Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 26(3): 321 – 346 (2022)
www.ejabf.journals.ekb.eg

Experimental studies on the toxicity of certain heavy metals and persistent organic pollutants on the Nile tilapia health

Hanaa M. M. El-Khayat[1]; Hanan S. Gaber[2]; Hassan E Flefel[1,*]

1- Department of Environmental Research,Theodor Bilharz Research Institute, Giza, Egypt.
2- National Institute of Oceanography and Fishing, Cairo, Egypt.

[*] **Corresponding author: h.flefel@tbri.gov.eg**

ARTICLE INFO

Article History:
Received: March 13, 2022
Accepted: May 3, 2022
Online: May 29, 2022

Keywords:
Zinc,
Cadmium,
Copper,
Lead,
Oreochromis niloticus,
Bioaccumulation,
Biochemical parameters,
Histopathology

ABSTRACT

This study was carried out to experimentally assess the acute and chronic effects of heavy metals {HMs; Zn, Cu, and Pb} and the persistent organic pollutants {POPs; Aroclor 1254 (A) and Decabromodiphenyl ether 98% (D)} on bioaccumulation, biochemical parameters and the histology of the Nile tilapia "*Oreochromis niloticus*". The groups that were exposed to AD10 HMs showed alterations related to each of HMs and ADs groups, such as the reduction in Hb & RBC (related to HMs) and the increase in platelets and WBC (related to ADs). In addition, a significant increase was recorded in ALT, ALP, and glucose and a significant reduction in total protein (related to HMs) associated with a significant reduction in total bilirubin and an elevation in GGT (related to ADs). Histopathological investigations showed muscle neoplasia indicating early warning for carcinogenic risks. Severe liver focal areas of necrosis and fibrosis were noticed, indicating the destruction of liver cells with an increase in liver enzymes' levels. Accumulation of hemosiderin in the liver and spleen due to excessive red blood cell destruction (haemolysis) explained the reduction in Hb and RBCs observed in fish groups exposed to HMs and AD10HMs mixtures. Liver & spleen lymphocyte infiltration may be associated with a massive elevation in lymphocytes in HMLC10, ADs & AD10HMLC5 mixtures. Thus, the present work would provide a measurable simulating model for the effects of environmental pollutants by using different chemical mixtures and responsive parameters for many physiological functions and histological structures as biomarkers for toxicity.

INTRODUCTION

Freshwater contamination with a wide range of pollutants has become a matter of concern over the last decades **(Zhang *et al.*, 2016; Sasakova *et al.*, 2018; Canal *et al.*, 2019)**. The natural aquatic system may extensively be contaminated with heavy metals released from domestic industrial and other man-made activities **(Sciences *et al.*, 2016; Ghorab, 2018; Jia *et al.*, 2018)** and the frequent use of pesticides. At present, there are more than 200 types of organic pesticides which are available in thousands of different

products. These pesticides contain various heavy metals such as iron (Fe), copper (Cu), chromium (Cr), cadmium (Cd), zinc (Zn), lead (Pb), nickel (Ni) and manganese (Mn) as active ingredients **(Sharma & Agrawal, 2005)**. These heavy metals ultimately reach the water bodies and adversely affect the growth, reproduction, physiology and the survival of aquatic life. Therefore, heavy metals have been recognized as strong biological poisons for their persistent nature, toxicity, tendency to accumulate in organisms, undergoing food chain amplification **(Oruambo *et al.*, 2014; Bo *et al*., 2015; Edokpayi *et al*., 2018)**.

Moreover, persistent organic pollutants (POPs) are globally concerned pollutants due to their widespread occurrence, long-term persistence, strong resistance, long-range transportation, high bioaccumulation, and potentially significant impacts on human health and ecosystems **(Xu *et al*., 2013)**.

The tool of toxicity testing has been widely used to identify suitable organisms as a bio-indicator and derive water quality standards for chemicals. It is also considered an essential tool for assessing the effects and fate of toxicants in aquatic ecosystems **(Shuhaimi-Othman *et al.*, 2010)**. Besides toxicity, studies quantify an organism's response to the biologically active materials **(Ali *et al.*, 2019)**. They are useful in determining water quality, it is, therefore, crucial to restore and resolve chemical pollution through environmental monitoring. Fish are relatively sensitive to changes taking place in the surrounding environment, and they play a vital role in the food-web; unfortunately, they are prone to be contaminated by chemicals dissolved in their surrounding water, among which heavy metals and POPs are considered **(GÜVEN *et al.*, 1999)**. The bioaccumulation and magnification of these chemicals can reach toxic levels in fish, even when the exposure level is low **(Jayaprakash *et al.*, 2015)**. The presence of toxic metals in fresh water is known to disturb the delicate mineral balance of the aquatic system which may adversely affect the freshwater fish **(Isangedighi & David, 2019)**. Since the mechanisms of heavy metals excretion, deposition and detoxification in fish are not capable of being handled in a short time, heavy metals tend to accumulate specifically in metabolically active tissues **(Younis *et al.*, 2012)**.

Accumulations of the heavy metals adversely affect the histology and functioning of liver, kidney, muscles and other fish organs **(Isangedighi & David, 2019)**.Consequently, histopathology can serve as a sensitive tool to find out the effect of pollutants, including copper and lead on fish tissues **(Atamanalp *et al*., 2008; Leonardi *et al*., 2009; Pathan *et al*., 2009; Yasser & Naser, 2011)**. Many authors **(Taweel *et al*., 2011; Wang *et al.*, 2014; Wei *et al.* 2014; Arantes *et al.*, 2016**) considered the gills, liver, spleen and kidney to be the responsive organs that respond to toxic pollutants and used them as biomarkers for environmental pollution assessment.

In the present study, the economically important freshwater tilapia fish, *Oreochromis niloticus*, was used as a biological indicator of environmental

contamination. The toxicity experiments were conducted to investigate 96h LC_{50} of the heavy metals (Cu, Zn, and Pb) and that of POPs (Decabromodiphenyl ether 98% and Aroclor 1254) against *O. niloticus*. Additional chronic toxicity studies using sublethal concentration values of the tested chemicals were administered individually or in mixtures to investigate their effects on liver and kidney functions, oxidative stress enzymes, organ bioaccumulation and histology.

MATERIALS AND METHODS

Chemical substances

Zinc sulphate (Zn SO_4 $7H_2O$) and copper sulphate (Cu SO_4 $5H_2O$) were supplied by El-Nasr Pharmaceutical Chemicals Co., Abu Zaabal, Egypt. Lead (II) nitrate [Pb $(NO_3)^2$] was supplied by Sigma-Aldrich, United Kingdom. Aroclor 1254 (A1254) was supplied by Supelco analytical, Bellefonte, PA, USA. Decabromodiphenyl ether 98% (DBDPE) was supplied by Aldrich Chemistry, USA. Tween 80 was used for dissolving A1254 & DBDPE.

Experimental Fish

Healthy specimens of *O. niloticus*, with an average body weight of 47.86±6.30g and length of 12.02±1.92cm, were obtained from Abbassa Fish Farm, Sharkia Governorate, Egypt. Fish samples were acclimatized to the laboratory conditions for two weeks in large fiberglass tanks containing well aerated tap water (temperature, 25±2°C; pH, 7.64±0.06; oxygen concentration, 6.7±0.01mg/L and total hardness, 134.3 ± 2.4 ppm). During acclimatization, the fish were fed on commercial pellets (28% protein) once per day. Waters were renewed every 24h with the routine cleaning of the aquaria, leaving no fecal matter or unconsumed food. Two days prior to the application of heavy metals and POPs, fish samples were transferred to 60 L water capacity glass aquaria, filled with 35 L of dechlorinated aerated tap water.

Toxicity bioassay

Preliminary experiments were conducted to determine the median lethal concentration after 96h (96h-LC_{50}) for Zn, Cu and Pb (**APHA 2005**) without feed. The current experiment used various concentrations of Cu (as Copper sulphate) at concentrations of 0.3, 0.6, 1.2, 1.5 and 2.0 mg/L, Pb (as Lead nitrate) at concentrations of 0.375, 0.7, 1.5, 1.8 and 2.0 mg/L, Zn (as Zinc sulphate) at concentrations of 4.0, 8.0, 16.0, 20.0 and 24.0 mg/L, A1254 at concentrations of 1, 2, 4, 6 and 10 mg/L and DBDPE at concentrations of 6, 8, 12, 50, 100, 200 mg/L. m. Mortality regression lines were done using SPSS Computer Program 20.0. Mortality was calculated according to the method of American Public Health Association **(APHA 1995**; **Geypens *et al.* 2012)** and regression lines were established by SPSS Computer Program 20.

Long term exposure or chronic exposure to heavy metals and POPs

The fish were daily fed during the experiment with artificial food. 10 fish samples were exposed to sub-lethal concentrations of the tested chemicals; 35 L of dechlorinated aerated tap water was adjusted to 60L water capacity glass aquaria. Twelve experimental groups were conducted for four weeks as shown in Table (1). Then, the specimens were subjected to the examination of different parameters, viz. bioaccumulation, biochemical parameters and histological alterations.

Residual analysis of heavy metals

In the Environmental Research Laboratory, Theodor Bilharz Research Institute, fish specimens were analyzed for the levels of copper, zinc and lead using Avanta Atomic Absorption Spectrophotometer. The tissues of the fish muscle, liver, kidney and whole fish samples were dried at 105°C in an electric oven for 36hrs. Then, one gram of dried tissue was transferred to clean screw capped glass bottle and digested with 10ml of solution HNO3-HClO4 (4:1 v/v) **(FAO 1983; Yi *et al.*, 2011)**. Initial digestion was conducted for four hours at room temperature, followed by heating at 40-45ºC for one hour in water bath, and then heat temperature was raised to reach 70ºC until the end of digestion. After cooling at room temperature, the digest was diluted to 25ml with deionizer water and filtered in volumetric flask to determine the concentrations of the examined heavy metals

Table 1. Experimental design of *Oreochromis niloticus* fish exposure to copper (Cu), lead (Pb), zinc (Zn), Aroclor 1254 and Decabromodiphyneyl Ether

Treatments		**Exposure time**
Controls	**Non-exposed**	**0 weeks**
	Nile samples	**0 weeks**
1	**LC_{50}Cu**	**4 weeks**
2	**LC_{50}Pb**	**4 weeks**
3	**LC_{50}Zn**	**4 weeks**
4	**1/5LC_5Cu, Pb&Zn**	**4 weeks**
5	**1/5LC_{10} Cu, Pb&Zn > 10g**	**4 weeks**
6	**1/5LC_{10} Cu, Pb&Zn < 10g**	**4 weeks**
7	**LC_{50} A**	**4 weeks**
8	**D 50 ppm**	**4 weeks**
9	**AD LC_{10} A & 10 ppm D**	**4 weeks**
10	**AD LC_{25} A & 25 ppm D**	**4 weeks**
11	**Pre –exposure to AD10 then 1/5LC_5 Cu, Pb &Zn**	**2+2 weeks**
12	**Pre- exposure to AD10 then 1/5LC_{10} Cu, Pb &Zn**	**2+2 weeks**

Biochemical studies

1-Determination of liver and kidney functions

The aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total and direct bilirubin (TB, DB), Albumin (ALB), total protein (TP), urea and creatinine were assessed in fish serum samples. They were biochemically assayed using biosystems auto-analyzer, Backmann at Theodor Bilhaz Institute Hospital Laboratories. **Fish serum**: Blood was collected from heart ventricle of the tilapia fish. Blood was left to clot at 20°C for 30min and then cooled at 0°C for 1h. Serum was obtained by centrifugation at 1000 × g for 8min. Sera were frozen at -20°C until used.

2-Determination of antioxidant enzymes

The antioxidant enzymes catalase, glutathione-s-transferase (GST) and gamma glutamyl transferase (GGT) were assayed using spectrophotometer in fish liver extracts. The fish were dissected, and the liver samples were removed, washed in an ice cold 1.15% KCL solution, blotted and weighed. They were then homogenized with 0.15%M of KCL; the resulting homogenates were centrifuged, at 2500rpm speed for 15mins, and each supernatant was decanted and stored at -20°C until analysis **(Habbu *et al.* 2008; Djuissi *et al.* 2021)**.

3-Determination of complete blood components

Fish blood samples were collected, after anesthetizing the fish, by cardiac puncture from the heart ventricle by inserting needle perpendicular to the ventral surface of the fish in the center of an imaginary line between the anterior most parts of the base of the pectoral fins. Complete blood picture was made by Coulter Counter apparatus for sample that was experimentally exposed to chemical treatments and control.

Histopathological studies

Specimens from experimentally control and exposed fish organs (muscle, gills, liver, spleen) were dissected and fixed in 10% buffered neutral formalin solution, dehydrated, cleared and embedded in paraffin wax. Five-micron thick paraffin sections were prepared, stained by hematoxylin and eosin (HE), and then microscopically examined for histopathology **(Bancroft & Stevens, 1996)**.

Statistical analysis

Data were expressed as means ± SD. The results were computed statistically (SPSS software package, version 20) using the T-test analysis. Values of $P<0.05$ were considered statistically significant.

RESULTS

Acute toxicity test

Results of the median lethal concentration after 96h (96h-LC_{50}) are presented in Table (2). Cu was the most toxic followed by Pb, Zn and aroclor 1254 (96h-LC_{50} were 1.4, 2.11, 17.0 & 3.8 mg/l, respectively**)**, while decabromodiphenyl ether was not toxic till recording 200mg/L.

Table 2. Probit analysis of the toxic effect of decabromodiphenyl ether 98% (D), aroclor 1254 (A), lead (Pb), copper (Cu) and zinc (Zn) against *Oreochromis niloticus* fish

	Toxicity (mg/l)							
Chemical	**LC_5**	**LC_{10}**	**LC_{16}**	**LC_{25}**	**LC_{50}**	**LC_{84}**	**LC_{90}**	**Slope**
D	**Not toxic till 200 mg/l**							
A	2.0	2.3	2.7	3.4	3.8	5.9	6.8	1.48
Cu	0.44	0.64	0.8	0.9	1.4	2.7	3.2	1.84
Pb	1.1	1.3	1.5	1.7	2.11	3.4	3.9	1.51
Zn	5.9	7.2	8.9	11.0	17.0	33.0	37.0	1.93

Bioaccumulation

The impact of exposure to sub-lethal concentrations of individual Pb, Cu, Zn, their mixtures HMs LC_5 & LC_{10} and their mixtures with the POPs, AD_{10}HM LC_5 & LC_{10} for a period of 4 weeks on their bioaccumulation were determined in muscle, liver and kidney of *O. niloticus* fish samples (Table 3). Results showed that Pb was most accumulated in the kidney of fish groups exposed to AD_{10}HM LC_{10}, LC_{50} Pb & AD_{10}HM LC_5 (VI, I & V) with 346, 125 & 103 folds, respectively, compared to the non-exposed control, followed by the liver of samples of fish group exposed to LC_{50} of Pb, 214 folds. Cu was most accumulated in samples of fish group exposed to LC_{50} of Cu and group VI (AD_{10}HM LC_{10}) with folds 2248 & 920, respectively, compared to the control, followed by Kidneys of fish group II (HM LC_{10}), liver of group I (HM LC_5) then kidney in samples exposed to LC_{50} of Cu with folds, 784, 305 and 103, respectively. Zn was most accumulated in the kidney & liver of group VI (AD_{10}HM LC_{10}) with 547 & 262 folds, respectively, followed by the liver & muscle in fish samples exposed to LC_{50} of Zn with 247& 189 folds, respectively. Then, all treatments in the descending order of kidney followed by liver then muscle V, I & II.

Biochemical Measurements:

From data present in **Tables (4-7),** all the examined groups showed significant increase in AST, urea and GST. The groups exposed to heavy metal mixtures, HM LC_5& LC_{10} were the most affected, had the most increase in ALT, ALP, creatinine, glucose,

total bilirubin, and the most decrease in total protein, Hb and RBC while showed normal CAT and GGT.

Groups exposed to POPs mixtures, AD10 & AD25 showed slightly higher Hb than control non-exposed with change of 17% & 13%, respectively; increase in the platelets with percentage of 625 & 255 %; increase in WBC with change of 80% & 57%, respectively; the least change in ALT and ALP activities (290 & -20 % and 7 & 93%, respectively), and the highest reduction in CAT & GGT levels (-26 & -34 % and -51 &-57 %, respectively).

Groups that exposed to POPs and heavy metals, $AD_{10}HM$ LC_5 & LC_{10}, showed the alterations related to each of heavy metals and POPs groups, like reduction in HB& RBC (related to heavy metal exposure) and increase in WBC (related to POPs exposure), significant increase in ALT, ALP, and glucose and significant reduction in total protein (related to heavy metals) while significant reduction in total Bilirubin and significant increase in platelets (related to POPs exposure).

Table (3): Residues (R) of lead (Pb), copper (Cu) and zinc (Zn) and their folds in comparable to control (C) in muscle, liver and kidney of *Oreochromis niloticus* fish samples; experimentally exposed to Cu), Pb, Zn, Aroclor 1254 (A) and Decabromodiphenyl ether 98% (D) through different experimental designs

Groups	Treatments	Tissue	Pb		Cu		Zn	
			Accumul-ated	Folds to control (R/C)	Accumul-ated	Folds to control (R/C)	Accumul-ated	Folds to control (R/C)
C	Control unexposed	Muscle	0.14		0.08		0.63	
		Liver	0.61		0.31		0.69	
		Kidney	2.98		2.19		4.29	
LC_{50}	Pb_{50} or Cu_{50} or Zn_{50}	Muscle	3.00	22	5.73	75	156.62	247
		Liver	129.65	214	687.12	2248	--	--
		Kidney	3.18	1	224.15	103	808.82	189
I	1/5LC5Cu, Pb&Zn	Muscle	3.57	26	2.94	39	42.54	67
		Liver	4.84	8	93.32	305	19.59	28
		Kidney	371.79	125	82.86	38	897.44	209
II	1/5LC10Cu, Pb&Zn	Muscle	2.34	17	0.87	11	29.39	46
		Liver	2.99	5	1.07	4	104.73	152
		Kidney	22.62	8	1715.08	784	535.71	125
V	Pre –exposure to AD10 then 1/5LC5Cu, Pb&Zn	Muscle	6.06	45	1.76	23	81.21	128
		Liver	19.20	32	29.70	97	126.48	183
		Kidney	307.69	103	26.57	12	769.23	179
VI	Pre –exposure to AD10 then 1/5LC10Cu, Pb&Zn	Muscle	7.55	56	0.55	7	70.14	111
		Liver	6.00	10	281.34	920	181.03	262
		Kidney	1031.25	346	44.42	20	2343.75	547

Table (4): Aspartate amino transferase, Alanine amino transferase, Alkaline phosphatase, glucose and creatinine in serum of *Oreochromis niloticus* fish samples (Family Cichlidae); exposed to copper (Cu), lead (Pb), zinc (Zn), Aroclor 1254 (A) and Decabromodiphenyl ether 98% (D).

Parameters / Treatments		Aspartate Amino Transferase (AST) (Unites/Ml)		Alanine Amino Transferase (ALT) (Unites/Ml)		Alkaline Phosphatase (ALP) (IU/L)		Total Bilirubin (Mg/Dl)		Glucose (Mg/Dl)	
		Serum level	% of change	Serum level	% of change	Serum level	% of change	Serum level	% of change	Serum level	% of change
C	Control unexposed	30.0±0.1		5.0±0.0		7.0±0.1		0.20±0.01		53.0±0.1	
I	1/5LC_5 Cu, Pb&Zn	100.0±0.0***	233	234.0±0.0**	4580	20.0±0.0*	186	0.70±0.00**	250	256.0±0.0***	383
II	1/5LC_{10}Cu, Pb&Zn	301.5±20**	905	71.5±2.5***	1330	46.5±2.0***	564	0.15±0.05	-25	443.5±37.8**	737
III	AD LC_{10} A & 10PPm D	136.0±8.1**	353	19.5±1.5**	290	7.5±1.5	7	0.45±0.05**	125	122.5±8.0**	131
IV	AD LC_{25} A & 25PPm D	170.0±5.5***	467	4.0±2.0	-20	13.5±1.5*	93	0.05±0.05	-75	44.5±4.5	-16
V	AD_{10} then 1/5LC_5Cu,Pb&Zn	590.0±0.0***	1867	83.0±0.0***	1560	9.0±0.0*	29	0±0.00	-100	231.0±0.0***	336
VI	AD_{10} then 1/5LC_{10}Cu,Pb&Zn	573.0±0.0***	1810	90.0±0.0***	1700	25.0±0.0***	257	0±0.00	-100	57.0±0.0*	8

A: Arocolr 1254**; D:** Decabromodiphenyl ether **(DBDPE)**

Table (5): Urea, total Bilirubin, total protein, Albumin (A), globulin (G) and A/G ratio in serum of *Oreochromis niloticus* fish samples (Family Cichlidae); exposed to copper (Cu), lead (Pb), zinc (Zn), Aroclor 1254 (A) and Decabromodiphenyl ether 98% (D) through different experimental design.

Parameters / Treatments		Urea (mg/dl)		Creatinine (Mg/Dl)		Glucose (mg/dl)		Total protein (g/dl)		Albumin (g/dl)		Globulin (g/dl)		A/G Ratio
		Serum level	% of change	Serum level	% of change	Serum level	% of change	Serum level	% of change	Serum level	% of change	Serum level	% of change	
C	Control unexposed	3.23±0.24		0.09±0.006		53.0±0.1		3.1±0.01		1.9±0.01		1.2±0.01		1.58
I	1/5LC_5 Cu, Pb&Zn	6.4±0.0**	98	0.24±0.00*	167	256.0±0.0***	383	2.5±0.00**	-19	0.9±0.00**	-53	1.6±0.00*	33	0.56
II	1/5LC_{10}Cu, Pb&Zn	7.5±0.32**	132	0.40±0.22**	344	443.5±37.8**	737	2.2±0.50	-29	1±0.30	-47	1.2±0.20	0	0.83
III	AD LC_{10} A & 10PPm D	8.55±0.22**	165	0.20±0.050	122	122.5±8.0**	131	2.05±0.45	-34	0.95±0.15*	-50	1.1±0.60	-8	0.86
IV	AD LC_{25} A & 25PPm D	4.3±0.0*	33	0.14±0.00	56	44.5±4.5	-16	2.95±0.65	-5	1±0.10**	-47	1.95±0.55	63	0.51
V	AD_{10} then 1/5LC_5Cu,Pb&Zn	6.4±0.0*	98	0.06±0.00	-33	231.0±0.0***	336	1.8±0.00**	-42	0.8±0.00**	-58	1±0.00*	-17	0.80
VI	AD_{10} then 1/5LC_{10}Cu,Pb&Zn	6.4±0.0*	98	0.16±0.00*	78	57.0±0.0*	8	1.2±0.00**	-61	0.5±0.00**	-74	0.7±0.00**	-42	0.71

Table (6): Catalase (CAT), Glutathione-S-transferase (GST) and Gamma-glutamyl transpeptidase (GGT) in liver of *Oreochromis niloticus* fish samples (Family Cichlidae); exposed to copper (Cu), lead (Pb), zinc (Zn), Aroclor 1254 (A) and Decabromodiphenyl ether 98% (D) through different experimental designs.

Groups	Parameter	Catalase (CAT) (Unites/g)		Glutathione-S-transferase (GST) (Unites/g)		Gamma-glutamyl transpeptidase (GGT) (Unites/g)	
	Treatments	Level	% of change	Level	% of change	Level	% of change
Control unexposed		0.83±0.005		0.35±0.16		549±24	
LC_{50}	$PbLC_{50}$	0.80±0.00	-3	5.62*±0.00	1506	176*±0	-68
	ALC_{50}	0.83±0.00	1	0.94±0.00	169	182*±0	-67
	D 50ppm	0.40*±0.125	-52	0.48±0.08	36	195**±15.3	-92
I	$1/5LC_5Cu$, Pb&Zn	0.84±0.009	2	2.51*±0.29	616	545±43	-1
II	1/5 $LC_{10}Cu$, Pb&Zn	0.84±0.00	2	1.92*±0.16	447	743±0	35
III	AD10, LC_{10} A & 10PPm D	0.61*±0.066	-26	0.89±0.43	155	267±107	-51
IV	AD25, LC_{25} A & 25PPm D	0.55*±0.061	-34	1.39*±0.14	298	239**±20	-57
V	AD10 then 1/5LC5Cu, Pb&Zn	0.84±0.019	2	2.65*±0.70	656	181**±10	-67
VI	AD10 then 1/5LC10Cu, Pb&Zn	0.84±0.014	1	5.84**±0.28	1569	216**±12	-61

Table (7): Complete blood picture of *Oreochromis niloticus* fish samples (Family Cichlidae); exposed to copper (Cu), lead (Pb), zinc (Zn), Aroclor 1254 (A) and Decabromodiphenyl ether 98% (D) through different experimental designs.

Groups	Parameter	Hemoglobin (mg/dl)		Red blood cell ($10^6 mm^{-3}$)		Platelet (10^3 /mm^3)		WBC ($10^6 mm^{-3}$)		LYM%
	Treatments	HGB	% of change	RBC	% of change	PLT	% of change	Mean ±SD	% of change	
Control		8.4 ±3.68		1.48 ±1.31		14 ±0.0		16.4 ±0.0		92
I	1/5LC5 Cu, Pb&Zn	4.9 ±0.0	-42	0.57 ±0.0	-61	9 ±0.0	-5	15.7 ±0.0	-4	95
II	1/5LC10 Cu, Pb&Zn	6.2 ±0.0	-26	0.84 ±0.0	-43	8 ±0.0	-6	41.4 ±0.0	152	89
III	AD LC10 A & 10PPm D	9.8 ±0.0	17	0.88 ±0.0	-40	639 ±0.0	625	29.6 ±0.0	80	97
IV	AD LC25 A & 25PPm D	9.47 ±3.7	13	1.00 ±0.11	-32	269.0 ±92.8	255	30.1 ±0.0	84	92.4
V	Pre –exposure to AD10 then 1/5LC5Cu, Pb&Zn	4.7 ±0.0	-44	0.65 ±0.0	-56	40 ±0.0	26	46.6 ±0.0	84	92.5
VI	Pre –exposure to AD10 then 1/5LC10Cu, Pb&Zn	5.7 ±0.0	-32	0.02 ±0.0	-99	809 ±0.0	795	14.5 ±0.0	130	82.3

Histopathological investigation:

Results of chronic impact of the individual HMs; Zn, Cu and Pb; and POPs Aroclor 1254 (A) and Decabromodiphenyl ether 98% (D) and six different mixtures of these chemicals (HM LC_5 & LC_{10}, AD LC_{10} & LC_{25} & AD_{10}HM LC_5 & LC_{10}) for a duration of four weeks on the histology of different organs of *O. niloticus* are presented in **Figures (1-6)**.

Fig. (1) illustrated the histopathological alterations in muscle sections of fish samples of all the examined groups. Samples from the control group showed normal muscle histology **(Fig. 1a)**.The muscle of studied fish exposed to heavy metals (Cu, Pb and Zn) showed severe edema, atrophy of muscle bundles and neoplsia **(Figs. 1b, c, d, e & f)**. Atrophy was observed

In muscle bundles of fish exposed Decabromo, Alochlor and $HMLC_5$ **(Figs. 1g, h & i)**. Exposure of fish [$HMLC_{10}$ and$ADLC_{10}$] resulted in splitting of muscle fiber **(Figs. 1j)**. However, severe degenerative changes in muscle bundles accompanied by focal areas of necrosis as well as edema between muscle bundles **(Fig. ll)**, edema between muscle bundles was observed in fish exposed to AD10+HMLC10 **(Fig. 1n)**.

Histopathological alterations in gills of studied fish **are** illustrated in **Figure (2.** The control group showed normal gills tissues **(Fig. 2a)**. After 45 days of exposure to Cu, Pb and Zn the gills revealed hyperplasia of the epithelium in between gill lamellae, epithelial lifting, congested blood vessel of the filament and sloughing **(Figs. 2b, c, d, e, I, j, m)**. Shortening, epithelial lifting, and curling elongated lamellae resulted in samples exposed to Decabromo and Aloclor **(Figs. 2f, g)**. Moreover, after exposure to AD10 and AD25, hyperplasia accompanied by fusion of the lamellae and focal areas of necrosis **(Fig. 2j)** and elongated lamellae with epithelial lifting (p AD10+LC5) **(Fig 2k)**. Besides proliferation of mucus and chloride cells to the top of lamellae were noticed in fish exposed to HMLC5 **(Fig. 2h)** and intensive vasodilation with congestion in the secondary lamellae telangiectasis "aneurism" **(Fig. 2l)**.

Liver sections from the control group showed normal liver architecture **(Fig. 3a)**. Many lesions exhibited in the liver of fish exposed to Cu, Pb and Zn showed dilation and congestion in hepatic sinusoids **(Figs. 3b, c & d)**, dilated vein with hemorrhage, surrounded with fibrous tissues was noticed. Vacuolar degeneration of the hepatocytes with focal areas of necrosis resulted from Decabromo, alochlo, $HMLC_5$ and HMLC10 **(Figs. 3e & g)**, dilation and congestion in hepatoportal blood vessels **(Figs. 3e, f, g, h & k)** and coagulative necrosis in case of AD_{10} **(Fig. 3i)**. In addition, there are dilation and thrombosis formation in hepatic blood vessels **(Figs. 3e & f)**.

Spleen is one of the most important hematopoietic centers. Increase of MMCs in the spleen was observed in all the experimental groups. Histological changes of spleen cells were summarized in hemorrhage, hemolysis, hemosidrin, focal areas of necrosis and degeneration in splenic tissues, **(Figs. 4b - k)**.

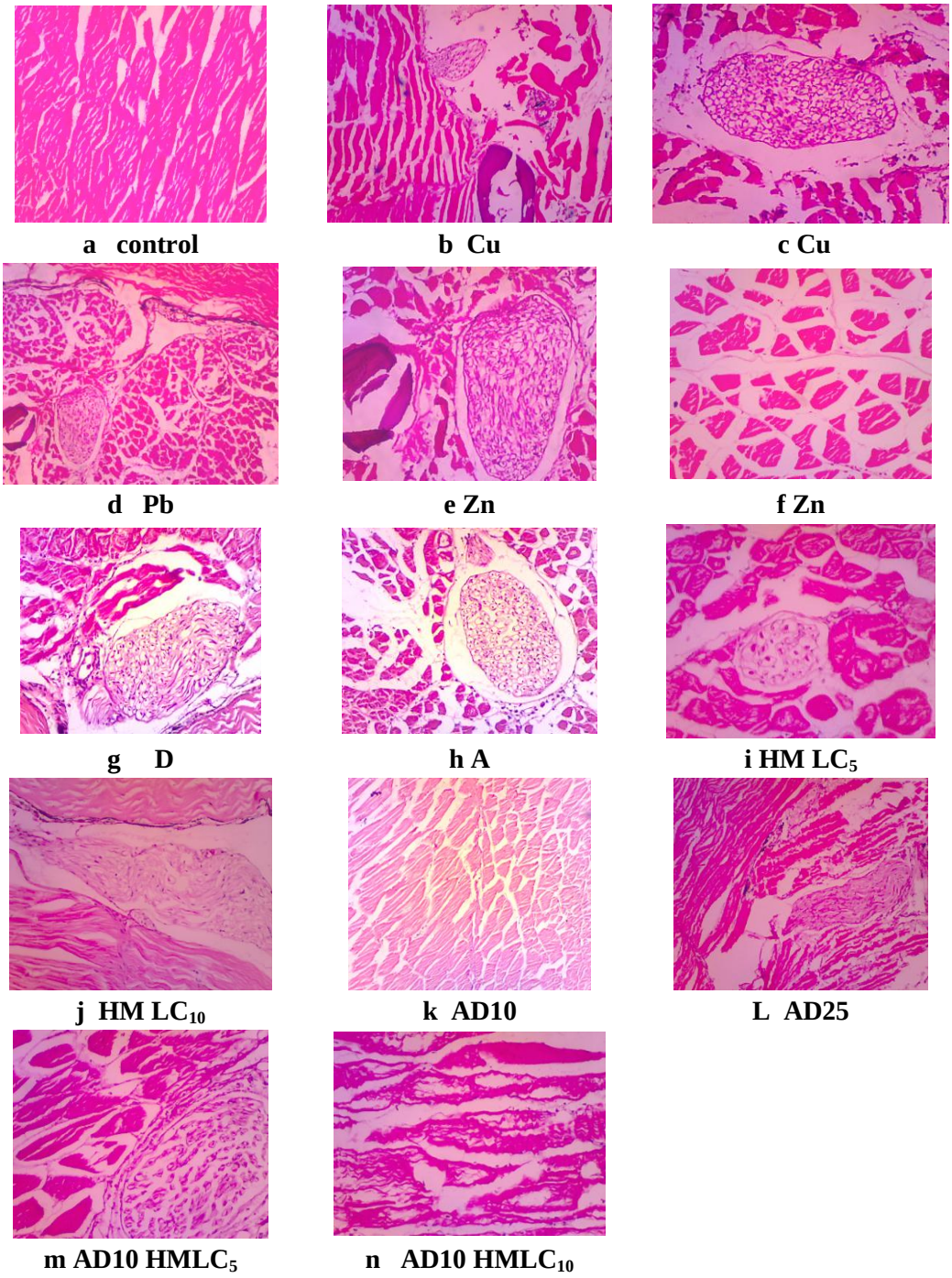


Fig. (1). A longitudinal sections of the muscles of *Oreochromis niloticus* showing normal structure (a) (X100);muscle of fish showing edema, necrotic change and neoplsia (b) (X100) & (c) (X400), Cu; severe necrotic change, severe splitting of muscle bundles and neoplasia (d) (X100), Pb ; severe edema, splitting of muscle fiber and neoplasia (e) (X400) & (f) (X400), Zn; neoplsia (g) (X400) D (Decabromodiphenyl ether 98%); edema, splitting muscle bundles (h) (X400), A (Aroclor 1254); severe edema and neoplasia (i) (X100), HM LC_5 (heavy metal mixture of LC_5 Cu, Pb & Zn) and (j) (X 400), HM LC_{10} (heavy metal mixture of LC_{10} Cu, Pb & Zn); mild edema (k) (X 100), AD10 (ALC_{10} & D 10ppm); neoplasia and focal area of necrosis (L)(X 100), AD25 (ALC_{25} & D 25ppm); edema and neoplasia (m)(X 400), AD10 $HMLC_5$; severe vacuolar degeneration of bundles fibers (n)(400X), AD10 $HMLC_{10}$.

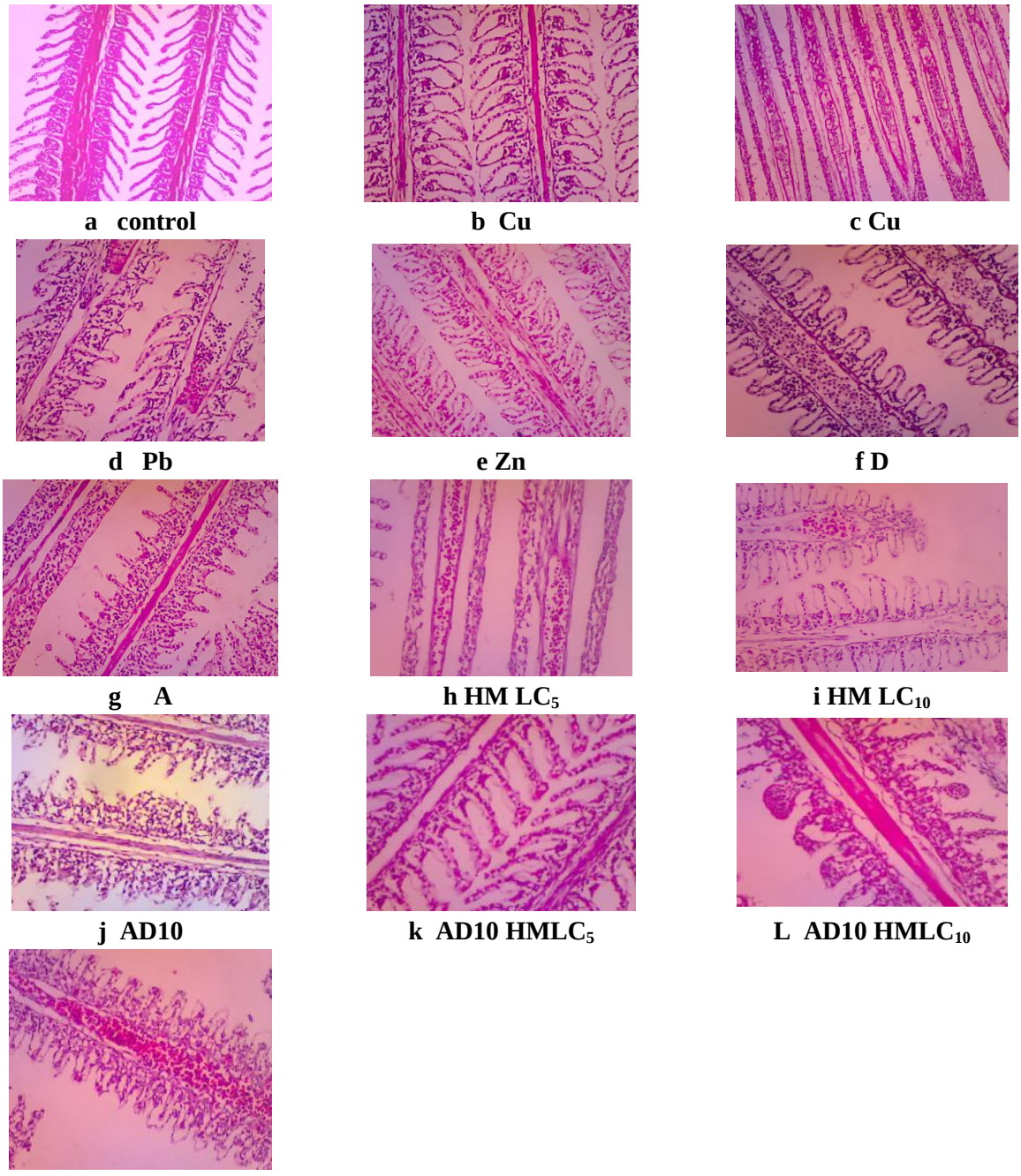
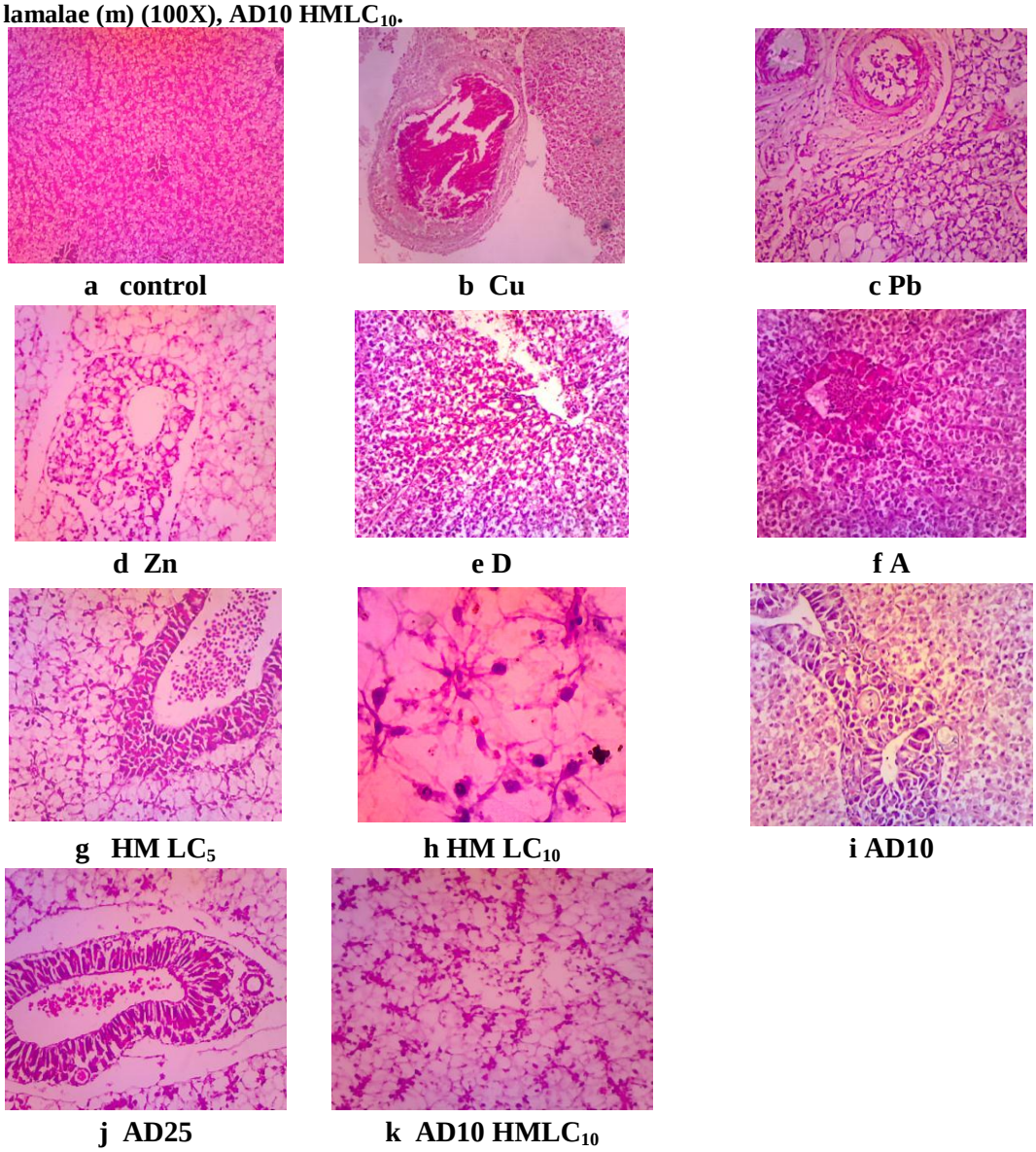


Fig. (2). Sagittal sections of the gills of fish showing normal structure, filament and primary lamellae (a) (X100), mild dilated and congested blood vessel of the filament, epithelial lifting of lamallae and sloughing (b) (X400) & (c) (X100), Cu; severe dilated and congested filament (d) (X400), Pb; severe edema and epithelial lifting (e) (X400), Zn; dilated primary filaments with lymphocyte infiltration, severe edema and shortening of gill lamellae (f) (X400) D (Decabromodiphenyl ether 98%); sloughing and epithelial hyperplasia and necrotic change (g) (X100), A (Aroclor 1254); sloughing (h) (X400), HM LC_5 (heavy metal mixture of LC_5 Cu, Pb & Zn); severe epithelial lifting and congested of filament (i) (X100), HM LC_{10} (heavy metal mixture of LC_{10} Cu, Pb & Zn);hyperplasia and fusion of adjacent lamellae (j) (X 100), AD10 (ALC_{10} & D 10ppm);curling and clubbing of lamellae (k) (400X), AD10 $HMLC_5$; aneurism (l) (X400), AD10 $HMLC_{10}$; severe congestion, epithelial lifting and shorting

lamalae (m) (100X), AD10 HMLC_{10}.



a control **b Cu** **c Pb**

d Zn **e D** **f A**

g HM LC_5 **h HM LC_{10}** **i AD10**

j AD25 **k AD10 HMLC_{10}**

Fig. (3). Sections in the liver of The Nile Tilapia showing normal structure (a) (X100), mild dilated veins and intravascular heamolysis (b) (X400), Cu; severe hydropic vacuolation, focal area of necrosis and haemorrhage between the hepatocytes (c) (X400), Pb; vacuolar degeneration and necrotic change (d) (X400), Zn; thrombosis and focal area of necrosis (e) (X400), D (Decabromodiphenyl ether 98%) and (f) (X400), A (Aroclor 1254); severe hydropic vacuolation, fibrosis and intravascular haemolysis and lymphocyte infilteration (g) (X400), HM LC_5 (heavy metal mixture of LC_5 Cu, Pb & Zn) and (h) (X1000), HM LC_{10} (heavy metal mixture of LC_{10} Cu, Pb & Zn); coagulative necrosis, aggregations of inflammatory cells as well as haemosiderin between the hepatocytes (i) (400X), AD10 (ALC_{10} & D 10ppm); vacuolar degeneration and lymphocyte infilteration (j and k) (400X), AD25 (ALC_{25} & D 25ppm) and AD10 HMLC_{10}, respectively.

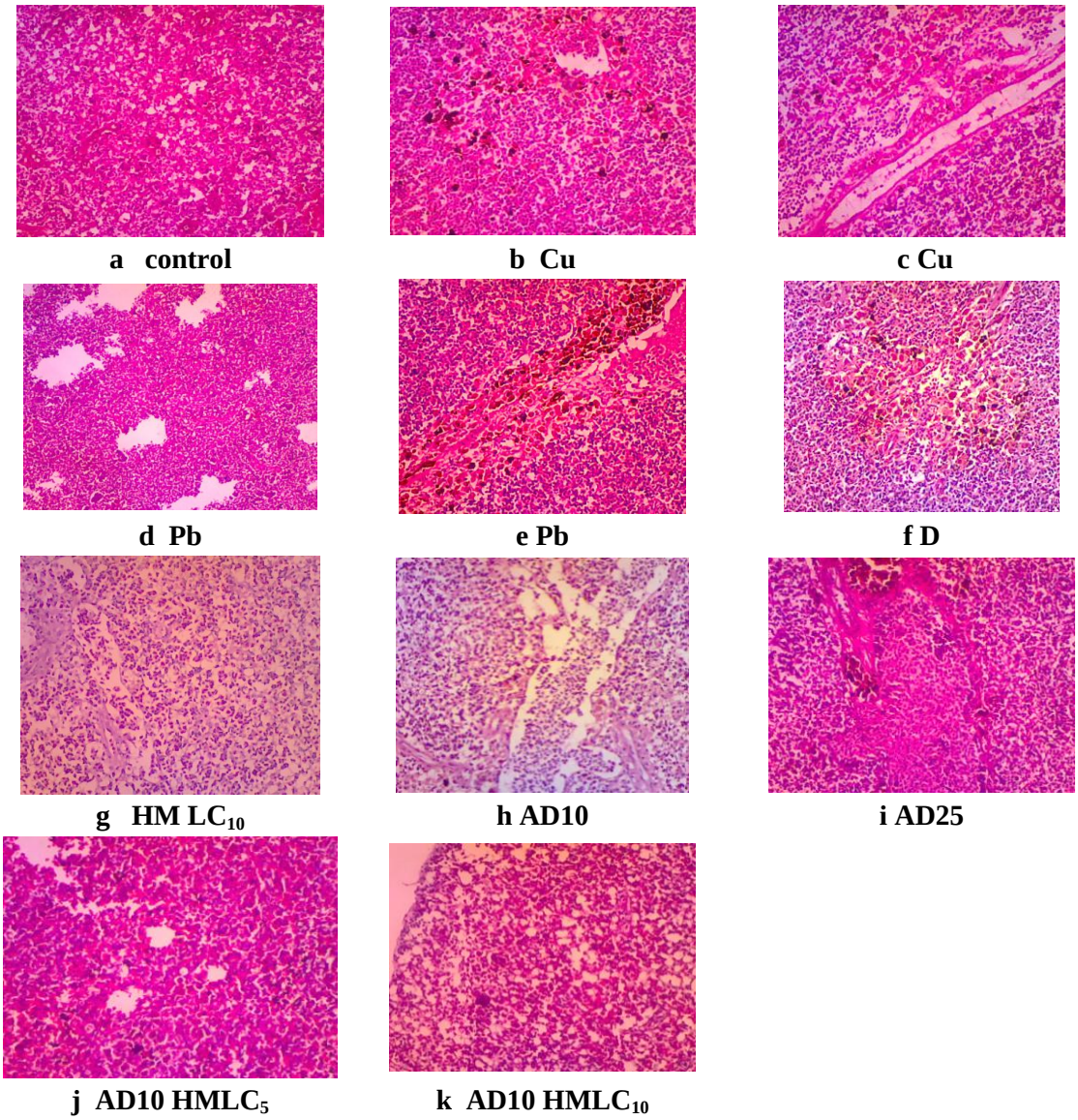


Fig. (4). Transverse sections of the spleen of fish showing normal histological structures (a) (X400); mild MMc, dilated blood vessels, focal areas of necrosis and haemosidrine (b&c) (X400), Cu; severe degenerative change and focal area of necrosis and hemorrhage (d&e) (X400), Pb; necrotic change and haemosidrosis (f) (X400), D (Decabromodiphenyl ether 98%); necrotic change and lymphocytes infiltration (g) (X400), HM LC_{10} (heavy metal mixture of LC_{10} Cu, Pb & Zn) and (h) (X400), AD10 (ALC_{10} & D 10ppm); coagulative necrosis and mild hemosidrosis(i) (400X), AD25 (ALC_{25} & D 25ppm); focal areas of necrosis(j) (X400), AD10 $HMLC_5$ and (k) (X400), AD10 $HMLC_{10}$.

DISCUSSION

In fish, metals uptake is taking place mainly via three routes namely, gills, skin and intestinal wall **(Murugan *et al*., 2008 and Guo *et al.* 2018)**. However, absorption via the gastrointestinal tract and skin is significantly limited. The present distribution of Pb, Zn & Cu residuals revealed that the kidney is the prime site of accumulation, which followed by liver then muscle in most studied samples. This agrees with that previously reported founding by **(Jayakumar & Paul, 2006; Igberaese, 2008 and Traina *et al.*, 2019)**.

The present results were in line with other studies, indicated that the accumulation of Cu, Zn, and Pb in the studied tissues increased with increasing exposure concentrations **(Karakoç & Dinçer, 2003 and Igberaese, 2008)**. The elevation of urea level in the blood with the significant increase of creatinine that observed in HMs & AD10HMLC10 groups and non-significant increase in ADs, confirms the more affected kidney of HMs & AD10 HMs LC10 groups. Many authors commented that the increase in urea and creatinine levels in lead (Pb) intoxicated fish group, might be due to the glomerular insufficiency and the increase in the production of creative oxygen species and kidney injury **(Upasani & Balaraman, 2003; Yu *et al*., 2004; Fırat *et al.*, 2011** and **Germoush *et al.* 2021)**..

The present elevation of GST in all fish groups was in accordance with other findings, GST activity in hepatopancreas of crustacean and mollusks and in fish liver has been suggested as biomarker of organic pollution of water environments **(Filho, 2001; Ahmad *et al*., 2004** and **Farombi *et al*., 2007). Hansson *et al*. (2006)** stated that induction of GST activity in some aquatic organisms such as mussels which found in high polluted marine environments after oil spills of the tankers **(Martinez-porchas *et al*., 2011)**.

In the present investigation, the increase of aminotransferases activity (ALT& AST) and ALP that shown in O. niloticus impacted to HMs and AD10HMs mixtures was in agreement to the finding of **Martinez-Porchas *et al*. (2011)** who documented an increase of aminotransferases activity in blood serum, plasma and other extracellular fluid in the organisms impacted to unfavorable conditions which were related to liver dysfunction or internal lesions in tissues. Also, the increase of serum ALT activity was demonstrated in the common carp impacted heavy metals (Cd, Pb, Ni and Cr) **(Rajamanickam 2008)** and exposed to herbicide **(Abd-Algadir *et al.*, 2011)** and demonstrated in tilapia after injection of Benzo[a]pyrene (PAH), a polycyclic aromatic hydrocarbon pollutant that used as a chemical carcinogen in experimental models of cancer **(Martinez-Porchas *et al.*, 2011)**. **Mohamed & Gad (2009)** stated that the increase of serum GOT; GPT and ALP may be related to the hepatocellular damage or cellular degradation, perhaps in liver, heart or muscle.

Blood glucose level known as a general secondary response to stress of fish to acute toxic effects and is considered as a reliable indicator of environmental stress **(Cicik**

& Engin, 2005 and **Sepici-Dinçel *et al.*, 2009)**. Hyperglycemic response illustrated in the present study is an indication of a disruption in carbohydrate metabolism, possibly due to enhanced glucose6-phosphatase activity in liver, elevated breakdown of liver glycogen, or the synthesis of glucose from extra hepatic tissue proteins and amino acids **(Raja *et al.* 1992; Almeida *et al.* 2001)**. On the other hand, The decrease in plasma total protein level (hypoproteinemia) that shown in the present study was in agreement with the findings of **Al-Asgah *et al.* (2015)**. Who studied the exposure of *O. niloticus*, weighing 36.45 ± 1.12 g to 10%, 20% and 30% of the LC50 of CdCl2 and recorded significant reduction ($p > 0.05$) in fish serum total protein levels for all the exposed treatments. Also, **Mekkawy *et al.* (2011)** showed that *O. niloticus* exposed to 4.64 mg/l (25% of 96 h LC_{50}) Cd for 15 and 30 days showed a reduction in serum protein levels. This decrease of total protein may be due to the destruction of protein-synthesizing subcellular structures and inhibition of the hepatic synthesis of blood protein **(Fontana *et al.*, 1998)**. Loss of protein from damaged kidneys could contribute further to the observed hypoproteinemia **(Mohamed Ali, 2008)**.

The present groups exposed to POPs mixtures, ADs and AD10HMs showed massive elevations in the platelets and WBC; indicating metabolic syndrome. Also, the present ADs groups showed the highest reduction in total bilirubin & GGT levels indicating cholestasis disorder, by the support of **(Isomaa *et al.*, 2001**; **Lakka *et al.*, 2002; Chen *et al.*, 2004** and **Jesri *et al.*, 2005)** who found that both platelet and WBC counts are positively related to the number of metabolic syndrome risk factors which in turn strongly and independently increases risk for heart disease, stroke, and chronic kidney disease.

This study presents an overview on the application of histopathology in evaluation of the health of fish subjected to measurable concentrations of heavy metals, persistent organic pollutants and their mixtures. The present muscle pathological alterations severe edema, neoplasia, necrotic change, fat vacuoles and splitting of muscle fiber were in agreement with observations in fish muscle due to the exposure of different pollutants **(Nour & Amer, 1995). Da Rocha *et al.* (2018)** stated that spontaneous neoplasms in fish may be related to the water pollution; the fish in this case will use as indicators of the presence of environmental carcinogens and the mechanisms of mutagenesis and carcinogenesis in fish are interconnected and influenced by environmental chemical or physical agents, or associated with infectious agents, especially retrovirus.

Microscopic examination of the liver of *O. niloticus* showed several lesions in samples exposed to metals, such as dilated of sinusoid and vacuolation of hepatocytes and congestion of blood vessel surrounded with fibrosis, the finding corresponded with **Thophon *et al.* (2003)** who studied the effects of CdCl2 on liver of Sea bass. Moreover, Focal areas of necrosis and coagulative necrosis and aggregations of inflammatory cells as well as haemosiderin between the hepatocytes were noticed in fish exposed to ADs and AD10 HMs mixtures. It is associated with a variety of clinical disorders that proved

by biochemical study, massive elevation in the platelets and WBC; indicating metabolic syndrome and the highest reduction in total bilirubin & GGT levels indicating cholestasis disorder in fish groups that exposed to ADs and AD_{10} HMs mixtures.

Also, present spleen sections of fish exposed to HMs and Ads mixtures showed the histopathological alterations; focal areas of necrosis, hemorrhage with hemosidrin and coagulative necrosis. **Kaleeswaran *et al.* (2012)** suggested the increased severity in the MMC as a homeostatic mechanism of the fish spleen to phagocytose. The increasing deposits of haemosiderin and other debris resulting from the destruction of tissues **(Loumbourdis & Danscher, 2004** and **El-Kasheif *et al.* 2013)** and this matches with the present study In addition, the present the histological configurations of *O. niloticus* exposed to HMs and A Ds and their mixture, demonstrated a pronounced decline in gonad activity of the studied fish which reflected by disturbed development of germ cells **(Bobek *et al.* 1996** and **Mohamed & Gad, 2008)**.

CONCLUSION

One could prove that, conserving the environment is not a pleasure or enjoyment any more, yet it became pivotal to protect our resources for the coming generations. Moreover, preservative the environment is a national duty and laws shall regulate the practices of keeping good environment.

ACKNOWLEDGMENT

This study is a joint project (Biomarkers as indicators of environmental pollution: Experimental approach and case studies), kindly funded by the Academy of Scientific Research and Technology through the Bilateral Agreement between Academy of Scientific Research and Technology of the Arab Republic of Egypt and Bulgarian Academy of Sciences, 2012- 2014.

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