cadmium levels exceeded the permissible limits in all the frogs. The highest cadmium level was detected in the liver (3.02±1.23 mg/kg). The erythrocyte parameters were significantly lower in the APL compared to the BPL group while the leucocyte parameters were higher in the APL than the BPL group. The histopathological lesions were consistent with pathological changes associated with renotoxic, hepatotoxic and reproductive features of cadmium toxicity. The study highlights the elevated cadmium levels in the tissue of the frog as a biomarker of exposure while the haematological and histopathological changes served as biomarkers of effect associated with cadmium toxicity in naturally exposed frogs. It also serves to underscore the importance of frogs as important sentinels of environmental cadmium toxicity, creation of public health awareness for cadmium toxicity and the evaluation of cadmium toxicity in the ecosystem.

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INTRODUCTION

Cadmium is a naturally occurring heavy metal that poses considerable detrimental biohazard effect on the ecosystem and poses severe health risk to human health. According to the International Agency for Research on Cancer (IARC) (2016), cadmium has been classified as one of the heavy metals (along with others like beryllium, nickel and their compounds) as a group one carcinogen. The ubiquitous presence and environmental pollution caused by cadmium has increased considerably over the years due to an increase in anthropogenic activities which utilizes them (Don-Pedro et al., 2004). Cadmium is usually obtained naturally as combined ores along with those of other heavy metals such as zinc, copper and lead. As such, tons of these heavy metals along with the industrial effluents are released during the extraction and smelting of some of these ores (Bernhoft, 2013).

The relatively high resistance to corrosion, low melting temperature, electrical exchange properties and excellent conductivity (thermal and electrical) of cadmium have also comparatively increased the utilization of cadmium in many industrial processes. Cadmium has thus found extensive application in the production of alkaline nickel-cadmium batteries, electronics, electroplating, nuclear fission cores, industrial pigments, alloys and as adjunct impurities in other metals (steel, zinc, lead and copper) (OECD, 1994 and Bernhoft, 2013).

This extensive use and the poor mechanism involved in the solid waste disposal of some of these products after their use have led to the abundance of cadmium in dumpsites and landfills globally. Water sources (groundwater, lakes, streams and rivers) can be polluted by cadmium leaching from industrial and consumer waste. The weathering of cadmium ore rich rock also serves as an example of natural activities contributing to environmental cadmium pollution (Environmental Protection Agency (EPA), 1985 and Morrow, 2001). These anthropogenic and natural activities therefore serve to encourage the mobilization and release of cadmium from non-bioavailable geological matrices into the ecosystem at concentrations far above the natural cycling process (Suru, 2008). The combination of different physical, chemical and microbial activities has been adduced by different researchers to be involved in the breakdown of some of these wastes and thus the release of cadmium into the environment (Leyval and Joner, 2001 and Gadd, 2010). The

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leaching of cadmium from dumpsites and rocks is also comparatively aided by acid rain which can exacerbate this process by enhancing the ease of cadmium release into the soil. This has inexorably led to an increase in cadmium pollution with dire deleterious impact on both the terrestrial and aquatic ecosystem (Worsztynowicz and Mill, 1995).

Cadmium serves no physiological role and despite the biotoxic effect it is not metabolized to less toxic forms in the body of the animal. Cadmium is also capable of accumulating in the tissues of the exposed animals when it is absorbed at a level in excess of the excreted volume (Britton, 1998 and Godt et al., 2006). Cadmium therefore tends to bioaccumulate in living organism with progressive increase in concentrations in biota several orders of magnitude greater than the environmental level. More so, the biomagnification of this metal also progressively increases with each trophic level. Humans, due to their position in the trophic level of the ecosystem, are therefore exposed to an elevated level of cadmium in excess of the environmental level (Wang, 2012 and Croteau et al., 2005).

The toxic bioaccumulation of cadmium in living organisms (plants and animals) has been widely studied as a vital indicator of environmental exposure. This bioaccumulation has been associated with toxic effects such as nephrotoxicity, carcinogenicity, teratogenicity and endocrine disruption (Serafim and Bebianno, 2007; Ikechukwu and Ajeh, 2011). These toxicities have been ascribed to the ability of cadmium to interfere with different enzymatic and biochemical processes in the body. Some of the mode of action employed in cadmium interference with physiological processes include: alteration of enzymatic processes and protein conformational changes. Cadmium is able to elicit these alterations due to the ability of cadmium to displace other metals (Copper, magnesium and zinc) and alter sulfhydryl groups in metalloproteins and metalloenzymes (Ikechukwu and Ajeh, 2011 and Haschek et al., 2013). Cadmium also induces oxidative stress through antioxidant enzymes binding, glutathione depletion, alterations of electron transport chain and metallothionein binding (Stohs and Bagchi, 1993; Hultberg et al., 2001 and Ercal et al., 2001). Other associated mechanism of cadmium toxicity includes: inhibition of heme synthesis (Chauhan et al., 1997; Nogueira et al., 2003 and Schauder et al., 2010), potentiation of apoptosis due to impairment of mitochondrial function (Cannino et al., 2009), oxidative DNA damage and epigenetic DNA changes (Martinez-Zamudio et al., 2011; Luparello et al., 2011 and Wang et al., 2012).

Frogs and toads due to their amphibious nature serve as an important link between the terrestrial and aquatic ecosystem thus making them invaluable as bio-indicator of different environmental contaminants (Hayes et al., 2002 and Unrine et al., 2007). This has also made them important sentinels both for the assessment of the presence and the evaluation of the toxic impact of environmental toxicants (Carey and Bryant, 1995; Loumbourdis et al., 2002; Papadimitriou et al., 2003; Rabinowitz et al., 2008 and Othman et al., 2009). Frogs have been extensively used in different studies as bio-indicators of pollutants accumulation due to their perpetual presence in water which keeps them directly in contact with the pollutants. The relatively thin nature of their skin and their position on the trophic level also makes them prone to persistent exposure to effluents in the water where they reside (Papadimitriou et al., 2003).

The crowned bullfrog (*Hoplobatrachus occipitalis*) is a species of frog in the Dicroglossidae family. It is found in the Sub-Saharan Africa. It lives in many habitats from dry savannahs to disturbed forest, using logging roads and rivers to penetrate deep into lowland forest. It naturally inhabits both aquatic and terrestrial areas therefore making it an excellent sentinel animal (McDiarmid and Mitchell, 2000; IUCN SSC Amphibian Specialist Group, 2014). The crowned bullfrog is often used as important sentinels because they serve as typical representative amphibians that could be used for assessment of environmental toxicity. This along with their categorization under the "Least Concern" or LC category also makes them a suitable candidate as sentinel animals (IUCN SSC Amphibian Specialist Group, 2014). More so they are highly prolific, easy to handle and comparatively economical to use for field evaluation and as an experimental model (Ezemonye and Enuneku, 2012). The sensitivity of frog to cadmium toxicity also makes them an excellent sentinel candidate for the evaluation of biomarkers and evidences of cadmium toxicity in the environment (James and Little, 2003; Rosenberg et al., 2007 and Ezemonye and Enuneku, 2012).

Despite the volume of ecotoxicological research using amphibians in recent times, there is however still a dearth of information on the impact of cadmium toxicity on local African frog species. This study therefore serves to elucidate the bioaccumulation and associated biomarker of effect in the indigenous crowned bullfrog while also highlighting their important role as sentinel for ecotoxicological assessment. The findings from this research could thus serve as an important guide in the use of the crowned bullfrog as sentinels in designing remediation programmes, cadmium toxicity intervention and the assessment of the impact of cadmium toxicity on conservation, ecosystem and public health.

MATERIALS AND METHODS

Study area

This study was conducted in Ibadan (7º23' N, 3º5' E), Oyo State in the south western part of Nigeria. The bullfrogs (*Hoplobatrachus occipitalis*) were sourced from the swamp and marsh of the Ogunpa River, Ibadan, Oyo State, Nigeria. The bullfrogs were captured at night from their spawning sites using net traps and hand nets to prevent injury to them.

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Animals

A total of 50 frogs (26 male and 24 female) were captured and used for this study. The frogs were apparently in good health condition and active. The weight of the sampled frog ranged from 30 – 58 grams. Both male and female frogs were used for this study and sex identification was done using the secondary sexual characteristics (head morphology and vocal cord identification) for the male and the identification of the gonads.

Sample collection

Blood collection: The frogs were initially anaesthetized using chloroform soaked cotton wool in a fume chamber. Blood samples (2 – 3 ml) were then collected by cardiac puncture from the ventricles. 1ml of the collected blood samples was transferred into lithium heparin bottles for complete blood cell count analysis. 1 – 2 ml of blood was preserved for cadmium blood level analysis using atomic absorption spectrophotometry.

Tissue collection: After the blood collection, the frogs were returned into the fume chamber for euthanasia in accordance with the institutionally approved procedures for humane handling of experimental animals. The frogs were afterwards dissected for collection of the tissue samples (liver, kidney, testes and ovaries). 1–2 grams of the liver, the kidney along with the testes or ovaries were collected in buffered formalin for fixation and histopathological sectioning. Some of the liver and kidney were also collected in plain bottles and stored in the freezer for heavy metal analysis.

Sample Analysis

Haematological parameters: Complete blood count and quantitative analysis of the haematological parameters were done immediately to prevent haemolysis and associated preanalytical errors. The haematological parameters considered in this study include the red blood cells counts, haemoglobin concentration, packed cell volume, platelet count, total white blood cell count and the absolute heterophil, eosinophil, basophil, monocyte and lymphocyte count as described by Jain (1986). The erythrocyte indices viz Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and Mean Corpuscular Haemoglobin Concentration (MCHC) were also calculated using the technique described by Jain (1986).

Histopathology: The selected formalinized tissues were processed after fixation and embedded in paraffin wax. The embedded tissues were then sectioned using a microtome to give 3µm thick tissue sections. The tissue sections were routinely stained with haematoxylin and eosin (H&E) before being examined under the light microscope (Olympus, Japan) for assessment of the histopathological changes (Lillie, 1965). The photomicrographs of the histopathological slides were taken using Toupview® histomorphometric camera and software.

Blood and Tissue Heavy Metal Analysis: The collected whole blood and tissues (liver and kidney) were digested using the method described by the AOAC (2004). The digested samples were then subjected to heavy metal analysis using the AAnalyst 200 Perkin Elmer Atomic Absorption Spectrophotometer (AAS) to determine the mean cadmium level for each of the samples.

Statistical analysis

The haematological parameters were expressed as Means ± SD. The blood, kidney and liver heavy metal concentration and haematological parameters were analyzed by analysis of variance (ANOVA) using SPSS (Statistical Package for Social Sciences version 20.0). Statistical significance was assumed at the p - value of $p < 0.05$.

RESULTS

Cadmium Level

Blood: 43 (86 %) of the examined frogs had blood cadmium levels above the maximum FAO permissible limit (0.5mg/kg) and constituted the Above Permissible Limit (APL) group while the remaining 7 (14%) constituted the Below Permissible Limit (BPL) group. The highest blood cadmium level observed in this study was 2.28 mg/kg while the lowest observed was 0.1 mg/kg (Table 1). As shown in figure 1. the blood cadmium level (0.96±0.46 mg/kg) was the lowest compared to the levels reported in the kidney and the liver. In this study, all the frogs with blood cadmium level below the permissible limits were female.

Kidney and Liver: The liver and kidney cadmium levels in all the sampled frogs presented high values which were higher than the FAO permissible limit in both tissues. The range of the kidney cadmium level was 0.8 – 3.36 mg/kg while the liver cadmium level range was from 1.44 – 6.4 mg/kg. As seen in figure 1, the Mean±SD tissue cadmium level is highest in the liver (3.02±1.23 mg/kg) followed by the kidney (1.93±0.68 mg/kg). The Mean±SD tissue cadmium levels in the male and female frogs is also presented in table 1 and shows a comparatively higher blood, liver and kidney cadmium level in the female frogs.

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Haematology

As shown in the table 2 there was a significant difference in the haematological parameters between the frog in the BPL group and those in the APL group. The erythrocyte parameters (PCV, Hb and RBC) and the platelet count were significantly higher in the BPL group than the APL frog group. The leucocyte parameters (white blood cell count, heterophil, lymphocyte, basophils and eosinophils) were higher in the frogs in the APL group while the monocyte count was higher in the frogs in the BPL group.

Histopathology

Histopathological examination of the liver tissue revealed marked loss of the normal hepatic architecture and diffuse hepatocellular degeneration. There was also a marked accentuation of the melano-macrophage centre in the liver (Figure 3). In the kidney (Figure 4), there was a mild degeneration of the renal tubules along with mononuclear infiltration. There was also a mild increase in the glomerular cellularity along with obliteration of the bowman space in a few glomeruli and mild glomerular capsule congestion. There was a characteristic denudation of the seminiferous tubules with loss of the germ cells and exposure of the Sertoli cells and the basement membrane (Figure 5).

Table 1. Means ± SD of the blood, kidney and liver cadmium level in male and female crowned bullfrog (*Hoplobatrachus occipitalis*) captured in Ibadan, Oyo State, Nigeria

Sex (n = 50)	Mean Cadmium Level (mg/kg)		
	Blood	Liver	Kidney
Male	0.94±0.39	3.00±1.27	1.54±0.41
Female	0.98±0.54	3.03±1.25	2.17±0.72

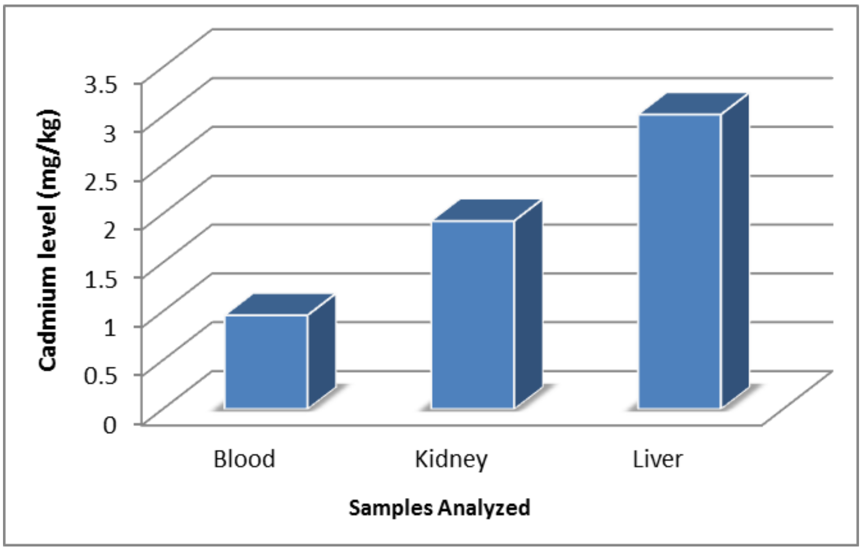

Figure 1. Mean blood, kidney and liver cadmium level in crowned bullfrog (*Hoplobatrachus occipitalis*) captured in Ibadan, Oyo State, Nigeria

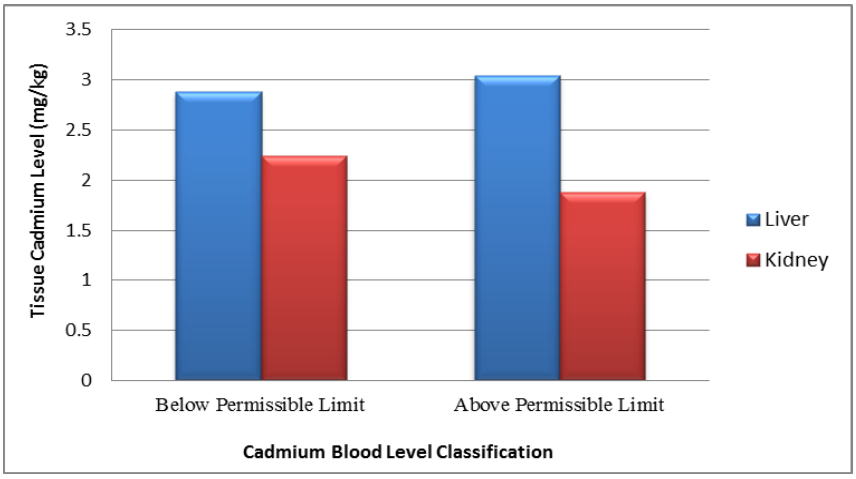

Figure 2. Mean tissue cadmium level for each blood cadmium permissible limit group in the crowned bullfrog (*Hoplobatrachus occipitalis*) captured in Ibadan, Oyo State, Nigeria

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Table 2. Means±SD of the haematological parameters of the crowned bullfrog (*Hoplobatrachus occipitalis*) captured in Ibadan, Oyo State, Nigeria with blood cadmium level permissible limit

Haematological Parameters	Cadmium Blood Level (mg/kg)	
	Below Permissible Limit n=7	Above Permissible Limit n=43
Packed Cell Volume- PCV (%)	31.57±4.61	23.00±6.60
Haemoglobin Concentration (g/dl)	8.57±2.04	7.39±2.20
Red Blood Cell ($\times10^3/\mu L$)	2.61±0.89	2.20±0.74
Mean Cell Volume (fl)	132.16±40.97	108.86±28.29
Mean Cell Haemoglobin (pg)	34.98±10.33	34.98±10.02
Mean Cell Haemoglobin Concentration (g/dL)	27.03±4.41	32.00±2.23
White Blood Cell ($\times10^3/\mu L$)	13.96±3.91	15.59±3.66
Lymphocytes ($\times10^3/\mu L$)	8.35±2.07	9.19±2.52
Heterophils($\times10^3/\mu L$)	4.61±1.72	5.18±1.56
Monocytes ($\times10^3/\mu L$)	0.51±0.36	0.47±0.29
Eosinophils ($\times10^3/\mu L$)	0.49±0.18	0.70±0.32
Basophils ($\times10^3/\mu L$)	0±0	0.05±0.08
Platelets($\times10^5/\mu L$)	1.68±0.82	1.37±0.37

FAO/WHO Permissible Limit (mg/kg) = 0.5mg/kg; n= number of samples

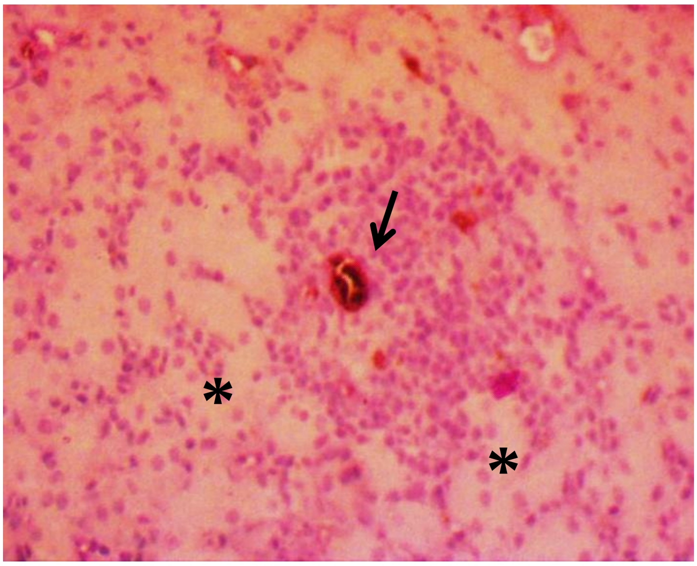

Figure 3. Liver tissue of the crowned bullfrog (*Hoplobatrachus occipitalis*) showing diffuse hepatocellular degeneration and loss of the normal hepatic architecture (asterisk). There is also a marked accentuation of the melano-macrophage centre (black arrow). ×1000 H&E

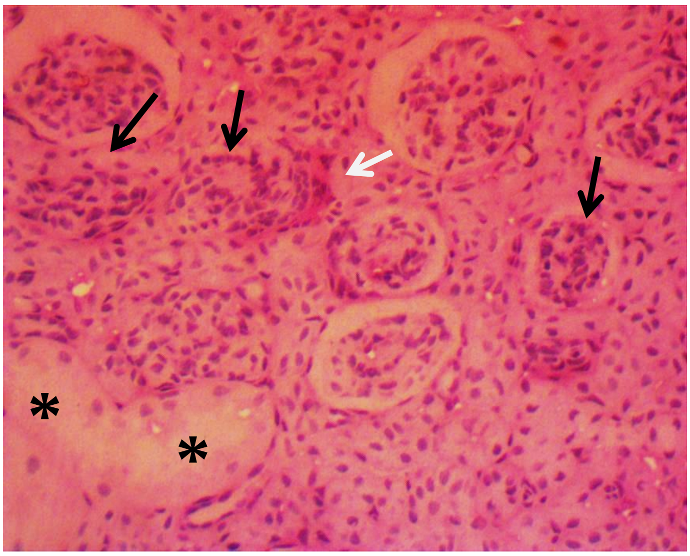

Figure 4. Kidney of the crowned bullfrog (*Hoplobatrachus occipitalis*) showing mild degeneration of the renal tubules (asterisk). There is also mild focal glomerulonephritis with increased cellularity of the glomeruli along with obliteration of the Bowman space (black arrows) in a few glomeruli. There is also a mild glomerular capsule congestion (white arrow). ×1000 H&E

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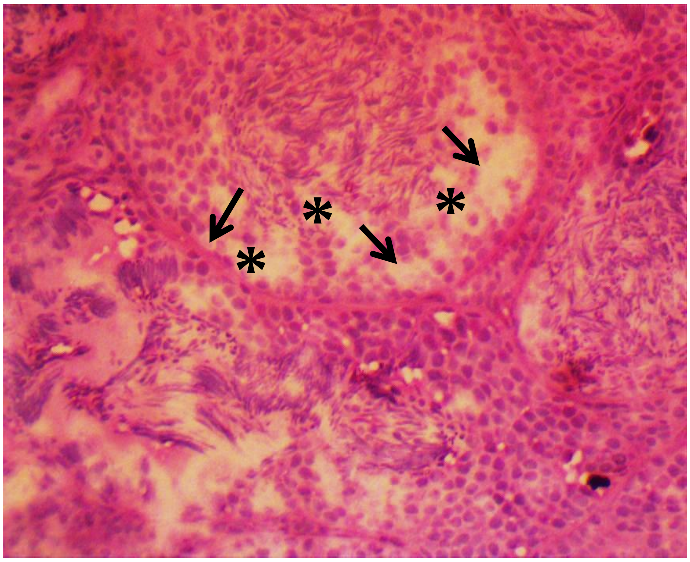

Figure 5. Testes of the crowned bullfrog (*Hoplobatrachus occipitalis*) showing loss of germ cells (asterisks) with exposure of the Sertoli cells and the basement membrane (black arrow). ×1000 H&E

DISCUSSION

Heavy metals such as cadmium, lead, copper and zinc are regarded as serious pollutants because of their toxicity and tendency to persist in the environment and be incorporated into the food chain (Kishe and Machiwa, 2003). Cadmium has been reported by different authorities as an important heavy metal pollutant in the environment with considerable ecotoxicological impact (International Programme on Chemical Safety (IPCS), 1992; International Agency for Research on Cancer (IARC), 1993 and Agency for Toxic Substances and Disease (ATSDR), 2012). Aquatic and amphibious organisms have been reported as perfect sentinel animals with the ability to accumulate heavy metal from various sources including soil, water bodies, erosion and waste water/effluents (Francis et al., 1984). With the spate of the increased discharge of cadmium in the environment, the use of frogs in this study is crucial as it helps in the assessment of the ecotoxicological impact of cadmium toxicity on the frog population. This is important due to the amphibious nature of frogs (exposing them to cadmium pollution both in the aquatic and terrestrial habitat) making them both an excellent sentinels for environmental toxicology and for the appraisal of the impact of cadmium toxicity on global frog population decline.

As shown in this study, there was an 86% prevalence of elevated blood cadmium level above the permissible limit (0.5mg/kg) in the sampled frogs. This observed high prevalence and elevated blood cadmium level corresponds to reports by other authors in which similar elevated blood cadmium levels were observed in frogs found in polluted environments and exposed to toxic environmental cadmium level (Loumbourdis et al., 2007 and Othman et al., 2009).

The tissue cadmium levels however were observed to be above the permissible limit in all the observed frogs in this study thus implying the severity of the cadmium exposure risk in the environment. The 100% prevalence of elevated tissue cadmium level observed corresponds with the similar elevated blood cadmium level observed although 14% of the sample frogs had blood cadmium level below the permissible limit. This could be associated with the tissue bioaccumulation of cadmium in frogs due to prolong exposure thus causing the tissue level to remain significantly high due to the slow release and excretion of the tissue residue over time (Agency for Toxic Substances and Disease Registry (ATSDR), 2013).

A comparatively higher cadmium level was detected in the female frogs compared to the male. This can be related to a similar finding by Vahter et al. (2007) who also reported a similar higher cadmium level in women compared to men. This observation has also been ascribed to be due to the higher intestinal dietary absorption of cadmium in women (Berglund et al., 1994; Vahter et al., 2007 and Järup et al., 2009) which according to Olsson et al. (2002) has been associated with the lower blood iron status of females.

In terms of the associated pathological changes in the frogs in the different blood cadmium permissible limit groups, the reported decrease in the erythrocyte (PCV, RBC and Hb) and platelet parameters observed can be ascribed to the previously documented decrease in haematopoiesis due to the cytotoxic effect of cadmium toxicity on haematopoietic precursor cells (Drastichová et al., 2004 and Witeska et al., 2010). More so, cadmium toxicity has been associated with haematotoxic effects on circulating blood cells thus causing accelerated destruction of the blood cells (Celik et al., 2005; 2009 and Van Den Heuvel et al., 2001). This is thus responsible for the characteristic normocytic and normochromic anaemia associated with early onset manifestation of cadmium toxicity. This anaemia can also be

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secondary to the reduction in iron absorption associated with cadmium toxicity (Campbell et al., 1984 and US - NRC, 2005).

The reported increase in the leucocyte parameters and reduction in the monocyte count in the frogs in the APL group is similar to the report by Enuneku and Ezemonye (2013) who also observed an increase in the leucocyte parameters in cadmium exposed frog *Hoplobatrachus occipitalis* and toad, *Bufo maculatus.* This finding is however in slight contrast to the report by Kondera and Witeska (2013) in which there was a general reduction in all the haematological parameters of common carp (*Cyprinus carpio L.)* exposed to cadmium. The elevation in the leucocytes could be associated with the increased demand for inflammatory response cells needed in response to the inflammation, degeneration and tissue damage caused by cadmium toxicity (Gill and Pant, 1985 and Davis et al., 2008). Furthermore, the lower cytotoxic sensitivity of the myeloid series precursor cells to cadmium toxicity compared to the erythroid series could be a likely reason why the leucopoietic process is minimally affected compared to erythropoiesis and the erythrocyte parameters (Van Den Heuvel et al. 1999 and 2001).

The highest mean cadmium tissue level in the sampled frogs was found in the liver (3.02±1.23 mg/kg) followed by the kidney (1.93±0.68 mg/kg) while the least level was observed in the blood (0.96±0.46 mg/kg). This finding is consistent with the report by Jezierska and Witeska (2006) who also reported a higher cadmium bioaccumulation in the liver than in the kidney and blood. This is in contrast with the report by Loumbourdis et al. (2007) in which the kidney was observed as the main site of bioaccumulation. This makes the liver a very important tissue for the assessment of heavy metal (especially cadmium, copper and lead) exposure since the rate of accumulation in this organ is usually proportionate to the environmental level (Jezierska and Witeska 2001 and 2006). Due to the excretory role of the kidney, the kidney tends to retain a high level of heavy metal and cadmium for a longer time. This makes the kidney an important tissue for the assessment of the tissue metal level even during remediation and depuration processes when the drop in the level of exposure is causing a drop in the metal level in other tissues (Jezierska and Witeska, 2006).

The histopathological findings in the liver, kidney and the testes were also consistent with the sequela of the cadmium toxicity mode of action via cellular membrane disruption via metallothionenin binding, oxidative stress induction due to glutathione binding and alteration of biochemical parameters. The observed histopathological changes in the liver of the frogs in the APL group were also similar to the observation by Ikechukwuu and Ajeh (2011) and Medina et al. (2016). The associated accentuation of the melano-macrophage seen in this study is consistent with the report from several authors as an important defense mechanism in response to xenobiotic exposure and increased phagocytic properties in response to inflammatory changes (Loumbourdis and Vogiatzis, 2002; Agius and Roberts, 2003 and Ribeiro et al., 2011).The renal lesions were similar to the reports by Jarup et al. (2000) in which proximal tubular cells damage and dose-dependent cadmium-induced renal pathologies were observed in cadmium exposed individuals. Åkesson et al. (2005) also reported that similar findings of both glomerular and tubular pathological changes have been associated with cadmium toxicity. The testicular pathological lesions were also consistent with the previous studies of the reproductive impact of cadmium toxicity on exposed animals and humans (Lee, 1983 and Thompson and Bannigan, 2008). All the histopathological lesions in this study were consistent with previous cadmium toxicity studies and further buttress the fact that the level of cadmium exposure and tissue bioaccumulation in the sample frogs was high enough to cause the observed pathological changes.

CONCLUSION

The study ascertained the bioaccumulation of cadmium in frogs, which is associated with deleterious effects on their tissues (blood, liver, kidney and testis) and ultimately could be responsible for the population decline of the species due to poor health and reproductive defects. It has also been further established that heavy metal toxicity is still a reality in our environment as observed in this bio-indicator species. Further research should still be carried out on the effect of exposure to this dangerous metal both on the ecosystem, human health and other wildlife species for a better understanding of the deleterious level. More so, there is a need for a more responsible utilization of cadmium and the safe disposal of cadmium containing products in order to reduce the release into the ecosystem and the impact on public health.

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Competing interests

The authors declare that there are no competing interest that might have influenced the presentation and the findings of this present study.

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