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ACUTE AND CHRONIC EFFECT OF LEAD NITRATE AND CADMIUM SULPHATE IN TADPOLES OF *AMIETOPHRYNUS REGULARIS* (COMMON AFRICAN TOAD)

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ABSTRACT
The relative acute toxicity of cadmium sulphate and lead nitrate acting singly against the common African toad *Amietophrynus regularis* were evaluated over a 96 hrs period. The effects of sub lethal concentrations of the metallic salts on the oxidative responses were also studied over a 28 day period using whole tadpole homogenate. On the basis of 96 hr LC_{50} value, cadmium sulphate with 96 hr LC_{50} of 1.513mg/l, was found to be 4.3 times more toxic than the lead salt with 96 hr LC_{50} of 6.456 mg/l. Exposures to sub lethal concentrations ($1/10^{th}$ and $1/100^{th}$ 96 hr LC_{50}) of both metals resulted in increase in the levels of lipid peroxidation product, malondiadehyde (MDA) compared to the control tadpoles at both day 14 and 28. Super oxide dismutase (SOD) activity was inhibited by day 28 relative to control for both metals but the activities of catalse (CAT) were often higher than those of the control tadpoles. Lead nitrate exposed tadpoles had reduced glutathione (GSH) which were significantly inhibited ($P<0.05$) in exposed groups in a dose dependent manner compared to the control. The consistency of MDA levels and SOD activity for both metal salt exposures was in agreement with previous findings and their combined use as biomarkers of exposure to pollutants in tadpoles is hereby supported.
Keywords: *Amietophrynus regularis,* metal toxicity, biomarkers, pollutants

INTRODUCTION
A variety of pollutants affect amphibians which include mainly pesticides, herbicides, fungicides, fertilizers and heavy metals (Sparling *et al*., 2001; Boone & Bridges, 2003). These pollutants may be spread globally or act on a local scale. They are transported atmospherically and have the potential to affect amphibians in remote, relatively undisturbed environments. Even low levels from atmospheric deposition are potentially harmful. In many cases, heavy metals from industrial and agricultural activities have been implicated (Maheswaran *et al*., 2008). Heavy metals are natural components of the Earth's crust. They occur naturally in the ecosystem but their concentrations may be enhanced by human activities (Oyewo, 1998). Heavy metals are present in rocks, soils and air in

small amounts from geological sources which in turn may influence the chemical composition and the nature of airborne particulates and dusts inhaled or ingested (Don-Pedro, 2009).

Heavy metals are metallic element which have atomic number greater than 20 or have density greater than 5g/cm^3. They have a relatively high density which is toxic or poisonous at low concentration, with major heavy metals of environmental health concern being arsenic, mercury, cadmium, lead, tin, chromium, zinc, nickel and a few others (Walker *et al.,* 2001). Most of these metals cannot be metabolized by the body and if accumulated can cause toxic effects by interfering with physiological function. Heavy metals often have variable valency which and are involved in electron transfer reactions which leads to the production of toxic radicals. Heavy metals have been associated with various toxic effects in humans and animals, ranging from oxidative stress and heat shock (Amaeze *et al.,* 2015) to histopathological effects, endocrine disruption (Kawai *et al.,* 2002), developmental abnormities and mortality (Kurdland, 1960). Exposure of animals to pollutants in their natural environment and laboratory conditions has been reported to result in oxidative stress (Esiegbe *et al*., 2012). High concentrations of toxicants or chronic exposures may overwhelm the anti-oxidative stress mechanisms resulting in oxidative stress (Reznick *et al.,* 1998). The presence and activities of enzymes such as superoxide dismutase, catalase and glutathione constitutes a formidable defense system against oxidative stress (Brucka-Jastrzębska, 2010). Heavy metals have also been associated with deleterious effect on living organisms such as change in the cell membrane structure, inhabiting enzymatic functions, functional change in the co-enzymes also destruction of non-target organisms which may lead to population imbalance of ecosystem (Don- Pedro, 1980), the destruction of wild life and aquatic resources (Hick, 1985). In spite of the biological effect arising from the use of heavy metals, the use continues unabated due to their numerous industrial applications and rapid urbanization.

One of the largest problems associated with the persistence of heavy metals is the potential for bioaccumulation and biomagnification causing heavier exposure for some organisms than is present in the environment alone (Idowu *et al.,* 2012). Bioaccumulation means an increase in the concentration of a chemical in a biological organism over time, compared to the chemical's concentration in the environment (Walker *et al.,* 2001). Heavy metal accumulation in have been associated with oxidative stress induction in amphibians (Idowu *et al.,* 2012)

Adult amphibians can take up heavy metals through their skin or orally by consumption and respiration. Larvae may also absorb them through their skin (Ezemonye & Enuneku, 2006). Amphibians have moist, permeable skin and unshelled eggs that are directly exposed to soil, water and sunlight, and that can readily absorb toxic substances. This further aided by their moist highly vascularized skin, which is an adaptation for cutenous respiration (Hickman *et al*., 2008). Amphibians are ideal for genotoxicity monitoring of aquatic ecosystem due to their high sensitivity of toxic substances and widely used bio-indicator to detect the presence of toxic substances in aquatic system and are value as indicators of environmental stress (Blaustein & Wake, 1995). Metals such as aluminum, lead, zinc, cadmium, mercury,

silver, copper, arsenic, manganese, molybdenum and antimony have been reported to induce a number of effects on amphibians. They can be lethal or induce sub lethal effects such as slowing growth and development, stress and altering behaviour (Lefcort *et al*., 1998, 1999; Raimondo *et al*., 1998; Blaustein *et al*., 2003).

Toxicity testing of chemicals on animals has been used for a long time to detect the potential hazard posed by chemicals to man and other living things (Don-Pedro, 2009). Toxicity can be defined as the study of adverse effect of chemical substances to living organisms. Toxicity is a resultant of concentration and time, modified by variables such as temperature, humidity, photoperiod and a number of environmental variables (Walker *et al.,* 2001). For this reason, it is important to carry out ecotoxicological studies on ubiquitous pollutant such as heavy metals aimed at establishing not only their toxicity levels, but to investigate the effect of sub lethal concentration on the behavior and physiology of living organisms over time.

Anuran species commonly inhabit many ecosystems in the southern Nigeria, with the common Africa toad, *Amietophrynus regularis* being particularly a common and abundant species (Onadeko & Rodel, 2009). Urbanization and forest clearance destroys their natural habitats and brings them closer to sites of human activities which further threaten their survival. Amphibians worldwide are reported to be on the decline and the drivers have been reported to include global warming (Houlahan *et al.,* 2000), disease (Kiesecker *et al.,* 2001) and in some cases aquatic pollution (Ezemonye & Tongo, 2010). It is commonplace to sight their tadpoles in ponds and open gutters which are receptacles and easy dumping sites for spilt petroleum products and spent engine oils. This therefore justifies a study evaluating the toxicities of these products to the tadpoles as well as the indicators of oxidative stress so as to ascertain the extent to which they pose threats to their survival.

In view of the above, this study aims to determine the relative acute toxicity of cadmium sulphate and lead nitrate on the tadpoles of *Amietophrynus regularis* and the anti-oxidative enzymes induce by exposures to sub lethal concentration of lead nitrate and cadmium sulphate on them.

MATERIALS AND METHODS

Experimental animals

Tadpoles of the anuran species *A. regularis* were used in this study to determine the effects of commonly detected heavy metals, Pb and Cd salts. The young amphibians used were collected with sweep net (0.9 x 0.3 mm mesh size) from Ogbe creek within University of Lagos campus, Akoka,Yaba, Lagos. They were collected at the advent of the rainy season when reproductive activities are at its peak among many anuran species. The tadpoles were transported to the laboratory in transparent plastic containers that were properly aerated. These tadpoles were kept in separate circular holding tanks (30 x 20 x 25 cm), in which they were allowed to acclimatize to laboratory conditions (temperature of 28±2ºC; humidity 70±2%) for 4 days before being used in the bioassays. During acclimatization, the tadpoles were fed with egg yolk and tiny pieces of cut cabbage.

About 24 hours before bioassay, active tadpoles of similar sizes were selected into separate pre-bioassay holding tanks with a stocking density of 10 tadpoles per liter to further enhance acclimatization to

experimental conditions. The bioassay lasted for four days (96 hours).

Test Compounds and Preparation of Test Media

The heavy metals investigated in this work were obtained as metallic salt from the Chemistry laboratory, University of Lagos, Akoka. The compounds used were; Cadmium sulphate- $CdSO_4$ and Lead nitrate- $Pb(NO_3)_2$.

Circular plastic containers (Volume; 2 litres) were used as bioassay containers. During the experiment, the test media were made up to 1 litre and 4 tadpoles were introduced per bioassay container. Each assay was replicated four times making a total of 16 animals per concentration. Predetermined amounts of heavy metal compound was weighed and made up to a fixed volume by adding appropriate volume of dechlorinated tap water to achieve a stock solution of known strength. The respective stocks were prepared by dissolving weighed out qunatities of the metallic salts in water to make up a 1000mg/l. The respective test concentrations were obtained from the stock and introduced into biossay tanks using syringes by way of dilution.

The concentration of exposure for the definitive test were as follows;
Lead Nitrate against tadpoles at 1, 5, 10, 15, 30mg/l and untreated control.
Cadmium Sulphate against tadpoles at 1, 2, 3, 4mg/l and untreated control.
Mortality was assessed once every 24hrs for 4days.

Assessement of Quantal Response (Mortality)

Four active tadpoles of similar sizes from pre-bioassay holding tanks were taken with a net and randomly distributed into bioassay tanks already holding heavy metals treated and untreated control medium. Tadpoles were taken to be dead when body or tail movements were no longer observed. Assessment of mortality was conducted every 24 hours for the 96 hour duration of the experiment. The responses obtained were documented and analysed using probit to determine the median lethal concentration (LC_{50}).

Effect of sub lethal concentration of test heavy metals against the tadpoles of *A. regularis*.

In this series of experiment, similar bioassays as described in above were conducted but this time, only sub lethal concentration of test heavy metals were employed, extrapolated as fractions of the 96hr LC_{50} concentration obtained in test conducted in the previous section as follows;

Sub Lethal Effects Assessment

Dilution was done from the stock solution to get the accurate sub lethal concentration. It was done by diluting the 1ml of stock solution with 1000ml of water twice and the sub lethal test concentrations were:
Pb^{2+} against the tadpoles:
6.5ml ($1/10^{th} \times$ 96hr LC_{50} value)
0.65ml- ($1/100^{th} \times$ 96hr LC_{50} value) and untreated control.
Cd^{2+} against the tadpoles:
1.5ml- ($1/10^{th} \times$ 96hr LC_{50} value)
0.15ml- ($1/10^{th} \times$ 96hr LC_{50} value)

In all these bioassay, 6 active tadpoles were exposed to each sub lethal concentration and untreated control. Semi static bioassay conditions were also adopted in which the test media were changed once every 96hrs to fresh media of the same concentration and untreated control due to feeding. After 14 and 28 days of exposure, the tadpoles were collected from the bioassay tank and subjected to biochemical

assessment for oxidative stress markers. The harvested tadpoles were stored using universal bottles in the refrigerator (-20^{o}C) and transported to the Biochemistry Lab for analysis in ice packs.

Determination of Superoxide Dismutase (SOD) Activity

Superoxide Dismutase activity was determined by its ability to inhibit the auto oxidation of epinephrine determined by the increase in absorbance at 480nm as described by Sun and Zigma (1978). The reaction mixture (3ml) contained 2.95ml 0.05 M sodium carbamate buffer pH 10.2, 0.02 ml of tadpole homogenate and 0.03ml of epinephrine in 0.005N HCL was used to initiate the reaction. The reference cuvette contained 2.95ml buffer, 0.03ml of substrate (epinephrine) and 0.02 ml of water. Enzyme activity was calculated by measuring the change in absorbance at 480 nm for 5minutes.

Determination of Catalase (CAT) Activity

Catalase activity was determined by measuring the decrease in absorbance at 240nm due to the decomposition of H_2O_2 in a UV recording spectrophotometer. The reaction mixture (3ml) contained 0.1 ml of serum in phosphate buffer (50mM pH 7.0) and 2.9ml of 30mM H_2O_2 in phosphate buffer pH 7.0. An extinction coefficient for H_2O_2 at 240nm of 40.0 M -1cm-1 (Aebi, 1984) was used for the calculation.

Determination of Reduced Glutathione (GSH)

The reduced glutathione content (GSH) content of the earthworm as non-protein sulphydryls was estimated according to the methods described by Sedlak & Lindsay (1968). 10% TCA was added to the homogenate, and the mixture was centrifuged. 1.0ml of supernatant was treated with 0.5ml of Ellimans reagent (19.8mg of 5, 5-dithiobisnitro benzoic acid (DTNB) in 100ml of 0.1% sodium nitrate) and 3.0ml of phosphate buffer (0.2, pH 8.0). The absorbance was read at 412nm.

Malondialdehyde (MDA)

Malondialdehyde (MDA) an index of lipid peroxidation was determined using the method of Buege and Aust (1978). 1.0ml of the supernatant was added to 2ml of (1:1:1ratio) tricarboxylic acid-thiobarbituric acid-hydrochloric acid reagent TCA-TBA-HCl reagent (thiobarbituric acid 0.37%, 0.24N HCl and 15% TCA) boiled at 100^{o}C for 15 minutes, and allowed to cool. Flocculent materials were removed by centrifuging at 3000 rpm for 10 minutes. The supernatant was removed and the absorbance read at 532nm against a blank. MDA was calculated using the molar extinction coefficient for MDA-TBA complex of 1.56×10^{5} M^{-1} CM^{-1}.

Statistical Analysis

The dose-response data was analyzed by probit analysis after Finney, (1971). Probit analysis was based on a programme by Ge le-Pathovriel, Imperial College, London, run by an IBM computer and adapted by Don-Pedro, (1989). The levels of significance between results were determined using ANVOVA, SPSS Version 16.

RESULTS

Relative Acute Toxicity of Heavy Metals Acting Singly against Tadpoles of *A. regularis*

On the basis of the 96hrs toxicity value Cadmium sulphate with LC_{50} value of 1.513 mg/L was found to be more toxic to *A. regularis* than Lead nitrate with LC_{50} of 6.456 mg/l. Base on toxicity factor (TF) assessment, Cadmium sulphate was found to

be 4.3 times more toxic than Lead nitrate (Table 1).

Table 1. Relative acute toxicity of Cadium sulphate and Lead nitrate acting singly against tadpoles.

Treatment	LC_5 (95 %)	LC_{50} (95 %)	LC_{95}(95%)	S.E	Equation of line	df	TF
Cd^{2+}	0.870 (0.316-1.18)	1.513 (1.079-1.970)	2.631 (2.009-6.043)	0.565	y= -1.230× -2.179	1	1
Pb^{2+}	0.297 (0.000-1.3530	6.456 (1.525-23.57)	140.498 (31.940-4163 906.878)	0.467	y= -0.996× -2.134	2	4.3

Biochemical Responses in Tadpoles of *A.regularis* Exposed To Cadmium Sulphate and Lead Nitrate

Lead Nitrate

The result indicate that the lipid peroxidation product, Malodiadehydehe (MDA) levels were higher at day 14 compared to day 28. Also MDA levels were significantly lower ($P<0.05$) in the tadpoles of control compared to those exposed to $1/100^{th}$ LC_{50} and $1/10^{th}$ LC_{50} concentrations. Also, MDA level were higher in tadpoles exposed to $1/100^{th}$ LC_{50} compared to $1/10^{th}$ LC_{50} concentration (Fig. 1A). Catalyse (CAT) levels were higher at day 14 compared to day 28 as well. Also CAT activities were lower in the control tadpoles compared to the exposed group except $1/10^{th}$ of 28days. CAT activities were higher in tadpoles exposed to $1/100^{th}$ LC_{50} compared to $1/10^{th}$ LC_{50} concentration as well. Superoxide diamutase (SOD) activities were higher at day 14 compared to day 28 (Fig. 1B). Also, SOD were significantly lower ($P<0.05$) in the control tadpoles at day 14 compared to exposed group but higher at day 28 compared to the exposed group. SOD activities were higher at $1/100^{th}$ LC_{50} concentrations exposed tadpoles compared to those exposed to $1/10^{th}$ LC_{50} concentration (Fig. 1C). The result indicates that Glutathione (GSH) level were significantly higher ($P<0.05$) in the control tadpoles compared to those exposed to $1/100^{th}$ and $1/10^{th}$ LC_{50} at both day 14 and 28 of the bioassay (Figure 1D).

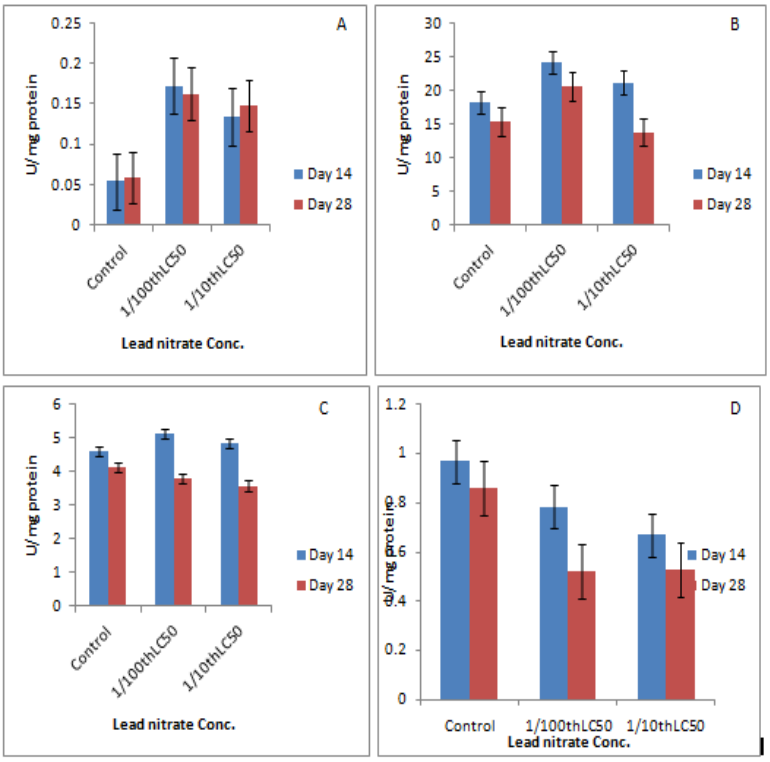

Fig. 1(A-D): Levels of (A) Malodiadehydehe and activities (B) Catalyse (C) Superoxide diamutase and (D) Glutathione in the whole body of *A.regularis* exposed to sub lethal

concentrations (1/10th and 1/100th LC_{50}) Lead nitrate.

Cadmium Sulphate

The result indicate that MDA level were also generally higher at day 14 compared to day 28 of exposure to sub lethal concentrations of Cd. Also, MDA levels were significantly lower ($P<0.05$) in the control tadpoles compared to those exposed to $1/100^{th}$ LC_{50} and $1/10^{th}$ LC_{50} concentration and the $1/10^{th}$ LC_{50} exposed group had higher levels than those exposed to $1/100^{th}$ LC_{50} (Fig. 2A). CAT activities were higher at day 14 compared to day 28 and was similarly lower in the control tadpoles compared to sub lethal concentrations of the cadmium salt. CAT activities in those exposed to $1/10^{th}$ LC_{50} were higher than $1/100^{th}$ LC_{50} concentration. (Fig. 2B). SOD activities were higher at day 14 compared to day 28, with lower activities also reported in the control tadpoles compared to the exposed ones. Also, SOD activities was higher in those exposed to $1/10^{th}$ LC^{50} compared to $1/100^{th}$ LC_{50} concentration of the cadmium salt (Fig. 2C). The result also revealed that GSH level were higher at day 14 compared to day 28. Also GSH level in the control tadpoles were significantly different ($P<0.05$) from and those exposed to $1/10^{th}$ LC_{50} concentration and $1/100^{th}$ LC_{50} concentration. GSH level were higher in those exposed to $1/10^{th}$ LC_{50} compared to $1/100^{th}$ LC_{50} cadmium sulphate and the difference was significant at day 14 (Figure 2D).

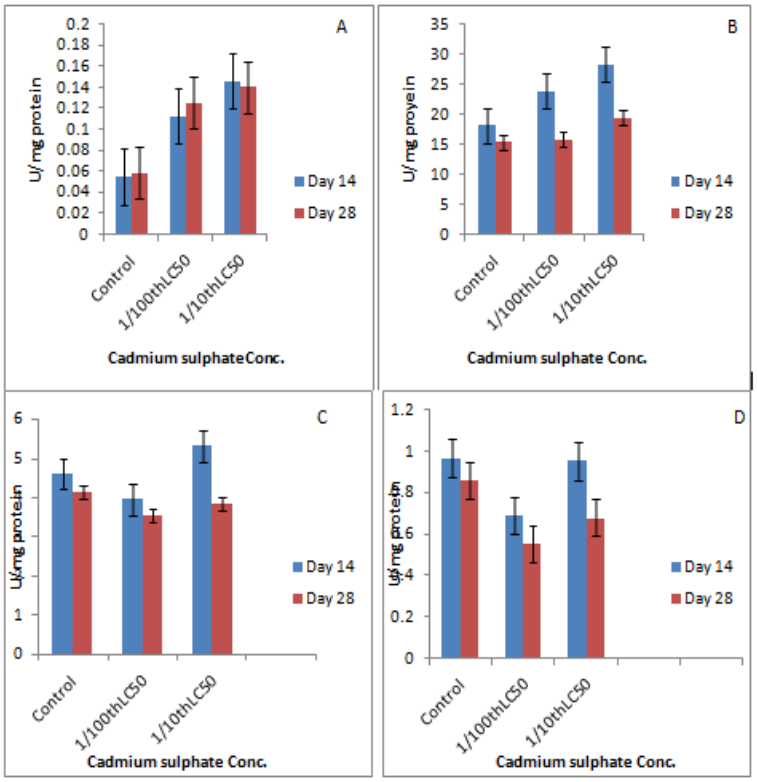

Fig. 2 (A-D): Levels of (A) Malodiadehydehe and activities (B) Catalyse (C) Superoxide diamutase and (D) Glutathione in the whole body of *A.regularis* exposed to sub lethal concentrations (1/10th and 1/100th LC_{50}) of Cadium sulphate.

DISCUSSION

In this study, the cadmium salt was found to be significantly more toxic than lead being 4.3 times higher in toxicity when acting singly against *Amietophrynus regularis*; this is in agreement with the findings of Otitoloju and Don-Pedro (2005) who also showed that cadmium was the most toxic chemical out of other heavy metals like Cu, Zn, and Pb tested against lagoon animals like *Clibaranius africanus*, *Tympanotonus fusctus* and *Seserma sp.* Others like Khangarot and Ray, (1987), Mackie, (1989) and Oyewo, (1998), had similar observation and recorded differential

toxicity of the heavy metal against different test organisms.

The observed differential toxicity of heavy metals as reported in this study can be attributed to several factors such as the relative solubility of the metallic salts including ionization, physic-chemical characteristics of the test solution and the mechanism of action. All of these factors determine the availability including the penetrability of the metals into the test animals and hence may bring about difference in toxicity. The higher acute toxicity to the tadpoles by cadmium compared to lead reported in this study against *A. regularies* is very much in agreement with the findings of Ezemonye and Enuneku (2005) who reported the reverse in tadpoles of *Bufo maculatus Ptychadena bibroni*. These differences may be resulting from a range of factors as indicated above. However, whatever the differences in the acute toxicity levels of both metals, what remains of concern is their effects on key fauna such as amphibians, whose decline is already well documented. The differential toxicity of the cadmium sulphate and lead nitrate to the tadpoles can be linked to their respective physical characteristics. The cadmium salt was more soluble than lead salt during the bioassay making it more available unlike lead. This may account for lead nitrate's lower acute toxicity compared to the cadmium sulphate. However, their relative toxicity should not override the fact that they all constitute environmental hazards. This raises important ecotoxicological concerns given the ubiquity of industrial effluent, smelting and mining, and agricultural run-offs in major cities and highways in Nigeria. These facilities often leave little consideration to waste management in their design. Surrounding drainages and ponds becomes recipients of their wastes either by deliberate introduction or when they are washed off as run off after rainfall. Dumping of cadmium batteries, phosphate fertilizers, detergents and refined petroleum products in gutters and drains is common activity in Nigeria. There are no measures put in place for collection and management of batteries and petroleum products from these workshops which are distributed across streets corners and major roads of the country thus, resulting in pollution concerns to animals inhabiting urban ecosystems.

This study has shown that cadmium possesses higher toxicity risk and should be controlled with safe limits. This was highlighted with the sub lethal concentrations which caused growth retardation and induced biochemical effects after 28 days exposures. This was also the case in those exposed to lead ions. In Nigeria, the safe limit for both metals is less than 1 mg/L as stipulated by the Federal ministry of environment through the environmental protection agency (FEPA, 1991). These limits are designed to minimize pollution inputs into the environment as tools of enforcement and compliance monitoring in industries. However, much still needs to be done in Nigeria to enforce compliance with stipulated safe limits in order to protect our valuable and most vulnerable species.

The assessment of MDA, the by-product of oxidative damage to the phospholipids of cell membranes indicated considerable damage to cells in the tadpoles exposed to the cadmium sulphate relative to the control individuals. Lipid peroxidation damage is one of the first indicators of damage to cells by toxicants and represents a key biomarker of oxidative stress (Cini *et al.,* 1994). Avci *et al*. (2005) have earlier

reported lipid peroxidation in the muscles and liver of fishes obtained from a river contaminated petroleum products from a nearby refiner. This study therefore provides an opportunity to extend the knowledge of the oxidative stress impacts of lead and cadmium and lead on tadpoles of the common African toad. The results from the biochemical assays indicated that there was inhibition of SOD activities in the exposed tadpoles relative to the control by the 28th day of exposure for both heavy metals. Inhibition of SOD activities have been reported in the African sharp tooth catfish (*Clarias gariepinus*) exposed to polycyclic aromatic hydrocarbons (Otitoloju and Olagoke, 2011).This gives credence to the possibility of oxidative stress resulting from the exposure of the metals and confirms results from the lipid peroxidation assay in this study. SOD, though involved in the protection of biological systems from the actions of free radicals and may be overwhelmed in the event of excessive toxic onslaught, resulting in oxidative stress, a condition that may be characterized by its eventual inhibition. Catalse however was not inhibited across the test relative to control. Although the inhibition of catalse is often associated with SOD inhibition owing to the reduction in available substrate concentrations, the findings from this study may imply that oxidative damage has not fully set in. Reduction in GSH levels in exposed group relative to control was observed in a dose dependent manner in tadpoles exposed to lead nitrate, implying an active uptake of free anions by cellular glutathione. This therefore justifies its use as a biomarker for assessing the toxic effects and responses to toxicants in this study.

CONCLUSION

This study showed the relationship between the toxicity of cadmium sulphate and lead nitrate and their respective CAT and SOD activity, as well as CAT, GSH and MDA levels. The consistent dose dependent increase in MDA levels in tadpoles exposed to both heavy metals was consistent with findings from several studies by previous authours. The relatively consistent relationship between SOD and MDA reported in this study was also consistent with previous findings. The importance of anti-oxidative enzymes as sensitive biomarkers in monitoring environmental pollution therefore cannot be downplayed owing to the large number of investigators who have demonstrated this in a variety of animal groups. This study therefore justifies the use of MDA levels and SOD activity as suitable compliments for monitoring oxidative stress resulting from exposure to non-essential heavy metals.

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