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HEAVY METAL INDUCED TOXICITY IN THE INDIAN GREEN FROG: BIOCHEMICAL AND HISTOPATHOLOGICAL ALTERATIONS

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Abstract

Heavy metal contamination may impart adverse effects on wetland biota, particularly on amphibians. Severe immunotoxic effects elicited in *Euphlyctis hexadactylus* (Indian green frog) due to metal exposure (Cd, Cr, Cu, Pb and Zn) in the Bellanwila Attidiya sanctuary, a polluted, urban wetland in Sri Lanka, provided the rationale for the current study. This study evaluated the biochemical and histopathological effects of this metal contamination with a reference *E. hexadactylus* population and a laboratory exposure group i.e subjected to 28 days exposure to a mixture of Cd, Cr, Cu, Pb and Zn (each in 5 ppm). A histopathological scoring for the semi quantification of tissue damage was established. Results of the biochemical and histopathologicl markers were remarkably consistent between the two exposure scenarios, providing a validation for the heavy metal exposure hypothesis. Damage to liver, kidney, lung and skin of metal exposed *E. hexadactylus* quantified multiple impairments absent in the reference frogs. Liver injuries complemented significantly elevated aspartate transaminase (AST), alanine transaminase (ALT), γ-glutamyltransferase (γ-GT) and alkaline phosphatases in frog liver homogenate, indicating hepatocellular leakage and loss of functional and structural integrity of the hepatocyte membrane in both field and laboratory exposed frogs. Significant elevation of *Kupffer* cell hypertrophy, pigmentation, inflammatory cell infiltrates & hepatic inflammation, extra medullary haematopoiesis and karyocytomegaly of hepatocytes ($p<0.05$) of the liver and degeneration of epithelia and necrosis of the lung, manifested as impairments in both metal exposure scenarios. Significantly reduced serum total protein and albumin, and significantly elevated urea and creatinine in metal exposed frogs were indicative of hepatic and renal dysfunction, respectively. The study affirms histopathology related biochemical alterations as potential biomarkers for heavy metal toxicity in amphibians. This article is protected by copyright. All rights reserved

Keywords: Heavy metals; semi quantitative histopathology; biochemical alterations; hepatotoxicity; nephrotoxicity

Abbreviations:

Alanine transaminase (ALT), aspartate transaminase (AST), γ-glutamyltransferase (γ-GT), alkaline phosphatase (ALP), thiobarbituric acid reactive substances (TBARS), glutathione peroxidase (GPX), glutathione reductase (GRD) and superoxide dismutase (SOD), Liver tissue homogenates (LTH), malondialdehyde (MDA), melano-macrophage centres (MMC), lipid peroxidation (LPO), catalase (CAT), hepatosomal index (HSI), graphite furnace atomic absorption spectrometry (GFAAS), Bioconcentration factor (BCF), dissolved oxygen (DO), biological oxygen demand (BOD), Analysis of variance (ANOVA), Bellanwila Attidiya Sanctuary (BAS), metallothionine (MT). International Harmonization of Nomenclature and Diagnostic Criteria (INHAND), Chronic Progressive *Nephropathy* (CPN),

BACKGROUND

Heavy metal contamination in wetland ecosystems is a prevailing environmental issue of global dimension, due mainly to the persistence and biomagnification of metal species through food chains [1]. Being sensitive inhabitants of wetland ecosystems, amphibians are at great risk of heavy metal toxicity [2-4]. Amphibians acquiring metals through their highly permeable egg membranes during embryonic stages, and orally or through skin by adult and larval stages, may serve as effective biomonitors for heavy metal pollution in wetland ecosystems [5]. The present study was designed to determine the heavy metal associated biochemical and histopathological alteration in an anuran amphibian, inhabiting a polluted urban wetland ecosystem.

Heavy metals accumulated in vertebrates elicit diverse effects including immunotoxic, genotoxic, cytotoxic, hepatotoxic, nephrotoxic and endocrine disruption [6]. Biochemical parameters were recognised as good indicators of stress induced effects of heavy metal toxicity [7] as these directly altered with the oxidative stress mechanisms within the animal. Measuring

aspartate transaminase (AST), alanine transaminase (ALT), γ-glutamyl transferase (γ-GT) and alkaline phosphatase (ALP) as functional biomarkers of hepatotoxicity [8-10] and urea, creatinine and phosphatase levels as nephrotoxicity biomarkers are considered extremely sensitive measures of oxidative stress, mediated by various stressors including heavy metals [11]. Despite the inability to generate free radicals directly, lipid peroxidation (LPO) is considered as a key mechanism for heavy metal toxicity [12]. Hence, thiobarbituric acid reactive substance (TBARS) - malondialdehyde formation (MDA) as an LPO measure has been analysed frequently [10] jointly with the antioxidant enzyme system including Superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), glutathione reductase (GRD) and glutathione (GSH)[13, 14].

Histopathological alterations, such as lesions and tissue disturbances in the liver, ovary, skeleton and skin, were used as common biomarkers in ecotoxicological studies [15]. Ezemonye and Enuneku [16, 17] reported excessive bile secretion and dilation of sinusoids in the liver of *Hoplobatrachus occipitalis* exposed to Cd and haemorrhages in the liver of *Bufo maculatus* exposed to lead . Elevation of the area occupied by the *Kupffer* cells and karyomegaly in the liver [18-20] and increased apoptosis in the kidney [21] of *Rana ridibunda* in response to metal exposure was reported.

Melanomacrophage centres are reported to increase in size or percentage under environmental or physiological stress and therefore were suggested as a biomarker for xenobiotic mediated toxicity studies [19, 20]. Extracutaneous pigmentary system of lower vertebrates such as fishes and amphibians involves functions which are not yet fully elucidated [22]. Among these, melano-macrophage centres (MMC)/ macrophage aggregates are distinctive groupings of pigmented cells, particularly located in the organs such as the liver. Melanomacrophage centres

appear as large nodules, composed of macrophages that accumulate melanin, synthesised elsewhere [23]. Since, MMC are devoid of tyrosinase activity, melanin synthesis was not reported from these centres [24]. Further, MMC contains pigments such as lipofuscin, melanin, and haemosiderin in the cytoplasm [18, 25].

Most of the histopathological analyses conducted to date are qualitative in nature [26]. An accurate quantification of the histopathology is critical for evaluating the factual effect of the causative agent, toxins, disease, etc. Semiquantitative scoring techniques practiced in some other instances lack sensitivity and are subject to scoring bias [26]. Conversely, quantitative assessments such as evaluation of histopathological alterations in Ohrid trout [27] and Nile tilapia [28] exemplified comprehensive, quantitative studies. Thus, there is a growing need for the development of more sophisticated techniques which are reliable, sensitive, reproducible and easy to use. Ashcroft scale [29] addressed this issue by assigning a numerical scale with grades from 0 to 8, for the amount of fibrotic tissue in human lung. Even though, this scale was originally designed for human lung it was widely used in lung fibrosis in murine models as well [30-32]. Moreover, modifications of the Ashcroft scale were used in various instances to quantify histopathological damages in the lung tissue [33-36]. However, this scale was not validated for other organs to reveal the extent of histopathological damage. Thus, in this study we modified and extended this scoring method for the semi quantification of tissue damage observed in the liver, kidneys, skin and lungs of an amphibian species.

Growing concern of amphibian decline and malformation issues worldwide, demand basic research to study toxic effects of xenobiotics on amphibians. Heavy metals as xenobiotics have not been tested adequately in the amphibian decline and malformation issue. Sri Lanka, being a biological hotspot, harbouring the highest number of amphibian species per unit area

[37] in South East Asia, presently reports 119 described species with more than 85% endemic species and the loss of 19 species [38, 39].

Euphlyctis hexadactylus, the Indian green frog, is a common amphibian species in the wet zone of Sri Lanka, particularly inhabiting wetlands such as Bellanwila Attidiya sanctuary (BAS). *E. hexadactylus* inhabits in the BAS sympatrically with 10 other amphibian species [40]. However, a survey conducted in 2005-2006 reported a decline in biodiversity in the BAS due to habitat destruction, industrial toxic waste and garbage dumping [41]. A pilot study conducted in 2010-2012 in the BAS reported significant levels of heavy metals, Zn, Pb, Cu and Cd, in wetland water [Zn-2.71ppm, Pb-0.955ppm, Cu- 0.04ppm, Cd- 0.019ppm] as well as in *E. hexadactylus* tissue [42]. The use of functional and non-functional immunologic biomarkers, including cytokine as indicators of heavy metal pollution in wetland ecosystems was reported in Jayawardena *et al*., [43]. Biochemical and histopathological alterations of any Sri Lankan amphibian species have thus far not been evaluated in relation to heavy metal pollution. Therefore, the biochemical alterations of *E. hexadactylus*, in the polluted site at the BAS in comparison with reference specimens collected from a relatively pristine environment in the Labugama reservoir and the catchment area, were evaluated by measuring frog serum AST, ALT, γ-GT, SOD, GPX, GRD, total protein, albumin, bilirubin, urea and creatinine levels. And the histopathological alterations of the major organs of *E. hexadactylus*, in the two study sites were evaluated by examining stained formalin fixed paraffin embedded sections of organ tissues. Apart from the qualitative analysis, semi quantification of the tissue damages was also assessed with a histopathological scoring method. Field studies were validated with a laboratory study of 28 days laboratory exposure of frogs to heavy metals.

MATERIAL AND METHODS

Approval was obtained for the collection of wildlife specimens from the protected areas, and for conducting animal research, from the Department of Wildlife Conservation, Sri Lanka (WL/3/2/10/13) and from the Ethics Review Committee, Faculty of Medicine, University of Colombo (ERC/12/176), respectively. Hence, all experiments conducted were in compliance with ethical guidelines provided by these two authorities.

Study sites and sample collection

The Bellanwila-Attidiya sanctuary, (6^o 48'-52' N and 79^o 52'-56' E), situated within the upper catchment of the Bolgoda river basin in the Western province of Sri Lanka [41, 44] was selected as the polluted site while Labugama reservoir and the catchment area (7^o 1'-60' N and 79^o 52'-0' E) with relatively pristine environmental setting, served as the reference site. As situated in the low country wet zone with a tropical monsoon climate, these study sites receives mean annual rainfall of 2800 mm with approximately 28 ^{o} C ambient temperatures throughout the year [45, 46].

Physicochemical parameters of water, pH, temperature, DO (Dissolved Oxygen) and BOD (Biological Oxygen Demand) were measured at each site during sampling carried out bimonthly from Feb. 2013- Feb. 2015. Water samples were collected in to thick plastic bottles and were preserved with 3% (v/v) conc. HNO_3 acid for heavy metal analysis [47].

The test animal, adult Indian green frog, *E. hexadactylus*, (N=15) from the polluted site, N=30 from the reference site) were randomly captured from water ways using bait. Animals were transported to the laboratory in aerated plastic bags half filled with water, for further analysis. Collected adult frogs were acclimatised to aerated glass tanks, containing dechlorinated

tap water for two days prior to further analysis, while providing them with chopped carp meat (~ 10% of their body weight) once daily [48]. These wild collected frogs (N=15, each from polluted and reference sites) were then utilised for biochemical and histopathological investigations.

Laboratory exposure

Adult *E. hexadactylus*, (N=15) collected from the reference site were subjected to laboratory exposure of a mixture of heavy metals (Cu, Cd, Cr, Zn and Pb at 5 ppm each) for a period of 28 days. This concentration was selected to simulate the average levels of each heavy metal {which varied from 3.8 to 18.4 ppm; Cu (18.39 ppm), Cr (9.51 ppm), Pb (7.24 ppm), Zn (4.68 ppm), Cd (3.75 ppm) [46]} present in the water samples collected from the polluted site. The adult frogs were reared in glass aquaria (40×40×70 cm) containing 4L of the exposure medium. The medium was renewed every other day, and the frogs were fed with chopped carp meat (10% of the bodyweight; [48]) once daily throughout the exposure period. The temperature and pH were maintained approximately at 26-30^{o}C and 6.5-7.0, respectively under a natural photoperiod of approximately 12:12 h. The heavy metals were supplied only through the water medium; other routes such as sediment, food contamination, etc. were kept metal free.

Tissue sampling and processing

Frogs were sacrificed with MS222 (0.2% solution) for analyses. Body weights were recorded using a digital weighing balance with up to 0.01 g accuracy (LP 202A, Ningbo Hinotek Tech, Zhejiang, China). Standard external morphometric parameters, snout-vent length (SVL) and body width (W) were measured using an electronic digital caliper (Grade 03, Control Company, Friendswood, USA). Blood was drawn by heart puncture and dispensed in to glass vials to obtain serum, according to standard methodology [49]. Liver weight was measured to

calculate the hepatosomal index (HSI), where HSI= (liver weight/ total body weight× 100). Portions of frog liver and gastrocnemius muscle from the left leg were preserved at -20ºC for heavy metal analysis. The major organs, *i.e.* liver, kidney, skin and lung were excised and preserved in 10% buffered formalin for histopathological investigations.

Heavy metal concentrations in water and frog tissue samples

Water samples (N=18) were filtered to remove any solid particles and analysed under graphite furnace atomic absorption spectrometry (GFAAS, AA-6650, Shimadzu, Japan). Frog liver and gastrocnemius muscle tissue were burnt to ash (450ºC) in a muffle furnace (JSMF-45J, research Inc, Seoul, Korea) and acid digested ($1HNO_3$: $1H_2SO_4$ [47]). The digested samples were filtered to remove any solid particles and analysed under GFAAS. Bioconcentration factor (BCF) denoted by the ratio between heavy metal concentration in animal tissue and that in water was determined for each test individual from both natural and laboratory exposure.

Estimation of serum biomarkers

The activities of serum AST, ALT, γ-GT, ALP, MDA, SOD, GPX, GRD, total protein, albumin, total bilirubin and creatinine were assayed spectrophotometrically using commercially available diagnostic kits (Randox diagnostics Pvt Ltd, Antrim, UK) according to the manufacturer's protocols. Liver tissue homogenates were prepared with ice cold STKM (250 mM sucrose, 50 mM TrisHCl, 25 mMKCl, 5 mM $MgCl_2$) buffer (16-20 mg/mL) using a glass homogeniser. The homogenates were immediately stored at -80ºC until further analyses for the above specified hepatic biomarkers.

Histopathological Investigations

For qualitative analysis of tissue histology, frog tissue samples fixed in 10% buffered formalin were processed after 48 hrs. At the outset, tissue samples were dehydrated through an ascending series of ethanol; thereafter were cleared in Histoclear, clearing agent (H-2779, Sigma Aldrich, Taufkirchen, Germany) and embedded in paraffin wax. Tissue sections of 5-8 µm thickness were prepared by using a rotary microtome (Yamato, Kohki, Japan) and stained with standard double staining procedure using Haematoxylin and Eosin. Histological identification was carried out with the aid of a histology guide, Colour atlas of *Xenopus laevis* histology by Wiechmann and Wirsig-Wiechmann [50]. Histopathological manifestations were identified and categorised according to the International Harmonization of Nomenclature and Diagnostic Criteria (INHAND) described in Thoolen *et al*., [51], Frazier *et al*., [52], Mecklenburg *et al*., [53], Renne *et al*., [54] (Tables 1 and 2). Accordingly, a histopathological scoring method was established for semi quantitative analyses of tissue damage; A rank ranging from 0-5 was assigned to each of the organs (Tables 1 and 2), depending on the extent of the tissue damage observed. Forty sections from different areas of each organ were analysed and photographed by Infinity analyse software (version 6.3, Lumenera cooperation, Canada). Scores were assigned to tissue sections by overlapping a 100×100 µm grid over each photomicrograph and data were recorded separately to test the reproducibility with a second observer (consistency >92%). Cytological scoring of melanomacrophage aggregates/ hepatocytes, morphometrics of MMCs and nuclei of the hepatocytes, thickness of the tissue layers such as epidermis, etc. were obtained by `cell counts` and `measure` tools of the Infinity analyse software.

Statistical Analyses

The analyses were conducted with SPSS 20.0 (IBM, USA). Statistical comparisons were made among the three major test groups, *i.e.* polluted, reference and laboratory frog populations, by applying one way analysis of variance (ANOVA) followed by Tukey's post hoc analysis where appropriate or with two sample t-test by considering two test groups at a time. Physicochemical parameters of water (BOD, DO, pH and temperature), biochemical data and histopathological scoring were compared using Friedman test followed by Mood`s median test. Morphometric variables of the frogs (N=15) from the two study sites were compared using simple t test. Linear Pearson correlation coefficient tested for relationships between heavy metal concentrations in water and in frog tissue samples. The level of significance was set at $p<0.05$. The results were presented as mean ± SD.

RESULTS

Physicochemical parameters of water showed no significant differences ($p>0.05$; ANOVA) between the reference and the polluted sites where temperature varied approximately 29-30 ºC, pH (reference 6.5 ±0.4, polluted 6.9 ±0.1), dissolved oxygen (DO, reference 3.22 ±0.2, polluted 3.7 ±0.13 ppm) and biological oxygen demand (BOD, reference 1.8 ±0.2, polluted 2.0 ±0.2 ppm) showed no marked variations.

Since, metabolic activity varies with body size and affects biochemical and other parameters, use of specimens with similar body weights was essential for comparison purposes. No significant difference ($p>0.05$) was recorded between mean weight (BAS-124±5.34 g; reference site-110±7.5 g; t=1.36, p=0.187) of adult *E. hexadactylus* collected from the two study sites. Similarly, other morphometric parameters, SVL (BAS-108.4±11.37 mm; reference site-

103.9±10.89 mm; *t*=-0.98, p=0.346) and the body width (BAS-39.34±5.20 mm; reference site-40.38±5.22 mm; *t*=-0.48, p=0.638) also did not vary significantly between the test sites.

Hepatosomatic index (HSI) indicative of enlargement of the liver of *E. hexadactylus* collected from the polluted site (1.33±0.34; ($F_{(2, 44)}$ =1.23, p= 0.013, ANOVA) and the laboratory exposure study (3.58±0.53, p=0.001) were significantly higher than that of the reference site animals (1.03±0.23); Laboratory exposure resulted in significantly higher HSI than natural exposure ($p<0.05$).

Water samples from the polluted site were contaminated with, heavy metals in concentrations ranging the order of Cu>Cr>Pb>Zn>Cd i.e significantly higher concentrations ($p<0.05$) compared with the reference site (Table 3). Accumulation of Cu and Cr in liver and gastrocnemius muscle tissue of *E. hexadactylus*, collected from the polluted site and laboratory exposed frogs was significantly higher ($p<0.05$) than those of the reference site (supplementary data). Unlike laboratory exposed frogs, liver tissues of the naturally exposed frogs showed remarkably higher bioaccumulation of Cu and Cr (30.37 and 72.81 ppm, respectively) than their corresponding accumulation in the gastrocnemius muscle (~2 ppm). Similarly, bioconcentration of heavy metals, given by BCF showed significantly higher values for Cu and Cr levels of natural and laboratory exposed frogs compared to their reference levels ($p<0.05$, ANOVA; supplementary data). Further, the BCF of liver tissue was significantly higher compared to that of the muscle. Particularly, BCF value for Cu and Cr in liver tissues of metal exposed frogs was >3.5, with Cu reporting the highest BCF of 3.96 compared to all other metals tested.

Biochemical Parameters

Levels of hepatic marker enzymes, AST, ALT, γ-GT and ALP in serum and in liver tissue homogenates were significantly higher ($p<0.05$) in both groups of heavy metal exposed *E. hexadactylus* (Table 4, Fig. 1a) than in reference animals. On the contrary, other hepatic biomarkers such as total protein and albumin were significantly decreased ($p<0.05$) in the metal exposed groups (Table 4, Fig.1b). Hepatic marker enzymes showed strong positive correlation with histopathological manifestations detected in heavy metal exposed frogs with a significant relationship in hepatic ALP levels of the laboratory exposed frogs(Pearson $R= 0.95$, $p=0.05$). Conversely, protein and albumin as liver products, showed negative correlation with the corresponding histopathological scores of the two groups of heavy metal exposed frogs; Nevertheless, significant relationship with the hepatic albumin level of naturally exposed frogs ($R= -0.642$, $p=0.002$) was reported. Markedly increased lipid peroxidation as expressed by MDA formation was recorded in heavy metal exposed frogs compared with their reference counterparts ($p<0.05$), showing positive correlation with liver damage. Both circulating levels and hepatic levels of antioxidant enzymes, SOD, GPX and GRD showed an increase with metal exposure reporting SOD level 455 to 625 U/L, GPX level 4 to 7 U/L and GPX level 350 to 895 U/L in frog serum and reporting SOD level 285 to 1065 U/L, GPX level 18 to 25U/L and GPX level 472 to 860 U/L in the liver. GPX/SOD ratio increased with metal exposure reporting marked elevation in the serum of laboratory exposed *E. hexadactylus* (1.98 compared to the reference of 0.57, $F_{(2,44)}= 8.67$, $p= 0.016$, ANOVA, Table 4, Fig 2a). GRD/GPX ratio showed no substantial increase or decline with metal exposure except slight elevation in the liver tissue homogenate level in the *E. hexadactylus* collected from the polluted site (0.05 compared to the reference of 0.02; $F_{(2,44)}= 1.49$, $p=0.254$, ANOVA,Table 4, Fig 2b). Urea and creatinine levels in frog serum

were significantly higher ($p<0.05$) in the metal exposed frogs collected from both natural and laboratory exposure than in analogous frogs from the reference site (Table 4, Fig.3).

Histopathological Investigations

Histology of major organs of *E. hexadactylus*, from the reference site showed normal morphology without any histopathological manifestations. Affected tissues of frogs from the polluted site and from laboratory exposure showed histopathological alterations in vital areas of each tissue type. Sections predominantly with normal tissue arrangements without visible tissue/cell distortions were assigned zero on the histopathological score. All sections were examined on five separate occasions to ensure repeatability of the score. Since, higher repeatability was obtained in the hands of a single observer, the semi quantification was continued without applying multiple observer variability [55, 56].

Liver

Liver tissue of healthy frogs collected from the reference site comprised of clearly arranged hepatocytes which were joined together in cords and were separated by numerous sinusoids (plate 1A) as described by Fenoglio *et al*. [57]. Hepatocytes were of regular shape with a large spherical nucleus at the centre. Portal triads containing an artery, vein and bile duct were observed throughout the liver. Lymphoid cell aggregates/ haematopoetic cell aggregates were found near the hepatic portal vein and the hepatic triads (Plate 1-B1 and B2). The hepatic parenchyma contained numerous melano macrophage centres (MMC) or *Kupffer* cells of different sizes and shapes and heterogeneous in nature (Plate 2). These melanin phagocytised macrophage cells were grouped together, partially conserving their cytoplasmic membrane, and surrounded by a thin connective tissue capsule. Developing macrophages were distributed near

the hepatic portal vein and the haematopoetic tissue showing relatively low pigmentation compared to fully developed MMC (Plate 2 B & C). MMC were highly variable in size with an average of (507 × 339) µm while reporting the lowest of (189×146) µm and the highest of (840× 510 µm).

Among major histopathological manifestations in the liver of metal exposed frogs, pigmentation, hepatic inflammation, extramedullary haematopoiesis, karyocytomegaly and k*upffer* cell hypertrophy were prominent (Plates 3 and supplementary data). Pigmentation was identified as incidental occurrence of pigments such as Lipofuscin (pale yellow to deep granular brown) and Iron/hemosiderin (yellow to brown and may be finely granular) accumulated by altered haeme metabolism and lipid peroxidation of cellular membranes [51]. Inflammatory cell Infiltrates &hepatic inflammation were characterised as aggregations of immune cells including lymphocytes, plasma cells, macrophages, and neutrophils where cellular necrosis and haemorrhages were also associated occasionally [51]. Small aggregations of haematopoietic cells, randomly distributed in the hepatic sinusoids and around central veins were distinctly manifested as extramedullary haematopoiesis [51]. Karyocytomegaly diagnosed as enlarged nuclei in hepatocytes which may be tetraploid or octaploid in nature while k*upffer* cell proliferation or aggregation into melanomacrophage centres were identified under the k*upffer* cell hypertrophy.

Metal exposed liver tissue of the frogs collected from the polluted site showed marginal hitopathological manifestations where 1-2 foci affected with pigmentation, inflammatory cell Infiltrates &hepatic inflammation, extramedullary haematopoiesis, karyocytomegaly (2-3%), k*upffer* cell hypertrophy (% MMC aggregation = 13-14%, histopathological score 1.65 ±0.025, S = 7.12, p=0.031, Friedman). Moderate hitopathological manifestations, with 7-12 foci affected

(3.69±0.34, p=0.01) by pigmentation, inflammatory cell Infiltrates &hepatic inflammation, extramedullary haematopoiesis, karyocytomegaly (7-9%), k*upffer* cell hypertrophy (% MMC aggregation = 19-21%), were observed in laboratory exposed frogs. Heavy metal accumulation, as evaluated by BCF in the liver tissue of frogs from the polluted site positively correlated with observed histopathological changes, particularly with significant association with Cr (Pearson correlation, *R*= 0.999, p= 0.024). Conversely, no such correlation was evident in laboratory exposed frogs (p>0.05). Number of MMCs increased with metal exposure as the reference liver recorded≤ 9.5% (number of MMCs per 100 hepatocytes) and metal exposed liver recorded 13-21% elevation.

Kidney

Kidney tissues of reference site *E. hexadactylus* were composed of clearly arranged cortex and medulla regions where renal capsules and convoluted tubules were located in the cortex and loop of Henle and collecting tubules were located in the medulla. Both proximal and distal convoluted tubules (PCT and DCT), comprised of cuboidal epithelium were located near the Bowman`s capsules (Plate 4B).Thick limbs of loop of Henle and collecting tubules also comprised of cuboidal epithelia with much larger cuboidal cells.

Metal exposed frogs showed marginal to slight tubular degeneration, tubular necrosis, dilation of Bowman`s space (Plate 5), chronic progressive *Nephropathy* (CPN), mineralisation and inflammation in the kidney infiltrate (Plate 6) with a histopathological score of 0.79 ± 0.036 (S= 6.18, p= 0.064, Friedman) in frogs from the polluted site, and 2.33 ± 0.9 in laboratory exposed frogs (p= 0.01) compared with their reference counterparts. Tubular degeneration in the kidney appeared as several morphologic changes in renal epithelial cells associated with loss of

viability including vacuolation, blebbing, cellular sloughing (Plate 5A) and other alterations as described by Frazier *et al*., [52]. Chronic progressive *Nephropathy* of the frog kidney was characterised by tubular basophilia and crowding as shown in the plate 5 B and C. Inflammatory cell infiltrates were small foci of lymphocytes, plasma cells, and/or macrophages within the interstitium (Plate 5D).Mineralisation was observed as dystrophic calcification specifically in the renal tubules and collecting ducts of the metal exposed frogs (supplementary data).

Lung

As described by Wiechmann and Wirsig-Wiechmann [50], trachea of *E. hexadactylus* lung is divided into paired bronchi which in turn divided into many bronchioles ultimately producing numerous alveolar sacs. Bronchi were lined with a cartilaginous layer while bronchioles possessed smooth muscle supporting the epithelial mucosa (Plate 6 A & B). Alveolar sacs were blind end sacs, lined with alveolar capillaries and pneumocytes [50].

In contrast, marginal hitopathological manifestations with 1-2 foci affected by degeneration of epithelia and necrosis were evident in those from the polluted site, reporting 0.59± 0.11 on the histopathological score ($S = 5.37$, $p= 0.05$, ANOVA; Plate 6 C&D) while laboratory exposed frogs reported an elevated score of 1.68 ± 0.09 ($p= 0.001$). As described by Mecklenburg *et al*., [53] loss of cilia in the respiratory epithelium, epithelial blebbing or cytoplasmic vacuolation, rounding up of the cells were evident in metal exposed frogs.

Skin

The skin of *E. hexadactylus* is composed of epidermis and the dermis, where numerous secretary glands are located in the dermis (Plate 7A). Mucous and serous glands, submerged in the dense connective tissue matrix in the dermis, open to the epidermal surface through glandular

ducts. Similar to *Xenopus laevis* skin, described in Wiechmann and Wirsig-Wiechmann [50], epidermis of *E. hexadactylus* was a keratinised stratified squamous epithelium divide in to four distinct layers, stratum corneum, s. granulosum, s. spinosum and s. basale. Cutaneous epidermis showed no apparent disturbances in the reference animals (Plate 7A) whereas their counterparts from the polluted site showed marginal hitopathological manifestations with 1-2 foci affected by *Atrophy- epidermal* (epidermal thinning) and *Atrophy- adnexal* (glandular cell damage) (0.29± 0.027, S = 5.22, p= 0.06, Friedman).Skin damage manifested by the laboratory exposed frogs were significantly higher (1.56± 0.22, p= 0.01; plate 7B) compared with the reference skin sections.

DISCUSSION

Numerous instances suggest that environmental levels of heavy metals may affect the survival and the development of different animal taxa, extending the effects up to population-level damage [2-4]. The present study was undertaken to evaluate biochemical and histopathological alterations of a common anuran amphibian, *E. hexadactylus*, with natural and laboratory exposure to heavy metals, compared with their counterparts collected from a pristine reference site. The study revealed that the Bellanwila-Attidiya sanctuary is highly contaminated with various heavy metal species, in concentrations varying the order of Cu>Cr>Pb>Zn>Cd, and significant bioaccumulation of Cu and Cr in frog liver and gastrocnemius muscle tissue confirming previous observations [45, 46]. Histopathology of all the major frog organs revealed tissue disturbances as significant alterations from the general morphology, observed in the reference animals. Showing strong correlation with the histopathological alterations, the biochemical parameters tested may serve as hepatotoxic and nephrotoxic biomarkers.

Heavy metals enter the amphibian body both by ingestion through the oral cavity via semi-permeable skin, and through inhalation via lungs/gills. Hence, metal ions may possibly exert damage to any of the organ tissues, during/ after transportation or the accumulation process. Histopathological investigation of all the major organs thus inevitably provided a detailed landscape of heavy metal mediated tissue damage in the amphibian body. Liver and kidney as important organs involved in the metabolism, detoxification and biotransformation of xenobiotics are highly susceptible to heavy metal mediated damage. Hence, we specifically evaluated liver function by measuring plasma AST, ALT, γ-GT, ALP activities, total protein, albumin and total bilirubin levels, and kidney function by assaying creatinine and urea levels investigating the histopathology of these two organs in parallel.

Heavy metal accumulation data accrued in our study revealed relatively higher accumulation in liver tissue in concordance with previous studies [16, 58]. Overall, muscle and liver burden of Cu and Cr in *E. hexadactylus* was significantly higher than those of the other metals reported in the study. The accumulation of Pb in naturally exposed frogs was generally low, but was significantly accumulated in muscle tissue as indicated by their BCF value in laboratory exposed frogs. The current study confirmed low accumulation of Pb as reported in the tissues of *Rana catesbeiana* and *Rana clamitans* [59].

Histopathological alterations of vital organs are undoubtedly an important biomarker of toxic chemicals [60]. Importantly, such alterations observed in this study are in accordance with other heavy metal mediated toxicity studies of amphibian and fish species conducted elsewhere [16, 61]. Furthermore, histopathological assessments reported in most of the ecotoxicological studies are not quantitative measures. Application of the established histopathological scoring

method facilitated more accurate evaluation of morphological effects caused by heavy metals nonetheless semi-quantifying approach was the only possibility under the available instrumentation and facility. The level of injury between individuals and between groups in the present study was compared with ease with the use of this scale. To our knowledge this is the first study that established a histopathological scale to semi quantitatively assess organ alternations of amphibians. Thus, the study developed a reliable and reproducible semi quantitative tool to bridge this gap, which can be used for ecotoxicological studies, on condition that standard procedure is followed.

Increased melanomacrophage centres in the liver of metal exposed frogs is in accordance with various other studies conducted with xenobiotic and other stressors on amphibians and fishes reported elsewhere [24, 62-65]. This indicates increased phagocytosis in the liver tissue as MMCs consist of vacuoles and cellular debris. Definite function/s of MMC is/are not yet fully explained but, iron capture and storage in haemolytic conditions is described as a primary function together with various other apparent functions such as antigen trapping and presentation to lymphocytes, sequestration of cellular debris and potentially toxic tissue materials (eg. Melanin), free radicals [25]. Lee *et al* [66] described increase in MMCs in the liver as an indication of oxidative stress [66]. Since, melanin is capable of scavenging ROS [67] MMCs may protect liver tissue against oxidative stress. Moreover, melanin is also associated with the accumulation, stimulatory action, and ultimate detoxification of the metal ions [68]. RBC breakdown related iron stored inside MMCs as hemosiderin and ferritin serve as an intracellular storage and sequestration of metals, in addition to the metallothionein and glutathione systems [69]. Therefore, apparent functions of the MMC may include absorption and neutralisation of free radicals, cations, and other potentially toxic agents derived from the degradation of

phagocytised cells. This explains the elevated percentage MMC in the metal exposed *E. hexadactylus,* and in fact it is an indication of enhanced cell death.

Structural damage of vital organs due to heavy metal accumulation may impair the basic role of detoxification and biotransformation of heavy metal ions, leading to dysfunction of such organs. This reduces the efficacy of the organ by further lowering the production of functional biomarkers, such as enzyme and other products. Hence, histopathological biomarkers are closely related to biochemical markers of heavy metal mediated oxidative stress as the metal ions interfere with the biochemical pathways in order to aggravate cellular damage by producing other intermediate forms [60]. For instance, alteration of lipid peroxidation activity will lead to tissue necrosis which in turn may be used as a histopathological biomarker [70]. Elevated number or the area occupied by the MMC replaced the activity of antioxidant enzymes such as SOD [71]. This explains reduced activity of SOD in the liver tissue of metal exposed *E. hexadactylus*. Therefore, histopathology and biochemical parameters may be used as cognate measures of heavy metal toxicity.

Hepatocelluar injury is well established by elevated levels of serum hepatic marker enzymes AST and ALT, indicating cellular leakage and loss of functional and structural integrity of the hepatocyte membrane [10, 70]. As a membrane bound enzymes, AST and ALT leakage would inevitably confirm hepatocelluar damage. Increased level of serum bilirubin in the study was a clear marker of hepatic dysfunction [72, 73]. As a proximate measure of cellular architecture, metabolic function and patterns of gene expression, lowering total protein levels in the serum is an indication of tissue damage [5]. Apparent mechanism of these low protein levels may be due to the interference of Mg^{2+} ion dependent RNA synthesis process by various other heavy metal species, which replace Mg^{2+}ions [5]. Furthermore, heavy metals enhance protein

degradation as these species readily precipitate protein molecules [74]. This is further explained by marked elevation of LPO indices following heavy metal exposure, consistent with previous reports [10, 74]. Increasing serum urea and creatinine levels suggests decreased excretion from the kidneys, signifying renal malfunction. As a vital organ that maintains homeostasis under highly variable external environmental conditions, renal dysfunction for amphibians will be catastrophic.

Heavy metal intoxication and detoxification pathways in biological systems suggest the involvement of cellular thiol, generation of free radicals or reactive oxygen species (ROS), oxidative stress, antioxidant enzymes, metallothionine (MT) and activation of mitogen activated protein kinase (MAPK) pathways [75]. Hence, in the animal body, metal species can directly bind with MT or glutathione (GSH) in the cell membrane and interfere with their role in oxidative stress management [75]. Moreover, various metal ions can cause oxidative stress through several mechanisms such as the Fenton reaction, depletion of cellular GSH, alteration of mitochondrial electron transfer chain, etc. [75].When the amount of heavy metals in the kidney/liver exceeds the binding capacity of MT, then non-MT bound heavy metal ions are believed to cause nephro and hepatotoxicity [76]. One such mechanism causing injury is the production of ROS and lipid peroxidation [77]. Similarly, GSH can effectively bind with heavy metal species and alter metal ion uptake and elimination to prevent interactions of metal ions with cellular structures. Hence, low GSH will also enhance heavy metal mediated toxicity. Therefore, measuring GSH and MT levels is of vital importance as a measure of the animal's ability to shield against heavy metal mediated toxicity.

Most of the ecotoxicological studies conducted to date are single stressor studies rather than evaluating mixture effects. Assessing mixtures is crucial as the animals get exposed to mixtures under natural context instead of single metal or other xenobiotic variety. Further, compared to single metals, metals in mixtures are considered to be toxic, particularly at lower doses [78]. Horne and Dunson [79] evaluated naturally-occurring metals (Al, Cu, Fe, Pb, Zn) on survival of the Jefferson salamander, *Ambystoma jeffersonianum*, and the wood frog, *Rana sylvatica* revealing differential effects depending on the toxicity of each metal ion and pH of the medium. Moreover, some metal ions in a mixture affect bioaccumulation of other metal ions, for instance accumulation of Cr in the kidney tissue of *Rana ridibunda* was doubled when Cd was present [80]. Thus, heavy metal mixtures elicit differential effects, which are unpredictable and independent from their individual exposures.

In general, laboratory exposure of organisms to toxic compounds reported somewhat higher effects than corresponding natural exposure in the field [81]. Our study too reiterated this trend. Possible disparity of the experimental settings may influence these minor deviations. Exposure of animals to heavy metals in the laboratory was conducted by intensive exposure through a period of 28 days where the frogs were confined to a small enclosure. Yet in the natural context, frogs may have better chances to avoid toxic stressors, and natural fluctuations of heavy metal levels over time may provide them opportunity to compensate the damage. However, estimation of the overall effect of heavy metal exposure on the *E. hexadactylus* populations in the study sites were beyond the scope of the present study as it requires an extensive population census and a bigger sample size. Hence, the data of the present study cannot be extrapolated to predict the damage at population level. Since many ecotoxicological

studies are focused on early life stages of amphibians, studying an adult amphibian could be of great importance.

CONCLUSIONS

The outcome of the present study clearly showed significant biochemical and histopathological alterations of heavy metal exposed *E. hexadactylus* compared with their counterparts from the reference site. Results of the naturally exposed frogs were comparable with corresponding measures of laboratory exposure, corroborating heavy metal mediated damage on this sensitive amphibian species. This study thus reinforces the potential of histopathological aberration associated biochemical alterations as biomarkers of aquatic health, predominantly linked with heavy metal toxicity of a sentinel amphibian in a wetland ecosystem. The semi quantitative histopathological scoring method developed in the study may provide a valuable, reliable and precise tool for assessing histopathological damage in ecotoxicological studies.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

Acknowledgment—Authors declare that the experiments conducted, complied with the current laws of Sri Lanka. The authors declare that there is no conflict of interest. UA carried out the study under the guidance of DD, WD and PV. UA drafted the manuscript and DD, WD and PV reviewed it before the initial submission. All authors read and approved the final manuscript. We acknowledge financial assistance through grants from the Ministry of Higher Education, Sri Lanka (HETC/CMB/QIGW3/SCI/OS/2012/02) and the University of Colombo (AP/3/2/2012/RG/SC/04).

Data Availability—The data sets supporting the results of this article are included within the article and its additional files.

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Figure 1. Hepatic biomarkers of *E. hexadactylus*, (a) serum hepatic marker enzyme levels (b) serum levels of liver products (c) tissue homogenate levels of hepatic marker enzymes (d) tissue homogenate levels of liver products. *Significant elevation of hepatic marker enzymes and significant decline of liver products were reported in both serum and tissue homogenate levels of heavy metal exposed frogs, compared to the reference frogs (*ANOVA*, $P<0.05$).

Figure 2. Stress biomarkers, antioxidant enzyme system of *E. hexadactylus*, (a) glutathione peroxidase (GPX)/ superoxide dismutase (SOD) ratio (b) Glutathione reductase (GRD)/ GPX in both serum and liver tissue homogenate levels *Significant elevation of the ratios, compared to the reference level (*ANOVA*, $P<0.05$).

Figure 3. Renal biomarkers of *E. hexadactylus*. Significantly elevated serum urea and creatinine levels were recorded in natural and laboratory exposed frogs to heavy metals, compared with their reference counterparts (*ANOVA*, $P<0.05$). The analyses were conducted in duplicate.

Plate1. Representative photomicrographs of the liver tissue of frog showing (A) general morphology of *E. hexadactylus* liver with hepatocytes (HPC) arranged in double cords, sinusoidal spaces (SYN) spread in between, blackish brown aggregates of melanomacrophage centres (MMC), hepatic triads (HTRD) containing branches of hepatic portal vein (HPV), biliary duct (BD) and hepatic artery (HA); (B) localisation of haematopoetic/ lymphoid cell aggregates (LMPN) near the HPV (LMPN1) and among hepatocytes (LMPN2). Scale bar represents 150 µm.

Plate 2. Representative photomicrographs showing the development of melanomacrophage centres (MMC). (A) Developing MMCs (DMMC) appeared as light brown nodules near the

hepatic portal vein (HPV); (B & C) Developing MM centre with small melanocytes (MC) aggregating (Scale bar represents 150 µm).

Plate 3. Representative photomicrographs of *E. hexadactylus* liver cross sections showing (A1)*Kupffer cell* hypertrophy, aggregation of low % MMC in reference tissue, (A2) high % MMC in metal exposed tissue and karyocytomegaly, enlargement of the hepatocyte nuclei (EN) in metal exposed tissues (B1-B2; Scale bar, 200 µm).

Plate 4. Representative photomicrographs of kidney tissue of *E.hexadactylus* showing(A) tissue arrangement in cortex and the medulla; (B) cross sections of reference kidney with glomerulus (GL), Bowmen`s capsules (BC), thin loop of henlae (TLH), collecting ducts (CD), proximal convoluted tubules (PCT), distal convoluted tubules (DCT) (Scale bar represents 150 µm).

Plate 5. Representative photomicrographs of kidney tissue of metal exposed *E.hexadactylus*showing(A) tubular degeneration, tubular necrosis with dialted Bowman`s capsule (DBC), glomerulus (GL), collecting ducts (CD), proximal convoluted tubules (PCT), distal convoluted tubules (DCT), (B) Chronic progressive *Nephropathy* showing tubular crowding of collecting ducts (CD), and (C) basophilic cell aggregates (D), Inflammation in the kidney infiltrate (INFLTR) (Scale bar represents 150 µm).

Plate 6. Representative photomicrographs of cross sections of the lung tissue of *E.hexadactylus* showing (A &B) reference tissue with bronchus (BRC), bronchiole (BRO), alveolar ducts (AD) and alveolar sacs (AS); (C) affected lung tissue of metal exposed frogs showing degeneration of epithelia and necrosis, damaged alveolar wall (DAV); (D1-D3) Alveolar degeneration (D1) healthy and (D2 and D3) heavy metal exposed frogs with degenerating alveolar wall (arrows point) otherthan the areas containing blood vessels (BV). Scale bar represents 120 µm)

Plate 7. Representative photomicrographs of cross sections of the skin of *E.hexadactylus*showing (A) epidermis and the dermis (A1) reference tissue with squamous epithelium (SE) in the epidermis, poison gland (PG) and mucous gland (MG) located below the epidermis, melanocyte layer (MCL) located just below the epidermis; (B) affected skin sections of metal exposed frogs showing *Atrophy- epidermal* (Epidermal thinning) nor *Atrophy- adnexal* (glandular cell damage) with damaged squamous epithelim (arrow heads) (B1) damaged glandular cells (arrow head). Scale bar represents 130 µm.

Table 1: Grading scheme for focal and multifocal organ lesions (modified from World Health Organization, 1978; Hardisty and Eustis, 1990; Derelanko, 2000).

Severity	Proportion of the organ affected	Grade	Quantifiable finding
None	None	0	0
Marginal or minimal	Very small amount	1	1-2 foci
Slight or few	Small amount	2	3-6 foci
Moderate or several	Medium amount	3	7-12 foci
Marked or many	Large amount	4	>12 foci
Severe	Very large amount	5	Diffuse

Table 2. Ranking of tissue damage according to the histopathological scoring method, developed in the study [The scheme was based on the International Harmonization of Nomenclature and Diagnostic Criteria (INHAND) nomenclature]

Organ	Rank	Description of the tissue damage
Liver	0	No hitopathological manifestations; pigmentation, inflammatory cell Infiltrates & hepatic inflammation, extramedullary hematopoiesis, nor karyocytomegaly, *Kupffer* cell hypertrophy (% MMC aggregation ≤ 9.5%).
	1	Marginal hitopathological manifestations with 1-2 foci affected; pigmentation, inflammatory cell Infiltrates & hepatic inflammation, extramedullary haematopoiesis, karyocytomegaly (2-3%), *Kupffer* cell hypertrophy (% MMC aggregation = 13-14%)
	2	Slight hitopathological manifestations with 3-6 foci affected; pigmentation, inflammatory cell Infiltrates & hepatic inflammation, extramedullary haematopoiesis, karyocytomegaly (5-6%), *Kupffer* cell hypertrophy (% MMC aggregation = 17-18%)
	3	Moderate hitopathological manifestations with 7-12 foci affected; pigmentation, inflammatory cell Infiltrates & hepatic inflammation, extramedullary haematopoiesis, karyocytomegaly (7-9%), *Kupffer* cell hypertrophy (% MMC aggregation = 19-21%)
	4	Marked hitopathological manifestations with >12 foci affected; pigmentation, inflammatory cell Infiltrates & hepatic inflammation, extramedullary haematopoiesis, karyocytomegaly (10-12%), *Kupffer* cell hypertrophy (% MMC aggregation 21-25%)
	5	Severe hitopathological manifestations diffused in the liver; pigmentation, inflammatory cell Infiltrates & hepatic inflammation, extramedullary haematopoiesis, karyocytomegaly (>12%), *Kupffer* cell hypertrophy (% MMC aggregation ≥ 25%)
Skin	0	No hitopathological manifestations; *Atrophy- epidermal* (Epidermal thinning) nor *Atrophy- adnexal* (glandular cell damage)
	1	Marginal hitopathological manifestations with 1-2 foci affected; *Atrophy- epidermal* (Epidermal thinning), and *Atrophy- adnexal* (glandular cell damage)
	2	Slight hitopathological manifestations with 3-6 foci affected; *Atrophy- epidermal* (Epidermal thinning), and *Atrophy- adnexal* (glandular cell damage)

	3	Moderate hitopathological manifestations with 7-12 foci affected; *Atrophy- epidermal* (Epidermal thinning), and *Atrophy- adnexal* (glandular cell damage)
	4	Marked hitopathological manifestations with >12 foci affected; *Atrophy,-epidermal* (Epidermal thinning), and *Atrophy- adnexal* (glandular cell damage)
	5	Severe hitopathological manifestations diffused in the skin; *Atrophy, epidermal* (Epidermal thinning), and *Atrophy- adnexal* (glandular cell damage)
Kidney	0	No hitopathological manifestations; Tubular degeneration, tubular necrosis, Chronic Progressive *Nephropathy* (CPN), Mineralisation, Inflammation in the Kidney Infiltrate, nor dilation of Bowman`s space
	1	Marginal hitopathological manifestations with 1-2 foci affected; Tubular degeneration, tubular necrosis, Chronic Progressive *Nephropathy* (CPN), Mineralisation, Inflammation in the Kidney Infiltrate, and dilation of Bowman`s space.
	2	Slight hitopathological manifestations with 3-6 foci affected; Tubular degeneration, tubular necrosis, Chronic Progressive *Nephropathy* (CPN), Mineralisation, Inflammation in the Kidney Infiltrate, and dilation of Bowman`s space.
	3	Moderate hitopathological manifestations with 7-12 foci affected; Tubular degeneration, tubular necrosis, Chronic Progressive *Nephropathy* (CPN), Mineralisation, Inflammation in the Kidney Infiltrate, and dilation of Bowman`s space.
	4	Marked hitopathological manifestations with >12 foci affected; Tubular degeneration, tubular necrosis, Chronic Progressive *Nephropathy* (CPN), Mineralisation, Inflammation in the Kidney Infiltrate, and dilation of Bowman`s space.

	5	Severe hitopathological manifestations diffused in the kidney; Tubular degeneration, tubular necrosis, Chronic Progressive *Nephropathy* (CPN), Mineralisation, Inflammation in the Kidney Infiltrate, and dilation of Bowman`s space.
Lung	0	No hitopathological manifestations; Degeneration of epithelia nor necrosis
	1	Marginal hitopathological manifestations with 1-2 foci affected; Degeneration of epithelia and necrosis
	2	Slight hitopathological manifestations with 3-6 foci affected ; Degeneration of epithelia and necrosis
	3	Moderate hitopathological manifestations with 7-12 foci affected ; Degeneration of epithelia and necrosis
	4	Marked hitopathological manifestations with >12 foci affected; Degeneration of epithelia and necrosis
	5	Severe hitopathological manifestations diffused in the lung; Degeneration of epithelia and necrosis

(% MMC- number of melanomacrophage centers per 100 of hepatocytes, % karyocytomegaly- number of enlarged nuclei per 100 of hepatocytes)

Table 3. Heavy metal concentrations (ppm) in water samples collected from the reference and polluted sites (N=18 per each). *Significantly higher level compared with the reference value (t test, $P<0.05$).

Field site	Heavy metal concentration (ppm) ±SD				
	Cd	Cr	Cu	Zn	Pb
Labugama (Reference)	0.059±0.01	0.724±0.13	0.060±0.02	0.367±0.10	0.933±0.31
Bellanwila (Polluted)	3.75±0.85	9.52±0.49*	18.39±1.21*	4.68±0.76	7.24±1.01

Table 4: Hepatic and renal biomarkers of *E. hexadactylus*, in the reference (Labugama) and polluted (Bellanwila-Attidiya) sites and from laboratory exposure.

Biomarker	Serum levels			Liver tissue homogenate levels		
	Labugama (Reference)	**Bellanwila (Polluted)**	**Laboratory exposed**	**Labugama (Reference)**	**Bellanwila (Polluted)**	**Laboratory exposed**
Hepatic Biomarkers						
AST (U/L)	6.42±0.65	16.31±0.88[a]	15.33±1.4[b]	13.25±0.77	20.96±0.62[a]	24.67±4.6
ALT (U/L)	8.97±1.9	14.63±0.72[a]	15.48±1.1[b]	12.80±0.32	21.20±0.96[a]	29.73±2.9[b]
γ-GT (U/L)	19.33±2.7	31.7±3.6[a]	36.54±1.8[b]	3.90±0.64	11.00±0.47[a]	11.82±0.74[b]
ALP (U/L)	3.11±0.54	16.68±1.2[a]	30.4±12.5	20.08±3.5	32.6±2.7[a]	72.5±8.8[b, c]
TBARS (mol/g)				5.21± 0.04	15.95±1.8[a]	15.54±2.6[b]
Total protein (g/L)	23.21±2.9	14.53±1.9[a]	12.38±1.7[b]	11.09±0.73	4.43±0.73[a]	2.58±1.3[b]
Albumin (g/L)	144.36±2.1	9.22±1.0[a]	10.65±1.3	7.70±0.79	2.21±0.42[a]	3.36±1.2[b]
Total Bilirubin (mg/L)	1.27±0.335	1.97±0.14	2.533±0.35[b]	3.42±0.69	14.43±0.59[a]	14.74±2.2[b]
GPX/SOD	0.57± 0.12	1.86± 0.4	1.98 ± 0.12[b]	0.88±0.06	0.61±0.26	0.81±0.21

GRD/GPX	0.03	0.004	0.011	0.023	0.056	0.026
Renal Biomarkers						
Urea (mg/L)	11.95±0.7	18.61±1.2[a]	24.45±2.5[b]			
Creatinine (mg/L)	12.85±0.89	19.55±0.6[a]	23.21±1.0[b,c]			

[a, b, c] Significant alterations at $p<0.05$ (ANOVA) with pairwise comparisons of Tukey`s HSD; a- reference/polluted, b- reference/laboratory exposed, c- polluted/laboratory exposed

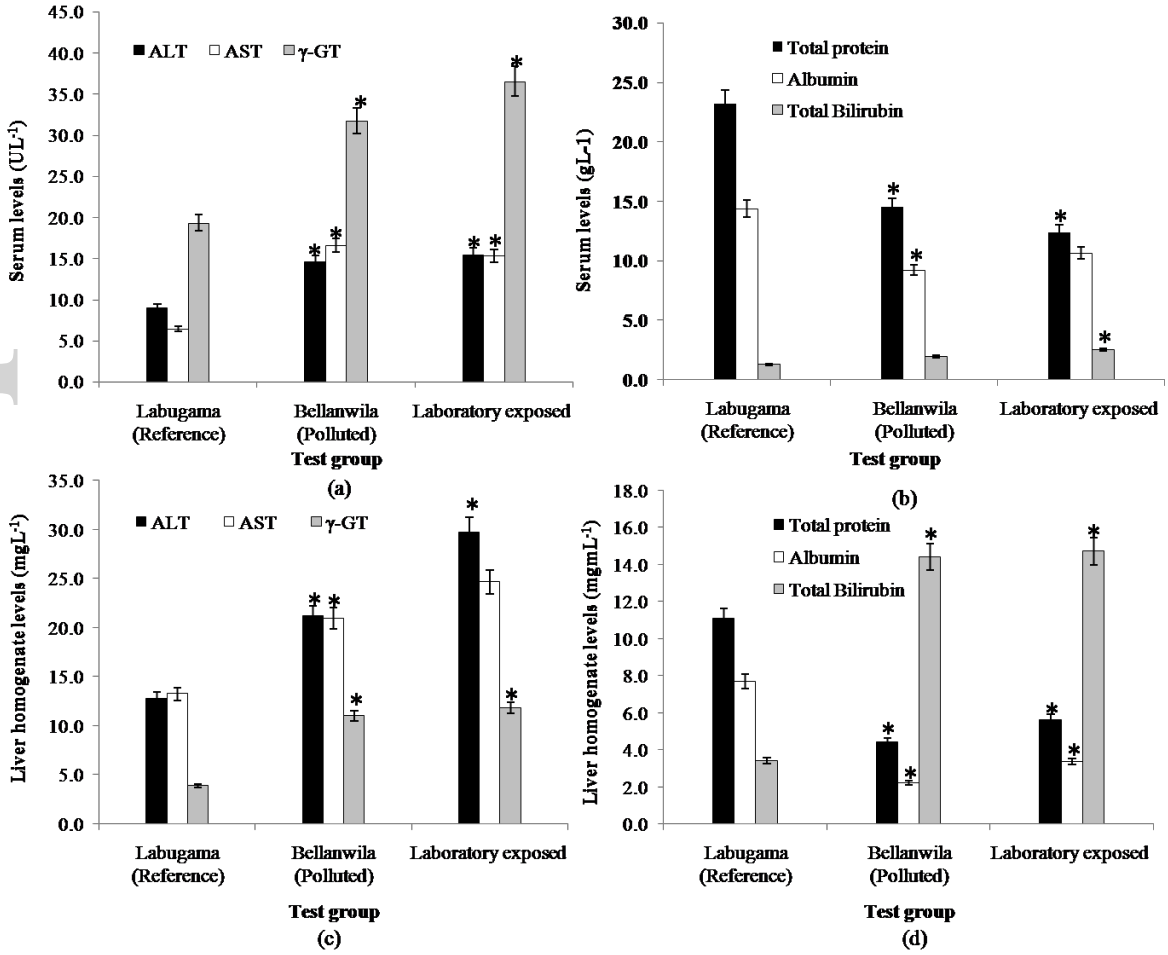

Fig. 1 Hepatic biomarkers of *E. hexadactylus*, (a) serum hepatic marker enzyme levels (b) serum levels of liver products (c) tissue homogenate levels of hepatic marker enzymes (d) tissue homogenate levels of liver products. *Significant elevation of hepatic marker enzymes and significant decline of liver products were reported in both serum and tissue homogenate levels of heavy metal exposed frogs, compared to the reference frogs (*ANOVA*, $P<0.05$).

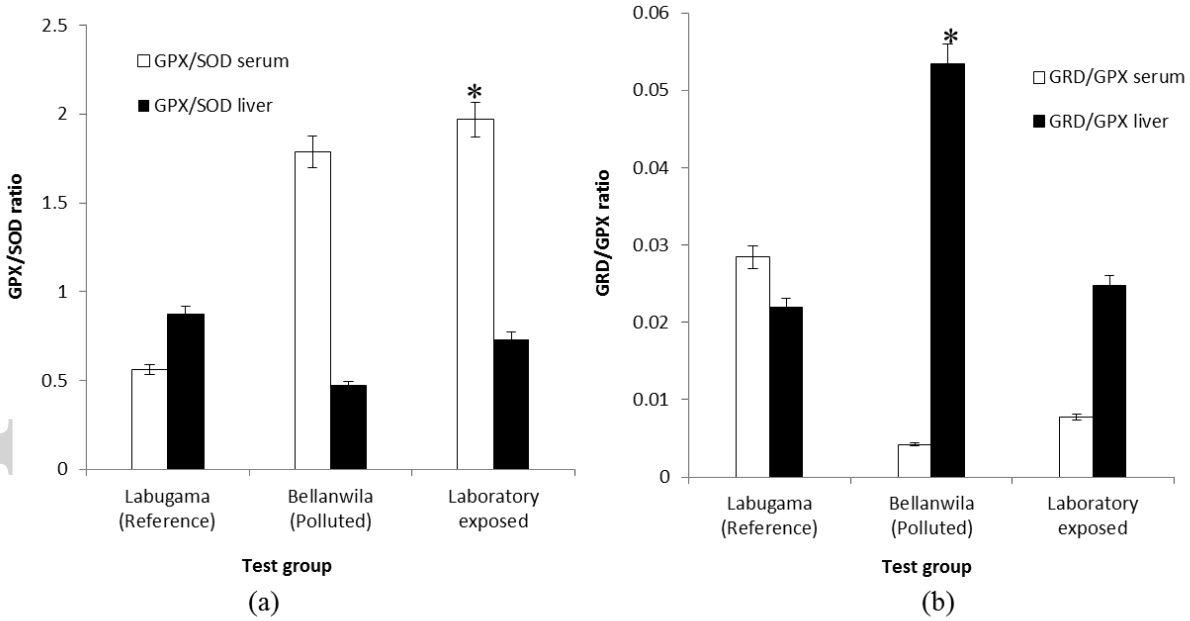

Fig. 2 Stress biomarkers, antioxidant enzyme system of *E. hexadactylus*, (a) glutathione peroxidase (GPX)/ superoxide dismutase (SOD) ratio (b) Glutathione reductase (GRD)/ GPX in both serum and liver tissue homogenate levels *Significant elevation of the ratios, compared to the reference level (*ANOVA, P<0.05*).

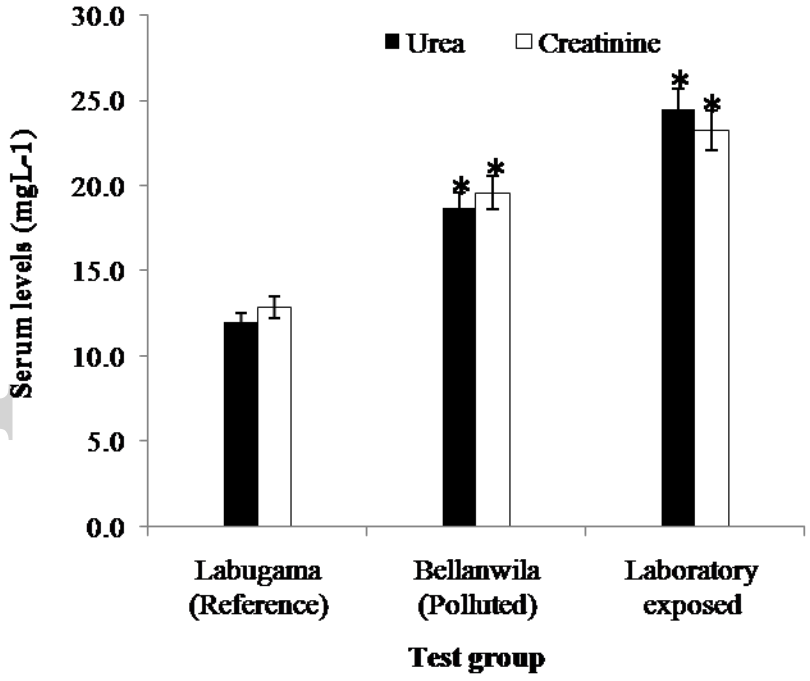

Fig. 3 Renal biomarkers of *E. hexadactylus*. Significantly elevated serum urea and creatinine levels were recorded in natural and laboratory exposed frogs to heavy metals, compared with their reference counterparts (*ANOVA*, *P*<0.05). The analyses were conducted in duplicate.

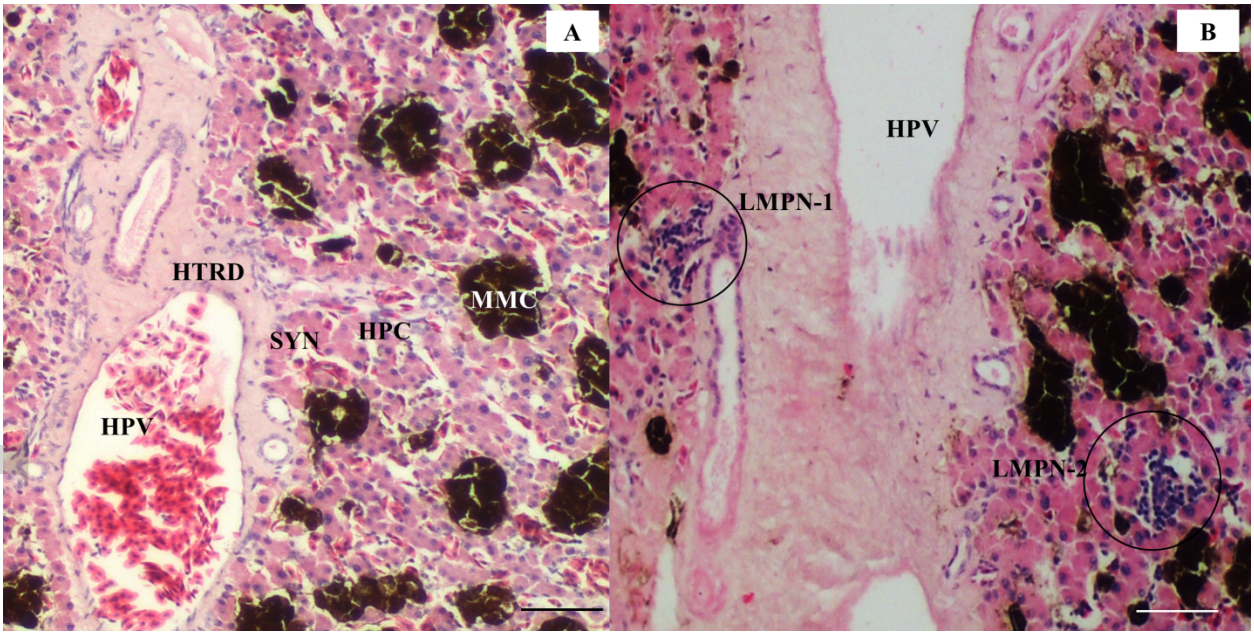

Plate1. Representative photomicrographs of the liver tissue of frog showing (A) general morphology of *E. hexadactylus* liver with hepatocytes (HPC) arranged in double cords, sinusoidal spaces (SYN) spread in between, blackish brown aggregates of melanomacrophage centres (MMC), hepatic triads (HTRD) containing branches of hepatic portal vein (HPV), biliary duct (BD) and hepatic artery (HA); (B) localisation of haematopoetic/ lymphoid cells aggregates (LMPN) near the HPV (LMPN1) and among hepatocytes (LMPN2). Scale bar represents 150 µm.

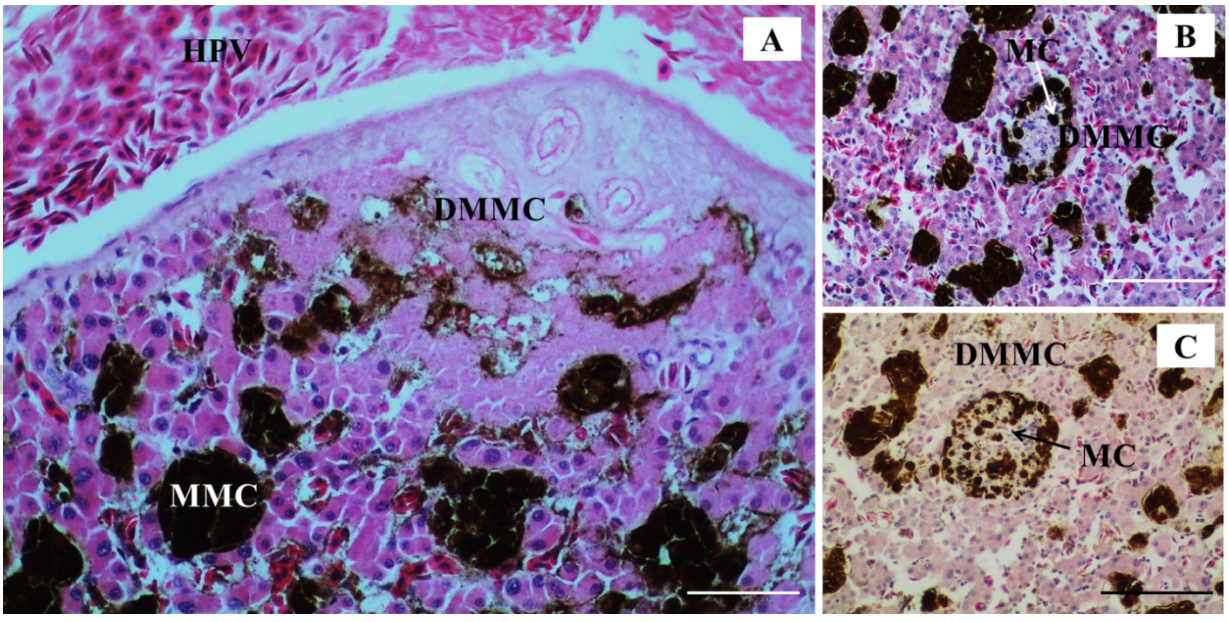

Plate 2. Representative photomicrographs showing the development of melanomacrophage centres (MMC). (A) Developing MMCs (DMMC) appeared as light brown nodules near the hepatic portal vein (HPV); (B & C) Developing MM centre with small melanocytes (MC) aggregating (Scale bar represents 150 µm).

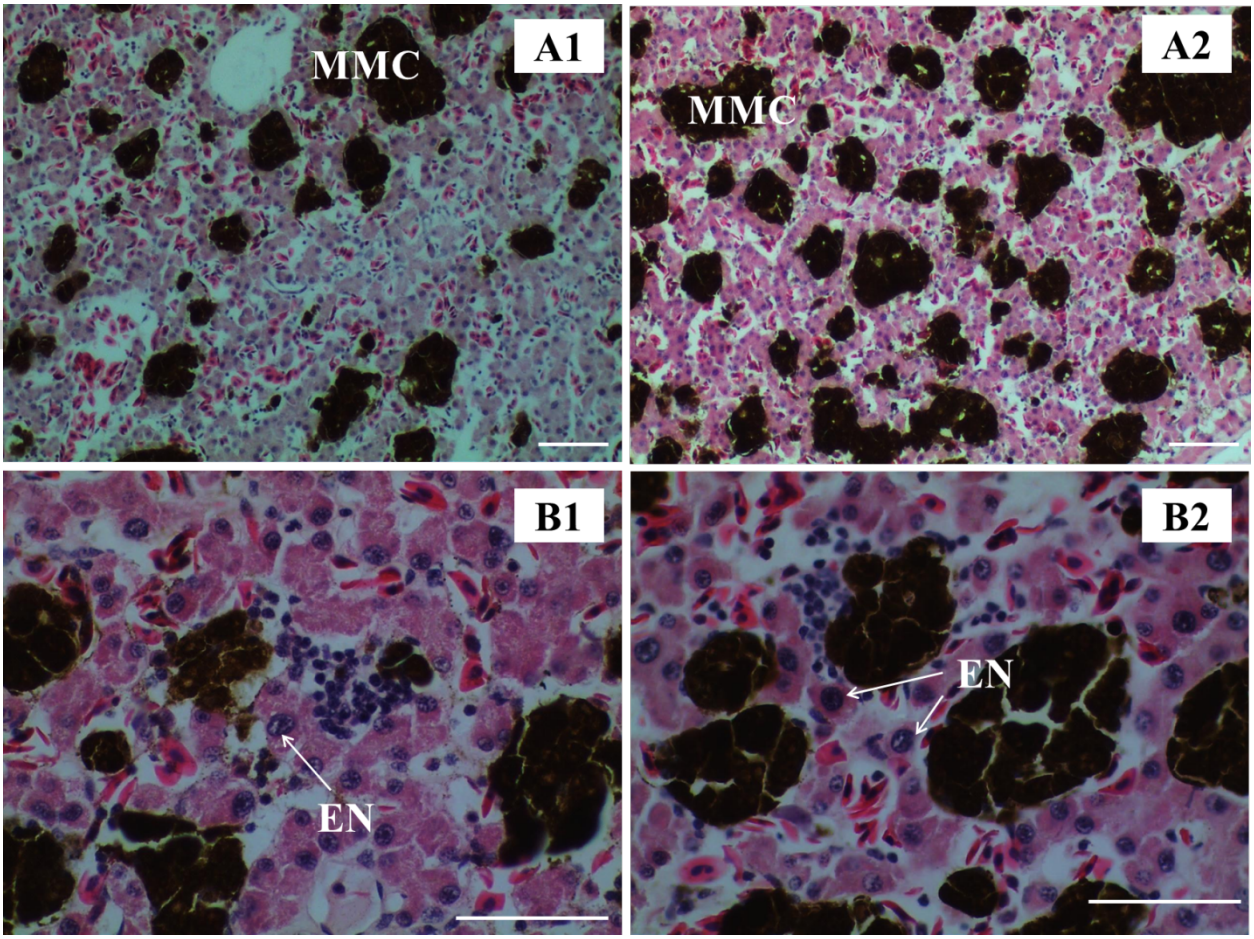

Plate 3. Representative photomicrographs of *E. hexadactylus* liver cross sections showing (A1) *Kupffer cell* hypertrophy, aggregation of low % MMC in reference tissue, (A2) high % MMC in metal exposed tissue and karyocytomegaly, enlargement of the hepatocyte nuclei (EN) in metal exposed tissues (B1-B2; Scale bar, 200 µm).

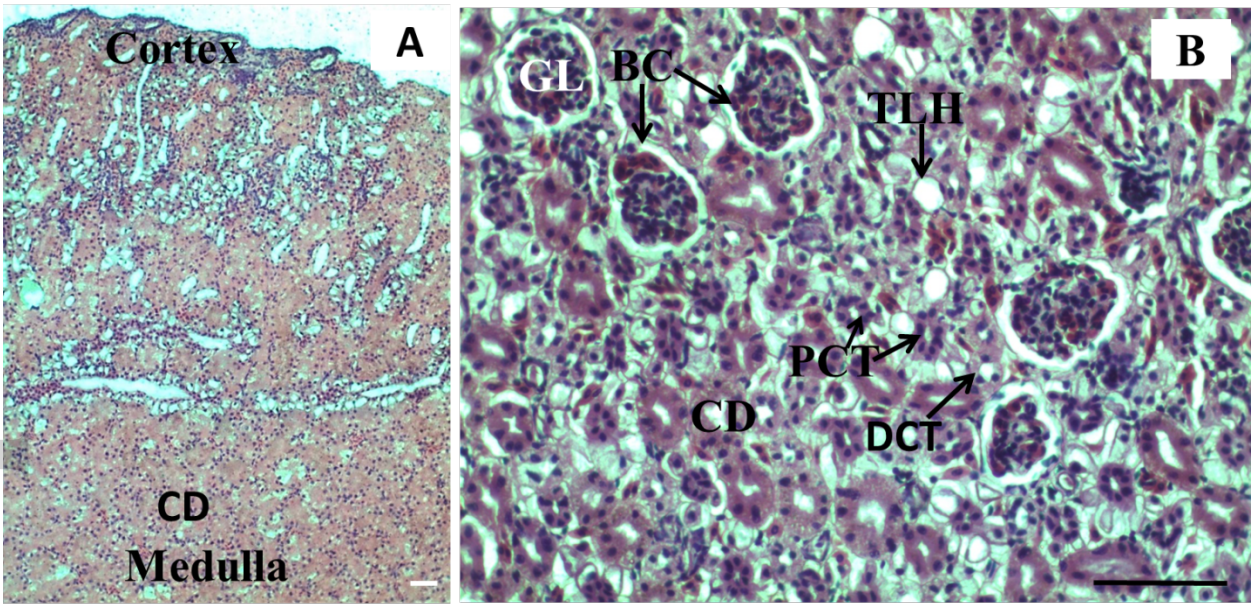

Plate 4. Representative photomicrographs of kidney tissue of *E.hexadactylus* showing (A) tissue arrangement in cortex and the medulla; (B) cross sections of reference kidney with glomerulus (GL), Bowmen`s capsules (BC), thin loop of henlae (TLH), collecting ducts (CD), proximal convoluted tubules (PCT), distal convoluted tubules (DCT) (Scale bar represents 150 µm).

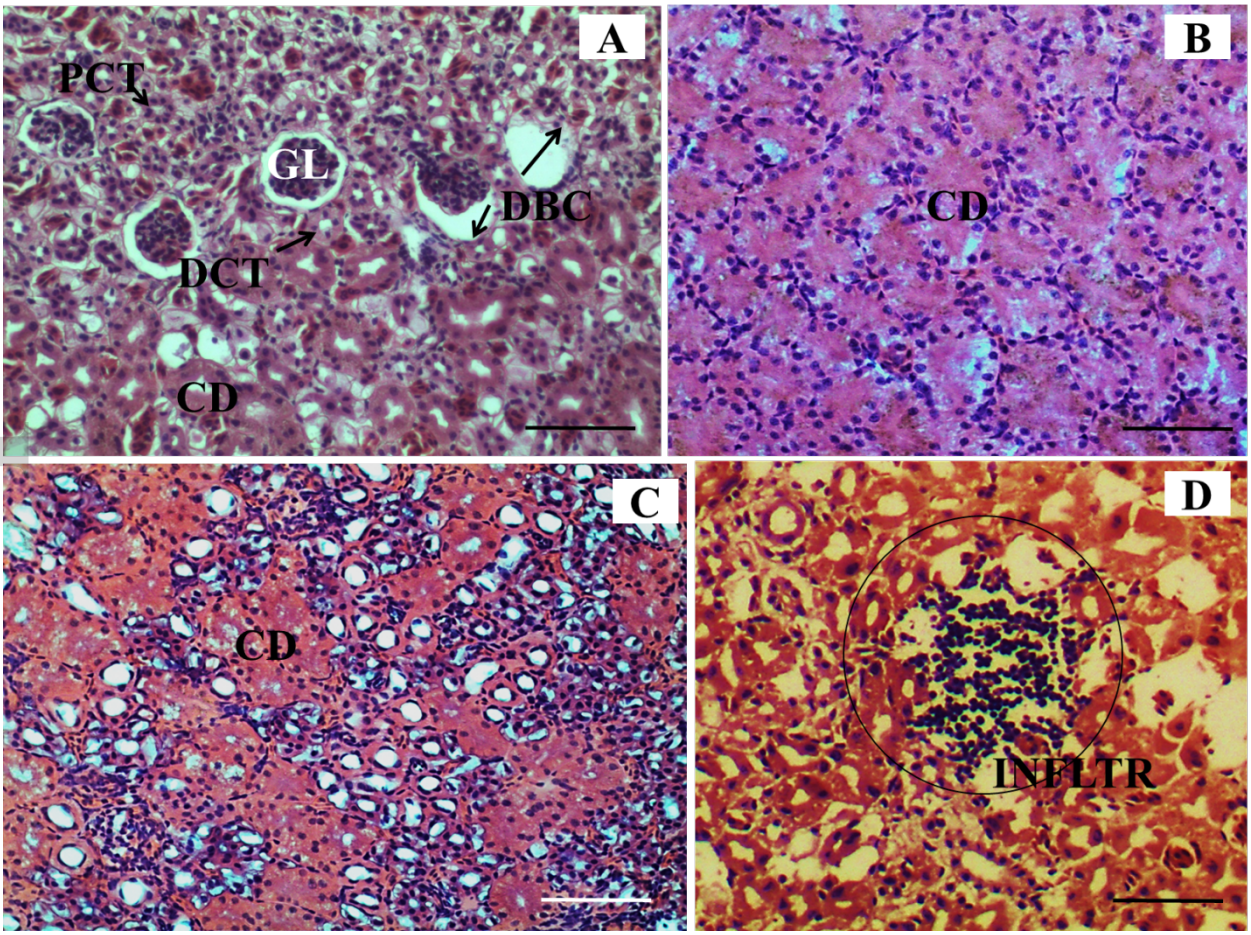

Plate 5. Representative photomicrographs of kidney tissue of metal exposed *E. hexadactylus* showing (A) tubular degeneration, tubular necrosis with dialted Bowman`s capsule (DBC), glomerulus (GL), collecting ducts (CD), proximal convoluted tubules (PCT), distal convoluted tubules (DCT), (B) Chronic progressive *Nephropathy* showing tubular crowding of collecting ducts (CD), and (C) basophilic cell aggregates (D), Inflammation in the kidney infiltrate (INFLTR) (Scale bar represents 150 µm).

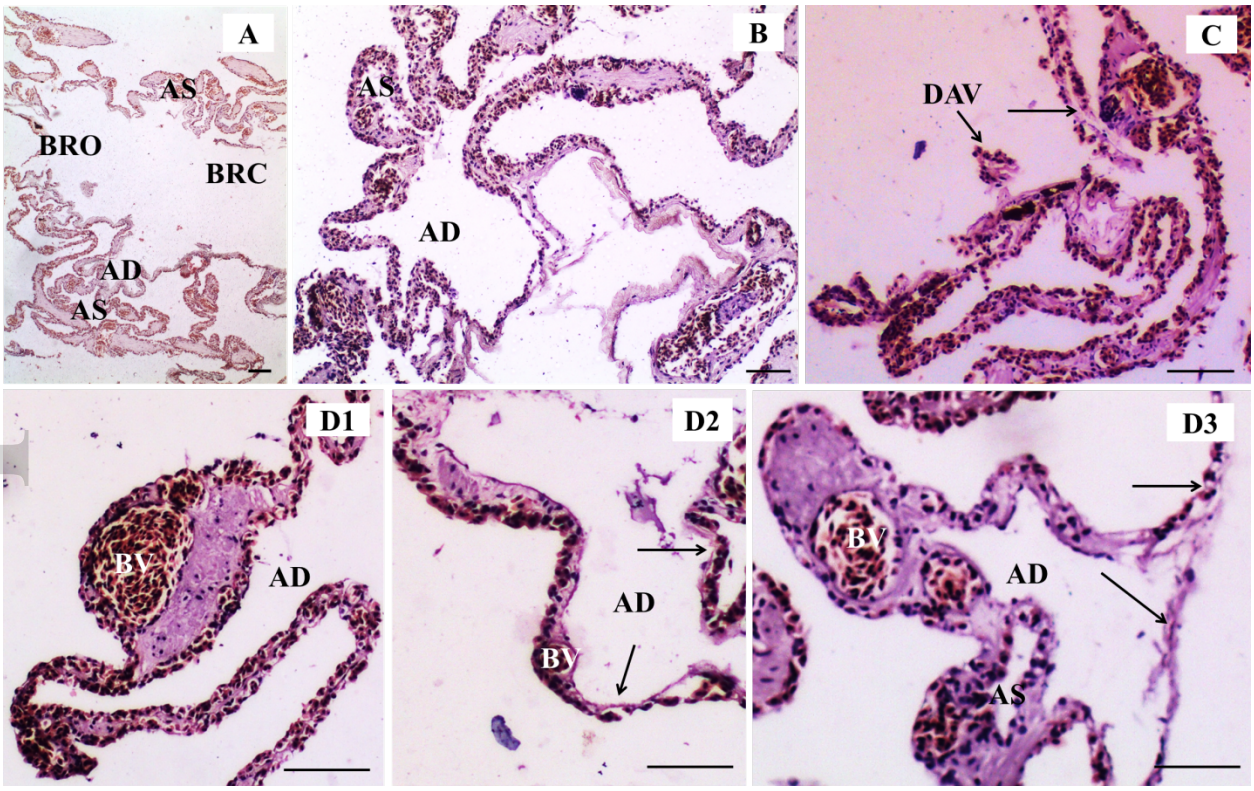

Plate 6. Representative photomicrographs of cross sections of the lung tissue of *E.hexadactylus* showing (A & B) reference tissue with bronchus (BRC), bronchiole (BRO), alveolar ducts (AD) and alveolar sacs (AS); (C) affected lung tissue of metal exposed frogs showing degeneration of epithelia and necrosis, damaged alveolar wall (DAV); (D1-D3) Alveolar degeneration (D1) healthy and (D2 and D3) heavy metal exposed frogs with degenerating alveolar wall (arrows point) otherthan the areas containing blood vessels (BV). Scale bar represents 120 µm)

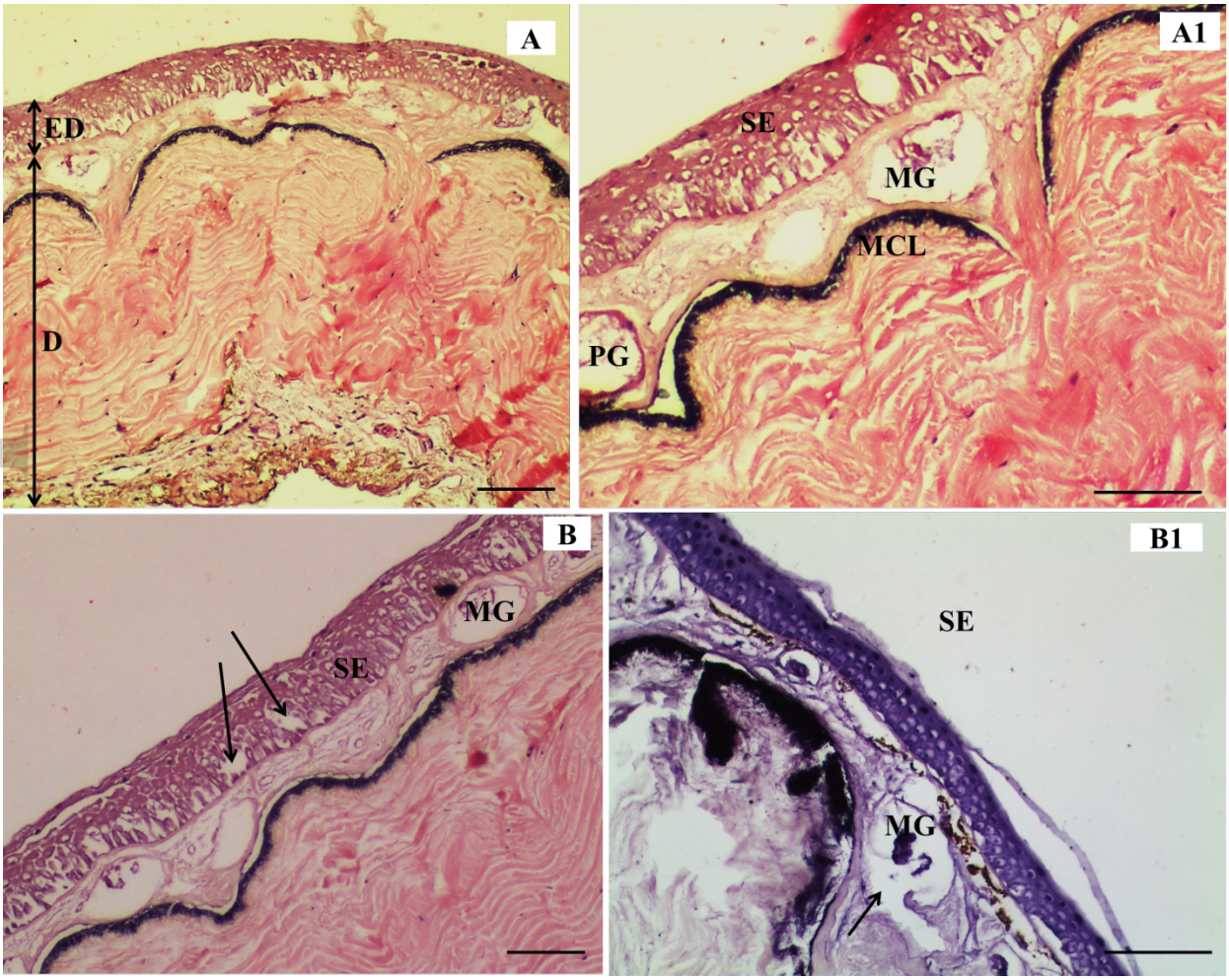

Plate 7. Representative photomicrographs of cross sections of the skin of *E.hexadactylus* showing (A) epidermis and the dermis (A1) reference tissue with squamous epithelium (SE) in the epidermis, poison gland (PG) and mucous gland (MG) located below the epidermis, melanocyte layer (MCL) located just below the epidermis; (B) affected skin sections of metal exposed frogs showing *Atrophy- epidermal* (Epidermal thinning) nor *Atrophy- adnexal* (glandular cell damage) with damaged squamous epithelim (arrow heads) (B1) damaged glandular cells (arrow head). Scale bar represents 130 µm.