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Hepatic Alteration of the Egyptian Toad Amietophrynus Regularis, as Biomarker to Environmental Deterioration

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Abstract: Based on available toxicological studies dealt with aquatic fauna in Egypt, none of these studies considered terrestrial stage of amphibian exposure and uptake of metal from surroundings habitat. Little is known about how contaminants affect amphibians at the population level. Three sites were selected scarily in order to comprise different types and sources of pollutants from one hand, and to prove the potential of selected animals to accumulate pollutants. The common Egyptian toad Amietophrynus Regularis was collected from different localities along the river Nile. The aim of our study was to evaluate the biochemical and histopathological biomarkers in this specie to a pollution gradient caused by domestic discharges and heavy metals and to verify the adequacy of the studied animals as appropriate bioindicator in their habitat. The results of this study revealed that deterioration of natural habitat of the selected amphibian produced manifested biochemical and histopathological changes in the liver.

Keywords: Amietophrynus Regularis, Histopathology, Heavy Metals

1. Introduction

The river Nile is the artery of the life in Egypt. As a sequence of increasing human activities, chemical pollution increased and adversely affected terrestrial and aquatic environments [1,2]. Among the more toxic pollutants are heavy metals [3]. Due to their toxicity and accumulation, heavy metals have gained a great ecological consideration [4]. One of the presumptions concerning the bioaccumulation of metals in amphibians is that the only significant route of metal uptake for terrestrial stage individuals is through diet and gaseous absorption of volatilized metals [5]. In some respects this assumption makes sense, metals tend to bind tightly to soil constituents and the skin of every vertebrate is a specialized barrier that only selectively allows transfer of elements and compounds from one side to the other [6]. Globally; many amphibian populations and species have declined or disappeared in the last few decades [7]. Chemical pollution was suggested to be one of the causative factors of amphibian declines [8]. The concept of heavy metals in fresh water ecosystem was reviewed in many previous studies. For example, [9,10] measured the concentration of (Fe, Mn, Zn, Cu, Ni, Co, Pb and Cd), to evaluate the river Nile water quality from Idfo to Cairo. Locally, [9] studied the pollution status of some areas along the river Nile, Egypt using Oreochromis Niloticus liver biochemistry and histology as biomarkers affected by heavy metals. References [11,12,13] investigated the water quality and heavy metal distribution along the Nile course from its start at Aswan to its estuary at Rosetta and Damietta. They evaluated the ectotoxicological border of the Nile pollution using two model species, Nile tilapia and African catfish considering physiological, biochemical, histological, and individual biomarkers. The damaging effect of heavy metals on amphibians has reviewed in several works e.g. [14,15,16,17,18]. Regarding their dual life and environmental impact, toads are exposing to terrestrial stressors. Despite their tendency to be stable and attached to the soil components, accurately predicting

terrestrial exposure to metals in soil remains problematic and is not well characterized [6]. Recently, agricultural soils contamination with heavy metal has markedly increased [19]. The composition of soil and the bioavailability of metals in the soil pore water solution were previously reviewed as important factors, governing the metal uptake and toxicity [20,21]. It is known that toads have been shown to discriminate between soils and prefer those with lower water withdrawing capacities, in addition adjust their tissues osmolarity to prevent dehydration by allowing water entry. Consequently, dissolved heavy metals will be taken up across this pathway from the soil porewater[6].

2. Materials and Methods

2.1. Study area: Three governorates (Sohag (site 1), Assiut (site 2) and El–Gharbia (site 3) were selected. Site 1: along the main stream at Tahta city (Sohag)Site 2: along Arab Al – Madabegh canal (Assiut) and Site3: along the main stream at Damietta branch, Zefta city (El–Gharbia) (Figure1) were selected to cover the purpose of the current work. The amphibian *Amietophrynus regularis* (5 individuals from each site) were collected in November 2010. This replicate was applied for water and soil samples.

2.1. Water Criteria

Some ecological parameters including water temperature (ºC), water pH, dissolved oxygen, O2 (mg/l) were measured by using water checker U–10 Horiba Ltd. In addition, water samples were taken in polyethylene bottles to determine totalsolids, TS (mg/l), nitrates No3 (mg/l) and total organic carbon, TOC (mg/l) were determined according to the standard methods [22].

2.2. Heavy Metals

Seven heavy metals were determined in water, agricultural soil and liver; Cadmium (Cd), lead (Pb), zinc (Zn), copper (Cu), chromium (Cr), Iron (Fe) and manganese (Mn) as follows:

2.2.1. In Water

Water samples were kept into acid–washed polyethylene bottle, acidified with nitric acid drops and transported to laboratory and stored until analysis. Water samples were digested according the method of [23].Water was digested by adding 5 ml of HNO_3 (65%) to 95 ml of water and heated until around 10 ml of the initial solution was obtained. This concentrate was transferred in a clean 100–ml flask; the digestion bottle was then rinsed three times with volumes of 20 ml of double distilled water, which were added to the concentrate, and finally the flask was filled up to the mark with distilled water.

2.2.2. In Agricultural Soil

In agricultural areas, three sediment cores were collected from three points; sediment was scooped from the top strata of sediment (10 cm) using 500 ml chemically clean glass jars according to [24]. In the laboratory, sediment samples were air dried, ground in fine mixture using mortar and pestle before sieved under 2 mm mesh. The samples were then stored in a polythene container ready for digestion and analysis [25]. For analysis, a mass of 1 g was digested using a mixture of HCl 37%, HNO_3 65% and H_2O_2 30% [26]. A volume of 3:1 ml concentrated nitric acid: hydrochloric acid was added to Pyrex tube containing soil sample. Mixture was heated gently till solution reduced to about 1ml, cooled at room temperature; then additional volume of digestive acids were repeated after each cool (3 times). Thereafter, 0.5 ml hydrogen peroxide H_2O_2 (30%) was added and the solution was heated and the volume was reduced to 0.5–1 ml. The addition of acid and hydrogen peroxide was repeated until the solution turns clear. Finally, the digested sample was diluted with bidistilled water to 10 ml and analyzed as the same for tissues below.

2.2.3. In Liver of Studied Animals

The liver was selected as the target organs for metal accumulation. After dissection of animals, tissues were stored in plastic bags in a freezer. Digestion of freezed tissues was performed according to [28], [29,30] with some modification (amount of chemicals and tissue samples). Concentrated nitric acid HNO3 (10 ml; trace metal grade) was added to 0.5 gm of the frozen tissue. The tissue was then heated gently till boiling and vortexed to help the tissue solubilization. The dissolved tissue was cooled at room temperature and additional 0.5 ml of the concentrated nitric acid was added. Then, the solution was heated until it starts to turn brown. The solubilized tissue was cooled. A third addition of 0.2 ml nitric acid was done and the volume was reduced to approximately 1 ml. Thereafter, 0.2 ml hydrogen peroxide H_2O_2 (30%) was added and the solution was heated and the volume was reduced to 0.5–1 ml. The addition of 0.2 ml concentrated nitric acid and 0.2 ml hydrogen peroxide was repeated until the solution turns clear. Then, 0.2 ml concentrated hydrochloric acid HCl was added and the solution was heated till the volume was reduced to 0.5 ml. Finally, the digested tissue was diluted with bidistilled water to 10 ml and analyzed. Heavy metal concentrations in water, tissues and sediments were determined by a Perkin–Elmer spectrometer with a specific–hollow cathode lamp for each metal. The metal concentration was calculated in µg/g wet weight for tissue, µg/g for sediment and µg/l for water. Metal concentration in tissue =

$$\frac{Reading \times dilution}{Weight\ (g)}$$

2.2. Histopathology

Liver were dissected out, fixed in 10% neutral formalin for 24 hours and then processed by conventional method [27], sectioned at 4-7 um and stained with Haematoxylin-eosine[12]. The sections were observed under light microscopy. The histological changes in the liver were examined in the randomly selected sections from each fish

and photographed using Leica Wild MPS48 microscope and CCD video camera (Sony, AVT-Horn).

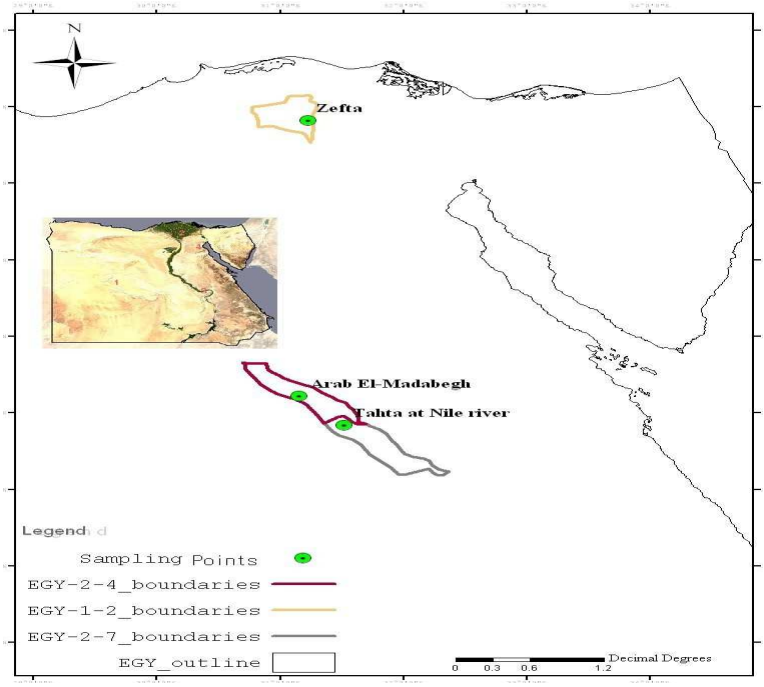

Figure 1. *Map showing the three selected localities for the current study.*

4. Results

4.1. Water Criteria

In the present work, the water temperature exhibited narrow fluctuation between the three sites. It fluctuated between (25.33 ºC), (27.66 ºC) and (23 ºC) in site 1, 2 and 3, respectively. The highest pH of water was recorded in site 1(8.36), followed by (8.20) and (7.13) in site 3 and 2, respectively. The lowest dissolved oxygen O2 (1.3 mg/l) was detected in site 2, and increased at site 3 (5.95 mg/l) and site 1(7.16 mg/l). Total solids (TS) showed remarkable increase from (196.663mg/l) in site 1, (223.663mg/l) in site 2 and (266.663mg/l) in site 3. The mean concentration of nitrates NO_3 was (1.13 mg/l) in site 1, (1.9 mg/l) in site 2 and (3.40 mg/l) in site 3. The total organic carbon (TOC) was observed in the samples from site 2 (57.33 mg/l), then exhibited a rapid decrease in site 3 (3.01 mg/l) and site 1 (0.44 mg/l). Mean ± standard deviation SD of water physico-chemical parameters at different sites were tabulated at Table 1.

Table 1. *Means ±SD of water physicochemical parameters at the investigated sites.*

Parameter	Site 1	Site 2	Site 3
Temperature	25.33±0.30	27.66±3.05	23±1.80
pH	8.36±0.15	7.13±0.05	8.20±0.2
Dissolved oxygen	7.16±0.12	3.31±0.44	5.95±0.06
Total solids	196.66±1.52	223.66±1.39	266.66±2.8
Nitrates	1.13±0.23	1.9±0.45	3.40±0.12
Total organic carbon	0.44±0.26	57.33±1.50	3.01±0.07

4.2. Heavy Metals

4.2.1. In Water (Table 2)

Site 2 showed high water metal level compared with site 1 and site 3, except in the case of iron (818.33±1.12 µg/l at site 3, 625.66±1.70 µg/l at site 2 and 124.33±4.04 µg/l at site 1). Results indicated that metals concentrations in water (Figure 3) were found in the following order: Fe > Zn > Cu >Mn> Cr >Pb = Cd in site 1, whereas they follow the order of Fe > Cu > Zn >Mn>Pb> Cr > Cd in site 2. In site 3, metals had the sequence of Zn > Fe > Cu >Mn>Pb> Cd > Cr.

Table 2. *Means ±SD of heavy metals concentrations (µg/l) in water at the investigated sites.*

Metal	Site 1	Site 2	Site 3
Cd	000±000	4.83±4.30	3.93±0.20
Pb	000±000	49.33±1.35	9.20±0.52
Zn	87.33±2.51	126.66±3.21	1395.33±5.23
Cu	83.66±2.51	444.66±2.50	420.66±3.50
Cr	0.25±0.00	36.66±1.54	3.73±0.41
Fe	124.33±4.04	625.66±1.70	818.33±1.12
Mn	23±1.20	59.66±8.73	19±2

4.2.2. In Soil (Table 3)

The mean concentration of cadmium in soil analyzed from site 1 was (0.51 µg/g), followed by (0.41 µg/g) in site 2 then (0.67 µg/g) in site 3. The measured lead in site 2 was twice that in site 3 (6.01 µg/g), but it was (8 µg/g) in site 1. Zinc peaked at site 1 (74.90 µg/g), decreased in site 2 (46.79 µg/g) and increased again in site 3 (50.61 µg/g).Copper was higher in site 1 (37.81 µg/g) and showed closed values in site 2 (17.12 µg/g) and site 3 (17.89 µg/g). Similar to copper; chromium peaked in site 1 (15.52 µg/g), then exhibited a

closed values in site 2 (5.67 µg/g) and site 3 (5.73 µg/g).The lowest concentration of iron was observed in site 1 with mean (275.36 µg/g) and gave negligible differences between site 2 (287.87 µg/g) and site 3 (288.31 µg/g). Also manganese peaked at site 1 (158.19 µg/g), but dropped in site 3 with the mean (83.92 µg/g) and site 2 with the mean (71.62 µg/g).

Table 3. *Means ±SD of heavy metals concentrations (µg/g) in agricultural soils of investigated sites.*

Metal	Site 1	Site 2	Site 3
Cd	0.51±0.26	0.41±0.19	0.67±0.11
Pb	8±3.70	12.03±0.6	6.01±1.37
Zn	74.90±4.80	46.79±8.27	50.61±1.02
Cu	37.81±0.67	17.12±5.34	17.89±2.76
Cr	15.52±0.45	5.67±3	5.73±0.88
Fe	275.36±0.91	287.87±5.53	288.31±2.66
Mn	158.19±1.34	71.62±3.4	83.92±1.02

4.2.3. In Liver (Table 4)

Regarding the three sites, the mean concentration of cadmium in liver was (0.33 µg/g) in site 1, (0.96 µg/g) in site 2 and (1.23 µg/g) in site 3. Lead concentration was at maximum value in site 2(16.37 µg/g), but exhibited a similar values at site 1 (10.42 µg/g) and site 3 (10.90 µg/g). Zinc peaked in site 2 (104.43 µg/g), then declined in site 3 (84.78 µg/g) and site 1 (51.52 µg/g). Copper increased as (42.2 µg/g), (70.95 µg/g) and (114.52 µg/g) from site 1 to site 3. Chromium increased from (1.55 µg/g) in site 1 to (2.73 µg/g) in site 3 and (3.40 µg/g) in site 2. (172.46 µg/g), (333.14 µg/g) and (208.33 µg/g) were the values of iron at site 1, 2 and 3; respectively. The mean concentration of manganese was high in site 2 with value (7.64 µg/g), followed by (5.91 µg/g) and (4.20 µg/g), in site 3 and 1; respectively.

Table 4. *Means ±SD of heavy metals concentrations (µg/g) in liver of toads collected from the investigated sites.*

Metal	**Site 1**	**Site 2**	**Site 3**
Cd	0.33±0.18	0.96±0.57	1.23±0.17
Pb	10.42±2.50	16.37±4.12	10.90±2.05
Zn	51.52±4.03	104.43±7.05	84.78±2.3
Cu	42.02±3.02	70.95±2	114.52±4.8
Cr	1.55±0.87	3.40±1.16	2.73±0.22
Fe	172.46±2.30	333.14±1.01	208.33±4.1
Mn	4.20±0.40	7.64±0.72	5.91±1.73

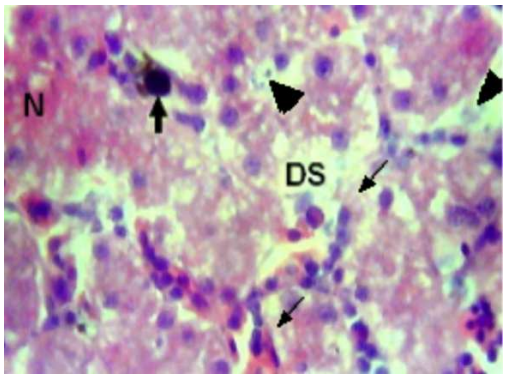

Figure 2. *Section in the liver of site 1 animals had a mild ulceration including dilation of sinusoids (DS), degenerated hepatocytes (thin arrows), parenchymal deposits (thick arrow), necrotic parenchyma (N), granular deposits (arrow heads), (H & E stain x400).*

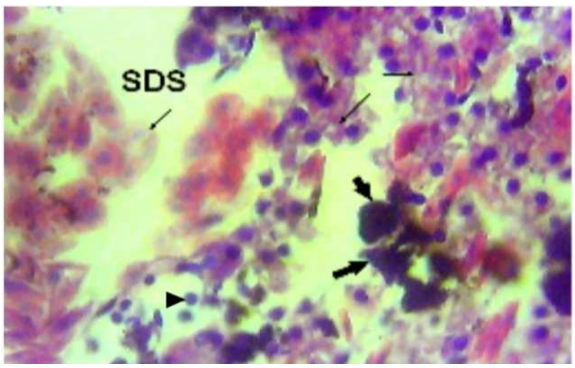

Figure 3. *Section in the liver of site 2 animals showing severe dilation of sinusoids (SDS), parenchymal deposits (thick arrows), vacuolated hepatocytes (thin arrows), hepatocytes with altered cytoplasm membrane (arrow-head), (H & E stain x400).*

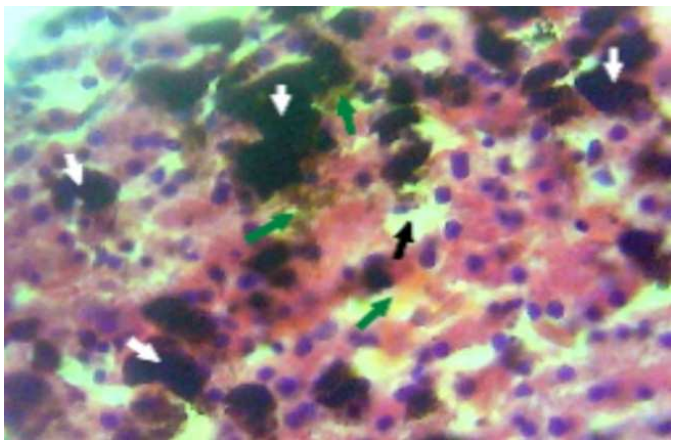

Figure 4. *Section in the liver of site 3 animals with multiple disorders including intensive parenchymal deposits (white arrows), excessive bile secretions (green arrows) and dilation of sinusoids (H & E stain x400).*

5. Histopathological Observations (Figures 2-4)

Based on light microscope investigation, the toad liver (site 1 samples) showed normal structure with some lesions (Figure 2). Regarding site 2 and site 3, the liver of investigated toads showed different degrees of disorders responding to impact degrees(Figures 3 and 4). Histopathological studies have revealed the presence of vacuolization of the cytoplasm, hepatic lesions such as fibrosis, inflammatory response, hepatic alterations necrotic and blood vessel congestion. Granular deposits, bile secretions, lipofuscin deposits were observed.

6. Discussion

Due to the excessive use of pesticides and fertilizers in agriculture, increasing discharge of heavily polluted domestic and industrial effluents into its waterways, the river Nile, Egypt is facing a rapid increase deterioration of its natural criteria [31]. Aluminum, agro-industrial, small private industries and sugar cane, in addition to many human activities on the coastal line of the Nile are the main source of Nile pollution in Upper Egypt. The Damietta branch also receives polluted water of a number of agricultural drains. The fertilizer company is considered as the major point source of industrial pollution at Damietta branch [31]. Generally, the increased pollution sources the decreased environmental quality the threaten life. In the present work, site 2 exhibited the lowest (Table 1) dissolved oxygen (3.31±0.44 mg/l) and the highest chemical oxygen demand (48±1 mg/l). These logic results attribute to the fact, that discharges of municipal wastes require higher amounts of oxygen for biodegradation of organic matters they have. Consequently, the availability of dissolved oxygen will decrease. Taking into account the mode of irrigation, the agricultural fields of site 2 are mainly depending on the various pollutants receiving water. From the morphological point of view, the crops of this site seem more prosperous, healthy and flourish; due to the richness of soils in organic matters coming from water. At this site, toads inhabit the agricultural lands which facilitate dermal contact to pollutants coming from water. On the other hand, toads perform their breeding activities in many water spots and pools, consequently, not only adults are facing impacted habitat, but also offspring expose directly. Most of the crops and vegetable species growing in metal polluted soils are unable to avoid the absorption of these metals [32]. Reference [33] stated that sludge used as fertilizers in agriculture has ability to induce genotoxic effect on the amphibian Xenopus Laevis. The present data of nitrates were higher than many previous records, for example [34,35,8,18,36,9,37,38].

According to world health organization, [39], Nitrate can reach both surface water and groundwater as a consequence of agricultural activity (including excess application of inorganic nitrogenous fertilizers and manures), from wastewater treatment and from oxidation of nitrogenous waste products in human and animal excreta, including septic tanks. Nitrite can also be formed chemically in distribution pipes by Nitrosomonas bacteria during stagnation of nitrate-containing and oxygen-poor drinking-water in galvanized steel pipes or if chloramination is used to provide a residual disinfectant and the process is not sufficiently well controlled [40,41]. Statistically, the physic-chemiclal parameters were significantly differed among the three sites (Data not tabulated). Intensive blooms of algae (Eutrophication) were observed at site 2. This level of eutrophication could be attributed to the higher water fertility enriched by nitrates and other nitrogenous compounds coming from agricultural fertilization. Since the mobility of water is slow at second site, this will reduce the process of recovery leading to bioavailability of pollutants and hence accumulation. In the present work the concentrations of Pb, Zn and Cu are higher than those measured by [6], but she recorded higher concentrations of Mn and Fe. In agreement with [6] and [58], higher concentration of Mn and Fe were determined. The present data of cadmium and lead are in coordination with [42], but they measured lower concentration of Zn, Cu, Cr, Fe and Mn compared to the present ones. The metal uptake from solution into aquatic organisms at their permeable surfaces is generally considered to be passive process not requiring the expenditure of energy [43,44,45] that might be controlled by the affinity of some metal ions for proteins and other cellular constituents [46]. Based on this assumption, the dissolved metal ions are bound on to transport protein from the external medium and the element will be transferred through the permeable membrane into the cell internally binding finally with ligand of high metal affinity [46]. Ecologically, up take of heavy metals by toads may affect them directly causing death or may be accumulated within their tissue, then pass on via predation to other organisms in the food web [6]. In the present work the mean concentration of copper and zinc are higher than those resulted by [32] from tissue of the caudate amphibian Proteus Anguinus. They found that the mean concentrations of copper ranged as (1.49 – 8.61 µg/g) in liver , while zinc was (6.40 – 49.17 µg/g) in liver. Several heavy metals lead to clastogenic effects as a result of DNA breakage and can induce the generation of reactive oxygen species (ROS), which can lead to biochemical, morphological, histopathological alterations and finally cell damage or death [47,48,49,50]. Vacuolation of the hepatocyte, pycnosis in many of the necrotic cells, necrosis of the parenchatous tissue, and disintegration of blood sinusoids [51] characterized the degenerative changes. Hepatocellular lipid vacuolation was previously resulted as a histopathological reaction to aquatic pollutants [52]. Totally, the studied metals here were found to accumulate in liver of animals collected from the second site compared to those collected from other sites (Table 5). The Degeneration and necrosis of hepatocytes may be attributed to the cumulative

effect of the metals and to the bimagnification and accumulation [53]. Among the observed histopathological lesions of liver, the melano-macrophage aggregates have been shown to be involved in a number of fish diseases, and as phagocytic cells [54]. Liver alteration observed in this work can be considered as a good biomarker for a long time environmental impact. This is due to the fact that tissue burdens of metals represent both past and recent impact, so that histopathological alterations of liver were considered here since it is more sensitive due to its biological functions. It must be taken into account that, liver ulcerations observed here may be caused not only by heavy metals but also many other toxicants were involved. Most previous works dealt with liver histopathology were focused of agricultural pesticides and insecticides; so these pollutants not only threat anurans in their terrestrial stages, but also in their aquatic stages through agricultural runoff into waterways. As it is known that heavy metals can't be degraded biologically and can persist in biotopes for long times, it was focused in the present work on its histopathological impact. Liver parenchyma stores large amounts of iron, which characterize installation of mesenchymal hemosiderosis, because iron is stored in Kupffer cells [55]. Lipofuscin, melanin (in large quantity) and ceroid (lipid like pigment) are found along with hemosiderin deposits. On the other hand, histological changes in pigmented macrophage aggregates (PMAs), suggest the involvement of these cells in the detoxification process. PMAs also known as melanomacrophage [56] aggregates or solely as macrophage aggregates, are distinctive groups of phagocytes, typically loaded with a yellowish to brown/black pigment content, supporting matrix of hematopoietic tissues, namely spleen and kidney, and also in other organs such as the liver and ovary [54]. PMAs are called so because of the presence of granular pigment. The content of the granular pigments varies, but the most frequent are melanin, hemosiderin and lipofuscin [57,54]. High contents of hemosiderin can be found in relevant to pollution or toxicants impacts which induce hemolytic disorders [58,54]). Hemosiderin granules are normally in close association with lipofuscin granules [54].Unfortunately, the available stain used here was only H&E, so differentiation between those (Hemosiderin, lipofuscin and granular pigments) contents was based on pervious investigations; they were termed as hepatic deposits. Field studies also support the hypothesis that pollutants have effects on PMAs regulation. Reference [59] revealed that PMAs resulted from the fish shiners (Notropis Hudsonius) is a good biomarker for assessing healthy status of their river habitat, which were remarkably affected by pollution. In the present study, extensive fibrosis is presumed to be attributed to carcinogenesis [60]. On the other hand, the appearance of granular aggregations in liver parenchyma might be attributed to metal storage, this finding agrees with [29,30,61]. The production of such granules is common in all major phyla [62]. Reference [63] stated that zinc was accumulated in barnacle Elminius Modestus as detoxified pyrophosphate granules; also [64] demonstrated the presence of conspicuous aggregations of granules in the mantle, palps and visceral mass of the freshwater bivalve mussel Hyridella Depressa, through examination of light microscopic sections. These granules are thought to have number of functions including metal detoxification and storage of elements [62].

7. Conclusion

It could be concluded that Amietophrynus Regularis has the tendency to bioaccumulate heavy metals in a polluted environment. The present data indicated that double impact represented by heavy metals, synergistically with water parameters have ability to weaken and impair tissues of Amietophrynus Regularis. As a site of detoxification Liver has shown as different degrees of damage and deteriorations. Consequently, the histopathological changes observed here demonstrated a high accumulation capability of the investigated animals.

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