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COMBINED SUBCHRONIC TOXICITY OF PHENANTHRENE AND BENZO[A]PYRENE MIXTURE IN JUVENILE CATFISH (*CLARIAS GARIEPINUS*)

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ABSTRACT

The effect of joint mixtures of phenanthrene and benzo[a]pyrene on selected haematological and histopathological parameters of the *Clarias gariepinus* was investigated. Healthy juvenile C. gariepinus (n = 90) weighing 19.7 ± 1.8 g were exposed to sublethal concentrations of joint mixtures of phenanthrene and benzo[a]pyrene for a subchronic period of 35 days. Acute toxicity studies showed that phenanthrene and benzo[a]pyrene had LC_{50} values of 1400 and 16 µg/L respectively while the safe limits of the chemicals varied in the ranges of 0.00016 to 1.6 µg/L and 0.014 to 140 µg/L for benzo[a]pyrene and phenanthrene

Introduction:

Human activities have led to an upsurge in the release of hydrocarbons such as those found in petroleum. These hydrocarbons though of economic benefit however pose significant risk to both the aquatic ecosystem and human health (Ramanpour *et al.*, 2014). Polycyclic aromatic hydrocarbons (PAHs) are a major component of these hydrocarbons. Although PAHs have low water-solubility and are short-lived in the water column, they persist in the sediment and can cause severe behavioural and

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respectively. The joint mixture significantly ($p < 0.05$) reduced growth rate in exposed fish. The joint mixtures also led to significant ($p < 0.05$) declines in the studied haematological parameters including blood cell count (RBC), haemoglobin (Hb) and haematocrit (Hct). The erythrocyte indices including mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) all showed significant ($p < 0.05$) declines in the presence of the joint toxicants. There were significant ($p < 0.05$) increases in white blood cells (WBC) count and platelet (PLT) count. Histopathological examination showed alterations in liver and gill sections of exposed fish

Keywords: subchronic, phenathrene, benzo[a]pyrene, safe level, haematology, histopathology, growth

Physiological alterations in aquatic organisms (Santos *et al.*, 2011). Phenanthrene and benzo[a]pyrene are among the sixteen PAHs that have been identified as priority contaminants by the World Health Organization (WHO) because they can bioaccumulate, in the environment and result in contamination of the food chain (Ramanpour *et al.*, 2014).

Both phenanthrene and benzo[a]pyrene occur in fossil fuels and are present in products of incomplete combustion. Some of the known sources of both compounds in the atmosphere are automobile emissions, coal burning, wood combustion and coke plants (U.S. EPA, 1988). They are widely distributed in the aquatic environment and have been identified in surface water bodies and tap water. They have also been identified in seafood collected from contaminated waters and in smoked fish and other foods (U.S. EPA, 1988).

Phenathrene and benzo[a]pyrene have served as markers of oil pollution (Ogata and Miyake, 1979) and are reasonably anticipated as carcinogens (Andelman and Snodgrass, 1974). Petroleum hydrocarbons have been

reported to cause structural damage to the respiratory lamellae of the gills (Prasad, 1991). The compounds tend to adsorb to organic or inorganic matter in sediments and can be trapped in long-term reservoirs. There is substantial uptake of these compounds by aquatic organisms through the diet, through exposure to contaminated water, and through direct contact with sediment (Johnson et al., 2002).

Clarias gariepinus was selected as the test organism, because of its hardiness, ecological and commercial importance in several tropical countries. It is also a model organism that has been used extensively in ecotoxicological research (Esenowo and Ugwumba 2010).

The aim of the present study is to study the effects of joint mixture of phenanthrene and benzo[a]pyrene on the growth, hematology and tissues of *C. gariepinus.*

Materials and methods

Chemicals

Phenanthrene and benzo[a]pyrene were obtained from Sigma Aldrich (Germany). Acetone was obtained from BDH chemicals (UK). Every other reagent was of analytical grade.

Animals

African catfish *C. gariepinus* (19.7 $\pm$1.8 g) were obtained from a commercial aquaculture and transferred to a 100 L maintenance tank. Fish were acclimated to the laboratory condition for 2 weeks prior to experiment. Fish were fed twice daily with commercial fish feed. Fecal matter and uneaten food were removed daily to prevent pollution and growth of algae. Fish were maintained on a 12-h light/dark cycle at all times.

Acute toxicity study

A range finding test was used to determine the appropriate concentrations of exposure. Based on the obtained pre-LC_{50} data, the 96 hour LC_{50} was determined for phenanthrene and benzo[a]pyrene according to the revised OECD guidelines for testing of chemicals (2019). Triplicate sets of 7 fish each were randomly exposed to varying concentrations of phenanthrene and benzo[a]pyrene separately. Acetone was used as solvent carrier. The fish were placed in 30L plastic tanks containing 20L of clean tap water. Another set of 7 fish was also maintained with equal amount of tap water (and solvent carrier) but without the test chemicals and considered as the solvent control. Fish was not fed throughout the experiment and lethality was the toxicity end point. Dead fish were removed and the mortality recorded at intervals of 24, 48, 72 and 96 h. The 96 hour LC_{50} value of naphthalene for the fish was determined by probit analysis.

Sublethal toxicity study

Stock solutions of phenanthrene and benzo[a]pyrene were prepared by dissolving the chemicals in distilled water and taking acetone as solvent carrier. Test solutions were prepared by dilution of stock solutions in tap water. During sublethal studies, fish were exposed to a mixture of 50% and 25% of the LC_{50} value of both chemicals (corresponding to treatment levels 1 and 2). A solvent control was included in the experimental design. Fish were kept in groups of 10 in 30L plastic tanks containing the test solutions. Experiments were performed in triplicates. Period of exposure lasted 5 weeks

Assays

At the end of the exposure period, hypothermia was used to anaesthetize the fishes. Blood was then collected from the immobilized fish by caudal vein puncture method as described by Argungu et al. (2015) using a 5ml sterile disposable syringe with a 22 gauge needle. The blood was transferred to EDTA tubes and transported to the lab for analysis.

The liver and gill tissues were dissected and place in 10% formal saline solution prior to histopathological investigation.

Haematology

Haematological parameters were determined using automated haematology analyzer machine (Mindray BC 2300, USA).

Weight gain (WG)

Weight gain was determined using the method of Thaller *et al* (2014):

$$WG = \frac{w_t \text{ (final weight)} - w_i \text{(initial weight)}}{w_i \text{(initial weight)}} \times 100$$

Statistical analysis

Results were expressed as mean $\pm$ standard error. Data from the different treatment groups were compared b a one-way analysis of variance (ANOVA) followed by a Scheffes test to determine statistically different groups. All differences were considered significant at $p < 0.05$. Statistical analysis was performed using the SPSS statistical package (ver. 24.0 SPSS Company, Chicago, IL, USA).

Results

The results for the acute toxicity tests are shown in figures 1 and 2, corresponding to phenanthrene (Phe) and benzo[a]pyrene (BaP) respectively. While the LC_{50} for Phe was 1400 µg/L, the LC50 for BaP was 16 µg/L. While there were no recorded mortalities in the control group, there was 100% mortality in the highest concentration groups corresponding to 3200 and 64 µg/L for the Phe and BaP groups respectively.

Tables 1 and 2 show the safe limit values for Phe and BaP ranged from 0.014 to 140 and 0.00016 to 1.6 µg/ L respectively.

Figure 3 shows the inhibitory effect of Phe and BaP joint compounds on growth of *C. gariepinus.* The result shows that joint compounds of Phe and BaP inhibited growth in a concentration dependant pattern.

The impact of the joint compounds on selected blood parameters is shown in table 3. The joint compounds lead to significant ($p < 0.05$) declines in red blood cell (RBC) count, haemoglobin (Hb) concentration, and haematocrit (Hct). Erythrocyte indices including mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCV), and mean corpuscular haemoglobin concentration (MCHC) all showed significant ($p < 0.05$) declines.

Significant ($p < 0.05$) increases where observed in white blood cell (WBC) count and plate (PLT) counts.

Plates 1 to 4 show the histopathology results. Plate 1 is the normal impression of the gills in the control group while plate 2 shows gill section for the group the second level of treatment. The gills in this group showed disorganized lamella. While plate 3 showed normal architecture for fish liver in the control group, plate 4 shows dense infiltrates for the liver tissues in the second level of treatment.

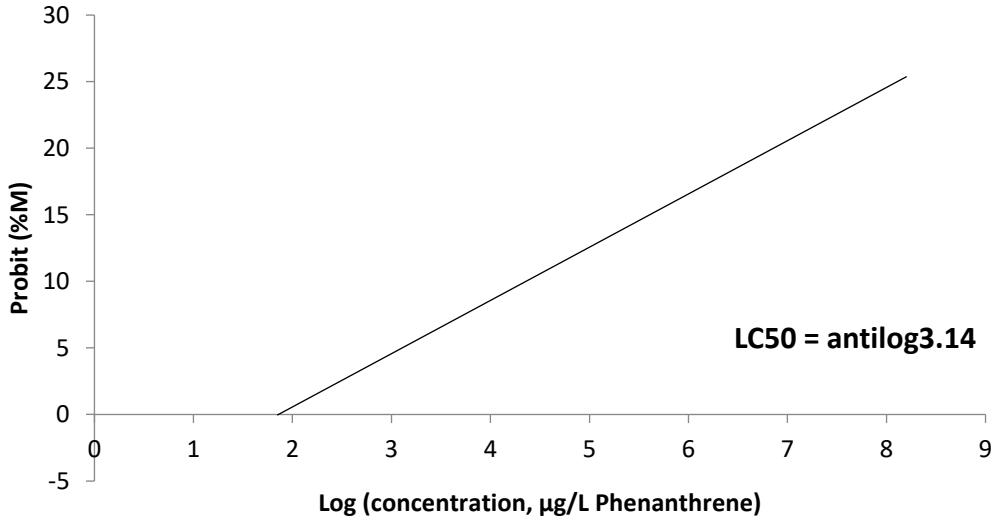

Fig 4.1.2 shows the linear regression curve of log10 concentration versus probit of phenanthrene induced mortality on catfish. $y = 3.99x - 7.43$. The LC50 was 1400 µg/L

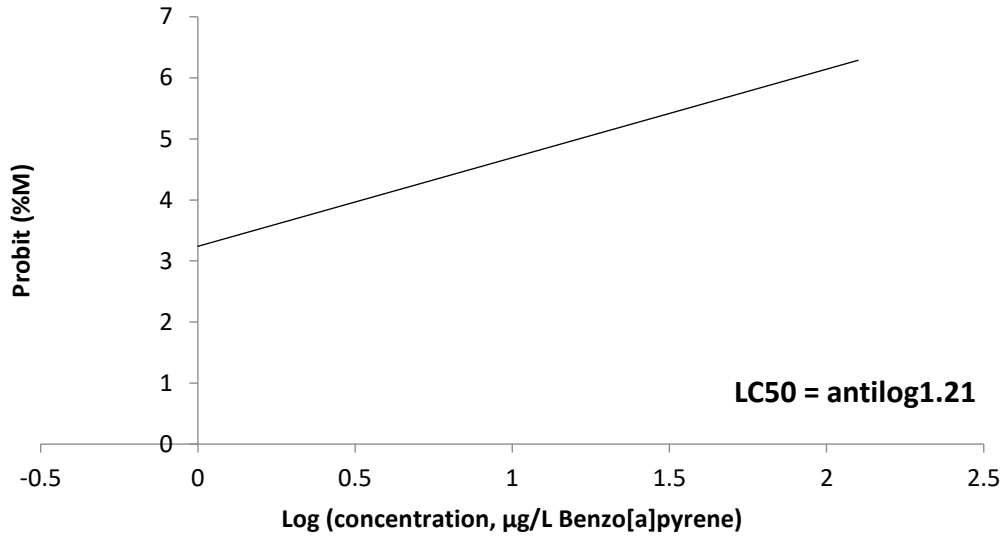

Fig 4.1.3 shows the linear regression curve of log10 concentration versus probit of benzo[a]pyrene induced mortality on catfish. $y = 1.451x + 3.24$. The LC_{50} was 16 µg

Table 1: Estimated safe level limits for phenanthrene

PAH	96h LC_{50} ($\mu g l^{-1}$)	Method	AF	Safe level ($\mu g l^{-1}$)
phenanthrene	1400	Sprague (1971)	0.1	**140**
		CWQC (1972)	0.01	**14**
		NAS/NAE (1973)	0.01-0.00001	**14 – 0.014**
		CCREM (1991)	0.05	**70**
		IJC (1977)	**5% of 96h LC_{50}**	**70**

Table 2: Estimated safe level limits for benzo[a]pyrene

PAH	96h LC_{50} ($\mu g l^{-1}$)	Method	AF	Safe level ($\mu g l^{-1}$)
Benzo[a]pyrene	16	Sprague (1971)	0.1	**1.6**
		CWQC (1972)	0.01	**0.16**
		NAS/NAE (1973)	0.01-0.00001	**0.16 – 0.00016**
		CCREM (1991)	0.05	**0.8**

IJC (1977) 5% of 96h LC_{50} 0.8

Growth inhibition

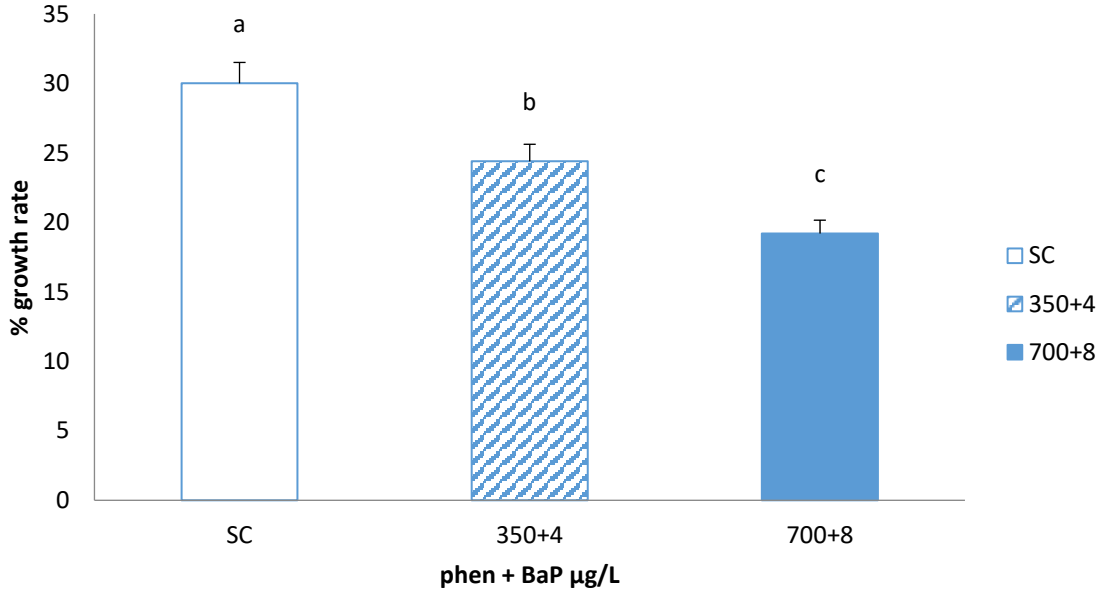

Figure 1: Growth rate of C gariepinus on exposure to joint mixtures of phenanthrene and benzo[a]pyrene. Means not sharing the same letter (a, b or c) are statistically different at $p < 0.05$

Table 3: Haematological parameters of *C. gariepinus* exposed to joint mixtures of phenanthrene and benzo[a]pyrene. Means not sharing the same letter (a, b or c) are statistically different at $p < 0.05$

Parameter	Control group	Phen + BaP joint concentration 350+4 µg/L	700+8 µg/L
RBC	2.41 ± 0.06^{a}	2.35 ± 0.13^{b}	2.17 ± 0.43^{c}
Hb	102.66 ± 3.71^{a}	99 ± 1.9^{b}	91.28 ± 2.8^{b}
Hct	34.85 ± 2.38^{a}	34.13 ± 2.13^{a}	30.1 ± 0.09^{b}
MCV	147.5 ± 2.5^{a}	143.8 ± 0.5^{b}	138.5 ± 0.96^{b}
MCH	43.3 ± 0.24^{a}	41.15 ± 0.52^{b}	39.6 ± 1.1^{b}

MCHC	305.33±4.4[a]	279.33±3.1[b]	272±1.2[b]
WBC	135.56±2.98[a]	146.86±3.3[b]	168.74±4.1[c]
PLT	12.66±1.33[a]	18.33±2.7[b]	19±1.82[b]

Plate 1: photomicrograph of a section of the gill from the control showing normal impression.

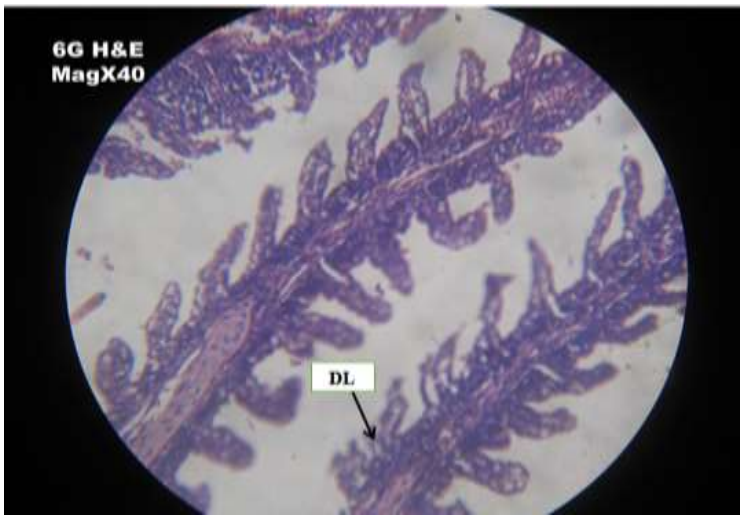

Plate 5: Photomicrograph of a section of the gill from the group exposed to Benzo[a]pyrene and phenanthrene (B+P) mixture showing disorganized lamella (DL)

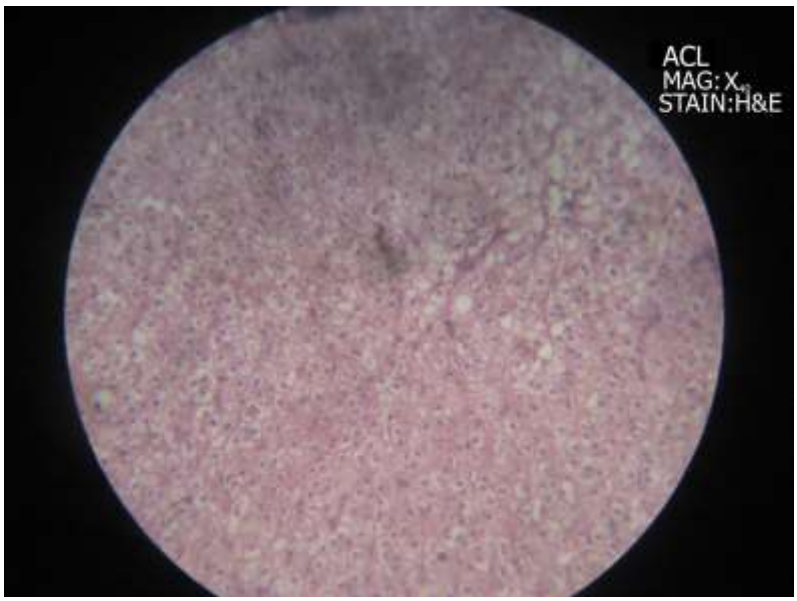

Plate 9: Photomicrograph of a section of the liver from the control group showing normal impression

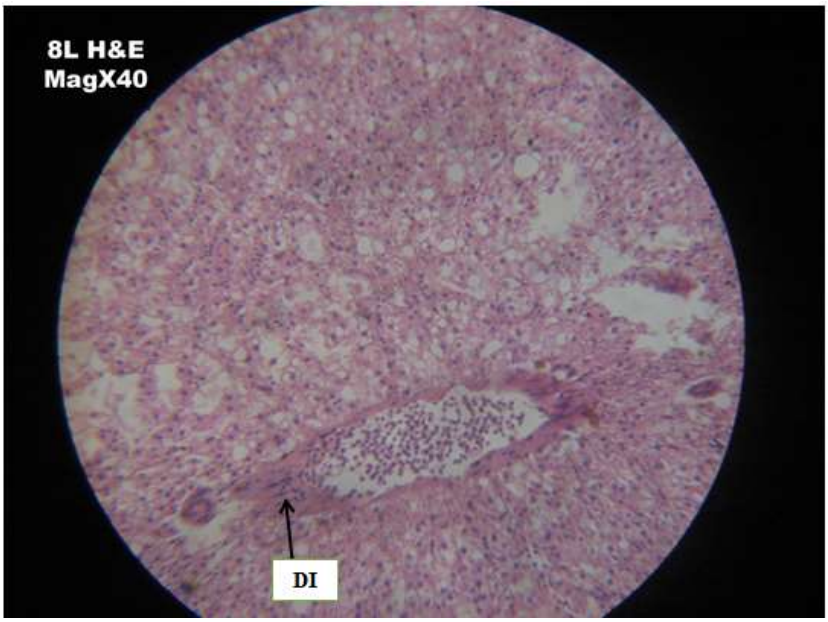

Plate 15: Photomicrograph of a section of the liver from the group exposed to benzo[a]pyrene and phenanthrene mixture (B +P), showing dense infiltrates (DI)

Discussion

The anticipated toxic effect of chemical substances usually represented as their LC_{50} describes the expected toxic effect of chemicals. The LC_{50} for phenanthrene and benzo[a]pyrene were found to be 1400 and 16 µg/L respectively. The LC_{50} for phenanthrene for different aquatic organisms as determined by other researchers are: 432 µg/L for *T. obscurus* (Lee *et al*., 2004); 940 µg/L for *C. macropomum* (Chavez-Ventemilla and Val, 2019) and 4676 µg/L for *C. fluminea* (Xiao *et al*., 2015). The LC_{50} for benzo[a]pyrene in *C. chanos* was 1.4 µg/L (Palanikumar *et al*., 2012). The observed variances in LC_{50} could be as a result of differences in metabolism of the different organisms. Environmental factors, size and regional influences could also play important roles in determining acute toxicities of PAHs (Palanikumar et al. 2012a).

There were huge variations in the estimated levels of safety for both toxicants. The estimated safe levels as determined by various methods obtained in the present study showed a large variation. Pandey *et al*. (2005) noted the controversy that surrounds estimated safe levels measurements and the attendant acceptability issues. There are noted variations in laboratory and field data which are not consistent and this may lead to difficulties in acceptance based on the method used. Kannega (1979) posits that calculation of application faction (AF) which is LC_{50} dependant may not be reliable.

Growth is a continuous increase in average weight or mass. The present study shows that growth rate was inhibited by sublethal concentrations of the joint mixture of phenanthrene and benzo[a]pyrene. It can be assumed that increasing sub-lethal concentration of the joint mixture led to a decrease in food utilization which ultimately led to reductions in growth rate. Surendranath (2003) posits that pollutants may induce changes at the biochemical level prior to cellular and tissue malfunction. Pavlov *et al*

(2004) reported significant decline in growth rate of Mozambique tilapia exposed to cadmium and naphthalene. Jee et al. (2004) demonstrated significant reduction in the relative growth rate of flounder exposed to varying concentrations of phenanthrene.

Red blood cells are essential in maintaining blood pH and regulation of blood flow to tissues and organs (Bloom and Brandt, 2008). Several chemical pollutants have been shown to reduce the measured quantities of red blood cells in circulation (Dey *et al*., 2019). Aquatic organisms exposed to phenanthrene have been shown to have low RBC counts. Mehrnaz *et al*. (2017) reported that phenanthrene-exposed yellowfin seabream had reduced red blood cell counts. This finding is in line with the results obtained in the present study. The observed decline in RBC count could be as a result of inhibition of erythropoiesis in hematopoietic tissues, internal hemorrhage or necrosis of blood cells (Khaniyan *et al*, 2016). Additionally, Kim *et al*. (2007) demonstrated that subchronic benzo[a]pyrene exposure in rockfish caused a significant reduction in erythrocytes as well as concentration of haemoglobin.

Haemoglobin which is synthesized in the bone marrow is the major protein of the red blood cells (Chhabra, 2013). The reduction in haemoglobin concentration as observed in the present study may be due to the disruptive effect of the PAHs on the erythropoietic tissues. Recently, Parmar and Shar (2021) demonstrated significant declines in both red blood cell count and haemoglobin concentration of *C. catla* exposed to RR 120 azo dye. Concentrations of haemoglobin can serve as a very reliable indicator of alterations in ecological conditions (Parmar and Shar, 2021).

The low HCT values observed in this work correlated with the reduction in red blood cell count as a result of PAH toxicity. Low levels of hematocrit level could be a direct consequence of low red blood cell counts (Mondal, 2021). The observed low HCT values in this work may be attributed to the

combined effects of erythropoiesis inhibition, iron metabolism alteration and destruction of red blood cells. (Eriegha *et al*., 2017). Glusczak *et al*. (2006) reported significant decline in HCT in *L obtusidens* exposed to glyophosate. Barcellos *et al*. 2003 showed that fish species exposed to toxic chemicals had lower Hematocrit, haemoglobin concentration and red blood cell counts.

Erythrocyte parameters can be utilized in understanding the etiology of anemias. Anemias are grouped, in accordance with the size of the erythrocyte, as being normocytic (normal MCV), macrocytic (increased MCV), or microcytic (decreased MCV) (Walker *et al*., 1990). There were significant declines all studied erythrocyte parameters.

There was increase in the levels of circulating white blood cells in exposed fish pointing to the immunotoxicity of the PAH compounds. White blood cells are part of the immune system and participate in immune responses. Ramesh and Saravanan (2008) reported increases in WBC count in *L. rohitato* exposed to deltamethrin at sublethal concentrations.

Platelets which are not regarded as cells play important roles in inflammation. Under stress conditions as that obtained in the present study, platelet counts are elevated to mitigate haemorrhage (Dey *et al*. 2019).

Histopathological alterations can be utilized to evaluate the health status of fish exposed to contaminants. In the present study, liver and gill sections were examined to assess the impact of the joint PAH mixtures on the tissues. While fish from the control group showed normal gill and liver architecture, exposed fish showed disorganized lamella of the gills and dense infiltrates in the liver tissues. Cell infiltrates which are seen in damaged tissues comprise plasma cells, lymphocytes and macrophages. The presence of dense cell infiltrates PAH exposure led to structural damage in the liver (Bluemel *et al*., 2015). The observed disorganized lamella in the present work could be a protective mechanism since it leads to a reduction in the total surface area of the gills (Kumar *et al*., 2010).).

Similar change has also been reported in *H. fossilis* after exposure to neem extract (Kumar *et al*., 2010).

In conclusion, the joint mixture of phenanthrene and benzo[a]pyrene had deleterious effects on the health status of exposed fish. There is need to intensive research in the area of PAH ecotoxicology especially using the tropical catfish as a model organism. The information obtained from this study will help to assess the effects of PAH mixtures on aquatic organisms and to establish water quality criteria in tropical countries.

Conflict of interest

The authors declare no conflict of interests.

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