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Snails and Fish as Pollution Biomarkers in Lake Manzala and Laboratory C: Laboratory Exposed Snails to Chemical Mixtures

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Abstract**—**Snails are considered as suitable diagnostic organisms for heavy metal–contaminated sites. *Biomphalaria alexandrina* snails are used in this work as pollution bioindicators after exposure to chemical mixtures consisted of heavy metals (HM); zinc (Zn), copper (Cu) and lead (Pb); and persistent organic pollutants; Decabromodiphenyl ether 98% (D) and Aroclor 1254 (A). The impacts of these tested chemicals, individual and mixtures, on liver and kidney functions, antioxidant enzymes, complete blood picture, and tissue histology were studied. Results showed that Cu was proved to be the highly toxic against snails than Zn and Pb where LC_{50} values were 1.362, 213.198 and 277.396 ppm, respectively. Also, *B. alexandrina* snails exposed to the mixture of HM (¼ LC_5 Cu, Pb and Zn) showed the highest bioaccumulation of Cu and Zn in their whole tissue, the most significant increase in AST, ALT & ALP activities and the highest significant levels of total protein, albumin and globulin. Results showed significant alterations in CAT activity in snail tissue extracts while snail samples exposed to most experimental tests showed significant increase in GST activity. Snail samples that exposed to HM mixtures showed a significant decrease in total hemocytes count while snail samples that exposed to mixtures containing A & D showed a significant increase in total hemocytes and Hyalinocytes. Histopathological alterations in snail samples exposed to individual HM and their mixtures for 4 weeks showed degeneration, edema, hyper trophy and vaculation in head-foot muscle, degeneration and necrotic changes in the digestive gland and accumulation in most tested organs. Also, the hermaphrodite gland showed mature ova with irregular shape and reduction in sperm number. In conclusion, the resulted damage and alterations in *B. alexandrina* studied parameters can be used as bioindicators to the presence of pollutants in its habitats.

Keywords**—***Biomphalaria,* Zn, Cu, Pb, AST, ALT, ALP, total protein albumin, globulin, CAT and Histopathology.

I. Introduction

A biological monitor or pollution biomarker is early diagnostic tool for biological effect measurement and environmental quality assessment and algae, macroinvertebrates and fish are considered as important indicators [1]-[3]. The most important HMs that polluted water are Zn, Cu, Pb, cadmium (Cd), mercury (Hg), nickel (Ni) and chromium (Cr). Some of these metals like Cu and Zn are essential to aquatic animals when they are found in trace amounts but become toxic at higher concentrations while others, such as Pb and Cd are toxic elements and have no known biological function [4]-[6].

Decabromodiphenyl ether is the primary component of commercial deca-BDE (typically≥97%), which constitute approximately 80% of the world market demand for PBDEs [7]. It is widely used in textiles and plastics industries as an additive flame retardant and could be released into the environment [8]-[13].

Several investigators [14]-[20] showed that snails are able to accumulate large quantities of metals in their tissues. This intoxication of the snail is a result not only of soil ingestion [21] but also of epithelial absorption of the contaminants [22].

Because the enzymatic activities are regarded as reliable indices of individual responses to environmental stress, they can be used to estimate pollution at population and ecosystem levels [23]. The use of physiological and biochemical parameters as indicators of histopathological changes have been widely used as biomarkers in the health evaluation of animal organisms [24].

The present study aims to assess the impact of some chemical pollutants on *B. alexandrina* liver and kidney functions; antioxidant enzymes; complete blood picture and histological observations as chemical pollution bioindicators. The tested chemical pollutants were represented by HMs zinc sulphate; copper sulphate, lead nitrate, organic pollutant Decabromodiphenyl ether 98% (DBDPE) and Aroclor (A1254).

II. Materials and Methods

A. The Chemical Compounds

The chemicals zinc and copper sulphate were supplied by El-Nasr Pharmaceutical Chemicals Co., Abu Zaabal, Egypt. Pb(II) nitrate was supplied by Sigma-Aldrich, United Kingdom. A1254was supplied by Supelco analytical, Bellefonte, PA, USA. DBDPE was supplied by Aldrich Chemistry, USA. Tween 80 was used as organic dissolver for each of A1254 & DBDPE.

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B. The Tested Animals

B. alexandrina snails were collected from irrigation canals in Giza governorate. Unhealthy and infected snails were excluded. The uninfected and healthy snails (8-10 mm) were maintained under laboratory conditions for one month (25 ˚C ± 1, pH = 7.2) in plastic aquaria fed twice a week on green lettuce leaves and water was changed once a week [25].

C. Toxicity Screening

A stock solution of each chemical (Cu, Pb, Zn, A1254 and DBDPE) was prepared on the basis of W/V using dechlorinated tap water (pH 7.0–7.5). Series of 6 concentrations that would permit the computation of LC_{50} and LC_{90} values were prepared from each stock solution [26]. Three replicates were prepared for each concentration in one liter to which 10 snails for exposure period of 24 hrs. at 25±1 °C. Snails were transferred to de-chlorinated tap water for recovery for another 24 hrs. Also, control group of three replicates were maintained in de-chlorinated tap water under the same experimental conditions as the exposed groups. Snails' mortalities in the various tests were corrected for control mortality using Abbott's Formula according to [27] for the calculation of the limits of confidence of the LC_5; LC_{10}; LC_{25}; LC_{50} and LC_{90}.

D. Biochemical and Histological Studies

Groups of 50 snails were exposed four weeks to ten different treatments of sub-lethal concentrations of individual and mixtures of Cu, Pb, Zn, A, and D (LC_{25}Cu, LC_{25} Pb, ¼ LC_{25} Zn, ¼ LC_5 Cu, Pb & Zn mixture, ¼ LC_{10} Cu, Pb & Zn mixture, A [25 ppm], D [25 ppm], AD mixture [10 ppm], AD mixture [25 ppm] and HMs and POPs mixture [D&A (2.5ppm) + ¼ LC_5 Cu, Pb & Zn]). Control groups were maintained under the same experimental conditions but without exposure to the tested material. After four weeks, specimens were taken for the following examinations.

1) Determination of Liver and Kidney Functions

The assessment of aspartate aminotransferase (AST), alanine aminotransferase (ALT), urea, creatinine, alkaline phosphatase (ALP), total and direct bilirubin (TB, DB), albumin (ALB), total protein (TP) were made in snail tissue extract samples. They were assayed biochemically using biosystem auto-analyzer, Backmann at Theodor Bilhaz Institute hospital laboratories. Snails were dissected then; each snail tissue from each treatment was homogenized in bi-distilled water (1:1 w/v) using motor homogenizer. Homogenates were centrifuged at 5000 rpm for 20 min at 4 ºC and the supernatants were taken and kept at -20 ºC till used as described by [28]. Urea was determined according to [29].

2) Determination of Antioxidant Enzymes

The activity of antioxidant enzymes; catalase, glutathione-s-transferase (GST) and gamma glutamyl transferase (GGT); were assayed using spectrophotometer in snail tissue extracts.

3) Determination of Complete Blood Component:

Hemolymph of treated and control snails was collected in accordance with techniques described by [30]. Total blood count was determined by Coulter Counter apparatus while differential blood count was determined by haemocytometer.

E. Histological Studies

Snail samples from control and exposed groups were dissected, gently removed from their shells then fixed in 10% buffered neutral formalin solution. Paraffin sections were prepared, stained with dyes; hematoxylin and eosin (HE); then microscopically examined and photographed to record histopathological observations [31].

F. Statistical Analysis

The results were computed statistically by SPSS software package, version 20; data are expressed as means ± SD and compared using T-test analysis and values of $p<0.05$ were considered statistically significant.

III. Results

The Probit analysis of the toxicity of POPs; DBDPE and A1254and HMs (Cu, Pb and Zn) against *B. alexandrina* snails are presented in Table I. Results indicated that both D and A are not toxic to snails till 500 mg/L while Cu was the highly toxic against snails than Zn and Pb; where their LC_{50} were 1.362, 213.198 and 277.396 ppm, respectively.

A. Effect of Tested Compounds on Snails' Survival Rate

Fig. 1 shows that the survival rate in snails exposed 4 weeks to the POPs was higher than that in snails exposed to HMs.

The impact of exposure to sub-lethal concentrations of the tested compounds in different modes for a period of 4 weeks on Pb, Cu and Zn bioaccumulation were determined in whole soft body tissue of *B. alexandrina* snails (Table II). Results of bioaccumulation in whole tissue of *B. alexandrina* showed that the highest Cu and Zn bioaccumulation recorded in the treatment in which snails exposed to the mixture of ¼ LC_5 Cu, Pb & Zn (108.907 = 45.4 folds).

B. Effect of Tested Compounds on Snails' Biochemical Parameters

Liver and Kidney Functions

Table III illustrates that snail group which exposed to HM mixture of LC_5 showed the highest significant activities of AST (159.7±3) and ALT (302.0±11), while snail group exposed to 25 ppm A showed the highest significant activity of ALP (181.3±11). The highest significant creatinine concentration (Table IV) was recorded in the snail group which exposed to HM-mixture of LC_5 concentration (16.25±1.4) followed by LC_{25} of Cu (5.70±0.63). Urea concentration showed significant increase in snails exposed to ¼ LC_{10} Cu, Pb & Zn (51.9±12) and high significance in snail group exposed to A 25 ppm (48.6±3.2). The determination of *TP*, *ALB*, globulin, A/G ratio & glucose in tissue extract of snails exposed to Cu, Pb, Zn, A and D through different experimental designs was illustrated in Table V. Snail group

exposed to ¼ LC_5 Cu, Pb & Zn showed very highly significant increase in *TP* concentration (37.78±0.3) and groups exposed to AD 25 ppm, D 25 ppm and A 25 ppm showed highly significant increase (25.21±2.1, 15.49±10.4 & 21.75±1.3, respectively). Also, *ALB* showed significant increase in group exposed to ¼ LC_5 Cu, Pb & Zn, AD 25 ppm and D 25 ppm (Table V). In one hand, globulin recorded significant increase in the group exposed to LC_{25} ppm of Pb; AD and A and ¼ LC_5 Cu, Pb & Zn (10.12±4.13, 11.08±5.80, 9.24±0.61 and 21.36±0.92, respectively). On the other hand, there are no significant differences in the A/G ratios (Table V).

TABLE I
TOXIC EFFECT OF DBDPE, AROCOLR 1254 (A1254), PB, CU AND ZN AGAINST *BIOMPHALARIA ALEXANDRINA* SNAILS AFTER 24 H EXPOSURE

Chemicals	LC_5	LC_{10}	LC_{25}	LC_{50}	LC_{90}	Slope
DBDPE	Not toxic till 500 mg/L					
A 1254	Not toxic till 2000 mg/L					
Cu	0.051	0.087	0.691	1.362	2.637	2.94
Pb	177.290	199.401	236.347	277.396	355.391	1.26
Zn	94.725	120.893	164.617	213.198	305.503	1.44

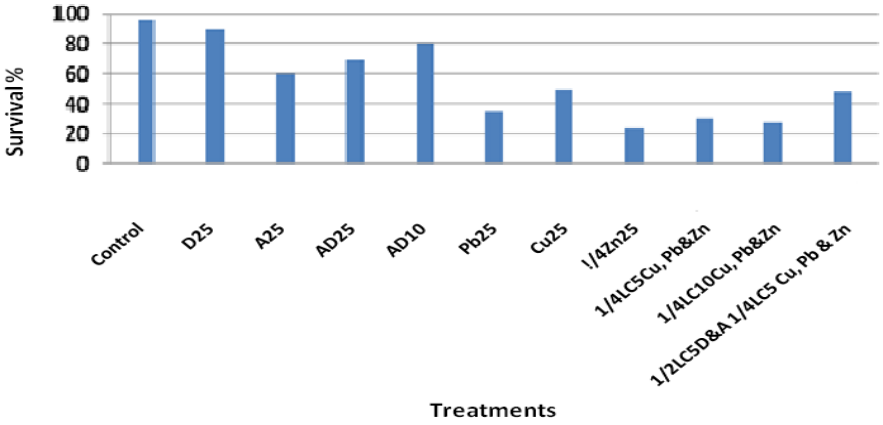

Fig. 1 The survival rate in *B. alexandrina* snails exposed to Cu, Pb, Zn, Aroclor 1254 (A), and DBDPE 98% (D) for a period of 4 weeks

TABLE II
THE BIOACCUMULATION OF PB, CU, ZN AND THEIR FOLDS COMPARED TO CONTROL IN *B. ALEXANDRINA* SNAILS TISSUE AFTER DIFFERENT TREATMENTS.

Concentration (ppm)	Pb	Folds	Cu	Folds	Zn	Folds
Control unexposed	19.76		0.96		14.93	
LC_{25} (Cu or Pb) or ¼ LC_{25} (Zn)	90.53	4.58	28.28	29.52	213.51	14.31
Mix of ¼ LC_5 (Cu, Pb & Zn)	85.14	4.31	104.32	108.907	398.55	45.40
Mix of ¼ LC_{10} (Cu, Pb & Zn)	69.86	3.54	3.36	3.51	182.34	12.22
Mix of ½ LC_5 D&A +¼ LC_5 (Cu, Pb & Zn)	76.24	3.86	8.65	9.03	148.20	9.93

TABLE III
AST, *ALT* AND *ALP* IN TISSUE EXTRACTS OF *BIOMPHALARIA ALEXANDRINA*; EXPOSED TO DIFFERENT TREATMENTS OF CU, PB, ZN, AROCLOR 1254 (A) AND DBDPE 98% (D)

Treatments	AST (Units/ml)	Change %	ALT (Units/ml)	Change %	ALP (IU/L)	Change %
Control unexposed	27.0±1		49.5±2		53.5±5	
LC_{25} Cu	55.7±6*	106	62.6±8	26	65.4±4	22
LC_{25} Pb	67.3±2.3**	149	84.8±4**	71	42.1±14	-21
¼ LC_{25} Zn	43.7±4*	62	77.4±13	56	101.7±12*	90
¼ LC_5 Cu, Pb & Zn	159.7±3***	491	302.0±11***	510	54.2±4	1
¼ LC_{10} Cu, Pb & Zn	52.7±18	95	90.8±3**	83	62.9±4.3*	18
A 25ppm	109.4±6.2**	305	183.5±11**	271	181.3±11	239
D 25ppm	69.7±4.4**	158	144.6±9**	192	41.3±28	-23
AD 10ppm	27.8±6	3	57.7±20	17	18.9±1*	-65
AD25ppm	132.1±11**	389	156.3±18*	216	87.7±5.7*	64
½ LC_5 D&A + ¼ LC_5 Cu, Pb & Zn	33.1±0*	23	62.2±15	26	21.4±11*	-60

Antioxidant Enzymes

Results of the examined antioxidant enzymes, catalase (CAT), GST and GGT, in snail tissue extracts treated with tested chemicals showed significant alterations in CAT activity. Exposure to $_{1/4}LC_{25}$ Zn, AD_{10}, POPs and HM mixture showed significant CAT increase while the other treatments

showed significant decrease (Table VII). Also, snails exposed to LC_5 & LC_{10} HM mixtures, A25, AD 10 & 25 mixtures and POPs+HM mixture showed significant increase in GST activity (Table VII).

Determination of Complete Blood Components

All exposed snail samples showed significant alterations in H count. Significant decrease was shown in RS and GS count obtained in samples exposed to all treatments except those exposed to POPs +HM in RS count and to POPs +HM and D25 in GS count. The exposed samples in all treatments showed decrease in Hb concentration except those exposed to AD25 and POPs +HM which increased by 28 and 6 folds, respectively (Table VIII).

TABLE IV
CREATININE, UREA AND BUN IN TISSUE EXTRACT OF *BIOMPHALARIA ALEXANDRINA*; EXPOSED TO CU, PB, ZN, AROCLOR 1254 (A) AND DBDPE 98% (D) AT DIFFERENT EXPERIMENTAL DESIGNS

Treatments	Creatinine (mg/g)	Change %	Urea (mg/g)	Change %
Control unexposed	0.58±0.27		9.6±2	
LC_{25} Cu	5.70±0.63**	883	16.3±4	70
LC_{25} Pb	0.85±0.0	47	23.2±13	142
¼ LC_{25} Zn	2.11.1±0.04*	264	20.1±6	109
¼ LC_5 Cu, Pb & Zn	16.25±1.4**	2702	42.1±7*	339
¼ LC_{10} Cu, Pb & Zn	1.80±1.04	210	51.9±12*	441
A 25ppm	2.18±0.13*	276	48.6±3.2**	406
D 25ppm	1.55±0.42	167	29.7±18	209
AD 10ppm	1.85±1.99	219	23.0±6	140
AD25ppm	4.58±0.574*	690	60.3±15*	528
½ LC_5 D&A+ ¼ LC_5 Cu, Pb & Zn	3.01±0.0**	419	8.1±2	-16

TABLE V
TP, *ALB*, GLOBULIN (G), A/G RATIO & GLUCOSE IN TISSUE EXTRACT OF *BIOMPHALARIA ALEXANDRINA*; EXPOSED TO CU, PB, ZN, AROCLOR 1254 (A) AND DBDPE 98% (D) AT DIFFERENT EXPERIMENTAL DESIGNS

Treatments	*TP* (g/dl)	Change%	*ALB* (g/dl)	Change%	Globulin (g/dl)	Change%	A/G Ratio
Control unexposed	6.13±0.8		2.66±0.45		3.47±0.32		0.760
LC_{25} Cu	12.02±1.8	96	5.35±1.01*	101	6.67±0.82*	92	0.796
LC_{25} Pb	15.62±5.7	155	5.50±1.60	107	10.12±4.13**	192	0.575
¼ LC_{25} Zn	10.33±1.8	69	4.69±0.81	76	5.65±0.97	63	0.830
¼ LC_5 Cu, Pb & Zn	37.78±0.3***	516	16.42±1.27**	517	21.36±0.92**	516	0.772
¼ LC_{10} Cu, Pb & Zn	10.92±3.9	78	3.86±1.36	45	7.06±2.55	103	0.548
A 25ppm	21.75±1.3**	255	10.67±0.70**	301	11.08±5.80**	219	0.872
D 25ppm	15.49±10.4**	153	6.25±0.43*	135	9.24±0.61**	166	0.652
AD 10ppm	5.81±1.5	-5	2.99±0.95	12	2.82±0.56	-19	1.032
AD25ppm	25.21±2.1**	311	13.82±0.85**	420	11.40±1.24*	229	1.218
½ LC_5 D&A+ ¼ LC_5 Cu, Pb & Zn	5.63±1.9	-8	2.77±1.03	4	2.86±0.93	-18	0.950

TABLE VI
TB, DIRECT & INDIRECT IN TISSUE EXTRACT OF *BIOMPHALARIA ALEXANDRINA*; EXPOSED TO CU, PB, ZN, AROCLOR 1254 (A) AND DBDPE 98% (D) AT DIFFERENT EXPERIMENTAL DESIGNS

Treatments	*TB* (Umol/l)	Change %	*DB* (mg/dl)	Change %	In-Direct Bilirubin (U/mg)	Change %	Glucose	Change%
Control unexposed	5.3±0.64		1.1±0.8		4.2±1.5		51.5±6.0	
LC_{25} Cu	10.3±1.7	5	0.7±0.1	-36	9.6±1.6	129	105.4±15*	105
LC_{25} Pb	8.4±0.0	3.1	0.4±0.0	-64	8.0±0.0	90	150.2±5.6**	192
¼ LC_{25} Zn	11.8±0.9*	6.5	0.5±0.2	-55	11.3±1.1*	169	84.5±13	64
¼ LC_5 Cu, Pb & Zn	33.5±0.8***	28.2	7.4±0.4*	573	26.1±5.0*	521	354.9±4***	589
¼ LC_{10} Cu, Pb & Zn	6.0±0.0	0.7	0.4±0.0	-64	5.6±0.0	33	96.7±32	88
A 25ppm	8.6±0.0	3.3	0.9±0.0	-18	7.7±0.0	83	180.6±10.6**	251
D 25ppm	23.5±0.0*	18.2	8.0±0.0	627	15.5±0.0	269	129.0±8.9**	150
AD 10ppm	3.8±0.0	-1.5	0.4±0.0	-64	3.4±0.0	-19	50.3±14	-2
AD25ppm	22.1±0.0*	16.8	3.8±0.0	245	18.3±0.0*	329	240.0±23**	366
½ LC_5 D&A+ ¼ LC_5 Cu, Pb & Zn	4.8±1.8	-0.5	0.3±0.3	-73	4.4±1.6	5	47.2±16	-8

TABLE VII
CAT, GST AND GGT IN TISSUE EXTRACT OF *BIOMPHALARIA ALEXANDRINA*; EXPOSED TO CU, PB, ZN, AROCLOR 1254 (A) AND DBDPE 98% (D) THROUGH DIFFERENT EXPERIMENTAL DESIGNS

Treatments	CAT (Units/g)	Change %	GST (Units/g)	Change %	GGT (Units/g)	Change %
Control unexposed	11.55±0.04		1.01±0.14		1133±5	
LC_{25} Cu	10.47±0.00*	-9	0.59±0.08	-42	1079±72	-5
LC_{25} Pb	9.21±0.44*	-20	0.56±0.21	-45	1077±185	-5
¼ LC_{25} Zn	13.36±0.00**	16	0.78±0.00	-23	1062±205	-6
¼ LC_5 Cu, Pb & Zn	8.88±0.00**	-23	0.52±0.04*	-49	915±157	-19
¼ LC_{10} Cu, Pb & Zn	5.22±0.00**	-55	4.09±0.27**	305	1031±255	-9
A 25ppm	8.16±0.00**	-29	5.06±0.15**	401	895±105	-21
D 25ppm	9.10±0.35*	-21	0.81±0.06	-20	805±29*	-29
AD 10ppm	13.38±0.22**	16	4.57±0.76*	352	1055±59	-7
AD25ppm	9.93±0.00*	-14	3.16±2.8*	213	1086±109	-4
½ LC_5 D&A+ ¼ LC_5 Cu, Pb & Zn	17.31±2.7**	50	5.78±0.09***	472	869±116	-23

TABLE VIII
HEMATOLOGIC PARAMETERS OF *BIOMPHALARIA ALEXANDRINA* EXPOSED TO CU, PB, ZN, AROCLOR 1254 (A) AND DBDPE 98% (D) THROUGH DIFFERENT EXPERIMENTAL DESIGNS

Treatments	Total cell count			Hyalinocytes (H)			Round small hemocytes (RS)			Granuolocytes spreading (GS)			Hb (g/100ml)	
	Mean±SD	Change %	%of (H)	Mean ±SD	Change %	%of RS	Mean±SD	Change %	%of (GS)	Mean±SD	Change %			Change %
Control unexposed	3.2±1.0		57	1.85±0.5		27	0.8±0.28		16	0.55±0.2			1.8	
LC_{25} Cu	2.6±0.0	-19	55	1.4±0.0*	-24	32	0.8±0.00	0	13	0.40±0.0	-27		1.1	-39
LC_{25} Pb	1±0.0***	-69	68	0.6±0.0***	-68	23	0.2±0.00***	-75	10	0.20±0.0***	-64		1.1	-39
¼ LC_{25} Zn	1±0.0***	-69	59	0.6±0.0***	-68	27	0.2±0.00***	-75	15	0.20±0.0***	-64		1.6	-11
¼ LC_5 Cu, Pb & Zn	0.85±0.4***	-73	61	0.45±0.1***	-76	25	0.15±0.21***	-81	17	0.25±0.1**	-55		1.4	-22
¼ LC_{10} Cu, Pb & Zn	0.7±0.3***	-78	67	0.43±0.2***	-77	26	0.13±0.12***	-84	8	0.13±0.1***	-76		1.1	-39
A 25ppm	0.9±1.0***	-72	31	0.4±0.7***	-78	8	0.17±0.29***	-79	38	0.27±0.2*	-51		1.1	-39
D 25ppm	3.1±1.7	-3	69	2.25±0.16*	22	20	0.6±0.00	-25	11	0.35±0.1*	-36		2.2	-22
AD 10ppm	2.8±0.4	-13	76	2.15±0.5	16	20	0.5±0.14*	-38	4	0.15±0.1***	-73		1.8	0
AD25ppm	2.15±0.1*	-33	65	1.35±0.1*	-27	21	0.4±0.14**	-50	13	0.40±0.0	-27		2.3	28
½ LC_5 D&A+ ¼ LC_5 Cu, Pb & Zn	4.9±4.7***	53	72	3.4±3.1***	84	16	0.85±0.92	6	12	0.65±0.6	18		1.9	6

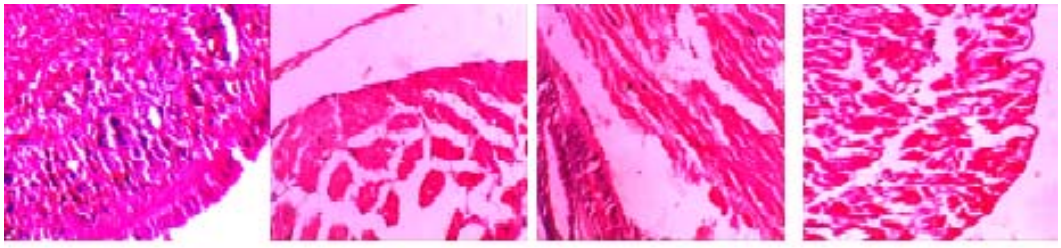

Fig. 2 Headfoot of *Biomphalaria alexandrina* showing control ((a), X400), splitting of head muscle bundle and edema due to treatment with Cu ((b), X400), edema in muscle fiber, hyper trophy and vaculation when treated with Mix of AD LC_{10} ((c), X400) and splitting of head muscle bundle and edema when treated with Mix of AD LC_{25} ((d), X400).

C. Effect of Tested Compounds on Snails' Histology

Head foot

The head foot composed of transversely interwoven muscle fibers, called as longitudinal muscle fibers and mucus glands and stain spots. The major part of the foot muscles is made up of thickly arranged oblique muscle fibers (Fig. 2 (a)).

Snail samples exposed to HM showed degeneration and splitting in muscle fibers (Fig. 2 (b)). Besides, edema, hyper trophy, vaculation and necrotic change were shown in samples treated with AD (Lc_{10} &Lc_{25}) and AD+HM (Figs. 2 (c), (d)).

Ganglia

Snail ganglia represent modified neurons in the form of neurosecretory cells (Fig. 3 (a)). The histopathological alterations showed an increase in the neurosecretory material synthesis and neurosecretory axons too are fully laden with neurosecretory material flakes especially in snails treated with A1254 and DBDPE (Figs. 3 (d), (e)). Also, there is an accumulation of neurosecretory material in the neuropilar region of the central ganglion. The other treatments showed degeneration in neurosecretory cells (Figs. 3 (f)-(i)).

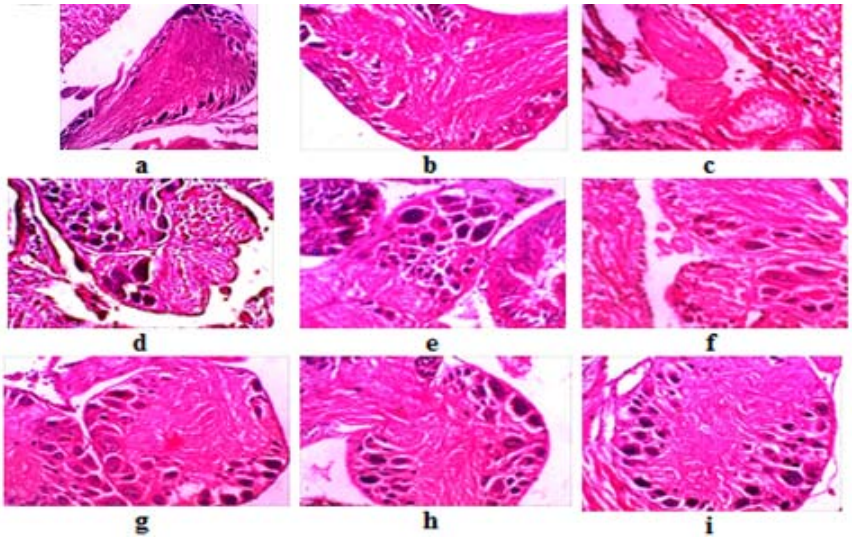

Fig. 3 Ganglia of *Biomphalaria alexandrina* showing the control ((a), X400), loosened neurosecretory neurons (Cu [(b), X400]), degenerated cytomorphlogical characters (Zn [(c), X400]), enlarged and increase of neurosecretory cells (A1254 [(d) X400]), accumulation of neurosecretory material in the neuropilar region of the central ganglion (DBDPE [(e), X400]), degeneration in neurosecretory cells (mix.HMLC$_5$ [(f), X400], Mix LC$_{10}$ [(g), X400], Mix HM & AD [(h) & (i) X400])

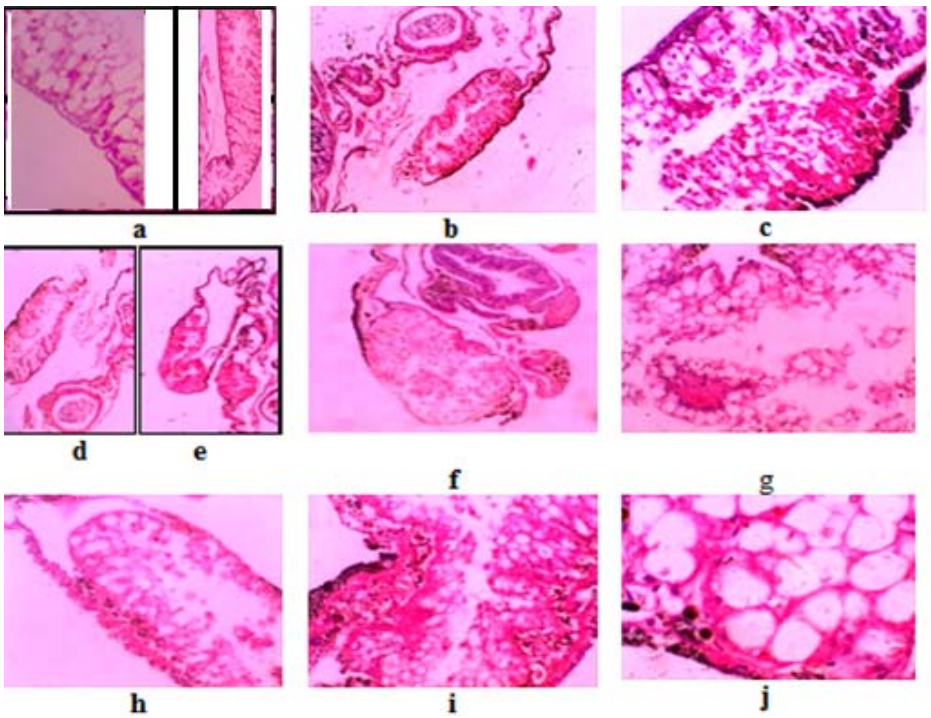

Fig. 4 Albumen gland of *B. alexandrina* showing the control ((a), X100 & X400), accumulation in outside wall (Pb [(b) X100]), degenerated secretory vesicles (Zn [(c), X400], Cu [(d), X100] and Aoclor [(e), X100]), enlarged albumen gland and degeneration in connective tissue (DBDPE [(f), X100]), degenerated secretory vesicle and focal hemorrhage (DBDPE [(g), X400), vaculation and degeneration (HM mix LC$_5$ [(h), X100) and accumulation in the wall of albumen glands (HM&AD mix [(i), X400 & (j), X1000])

Albumen Gland

The albumen gland is a compact organ lined by a glandular epithelium that contains large secretory vesicles which are different in size, shapes and structures. The tubules are separated from each other by thin connective tissue sheath which contains blood spaces and is continuous with the connective tissue that binds the gland peripherally. The cell boundaries of the secretory cells are distinct and the nuclei are basally placed (Fig. 4 (a)). In all treatments, albumen gland enlarged, secretory vesicles degenerated and vacuole formed (Fig. 4). After the treatment with both Zn and DBDPE showed the large secretory cells around the lumen had undergone hypertrophy (Figs. 4 (c), (f)). Degeneration of secretory cells was observed in Pb, Cu and Arochlor (Figs. 4 (b), (d), (e)).

Accumulation was more pronounced in snail samples exposed to mixture treatments (Figs. 4 (c), (i), (j)).

Digestive Gland

The digestive gland consists of numerous digestive tubules which have various shapes and sizes. The digestive tubules consist of epithelium with a single layer of cells, separated from surrounding connective tissue and muscle cells by basal lamina. The cells constituting the wall of the tubule are irregularly arranged around lamina. The cells are differentiated into digestive cells, calcium cells and excretory cells (Fig. 5 (a)). The digestive gland of *B. alexandrina* exposed to DBDPE showed enlarged tubules lead to stick and interfere to each other and enlarged lumen filled with hyaline substance (Figs. 5 (e), (f)). The severity of lesions was progressed by exposure to A1254 that caused severe vacuolar degeneration of digestive gland with focal areas of necrosis (Fig. 5 (g)). The digestive gland histological alterations were more evident in snail exposed to the mixture of AD, HM and their mixtures (Fig. 5 (k)).

Hermaphrodite Gland

The *B. alexandrina* hermaphrodite gland consists of large number of acini that are connected together with a connective tissue. The acini are lined with distinct germinal epithelium composed of cuboidal and flattened cells. This epithelium gives rise to spermatogenesis and oogenesis. The male cells are differentiated forming primary and secondary spermatocytes. The female oogonia cells are usually found in groups arranged along the periphery of the acinus as primary, secondary oocytes and mature ova. Developed sperms were observed in the lumen of the acinus, each has an oval small head and thread like long tail. Each acinus contains 1-2 mature ova, while sperms aggregated in large numbers inside the lumen (Fig. 6 (a)). Samples exposed to single HM showed lyses of cells and matrix' components. Also, the mature ova appeared atrophied, dense and irregular in shape (Figs. 6 (b), (c)). Samples exposed to A or D showed atrophied ova and sperms (Figs. 6 (d)-(g)). In samples exposed to HM mixtures, LC_5 (Fig. 6 (h-j) and LC_{10} (Fig. 6 (k & l)), the damage increases showing discharge, evacuation and disappearance of most of the components of the gonad cells. Moreover, complete destruction of gametogenic cells and sever damage of ovotestis gland were clear. Snails exposed to HM+AD mixture showed almost the same obvious effects (Figs. 6 (m)-(o)).

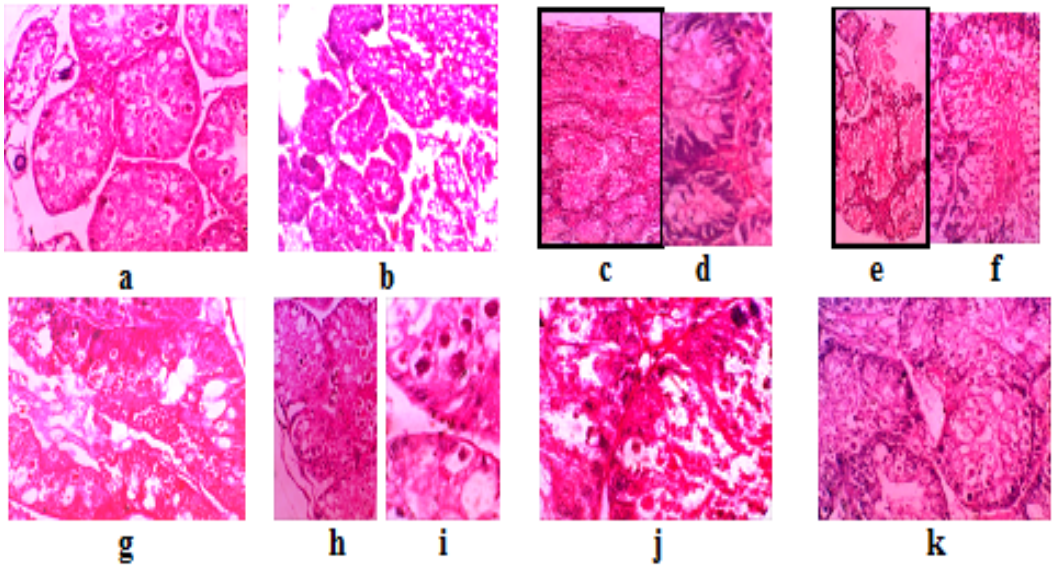

Fig. 5 Digestive gland of *B. alexandrina* showing the control ((a), X400), degeneration and edema in digestive tubules (Cu [(b) X100]), tubules stick to each other and accumulation in basement membrane (Zn [(c) X100, (d) X400]), tubules interference forming large lumen focal area of necrosis (DBDPE, (e) X100, (f) X400), vacuolar degeneration and necrotic change (A1254 [(g) X400]), coagulative necrosis and vacuolar degeneration and pyknotic nuclei (HM mix LC_{10}, (h) X100, (i) X400), necrotic change ($ADLC_{10}$ [(j) X400]), enlarged tubules and interfere with each other and lumen of tubules disappeared (HM&AD [(k) X400])

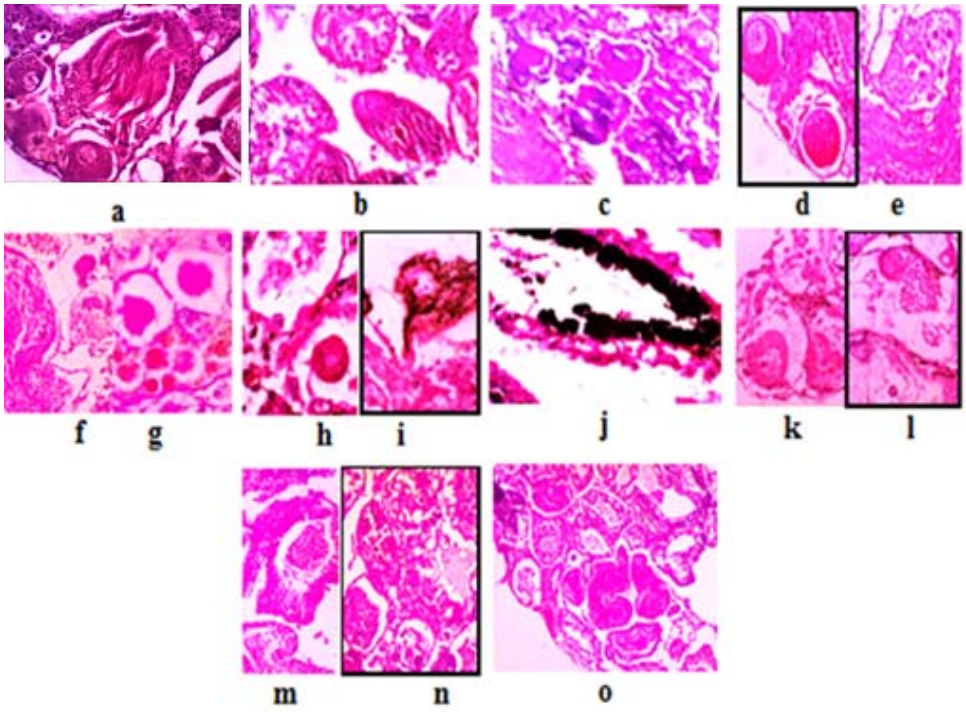

Fig. 6 Hermaphrodite gland of *Biomphalaria alexandrina* control ((a), X400). Alterations observed in the exposed snails showed atrophy and lyses of cells, matrix' components and the mature ova appeared dense and irregular in shape (Cu (b) & Pb (c), X400), ova lost their normal shape and reduction in the number of sperms was noticed (A1254 (d) & (e), DBDPE (f) & (g), X400), edema, atrophy of spermatogonia, necrotic spermtogonia and oocytes and accumulation (HM LC_5 [(h)-(j), x1000]), severe degeneration of ovitestis, spermatogonia & ova, and accumulation (HM10 [(k) & (l), X400]), and atrophy of sperms, clogged sperms, Degeneration of hermaphrodite gland, absorption of oocytes and necrotic change of digestive gland (HM+AD [(m) & (n), X400, (o) X100])

IV. Discussion

Metals can be highly reactive and they are toxic to most organisms [32] because of their ability to disturb various cellular processes, especially the functioning of cellular membranes [33], [34]. There are many authors studied the toxicity of metals (e.g. [32], [35], [36]).

This work studied the impact of zinc sulphate; copper sulphate, lead nitrate, organic pollutant DBDPE 98% and A1254 on liver and kidney functions; antioxidant enzymes; complete blood picture and tissue histology in *B. alexandrina* snails.

The Probit analysis of the toxicity of these chemicals against *B. alexandrina* snails indicated that Cu showed higher toxicity than Zn and Pb; where LC_{50} was 1.362, 213.198 and 277.396 ppm, respectively. These findings are compatible with those of [37] and [38] who recorded that Pb is commonly regarded as the least toxic metal for the majority of invertebrates and it is less toxic than Cu and Zn for *Cantareus asperses*. The survival rate in snails exposed to the POPs was higher than that in snails exposed to HM.

The antioxidant defense system plays a crucial role in animals' survival periods of reactive oxygen species overproduction. It consists of enzymatic and non-enzymatic components [39]-[41]. Few data are available concerning the antioxidant defense modulation in snails in response to metal exposure [32], [42], [43].

Results showed that the highest Cu and Zn bioaccumulation was recorded in *B. alexandrina* snails exposed to the mixture of ¼ LC_5 Cu, Pb & Zn (108.907 and 45.4 folds, respectively). Metal concentration in gastropods has been investigated in [44]. Reference [17] demonstrated that *B. alexandrina* snails exposed to Pb, Cd or Hg have increased concentrations of HM in their bodies within 4 weeks at 17–28 °C, so these species are considered bioindicators for HM pollution. Reference [45] studied the association between metals accumulation in selected tissues and the shell in *Helix aspersa* snails. They found that accumulation of Zn, Fe, Pb, Cd, and Mg was organ-specific and was the highest in the hepatopancreas.

Result illustrates that there was significant increase in AST, ALT & ALP activities in *B. alexandrina* exposed to the tested chemicals, which may indicate the high energy demand of the snail under stressful conditions of intoxication. The alteration in liver enzymes indicates mainly the hepatocellular disorder [46]. This agreed with [24], who found significant increase in the liver enzymes; AST ALT, ALP; in *Planorbis* and *Physa* snail samples, collected from Nasayma site in polluted Lake Manzala.

Significant elevations in the levels of acid phosphatase and ALP are noticed in *B. alexandrina* after exposure to the pesticide, profenophos, as in [47]. Also, [48] revealed an elevation in the activities of AST, ALT and ALP enzymes in snails' tissues post treatment with LC_{10} and LC_{25} of Basudin, Selecron and Bayluscide in comparison with control groups.

Under physiological stress conditions in animals, the catalytic activity of the urea pathway enzymes is also accelerated [49]. Urea and creatinine are synthesized in liver and kidney and major illness may increase their levels [50]. Snail groups exposed to HM-mixture showed significant increase in creatinine and urea concentrations. This agreed with [24], who recorded significant increase in urea and creatinine in snails collected from Port-Said and Dakahlya sites in polluted Lake Manzala. In accordance with this, the exposure of *Biomphalaria glabrata* to *Euphorbia splendens hislopii* latex caused urea increase in their hemolymph [51].

The determination of TP, ALB and globulin levels showed significant increase in snail groups exposed to HM-mixture. Reference [52] attributed the increase in TP level to the changes in hepatic protein synthesis due to the stress in the polluted habitat. In agreement with these results is [53] who recorded significant increase in the *TP* under the effect of a chemical stress at different biological models. Also, [54] recorded an increase in the *TP* concentration in *Helix* snails dependent in the presence of metal dust. On the same run, significant increase of TP, ALB and globulin levels were recorded also in samples collected from Lake Manzala [24].

The antioxidant defense system utilizes enzymatic and non-enzymatic mechanisms. The most important antioxidant enzymes are superoxide dismutase (SOD), CAT, glutathione peroxidase (GPX), GST and GGT. Results of the treatment with tested chemicals showed significant alterations in CAT activity in snail tissue extracts. Snail samples exposed to LC_5 & LC_{10} HM mixtures, A25, AD 10 & 25 mixtures and POPs+HM mixture showed significant increase in GST activity. Oxidative stress is an imbalance between pro-oxidant production and antioxidant defense mechanisms [55]. *H. aspersa* snail from polluted areas exhibited higher CAT activities compared with control snails [56]. Also, the hepatopancreatic CAT and GPX activities were increased in *Helix aspersa* snails exposed to HM [45]. In addition, [57] found a significant increase of CAT activity in the garden snail specimens (*Helix pomatia* L.) collected from polluted area. The decease of CAT recorded in *Achatina fulica* snails, may be due to over-production of active oxygen by the metals [42]. Also, [23] has shown that low activity of GST may be regarded as a compensatory mechanism. In association with these findings, GST activity appears to be altered also by exposure to xenobiotics and to be elevated in some field studies [58]. Also, [24] recorded alterations in CAT, GST and GGT activity in snail samples collected from polluted Lake Manzala. On the other hand, [59] showed that there was no correlation between Zn, Pb concentrations and enzyme activities

All exposed snail samples resulted in significant alteration in H count. Snail samples exposed to HM mixtures showed a significant decrease in total hemocytes count while snail samples exposed to mixtures containing A & D showed a significant increase in total hemocytes and hyalinocytes. Most of the exposed samples in all treatments showed decrease in Hb concentration. References [60] and [61] mentioned that haemocytosis represents a response to external stress or certain stimuli and may originate from a variety of biotic or abiotic sources. Reference [62] found that exposure to dyestuff and chemical effluent could result in decreases in RBC count and Hb content which are symptoms of anemia. Also, Snail samples collected from polluted water in Lake Manzala showed significant decrease in total hemolymph cell count [24]. The decrease in hemolymph cells may be considered as a haemolysis response to the multiple pollution elements in Lake Manzala.

Histopathological alterations in snail samples exposed to HM for 4 weeks showed degeneration and splitting in head foot muscle fibers. Besides, edema, hyper trophy, evacuation and necrotic changes were noticed in samples treated by AD (LC_{10} & LC_{25}) and AD+HM. Ganglia of treated snails showed an accumulation of neurosecretory material in the neuropilar region of the central ganglion and degeneration in neurosecretory cells. In all treatments, the albumen gland enlarged and secretory vesicles degenerated. Metal accumulation was more pronounced in snail samples exposed to mixture treatments. The digestive gland of *B. alexandrina* exposed to DBDPE showed enlarged sticky tubules interfered to each other and enlarged lumen filled with hyaline substance. The severity of lesions was progressed by exposure to A1254 and the mixture treatments causing severe vacuolar degeneration of digestive gland with focal areas of necrosis. Samples exposed to single HM showed lyses of cells and matrix' components. Also, samples exposed to A or D showed atretic ova and devoid of spermatozoa. Samples exposed to A1254 or DBDPE showed ova that lost their normal shape and reduction in the number of sperms. In samples exposed to HM mixtures, the damage increases showing discharge, evacuation and disappearance of most of the components of the gonad cells. Moreover, complete destruction of gametogenic cells and sever damage of ovotestis gland were clear. A prevalence of hepatocellular foci of cellular alterations in the hepatopancreas, basophilic adenoma and ovotesticular fibrillar inclusions in the ovotestis were observed in *Archachatina marginata* snails exposed to metals [63]. Similar observations were noticed by [24] in snail samples collected from Lake Manzala and referred these histopathological damages in snail organs due to the pollution of Lake Manzala water by HM which recorded by [20]. Also, [64] illustrated that stress responses in invertebrates can occur following acute or chronic exposures to contaminated environments. Treatment with pesticides showed degeneration of digestive tubules, fragmentation of the digestive cells and breakdown of basement membrane which leads severe damage in the tissues and alteration in the muscle layer [65]-[68]. Also, after treatment with pesticides the albumen gland showed degeneration of secretory tubules, vacuolation in connective tissue and degeneration of epithelial lining of duct [69]-[70]. The ovotestis showed reduction in number of acini. Sertoli cells, spermatozoa and oocytes also decreased and appear to be degenerated in the acinus. Severe damage in ovotestis tissues were observed in snails treated with chemical pesticides [52], [71]-[73]. Reference [74] noticed that the lumen of digestive gland tubule was shrunken; degeneration of

cells, secretory cells became irregular, necrosis of cells and atrophy in the connective tissue of digestive gland when exposed *Lymnaea luteola* to Paraquat (Gramoxone). Moreover, it is worthy to mention that in the freshwater snails' nervous system has been proved to be sensitive to many toxic materials and cytotoxicants that may induce injurious consequences [75]-[77].

In conclusion, there are severe alterations in the physiological hematological and histological parameters of *B. alexandrina* snails caused by exposure to HM and tested chemicals in the laboratory. These symptoms are similar to that were seen in snail samples collected from Lake Manzala which is definitive indicator to the pollution of its water and that we recommend treat this water for the sake of the public health. Results also, proved that *B. alexandrina* snails can used as important good bioindicators for their habitat abnormalities.

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