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Full Length Research Paper

Toxicity study of diethyl phthalate on *Clarias gariepinus* fingerlings

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Diethyl Phthalate (DEP) is used as a plasticizer, a detergent base, in aerosol sprays, as a perfume binder and after shave lotion. It is known to be a contaminant of fresh water and marine ecosystem. Therefore, a study was designed to determine the acute toxicity effects of DEP on a fresh water fish, *Clarias gariepinus* fingerlings. The fish was treated with 50, 75, 100 and 150 µg/l. DEP was dissolved in distilled water to determine the $LC_{50.}$ There was 100% mortality observed in 150 µg/l. The LC_{50} of DEP was estimated at log toxicant concentration as 2.217, 2.734, 3.435 and 3.931 µg/l at 24, 48, 72, 96 h and 1.871µg/l for the total death. This shows that the impacts are dose and time dependent with respect to marked reduction in mortality rate. At sub-lethal concentrations of the test substance at 30, 40, 60 and 80 µg/l in a renewal bioassay system, the water and the test compound were changed intermittently. One group was maintained as a control in dechlorinated water. There was significant difference ($P < 0.05$) in brain and muscle AchE activity compared to the control. The liver ACP activity was statistically significant ($P < 0.05$) at day 15 while the muscle ACP in other treatment groups showed no significant difference ($P > 0.05$). Liver AST showed no significance in all treated groups ($P > 0.05$) and liver ALT activity was statistically significant ($P < 0.05$) at day 30 only. The haematological parameters (HB, PCV, RBC and WBC) carried out showed that haemoglobin and erythrocyte levels estimated in all treatment groups to the duration of exposure showed no significant difference ($P > 0.05$) compared to the control. The park cell volume showed a significant difference ($P < 0.05$) at day 30 only. The leucocyte count throughout the exposure period showed that the mean values are statistically significant ($P < 0.05$) at day 15 only compared to the control. The mean cell volume (MCV) showed a significant difference at day 15 ($P < 0.05$) whereas mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) showed no significant difference ($P > 0.05$) throughout the exposure period. No significant difference was seen between the lymphocytes and the neutrophils. In day 0 and 15 only, the monocytes and the lymphocytes showed a significant difference ($P < 0.05$). The gill damages indicated toxicity of DEP with raised lamella, oedema of the lamella epithelia, loss of lamellar epithelium, mild oedema and raising of the filament. The liver damage showed focal necrosis and vacuolization, hepatocyte degeneration in the liver. These alterations may have long term effects on that that are continuously exposed to DEP in the aquatic environment.

Key words: Diethyl phthalate, *Clarias gariepinus*, acute toxicity, sublethal toxicity, heamatology, biochemical, histopathology, environment.

INTRODUCTION

Diethyl Phthalate (DEP) is an industrial chemical that originated from a variety of compounds of anthropogenic origin such as pesticides, detergents and plasticizers (Nivedita et al., 2002) used in products such insecticides,

mosquito repellants, camphor substitute, plasticizer for cellulose, bathing soaps, cosmetics, pharmaceutical coatings, after shave-lotion, detergent, esterplastic film and sheets, etc. (Kamrin and Mayor, 1991; Huang et al., 2008). Diethyl phthalate in aquatic environment originates from a variety of compounds of anthropogenic origin such as pesticides, detergents and plasticizers (Fatoki et al., 2010). Many reports have discussed the impact of man-made xenoestrogenic compounds on man and wildlife (Fatoki et al., 2010; Sekizawa et al., 2003).

A study carried out on Puerto Rican girls, for example, revealed that seven samples collected from the girls contained high levels of phthalate esters, which were suspected to be responsible for premature breast develop-ment (Colon et al., 2000). It has also been estimated that approximately 1% the phthalate ester content of plastic materials in direct contact with water or other liquids are released into the aquatic environment (Peakall, 1975). Besides, DEP may be discharged from industries as effluent.

The empty DEP containers are washed in freshwater bodies such as rivers and lakes and the container are used for domestic water storage. There is a growing awareness of the critical role, which changes in the blood parameters, biochemicals and tissue damage of fish could play in the assessment of the pollution status in aquatic environment. This is predicted on the fact that blood parameters respond rapidly to changes in water quality (Oluah and Njoku, 2001; Nussey et al., 1995; Van Vuren, 1986).

Vosylienė (1999) noted that changes in the hematological parameters, biochemicals are useful tools in assessment of the physiological status of fish. The use of biochemical and physiological responses in the biological assessment of the environmental impact of chemical substances has increased in the past few years. The present study was conducted to determine the toxic effects of DEP contamination of fresh water through an experiment on 150 fish *Clarias gariepinus*.

MATERIALS AND METHODS

Collection of test organism

One hundred and fifty fingerlings of mean wet weight, 13.13 ± 2.27 g were obtained from Aquafish Limited, Awka, Anambra State, Nigeria, identified using taxanoic key of Reed et al. (1967) and were treated with 0.05% $KMnO_4$ solution for 2 min to avoid any dermal infections. Fish was acclimated for 21 days and fingerlings were randomly distributed into fifteen 25 L glass containers (75 × 45 × 45 cm) filled to 20 L mark with unchlorinated water each containing ten fingerlings. Each container was covered on top with nylon mesh tied firmly around the top of the container with rubber band to prevent the fish from jumping out. Every effort was made to provide optimal conditions for fish; no mortality occurred during this period. Feeding was discontinued 48 h before the commencement of the experiment to minimize the production of water in the test container (Ezekiel and Benedict, 2008). The handling of the test organism conforms to the policy on Animal use by the American College of Toxicology.

Test chemicals

Analytical grade diethyl phthalate (99.97% purity) obtained from Sigma Chemical, Ohio, USA was used in this study. The chemical is usually used in manufacture of insecticides etc.

Range finding test

The exploratory range of concentration of test chemicals was determined with a series of range finding experiment (American Public Health Association, 1998). This was initially conducted using the geometric series of concentration values to identify the highest concentrations that will kill 100% of the test organisms and the least concentration that will have no effect on them. Thereafter, definitive acute toxicity bioassays (continous flow-through system) were conducted by exposing fish to different concentrations of DEP.

Test solutions

Stock solution was prepared by dissolving 1 g of DEP in 1000 ml of distilled deionized water because DEP is soluble in water and serial dilutions made from which different concentrations (0, 50, 75, 100 and 150 µg/L) corresponding to the treatments were made. Water quality of the experimental tank was determined according to standard procedures (APHA, 1998).

Acute toxicity tests

Acute toxicity test were conducted according to standard proce-0dures (American Society for Testing of Materials, 19990). The fish were monitored for 96 h and any mortality, morphological, behavioral changes and mortality were recorded. The definitive test was conducted using concentrations (0, 50, 75, 100 and 150 µg/L) of DEP earlier determined for the range finding test. Mean mortality from a particular dose and its replicate was calculated. A fish was considered dead when it did not respond after prodding with a glass rod; dead fish were removed from the tank immediately. Fish behavior was also observed during exposures. Fish were not fed during the experimentation period as recommended by Ward and Parrish (1982) and Reish and Oshida (1987). To ensure proper oxygenation of water throughout the experimentation period, during experimentation, it was observed that an enhanced concentration of particular test chemicals was required as a lethal dose in flow-through systems as compared with static tanks. As such flow-through system posseses many advantages over static tanks, the toxicity in flow-through system, however, provides chemical parameters consistency, which is not achievable in static. A continous-flow system not subject to build up of metabolites and depletion of toxicants clearly represents a more appropriate model of an open system in nature than does a static culture (Porcella, 1969; Davis, 1978). Flow-through toxicity tests are especially important for fish (Solbe, 1974; Spraque, 1964).

Mortality

Mortality was recorded at an interval of 24 h over a period of 4 days (96 h). Fingerlings were taken dead when they turned upside down

Table 1. Water quality parameters of exposure aquaria (30 days).

Dissolved oxygen (mg/L)	Concentration of DEP (µg/L)		
	Day 1	Day 15	Day 30
Control	4.60±0.10	4.30±0.20	4.20±0.10
30	1.40±0.10	1.80±0.10	2.00±0.10
40	1.70±0.10	2.20±0.00	1.90±0.00
60	1.90±0.10	2.20±0.00	2.50±0.10
80	2.50±0.10	2.30±0.10	2.40±0.10
pH			
Control	6.90±0.00	6.70±0.10	6.70±0.10
30	6.20±0.00	6.10±0.00	6.10±0.00
40	6.10±0.00	6.10±0.00	6.10±0.00
60	6.20±0.10	6.10±0.00	6.10±0.00
80	6.40±0.10	6.10±0.00	6.00±0.00
Temperature(°C)			
Control	28.0±3.01	28.40±3.03	28.50±3.03
30	28.0±3.03	28.00±3.03	28.30±3.03
40	28.0±3.03	28.2±2.03	28.20±2.03
60	27.0±2.00	28.2±3.03	28.20±2.03
80	27.0±2.71	28.5±3.03	28.00±3.03

*Each value is mean±SD of 3 observations.

and sank to the bottom of the tank or when their tail showed no form of movement even prodded with a glass rod (Mgbaeruhu, 2002).

Chronic toxicity tests

One hundred and eighty fingerlings (13.13 ± 2.27 g) were used and the fish were randomly divided into five treatments groups (A to E) of thirty six fish. Each group was further randomized into three replicate experiments containing twelve fish each. The fish in group A and B were exposed to 30 and 40 µg/L of DEP, respectively. Similarly, the fish in groups C and D were treated with 60 and 80 µg/L, respectively. The fifth group (control) was exposed to tap water as the control. The experiment lasted for 30 days, and at the beginning of every week fingerlings were collected to assess their heamatological, biochemical and histopathological changes. Water temperature, pH and dissolved oxygen, DO of the exposure aquaria were monitored using meecury in-glass thermometer, dissolved oxygen meter (Table 1) (Jenway, 9071) and digital pH meter (mettle Toledo 320).

Heamatological evaluation

Heamatological examination of fish followed the method described by Svobodova et al. (1991). 11/2 ml of blood were collected at the beginning (initial) of the experiment through weekly to the end of the experiment (week 4) from the caudal peduncle of both the test and the control fish as described by Stoskopf (1993) and Joshi et al. (2000). The blood samples were dispensed into tubes containing lithium heparin anticoagulant. Heamoglobin was estimated by cyanomethemoglobin method. Red blood cells (RBC) and white blood cell (WBC) were counted by Neubauer's improved heamocytometer using Hyems (Shah and Alltinday, 2005) and Turks solution (Mgbenka et al., 2003) as a diluting fluid respectively. Packed cell volume (PCV), mean corpuscular heamoglobin concentration (MCHC), mean corpuscular heamoglobin (MCH) and mean cell volume (MCV) were calculated respectively using standard formula described by Dacie and Lewis (1991) and Joshi et al. (2002).

Biochemical profile

At the end of each week, the fish were stunned by being placed on a block of ice and then dissected to obtain the brain, muscle and liver of the test animal (*Clarias gariepinus*); 10% homogenate in ice cold saline were prepared. The homogenates were used for estimating enzyme activity. The liver and muscle homogenates were used for estimating the activity of acid and alkaline phosphatase (ACP and ALP) (Bassey et al., 1946), aspartate and alanine aminotransferase (AST and ALT) (Reitman and Frankel, 1957). The muscle and brain homogenates were used for estimation of acetylcholinesterase (AchE) activity (Bergmeyer, 1974).

Histopathology

The tissue samples (liver and gill) were quickly excised from the fingerlings and fixed at 10% formal-saline. Slices of the organs were quickly prepared for histological examination to show if there were morphological change in the organs during the treatment (intoxication). Processing started with parking of the tissues in the tissue capsule. The tissues were dehydrated in graded levels of ethanol (70 to 100%) in ascending order. The alcohol was changed after soaking the tissues in them for 1 to 2 h. The tissues were cleared in chloroform and impregnated with paraffin wax and sectioned at 4 to 5 µ thickness using rotary microtone. The sections were floated on a water bath maintained at 2 to 3°C below melting point of paraffin wax. They were on a hot plate thermostatically maintained at a temperature of 2 to 3°C above the midpoint of paraffin wax. When properly dried (15 to 30 min), they were stained with haematoxylin and eosin (H and E), dehydrated, cleared and

Table 2. Cumulative percentage mortality of *Clarias gariepinus* exposed to graded concentrations of diethyl phthalate (DEP) for a maximum of 96 h.

DEP (µg/L)	% mortality (24 h)	% mortality (48 h)	% mortality (72 h)	% 96 h mortality	Total % mortality
Control	0	0	0	0	0
50	3.3	13.3	10	3.3	29.9
75	6.7	16.7	13.3	10	46.7
100	23.3	23.3	6.7	3.3	56.6
150	43.3	26.7	20	10	100

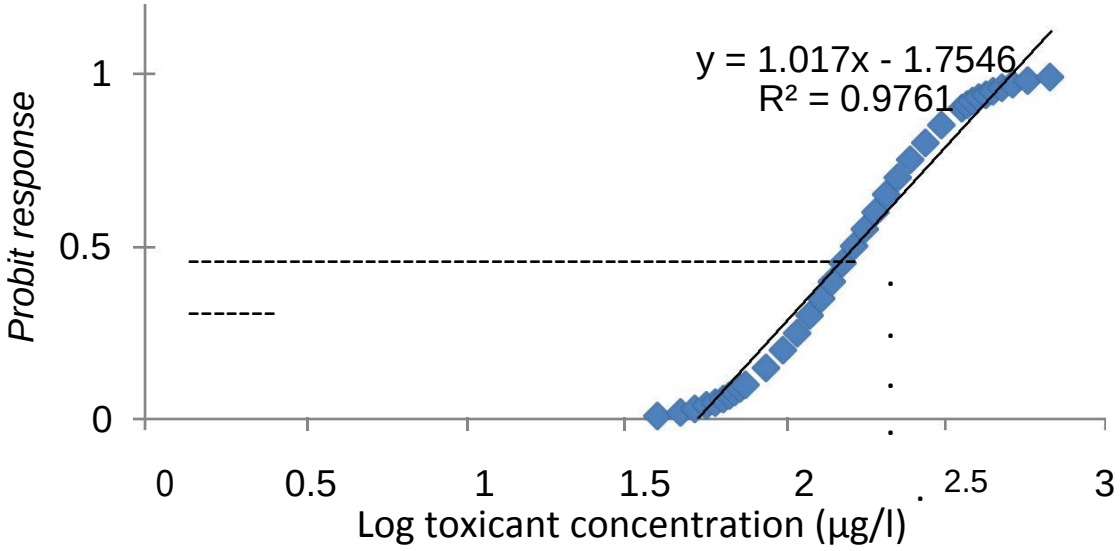

Figure 1. Probit transformed responses for 24 h exposure of *Clarias gariepinus* fingerlings to graded concentrations of diethyl phthalate (DEP).

mounted (D.C.M.) in a mountant, avoiding air bubbles. E staining was used for the demonstration of general tissue structures in various colours. The nuclei as well as some calcium salts and ureates were to take blue colour.

Other tissue structures were to appear red, pink or orange in color (eosinophilic). The permanent slides prepared were mounted one after the other and were viewed at different magnifications of the microscope. Photographs of each slides was taken and the results are shown.

Statistical analysis

Mean values were analyzed for significant differences ($P \leq 0.05$) using the ANOVA. Differences between means were partitioned using the Duncan New Multiple Range test. The Statistical Package for Social Sciences (SPSS) version 16 was used. The probit value was determined from the probit model developed by Finney (1971).

RESULTS

Acute test result

The mortality rate of *C. gariepinus* fingerlings exposed to diethyl phthalate for the period of 96 h is shown in Table 2. Mortality of fish treated with DEP concentrations (50, 75, 100 and 150 µg/L) was dose-dependent. The LD_{50} of DEP for the fish at 24, 48, 72 and 96 h are shown in Figures 1 to 5. The LD_{50} values for DEP were 2.22, 2.73, 3.44 and 3.93 µg/L after 24, 48, 72 and 96 h, respec-tively. The mean LD_{50} was 1.87 µg/L after 96 h. A chi-square goodness-of-fit did not indicate significant differ-rence ($P>0.05$) between the observed and expected responses. The observed percentage mortality which was dose dependent increased from 29.9 to 46.7% for the fingerlings exposed to 50 and 75 µg/L DEP while the mortalities were 56.6 and 100% for the fish exposed to concentrations of 100 and 150 µg/L, respectively. No mortality was recorded in the control group. The r^2 value was 97.6% indicating the adequacy of the model. Hae-morrhaging of the gill of some fish was observed in the fish exposed to 100 and 150 µg/L of the compound. Rapid opercula movement, restlessness, erratic swim-ming and loss of balance were observed in the fish expo-sed to DEP. The results of the study are shown in Tables 3 and 4. The haematological parameters in the control fish did not alter significantly throughout the study period. The haemoglobin concentration in the treatment groups were significantly lower than the control ($P < 0.05$) and also differed with between the treatment groups ($P < 0.05$). There was inverse relationship between the haemoglobin con-centration and DEP concentration at day 15 (Y = -0.025x

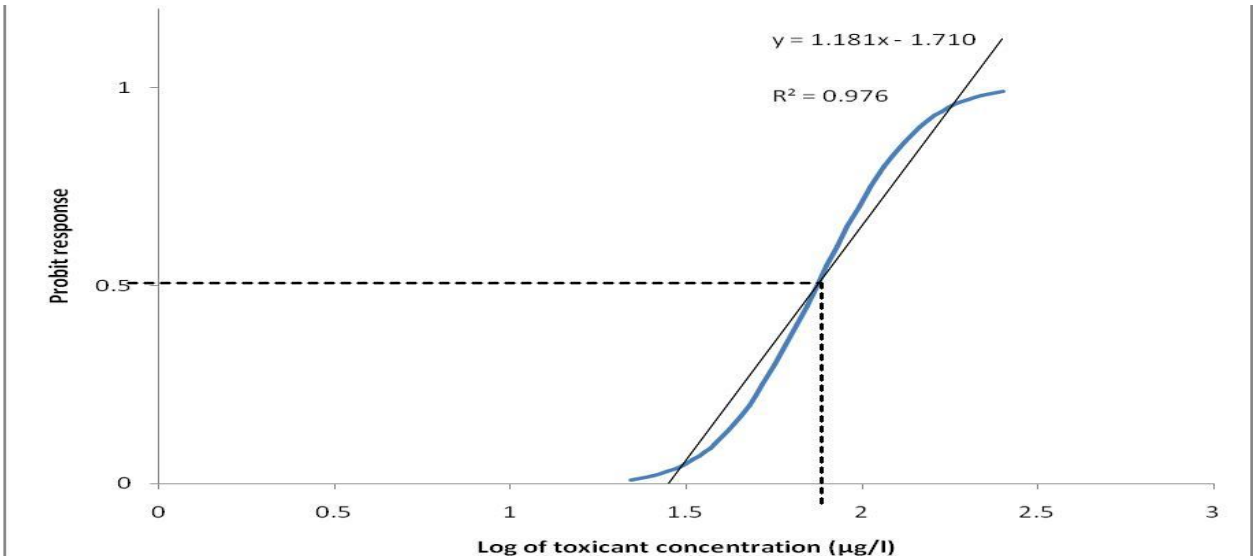

Figure 2. Probit transformed responses for total death of *Clarias gariepinus* fingerlings exposed to graded concentrations of diethyl phthalate (DEP).

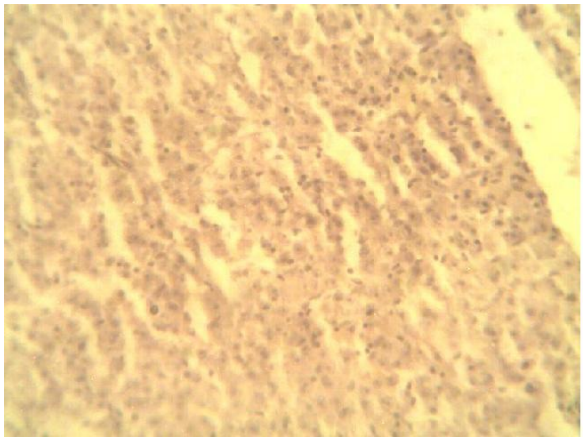

Figure 3. Photomicrograph of liver section from control juvenile *C. gariepinus* 80 µg/L diethyl phthalate at day 30 showing the central vein (V) and normal place of hepatocytes (arrows). H&E (mag ×100).

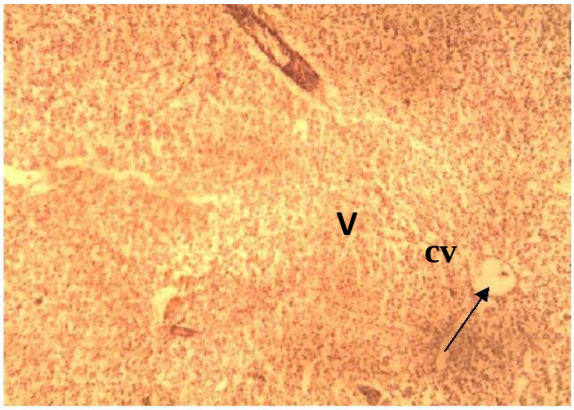

Figure 4. Photomicrograph of histologic section of liver of *Clarias gariepinus* treated with 30 µg/L of diethyl phthalate day 7 showing mild vacuolation of hepatocytes. Note the central vein (V). H&E (mag ×100).

+ 4.92; R = 0.051) and day 30 (Y = -0.0214x + 4.29; R = 0.937). This was an indicative that haemoglobin concentration decreased with increasing DEP concentration and with duration. There was a non-significant increase in Hb concentration at day 15 in fish exposed to 60 µg/L DEP when compared to the group treated with 30 µg/L DEP. The packed cell volume (PCV) of the DEP exposed fish was significantly lower ($P < 0.05$) than the control. The decrease in PCV was concentration dependent and with increasing duration of expo-sure. The erythrocyte count decreased significantly in the treatment group when compared with the control ($P < 0.05$). Also, the erythrocyte count differed in the treatment groups ($P< 0.05$). Similarly, the rate of decrease was DEP concentration dependent and with exposure time.

The morphological indices of the fish exposed to DEP were affected adversely. Both the MCV and MCH of the DEP exposed fish were lower than the control ($P <0.05$). The decrease in the morphological indices of the fish was neither DEP concentration dependent nor influenced by the duration of exposure. The leucocyte count in the fish exposed to DEP was significantly higher than the control ($P < 0.05$) although the rate of increase does not seem to be concentration dependent. Results of the biochemical changes of *C. gariepinus* exposed to the four doses (30, 40, 60 and 80 µg/L) of DEP were as follows. There was a significant increase in liver and muscle ACP levels com-pared to the controls (Tables 5 and 6) interestingly, liver ACP levels were significantly increased in treated groups (30 to 80 µg/L) compared to the control; whereas, muscle ACP levels also increased significantly in the experimental

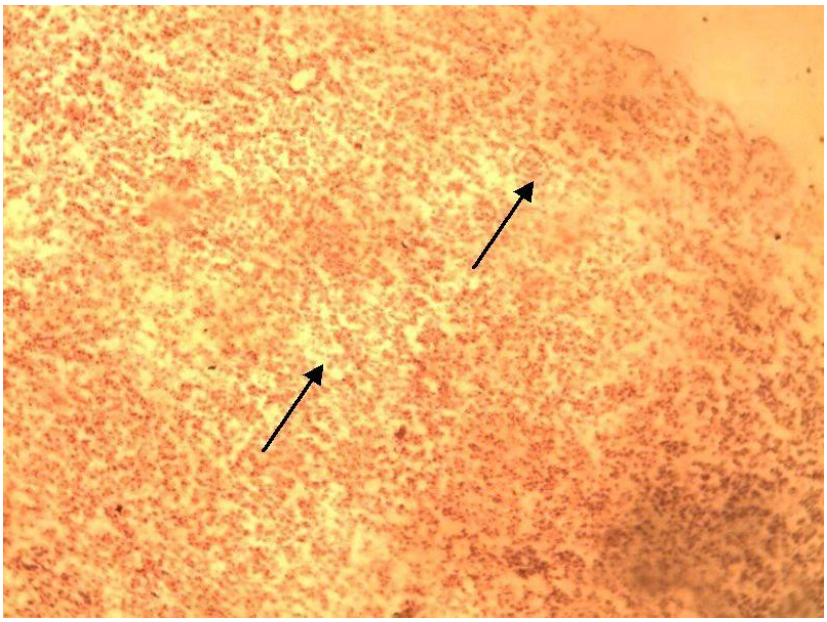

Figure 5. Histologic section of liver of *Clarias gariepinus* treated with 30 µg/L diethyl phthalate at day 15 showing multifocal areas of hepatocyte degeneration. (H&E) mag ×100.

Table 3. Changes in the haematological parameters of *Clarias gariepinus* fingerlings exposed to diethyl phthalate (DEP) for 30 days[1, 2, 3].

DEP (µg/L)	Time (day)	HB (g/dl)	PCV (%)	RBC (× 0^6/mm^3)	WBC (×10^4/mm^3)	MCV (fl)	MCH (pg)	MCHC (g/dl)
	1	4.92±1.47^a	18.2±1.30^a	2.714±0.59^b	1.3±0.10^a	9.70±4.25^a	18.56±5.10^a	0.24±0.07^a
Control	15	4.92±1.61^c	18.4±4.15^c	2.694±0.45^c	2.12±0.35^{ab}	9.6±.64^a	18.32±4.90^a	0.27±0.07^a
	30	4.42±.45	18.2±1.64	2.27±0.33	2.73±0.19	8.12±1.04	19.56±1.45	0.24±
	1	4.64±.98^a	19.6±2.40^a	2.20±0.58^{ab}	2.04±0.24^{ab}	9.20±1.7^a	21.6±4.92^a	0.24±0.08^a
30	15	3.64±1.25^{ab}	17.2±2.38^{ab}	2.34±0.70^{ab}	2.26±0.50^b	7.70±1.85^{ab}	14.31±5.40^a	2.02±0.96^a
	1	4.76±1.40^a	16.4±3.91^a	2.12±0.37^a	1.83±0.26^a	8.56±2.12^a	22.42±4.93^a	0.296±0.08^a
40	15	2.76±0.95^a	15.2±4.4^{ab}	1.612±.47^a	2.05±0.19^{ab}	6.79±2.60^b	17.46±4.60^a	0.192±0.07^a
	1	4.70±0.62^a	17.8±3.11^a	2.36±0.21^{ab}	1.92±0.18^{ab}	7.68±4.14^a	24.20±2.32^a	0.274±0.05^a
60	15	3.96±1.33^{ab}	14.8±3.70^{ab}	2.15±0.61^{ab}	2.35±0.13^b	6.30±0.16^a	16.51±1.79^a	0.27±0.03^a
	1	4.96±1.24^a	19.6±1.94^a	2.54±0.70^{ab}	1.92±0.98^{ab}	8.09±1.90^a	20.28±9.50^a	0.241±0.06^a
80	15	2.86±1.98^{ab}	12.4±4.39^a	2.014±0.67^{ab}	2.38±0.40^b	6.50±2.02^a	14.40±8.20^a	0.26±0.22^a

[1]Means within the same column followed by different letters are significantly different (P ≤ 0.05). [2]DEP = concentration of diethyl phthalate, HB = haemoglobin, PCV = packed cell volume, RBC = red blood cells, WBC = white blood cells, MCV = mean cell volume, MCH = mean cell haemoglobin, MCHC = mean cell haemoglobin concentration. Each value is mean±SD of 3 observations.

groups compared to the control. The liver ACP activity was statistically significant (P < 0.05) at day 15 respectively compared to the control. The muscle ACP in other treatment groups showed no significant difference (P > 0.05). Liver AST levels were significantly increased in DEP treated fish compared to the control. The liver AST levels were dose-dependent (Tables 5 and 6). On the other hand, liver ALT only increased significantly on day 15 and significantly decreased in day 30. Liver AST showed no significance in all treated groups (P > 0.05) and liver ALT activity was statistically significant (P < 0.05) at day 30 only. There was significant increase in brain AchE in day 15 among the treated groups and decreased at day 30. It can be seen that the initial values of AchE was maintained at the end of the day 30 which shows that DEP inhibits AchE activity for some period or re-mains in a constant level throughout the exposure. There was significant difference (P < 0.05) in brain and muscle

Table 4. Changes in the haematological parameters of *Clarias gariepinus* fingerlings exposed to diethyl phthalate (DEP) for 30 days[1, 2].

DEP (µg/l)	Duration (day)	Hb (g/dl)	PCV (%)	RBC (10^6/mm^3)	WBC (10^4/mm^3)	MCV (µm ^{3})	MCH (pg)	MCHC (g/dl)
	1	4.92±1.47^a	18.2±1.30^a	2.714±0.59^b	1.3±0.10^a	9.7±4.25^a	18.56±5.1^a	0.2442±.07^a
Control	30	4.42±.449^b	18.2±1.64^d	2.27±0.327^c	2.73±0.19^a	8.12±1.04^a	19.56±1.45a	0.24±.03^a
	1	4.64±.978^a	19.6±2.4^a	2.20±.578^{ab}	2.26±0.5^b	9.2±1.7^a	21.6±4.92^a	0.24±.08^a
30	30	3.36±1.2^{ab}	13.4±2.79^c	1.93±.477^a	2.58±0.30^a	7.1±1.2^a	17.32±2.4^a	0.25±.07^a
	1	4.76±1.4^a	16.4±3.91^a	2.12±.374^{ab}	2.05±0.19^{ab}	8.56±2.12^a	22.42±4.93^a	0.30±.08^a
40	30	3.5±1.17^{ab}	13.8±3.34^c	1.84±.450^a	2.64±0.36^a	7.8±2.95^a	18.76±1.67^a	0.26±.083^a
	1	4.7±.621^a	17.8±3.11^a	2.36±0.21^a	1.92±0.18^{ab}	7.68±4.14^a	24.2±2.32^a	0.27±.054^a
60	30	2.96±1.10^{ab}	11.6±2.96^{ab}	1.94±.501^a	2.45±0.48^a	5.42±1.02^a	14.76±7.8^a	0.29±.081^a
	1	4.96±1.24^a	19.6±1.94^a	2.54±.695^{ab}	1.92±0.98^a	8.09±1.9^a	20.28±9.5^a	0.24±.062^a
80	30	2.66±1.7^a	8.8±3.44^a	1.62±.644^a	2.87±0.32^a	6.24±2.7^a	17.8±9.4^a	0.3±.11^a

[1]Means within the same column followed by different letters are significantly different (P ≤ 0.05). [2]DEP = concentration of diethyl phthalate, HB = haemoglobin, PCV = packed cell volume, RBC = red blood cells, WBC = white blood cells, MCV = mean cell volume, MCH = mean cell haemoglobin, MCHC = mean cell haemoglobin concentration. Each value is mean±SD of 3 observations.

Table 5. Effect of diethylphthalate on the biochemical parameters of *Clarias gariepinus* fingerlings [1, 15].

Tissue	parameter	Duration	Control	30 µg/L	40 µg/L	60 µg/L	80 µg/L
		1	15.30±2.89^a	13.09±3.82^a	16.20±3.64^a	19.05±5.06^a	15.80±1.69^a
	AST	15	35.80±2.83^{ab}	32.40±3.82^{ab}	27.0±.01^c	39.15±9.55^c	47.30±1.25^a
		1	11.70±3.67^a	18.15±3.71^a	18.15±3.71^a	22.10±2.58^a	21.80±2.54^a
Liver	ALT	15	28.15±3.98^a	14.55±5.16^a	14.55±5.16^a	17.30±1.27^a	14.55±2.62^a
		1	1.11±.14^a	1.21±.14^a	1.62±.28^a	1.16±.21^a	1.67±.35^a
	ACP	15	1.54±.34^{ab}	2.22±.94^b	3.11±.097^c	1.09±.097^a	1.84±.36^{ab}
		1	1.06±.07^a	3.59±.78^a	3.84±.43^a	2.58±.78^a	2.53±.14^a
	ACP	15	2.39±.62^a	2.12±.27^a	2.55±.78^a	3.93±.21^a	2.02±.14^a
Muscle		1	1110.4±55.7^a	3322.10±2.54^a	1697.2±6.81^{ab}	2527.70±5.01^{cd}	2879.8±1.47^{cd}
	AChE	15	1184.4±8.9^{bc}	2446.4±8.63^a	1263.80±3.53^c	9505.90±20.78^b	5777.6±4.61^a
		1	5615.10±96.35^b	2049.20±25.7^a	6048.5±11.96^{bc}	9226.10±14.53^c	8327±16.12^{cd}
Brain	AChE	15	2903±6.30^u	5777.70±4.43^a	13433±19.71^v	6086.53±10.21^a	22014±8.53^c

[1]Means within the same column followed by different letters are significantly different (P ≤ 0.05). [2]ALT, AST, ACP, ALP and AchE are indicated as IU/l. Abbreviations: ALT, Alanine transaminace; AST, aspartate aminotransfarase; ALP, alkaline phosphotase; ACP, acid phosphatase. Each value is mean±SD.

AchE activity compared to the control.

Histopathological studies

Liver

The histology of control fish liver revealed normal (Figure 3) typical paranchymatous appearance. The liver was made up of hepatocytes, which were polygonal cells with a central spherical nucleus. There were degeneration of hepatocytes (Figures 4 and 5) in 30 µg/L treated fish at day 30 and mild vacuolation of hepatocytes and multi-focal areas of hepatocytes degeneration at day 15 and 30, respectively. Fish exposed to 40 µg/L DEP (Figures 6 and 7) showed a centrilobular degeneration of hepato-cytes for day 15 and 30, respectively. At 60 and 80 µg/L concentration, mild vacuolation of hepatocytes and severe necrosis were observed accordingly as shown in Figures 8 and 9, respectively. The histopathological changes were more pronounced after 30 days exposure period. The liver cells were degenerated with necrosis which appeared as focal areas with lymphocytic infiltration (Figure 10).

Table 6. Effect of diethylphthalate on the biochemical parameters of *Clarias gariepinus* fingerlings[1, 30].

Tissue	Parameter	Duration	Control	30 µg/L	40 µg/L	60 µg/L	80 µg/L
Liver	AST	1	15.30±2.89^{a}	13.08±3.82^{a}	16.20±3.64^{a}	19.05±5.06^{a}	15.30±1.69^{a}
		30	37.75±9.55^{a}	27.80±9.42^{a}	64.85±3.64^{a}	62.15±2.98^{a}	81.50±1.9^{a}
	ALT	1	11.70±3.67^{a}	18.15±3.76^{a}	18.15±3.71^{a}	22.10±2.55^{a}	21.85±2.54^{a}
		30	11.80±1.27^{a}	2.70±1.27^{c}	6.00±.56^{a}	7.30±2.55^{ac}	7.30±2.55^{c}
	ACP	1	1.11±.14^{a}	1.21±.14^{a}	1.62±.28^{a}	1.16±.21^{a}	2.53±.14^{a}
		30	1.53±0.31^{c}	143.70±5.27^{a}	127.7±5.27^{a}	136.63±17.7^{ab}	153.7±20.37^{ab}
Muscle	ACP	1	1.06±.0.7^{a}	3.59±.78^{a}	3.84±.43^{a}	2.58±.78^{a}	2.53±.14^{a}
		30	121.23±.051^{b}	175.30±7.29^{b}	142.2±9.87^{a}	140.65±13.64^{a}	153.70±20.37^{ab}
	AchE	1	1110.40±55.74^{a}	3322.10±2.54^{a}	1697.20±6.81^{ab}	2527.70±5.01^{cd}	2879±1.47^{cd}
		30	1110.40±26.74^{a}	3322.1±22.54^{d}	1697.2±26.80^{ab}	2527.7±15.01^{cd}	2879.8±11.47^{cd}
Brain	AchE	1	5615.10±96.35^{b}	2049±25.7^{a}	6048.50±11.96^{bc}	9226.10±14.53^{c}	8327±16.12^{cd}
		30	5615.10±96.35^{b}	2049±25.72^{a}	6048.50±11.96^{bcd}	9226.10±4.53^{b}	8327±16.12^{bdc}

[1]Means within the same column followed by different letters are significantly different (P ≤ 0.05). [2]ALT, AST, ACP, ALP and AchE are indicated as IU/I. Abbreviations: ALT, Alanine transaminace; AST, aspartate aminotransfarase; ALP, alkaline phosphotase; ACP, acid phosphatase. Each value is mean±SD.

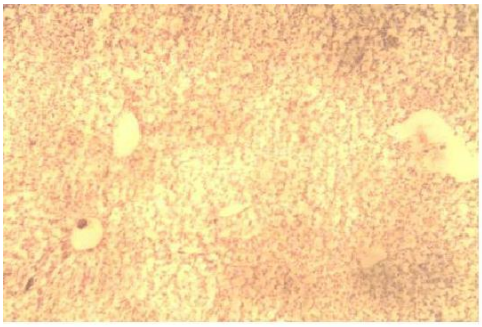

Figure 6. Photomicrograph of juvenile *Clarias gariepinus* treated with 30 µg/L diethyl phthalate at day 30 showing degeneration of hepatocytes. (H&E) mag ×100.

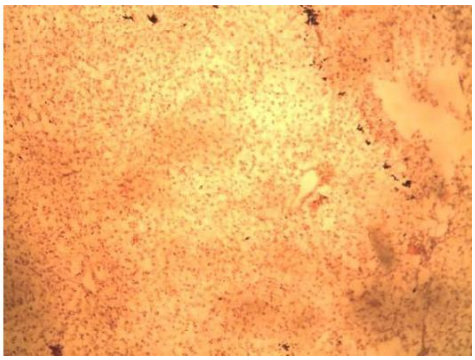

Figure 8. Photomicrograph of liver of *C. gariepinus* treated with 40 µg/ diethyl phthalate at day 30 showing centrilobular vacuolation of hepatocytes. H&E (mag ×100).

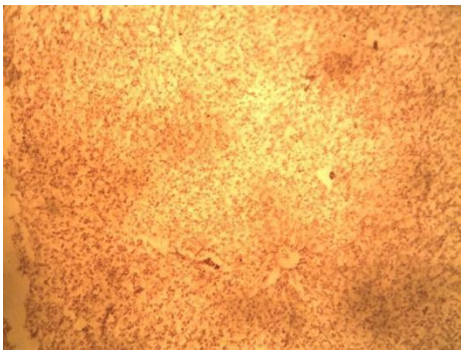

Figure 7. Liver section of juvenile fish treated with 40 µg/L diethylphthalate at day 15 showing centrilobular degeneration of hepatocytes. H&E 9mag ×100).

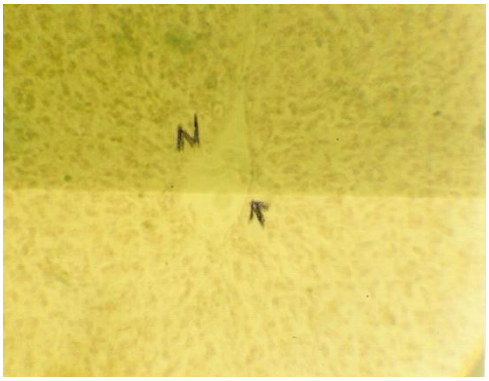

Figure 10. Liver section exposed to 80 µg/l diethyl phthalate for 30 days mag ×40 (severe necrosis).

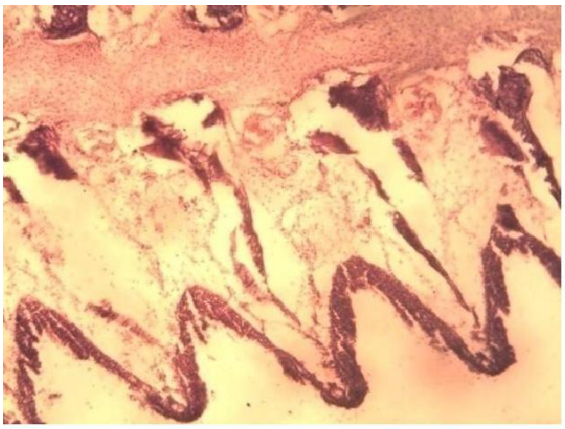

Figure 12. Photomicrograph of gills of adult *Clarias gariepinus* treated with 30 µg/ diethyl phthalate at 7 day showing raised lamellar. H&E (mag ×100).

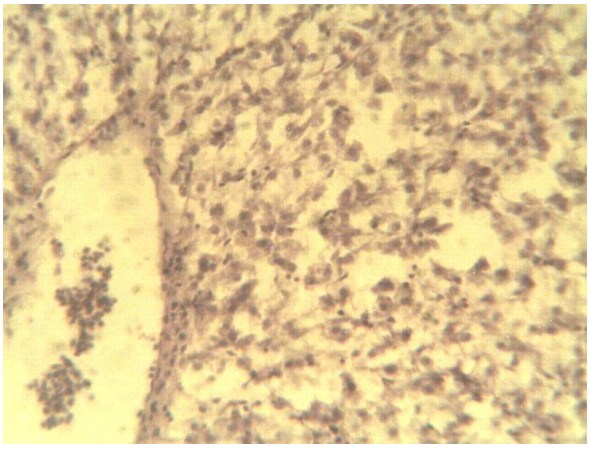

Figure 9. Photomicrograph of liver section of *C. gariepinus* treated with 60 µg/L for day 30 diethylphthalate showing mild vacuolation of hepatocytes.H&E (mag × 400).

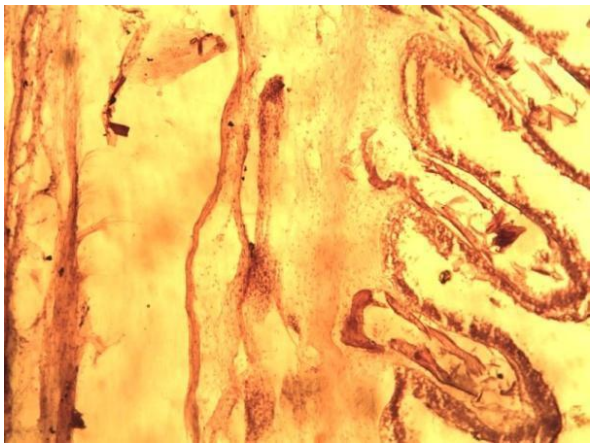

Figure 11. Histologic section of gills of control *C. gariepinus* showing normal filaments and well separated lamellar. H&E (mag ×100).

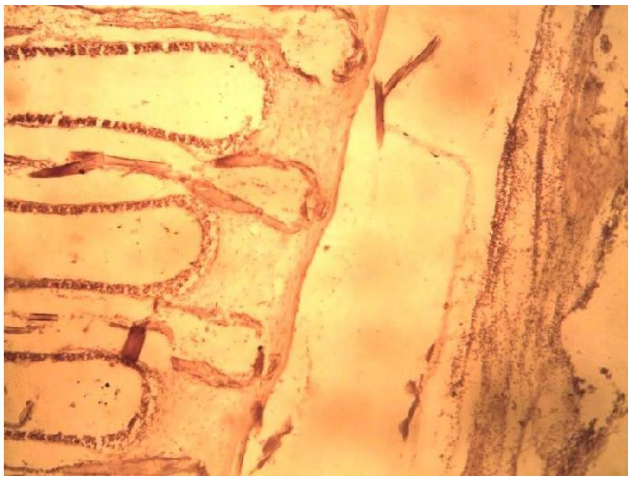

Figure 13. A section of gills of juvenile *Clarias gariepinus* treated with 30 µg/L diethyl phthalate at day 14 showing oedema (D) of the lamellar epithelia. H&E (mag ×100).

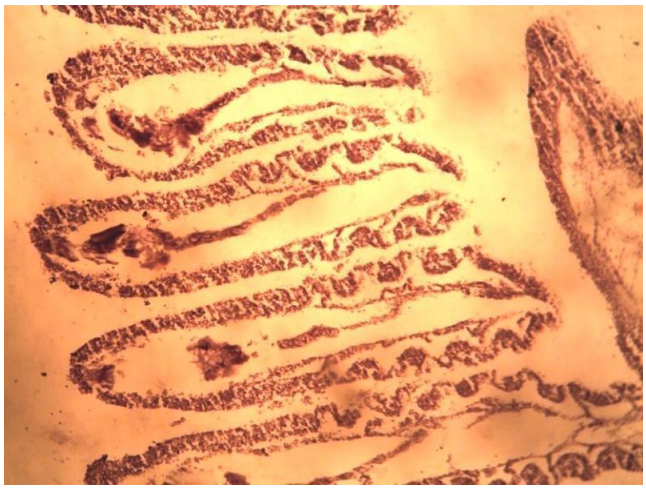

Figure 15. Photomicrograph of transverse section of gills of *C. gariepinus* treated with 60 µg/L diethyl phthalate day 21 sho-wing oedema (E) and raising of the filaments. H&E (mag ×100).

Gill

No recognisable changes were observed in the gills of the control fish. Each gill consisted of a primary filament and secondary lamella (Figure 11). At different concentration of DEP, there were raised lamellar (Figure 12). Most of the primary and secondary lamellae were distended with oedema in the lamellar epithelia (Figure 13). However, mild oedema and raising of the filament (Figures 14 and 15) and loss of lamella epithelim (Figure 16). These changes depicts possible gill damage that have affected the respiratory system of the fish. The degrees of the pathological changes in the gills were directly con-centration-dependent.

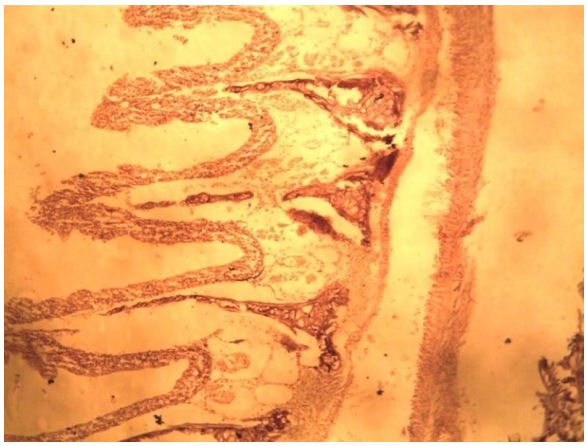

Figure 14. Photomicrograph of gills of juvenile *Clarias gariepinus* treated with 40 µg/L diethyl phthalate showing mild oedema and raising of the filaments. H&E (mag ×100).

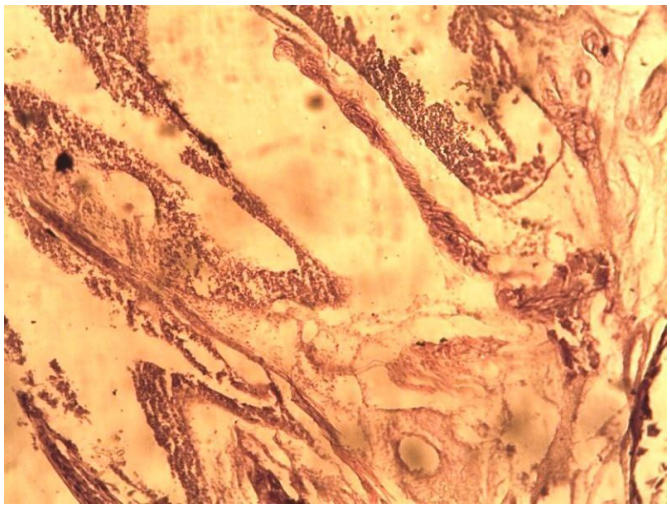

Figure 16. Histologic section of gills of *C. gariepinus* treated with 80 µg/L diethyl phthalate at day 21 showing loss of lamellar epithelium. H&E (mag ×100).

DISCUSSION

Results obtained from this study show that percentage mortality of *C. gariepinus* fingerlings increased with increase in concentration of diethyl phthalate and was dose dependent. This is in consonance with a similar study by Nivedita et al. (2002) on toxic effect of DEP on a freshwater fish, *Cirrhina mrigala*. The LD_{50} reported in this study is less than the observed field concentration in the water column (0.16 to 3.53 mg/L) and sediment (0.16 to 0.32 mg/L) of DEP in the Venda region of South African waters (Fatoki et al., 2010) where similar indiscriminate discharge of DEP-laden effluents and wastes take place as in Nigeria. In Nigeria, there is dearth of information about the field levels of DEP but it is expected to be higher than the LD_{50} reported in the present study. The LD_{50} of the carbofuran to juvenile fathead chubs was 1.96 mg/L (Fisher et al., 1999), a data that is comparable to the result in this study. Since DEP binds to the sediment and remains in the water column, it is possible that it could pose serious threat to fish and other aquatic life. The rapid opercula movement, erratic swimming and loss of balance observed in this study suggested possible nervous disorder. Haemorrhaging of the gill when the test fish were exposed to 100 and 150 µg/L of the compound is indicative of toxicity of the chemical. This is probably due to rupture of blood vessels of the gills and possible reduction in the haemotological parameters of erythrocyte count, haematocrit and mean corpuscular volume of the fish. Mgbenka et al. (2005) reported similar toxic effects with clogging of the gills with mucus of *C. gariepinus* due to lindane exposure. These observations are also in agreement with earlier reports by Okoli-Anunobi et al. (2002) on the lethal effect of the detergent (Elephant blue) on the Nile tilapia.

Haemorrhaging has also been reported in fathead minnows exposed to organic-based insecticide (Buckler et al., 1981). At higher concentrations of 0.16 to 4.04 mg/L DEP in the water of some rivers in the Venda region of South Africa, Fatoki et al. (2010) have suggested that DEP is toxic and could be carcinogenic to aquatic orga-nisms and man though it is less harmful than di-(2-ethyl-hexyl) phthalate (DEHP). The observed decrease in the haemoglobin concentration is in agreement with some earlier studies. Van Vureen (1986) reported that metasystox caused decreased haemoglobin concentration in *Labeo umbratus* while Omoregie et al. (1990) observed decre-ase in haemoglobin in *O. niloticus* exposed to gammalin 20 and actellic 25EC. Other studies (Santhakumar et al., 1999; Mgbenka et al., 2003) reported that monocro-tophos and acetellic exposures decreased the haemoglo-bin concentration in *Anabas sp.* and *C. albopunctatus*, respectively. The decreased haemoglobin concentration observed in this study is an indication of impaired oxygen delivery to the tissues. The reduction in erythrocytes count in *C. gariepinus* due to DEP exposure is in conso-nance with the report of Santhakumar et al. (1999) on *Anabas sp.* exposed to monocrotophos and in *C. albopunctatus* treated with actellic 25 EC (Mgbenka et al., 2003). Similar results were reported for *Oreochromis mossambicus* exposed to copper (Nussey et al., 1995). The general reduction in haemoglobin concentration, ery-throcyte count and PCV in the fish treated with DEP was an indication of anaemia.

The observed decrease in the PCV, Hb and erythrocyte count of *C. gariepinus* juvenile after 30 day exposure to DEP could be indicative of heamodilution due to erythrocyte sequesteration. Some workers have attributed changes in such blood parameters to erythrocyte swelling (Annune and Ahuma, 1998) or haemolysis (El-Domiaty, 1987; Annune et al., 1994). The fact that MCV similarly

decreased throughout the study suggested that the observed decrease in there parameters may not be due to erythrocyte swelling. Earler studies (B. C. Ikele, University of Nigeria, Nsukka, Msc thesis) showed that the kidney architecture in *C. gariepinus* juvenile was adversely affec-ted by DEP exposure. Erythropoeitin that is produced in the kidney of vertebrates (Gordon et al., 1967) control erythropoiesis and according to Reddy et al. (1992) activates pyridoxal phosphate in immature erythrocyte to facilitate haemoglobin synthesis; thus, the reported ammonia in this study could plausibly be due to impaired erythropoietin production following damage to the kidney tissue in the fish. Thakur and Bais (2000) also reported that erythrocyte count, MCV, MCH and MCHC decreased in *Heteropneutes* sp. treated with insecticides with con-comitant decline in oxygen transport. Leucocytosis is a usual response of the vertebrates to conditions or sub-stances that attempt to change their normal physiology; thus, the leucocytosis recorded in this study shows that DEP elicited the stimulation of the immune system of the fish to protect it against possible infection or secondary effect of DEP to predispose it to disease. Similar leukocytosis was reported in *C. albopunctatus* exposed to gammalin 20 (Mgbenka et al., 2003) and in Indian catfish (*Heteropneustes fossilis*) treated with sewage, fertilizer and insecticides (Srivastava and Narain, 1982).

Leucocytosis was also reported in *Anabas testudinus* exposed to monocrotophos (Santhakumar et al., 1999). Also, Trivedi et al. (1990) reported a similar trend in *C. batrachus* exposed to fertilizers. The reduction in the erythrocyte counts and the haemoglobin concentrations in *C. gariepinus* have demonstrated that DEP may have affected the erythropoietic centres in the kidney thereby limiting erythropoiesis in the fish. It was evident that liver and muscle acid phosphatase level were significantly increased in DEP treated fish at various concentrations and also decreased significantly at other groups. This increase is probably due to increased lysosomal activity in the liver and muscle tissues. This goes in consonance of Nivedita et al. (2002) that ACP is an inducible enzyme because its activity goes up when there is a toxic impact and the enzyme begins to counteract the toxic effect. Subsequently, the enzyme may begin to drop either as a result of having partly or fully encountered the toxin or as a result of cell damage. In a study of male Sprague-dawley rats treated with 50 ppm (w/v) DEP in drinking water for four months, there was significant increase in liver ACP (Sonde et al., 2000). It is apparent that DEP causes increased ACP activity in the liver and muscle by interacting with lysosome. Lowe et al. (1992) reported that alteration in the membrane permeability can have severe consequences such as leakage of hydrolytic enzyme including ACP, which could have detrimental effect on the cell.

Liver AST levels were significantly increased in DEP treated fish though not statistically significant compared with the control. This indicates that DEP stimulates gluta-mate transaminase activity in the liver which could be due to toxic injury caused by DEP, which may stimulate tissue repair through protein turn over and increased respire-tion. AST levels were comparatively lower in DEP treated group indicating that DEP does affect mitochondrial function. This agrees with Nivedita et al. (2002). This cor-relates well with increased AST activity in the liver of DEP treated fish. In this regard, it can be said that DEP toxicity leads to enhanced AST activity, which is indicative of high protein turnover and amino acid metabolism. This is in consonance with Nivedita et al. (2002) and Muthuviveganandavel et al. (2007). Acetylcholinesterase is of interest because it is the target site for organophos-phate and carbamate pesti-cides in the central nervous system and its role in choli-nergic synapses is essential for life. It is an enzyme that degrades the neurotrans-mitter acetylcholine, producing choline and acetate group. It is mainly found in neuro-muscular junction and cholinergic synapses in CNS, where it terminates the synaptic transmission. It is also found in red blood cell membranes, where it constitutes the Yt blood group antigen. Some studies reported evidence that AchE acti-vity may be inhibited by environmental contaminants other than organophosphate and carbamate compounds, including some metals, surface-tants agents and com-bustion hydro carbons (Guilhermino et al., 1994; Herbert et al., 1995; Payne et al., 1996; Labrot et al., 1996).

It was evident in this study that the AchE activity in the brain and muscle of diethyl phthalate treated fish was found to be significantly increased and also decreased based on the duration, indicating that DEP inhibit AchE activity. This could be due to the lipophilic nature of DEP, which may be taken up faster by the brain tissues. This correlates with the sluggish, non-motile behavior of the DEP treated fish. In previous studies, the sensitivity of AchE to endosulfan was similar to the activity of non-exposed animals. The higher the AchE level in the tissue, the more susceptible it is to inhibition and low concen-tration of toxicants can inhibits AchE, which leads to an accumulation of acetylcholine at the central cholinergic synapses and neuromuscular junction which was evident in group B. Cholinesterase inhibition in brain and muscle produces effect in the movement of fish because acetyl-cholinesterase participates in the neuronal and neuro-muscular transmission (Fernandev-Vega et al., 1999). The unexposed fishes (control) showed inhibition in the con-trol group in day 15 and higher specific activity in the brain compared to the muscle, this inhibition can be spe-cies dependent according to Sancho et al. (1998). Thus, this enzyme seems not to be sensitive to this chemical, results agree with those obtained by Inbaraj and Hainder (1988) in *Channa punctatus*.

The literature on histopathology effects of DEP on fish is still rare. Neskovic et al. (1996) conducted sub lethal toxicity test (14 days) of sub lethal glyphosate concen-tration on histopathological changes of carp organ such as gill, liver and kidneys. In the present study, damages

of the gills indicated that the sublethal concentration of DEP caused impairment in gaseous exchange efficiency of the gills. The major changes were raised lamellar, oedema of the lamellar epithelia, mild oedema and raising of the filament, and loss of lamellar epithelium. Histopa-thological changes of gill such as hyperplasia and hyper-trophy, epithelial lifting, aneurysm and increase in mucus secretion have been reported after the exposure of fish to a variety of noxious agents in the water such as herbi-cides, phenols and heavy metal (Nowak, 1992). Liver is especially a useful organ in assessing the possible impact of pollutant in fish. This is because che-mical tends to concentrate there. This is also a major site for biotrans-formation of toxic chemicals which usually makes them less toxic and more easily excreted. In the study of Risbourg and Bastide (1995), the exposure of fish to atrazine herbicide increased in the size of lipid droplets, vacuolisation in the liver. The most frequent encountered types of degenerative changes are those of hydropic degeneration, cloudy swelling, vacuolization and focal necrosis. This also agrees with Babu et al. (2007) in the exposure of fish to fenevalerate on the liver tissues of *C. mrigala*, when necrosis of tubular epithelium and pyc-notic nuclei in the hematopoietic tissue occurred.

Necrosis of the liver tissues in the study was observed, probably resulted from the excessive work required by the fish to get rid of the toxicant from its body during the process of detoxification by the liver. The inability of fish to regenerate new liver cells may also have led to necrosis.

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