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Sublethal concentrations of di-n-butyl phthalate promote biochemical changes and DNA damage in juvenile Nile tilapia (*Oreochromis niloticus*)

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Abstract
Increase in consumption of consumer items such as plasticizers have resulted in a sharp rise in the presence of xenobiotics like phthalic acid esters (PEs) in freshwater and marine environments due to contaminated runoff and improper release of effluents. The sublethal toxicity of Di-n-butyl phthalate (DBP) was investigated in juvenile Nile tilapia, *Oreochromis niloticus*, in an attempt to determine the biological effect of exposure to 1/2 and 1/3 median lethal concentration (96-h LC₅₀) which, in our study was experimentally determined to be 11.8 mg/l. Following four days of exposure, indices of the oxidative potential [Malondialdehyde content (MDA)], antioxidant parameters [superoxide dismutase activity (SOD) and reduced glutathione level (GSH)] and DNA damage were evaluated by single-cell gel electrophoresis (Comet assay). Hepato-renal markers [alanine aminotransferase activity (ALT), creatinine and urea level] and cortisol levels were also quantified in serum. Additionally, histopathological investigations of liver, kidney and gill tissues were conducted. Comparative results between the 1/2 96-h LC₅₀ group and the 1/3 96-h LC₅₀ group clearly showed that there was a significant elevation in MDA levels and a marked increase in DNA damage in addition to inhibition of antioxidant barriers as represented by attenuation of SOD activity and GSH level in the group that was exposed to higher concentration of DBP (1/2 96-h LC₅₀). The hepatorenal markers and cortisol levels were also observed to be elevated. Histopathological examination of the liver, kidney and gills showed pathological alterations that could be correlated with changes in the biochemical profile of the exposed fish. Additionally, anomalous clinical signs were noted. Based on these findings, we conclude from our study that exposure of juvenile *O. niloticus* to DBP has the potential to induce biochemical as well as tissue morphological alterations associated with oxidative injury and DNA damage.

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Introduction

Environmental contaminants, such as xenobiotic substances emitted as a byproduct of anthropogenic activities, inevitably lead to contamination of aquatic environments. They negatively impact the ecosystem through adverse effects on growth, development and reproduction of aquatic species\textsuperscript{25}, leading to a sharp fall in number as well as quality of the aquatic population\textsuperscript{45}. As a downstream effect, such contamination also impacts human and animal health especially in cases where fish is consumed or used as a food source. This is because fish are well known contaminant bioaccumulators and have the highest potential for transferring such residues to humans\textsuperscript{44}. One of the best examples of xenobiotics are endocrine disrupting compounds (EDCs) such as phthalate esters (PEs), which have the ability to perturb a variety of biological systems including the invertebrate, reptilian, avian, aquatic and also the mammalian systems\textsuperscript{36}.

PEs are a very important part of modern life by virtue of their usage in a wide variety of consumer products such as toys, paints, adhesives, lubricants, wrappings, building materials, personal care items, electronics, inks, coatings and even certain medical devices and enteric coatings of some pharmaceuticals. The annual worldwide production of PEs, for the purpose of production of soft polyvinyl chlorine and assorted plasticizers, is estimated to be nearly 6 million tonnes\textsuperscript{39}. PEs are not bound to the polymer backbone by a covalent bond which results in them ‘leaking’ into the surrounding environment throughout the processing period and even after disposal. Hence it comes as no surprise that PEs are found ubiquitously in a variety of water sources and sediments across the worldwide aquatic environment,\textsuperscript{24,50} largely due to defective waste disposal protocols followed by production units, accidental spillage from industries, effluents from wastewater treatment plants, agricultural run-off, faulty disposal of consumer products and empty laundry containers in freshwater bodies such as rivers and lakes and the use of such containers for water storage\textsuperscript{57}. It is a well documented fact that the PEs that accumulate in fish are sourced from the water column and sediments via gill respiration, contaminated food ingestion and dermal exposure, and that their accumulation results in reproductive and embryonic abnormalities\textsuperscript{40} as well as growth and developmental defects\textsuperscript{11}. Additionally, PEs’ ability to cause improper behavioral responses to external stimuli\textsuperscript{26} and by reducing fertility which in turn can affect fish population structure as well as size\textsuperscript{35}. The physicochemical characteristics of PEs and the fish life ways can also manipulate bioavailability and consequently their impact in fish\textsuperscript{23}. It is therefore imperative that the toxic effect of PEs, especially their effect on aquatic organisms, should be rigorously evaluated and characterized.

Di-n-butyl phthalate (DBP) plasticizers have been listed as a US Environmental Protection Agency priority pollutant and as EDCs; based on their high production, extended environmental persistence, and increased tendency for bioaccumulation. DBP has been documented to impact development at the early stages of life\textsuperscript{20,21,55,58}. Additionally antiandrogenic and estrogenic effects of DBP have been demonstrated in male rats as well as fish\textsuperscript{4,9,19,56}, which underline the relevance of the studies on the potential effects of DBP on various stages of fish lifecycle.

Nile tilapia (\textit{Oreochromis niloticus} \textit{L}.) is a highly invasive fish that plagues a variety of aquatic ecosystems particularly those located in the tropics. It populates a variety of freshwater and brackish habitats and is known to persist even in highly polluted environments. In recent years Nile Tilapia has attracted considerable worldwide
attention both for its potential as a bio-indicator for aquatic environmental contaminants as well as for its potential to be used as an intensive aquaculture species. It has earned the name “aquatic chicken” due to its extraordinary production capabilities which allow it to be priced within reach of low-income families and hence be abundantly available to consumers. Tilapia has a very high biological value and is a very good source of sustenance for humans. Consequently, it is considered to be of extreme economic significance worldwide.

Because there is a scarcity of published reports regarding the impact of DBP on Nile tilapia (O. niloticus), the current study was conducted in an attempt to assess the toxic effects of DBP on the juvenile stage of Nile Tilapia by computing the median lethal concentration value (96-h LC50). The study also aimed to evaluate the consequences of acute exposure to 1/2 and 1/3 96-h LC50 on the serum hepato-renal biochemical markers. Oxidative status was assessed as a possible toxicodynamic pathway through which DBP exerts its effect. In addition, DNA damage was assessed and a histopathological investigation of liver, kidney and gills, post-laboratory exposure was also conducted.

Materials and methods

Experimental fish and protocol: One hundred and ninety juvenile O. niloticus (10.62 ± 0.24 g BW) were obtained from the Abbassa Fish Hatchery at EL-Sharkia Province, Egypt. The juveniles were maintained in a glass aquaria filled with dechlorinated tap water and allowed to acclimatize to laboratory conditions for two weeks prior to exposure. Each aquarium was equipped with an aerator and was rigorously thermostatically controlled. All the aquaria were maintained under similar conditions of water temperature (25 ± 1.0°C), pH (6.9 ± 0.1), dissolved oxygen (7.4 ± 0.34 mg/l) and ammonia (0.035 ± 0.01 mg/l); the photoperiod (10 h light : 14 h dark) in the laboratory was also strictly controlled. Through the duration of the experiment the juveniles were fed three times a day with commercially available dry fish pellets at a rate that approximated 3% of their body weight. The experiments were undertaken in accordance with the Care and Use of Laboratory Animals Guidelines of the National Institutes of Health (NIH), and approved by the local authorities of Cairo University, Egypt.

Chemicals and reagents: Di-n-butyl phthalate (C16H22O4, DBP, CAS No. 84-74-2, 99% purity) was procured from Sigma- Aldrich Chemical Co. (St. Louis, MO, USA) as a colorless to faint yellow viscous liquid. The kits used for biochemical measurements of ALT activity, urea, creatinine and cortisol levels were obtained from Biodiagnostic Co., Egypt. All other chemicals were obtained commercially from local scientific distributors in Egypt.

Median lethal concentration assessment (96-h LC50) and toxicity symptoms of DBP in juvenile O. niloticus fish: Following range finding tests, DBP dissolved in dimethyl sulfoxide (DMSO) in concentrations that ranged from 0 to 24 mg/l. A 96-h acute toxicity test was conducted on 130 juvenile O. niloticus fish split into 13 groups of 10 fish each. Each group was exposed to varying concentrations of DBP and the resultant mortalities were counted and recorded at 24, 48, 72 and 96 h intervals. 96-h LC50 value was calculated using the method described by Behrens and Karber as shown in Table 1.

Sublethal exposure experiment: The toxic effects of 1/2 and 1/3 96-h LC50 value of DBP on juvenile O. niloticus for an exposure period of four days were assessed. Sixty fish were randomly divided into three groups each of two replicates, comprising of 10 fish each. Group I (control) fish received no treatment whereas groups II and III were exposed to DBP at a concentration of 1/2 and 1/3 96-h LC50 value (5.9 and 3.9 mg/l, respectively). The medium of the aquaria was renewed daily.
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and fresh solutions were spiked so as to maintain water quality with an appropriate DBP level. Throughout the period of exposure, fish were closely observed and clinical signs were rigorously followed, as well as post-mortem lesions of dead fish were monitored.

**Blood sampling and serum collection:** Following exposure to DBP, blood samples were collected from the caudal vein of each individual fish. These samples were centrifuged at 664 × g for 10 min in order to obtain serum that was then stored at −20°C till required for further biochemical analysis.

**Preparation of tissue homogenates:** The fish were sacrificed by decapitation following which they were dissected and gill tissues were homogenized in 10 volumes of potassium phosphate buffer (PBS) (pH 7.4). The homogenates were centrifuged at 664 × g at 4°C for 30 min and the supernatant was utilized for estimation of the DNA damage using the alkaline comet assay method.

**Evaluation of oxidative stress indices:** Serum GSH level was assayed using the method of Beutler et al. SOD activity was assayed using the method described by Nishikimi et al. Lipid peroxidation (MDA) was determined using a colorimetric assay as described previously.

**Single cell gel electrophoresis (SCGE); Alkaline Comet assay:** The Comet assay was performed according to a previously described standard method. The cells were examined by a 40X objective lens using an epi-illuminated fluorescence microscope (Olympus-Bx60, excitation filter: 515–560 nm; barrier filter: 590 nm) attached to a color CCD video camera and connected to an image analysis system (Comet II, Perspective Instruments, UK). The Comets were analyzed by a visual scoring method and computer image analysis. To assess DNA damage, tail length, tail DNA intensity (%) and tail moment were estimated using the Comet Assay Project Software (CAPS).

**Serum biochemical measurements:** Serum ALT activity, creatinine, urea and cortisol levels were spectrophotometrically estimated using commercially available kits following manufacturer's instructions.

**Histopathological examination:** Kidney, liver and gill specimens were fixed in 10% neutral-buffered formalin and processed for paraffin embedding. The sections were stained with Hematoxylin and Eosin (HE) for histopathological examination under a light microscope.

**Statistical analysis:** Data were expressed as mean ± SE. SPSS 16.0 computer program (SPSS) was used for analyzing results. Statistical significance between groups was evaluated using the analysis of variance (one-way ANOVA) test followed by Duncan's multiple range test for significant difference. P-values <0.05 were accepted as statistically significant.

**Results**

**LC₅₀ value and clinical observations**

The 96-h LC₅₀ value for DBP in juvenile *O. niloticus* was determined to be 11.8 mg/l (Table 1); and it was observed that associated fish mortalities increased with increments in DBP concentration when monitored within a 96 h. Throughout the exposure period, no clinical signs were noticed in control fish as well as in fish exposed to the lowest concentration of DBP (<2 mg/l) whereas in those exposed to higher DBP concentrations (>2 mg/l), anomalous clinical signs were noted immediately. The observed impairments included abnormal swimming movements and restlessness followed by convulsions. The fish under study exhibited difficulty in breathing as represented by speedy respiration accompanied by rapid operculum movements and failure to respond to escape...
reflex. Additionally, a thick layer of mucous associated with a dark discoloration of the skin was also noted. Severe congestion of the internal organs along with excessive slime deposition on the gills was observed during the postmortem investigation.

Sublethal exposure assay

During the 96-h exposure period used in the current study, three mortalities were encountered in the $\frac{1}{2}$ 96-h LC$_{50}$ DBP-exposed group while two mortalities were noted in the $\frac{1}{3}$ 96-h LC$_{50}$ group.

Antioxidants and lipid peroxidation indices

When compared with the control, significant ($P < 0.05$) decreases were observed in the GSH levels of the $\frac{1}{2}$ 96-h LC$_{50}$ treated group while no such significant difference was observed when results were compared in the $\frac{1}{3}$ 96-h LC$_{50}$ group. The SOD activity showed a significant reduction in case of fish exposed to $\frac{1}{2}$ 96-h LC$_{50}$ while it appeared significantly induced in the $\frac{1}{3}$ 96-h LC$_{50}$ DBP-exposed fish ($P < 0.05$). In contrast, the MDA contents of the serum from both intoxicated fish groups, was observed to be significantly higher when compared to the control groups; this observation was more pronounced in the $\frac{1}{2}$ 96-h LC$_{50}$ exposed fish (Fig. 1).

DNA damage level

The comet assay was used to determine the magnitude of DNA damage in gills of both treated and control groups. Results are depicted in Fig. 2 and 3. Data obtained from this experiment revealed that as compared to the control group the $\frac{1}{2}$ 96-h LC$_{50}$ DBP-exposed fish recorded significant alterations ($P < 0.05$) in values of all observed parameters (tail length, tail moment and DNA%), while comparatively less DNA damage was observed in the $\frac{1}{3}$ 96-h LC$_{50}$ DBP-exposed group.

Table 1. The median lethal concentration (96-h LC$_{50}$) of DBP in juvenile O. niloticus according to (Behrens and Karber 1953)

<table>
<thead>
<tr>
<th>Group</th>
<th>Fish number</th>
<th>DBP Concentration (mg/l)</th>
<th>Number of dead fish after 96 h</th>
<th>$a$</th>
<th>$b$</th>
<th>$\sum (a \times b)$</th>
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<td>10</td>
<td>Zero</td>
<td>0</td>
<td>0</td>
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<td>3</td>
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<td>2</td>
<td>8.5</td>
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<td>10</td>
<td>24</td>
<td>10</td>
<td>2</td>
<td>9.5</td>
<td>19</td>
</tr>
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</table>

$a$ = Constant factor between two successive doses  
$b$ = The mean of dead fish in the groups  
$n$ = Number of fish in each group  
$\sum$ = The sum of ($a \times b$).

$96$-h LC$_{50}$ = highest dose $- \frac{\sum (a \times b)}{n} = 24 - \frac{122}{10} = 11.8$ mg/l
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Serum biochemical parameters
Exposure to DBP resulted in significant ($P < 0.05$) changes in the values of indicative markers used for estimating hepato-renal damage (ALT activity, creatinine and urea levels). Statistically significant concentration dependent elevation of serum cortisol levels was observed for both the DBP exposed groups when observations were compared with the control fish (Table 2).

Histopathological findings
Light microscopical examination of liver, kidney and gills of control group showed normal histopathological structure (Figs. 4A, 5A and 5D). The lesions observed in both DBP- exposed groups were almost similar except that for the $\frac{1}{2}$ 96-h LC$_{50}$ group the lesions were marginally more numerous and prominent. The liver showed multifocal areas of coagulative necrosis as represented by pyknosis or disappearance of the nuclei. The observed necrosis was extended...
so as to include the pancreatic acini and melanomacrophages along with the invaded extravasated erythrocytes (Figs. 4B and C). Severe instances of vacuolations and hydropic degeneration were diffusely seen in the hepatocytes with the portal areas showing congested blood vessels infiltrated with lymphocytes and eosinophilic granular cells (ECGs) (Fig. 4D). Additionally, focal interstitial aggregations of lymphocytes and diffuse vacuolations were also observed (Fig. 4E).

The kidneys showed focal areas of coagulative necrosis as represented by pyknosis and karyorrhexis (Fig. 5B). Severe hydropic degeneration and vacuolation of the renal tubular epithelia along with a few interstitial round cell infiltrations (Fig. 5C) were also seen. Occasionally the renal tubules also exhibited cloudy swelling accompanied by cellular and nuclear hypertrophy. The glomerular tufts appeared contracted with dilated Bowman’s capsule. Extensive hemorrhage was also noticed among the degenerated renal tubules. Congestion of the renal blood vessels and edema were also seen.

### Table 2. Effects of 1/2 and 1/3 96-h LC50 value of DBP exposure on the hepato-renal markers (ALT activity, urea and creatinine levels) and cortisol level in juvenile *O. niloticus* throughout 96-h exposure period (n = 6)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (ALT 17 ± 0.21&lt;sup&gt;c&lt;/sup&gt;)</th>
<th>1/3 96-h LC50 (15.33 ± 0.33&lt;sup&gt;b&lt;/sup&gt;)</th>
<th>1/2 96-h LC50 (0.55 ± 0.15&lt;sup&gt;b&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea (IU/dl)</td>
<td>7.67 ± 0.33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>15.33 ± 0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.00 ± 0.57&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.22 ± 0.12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.55 ± 0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.79 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cortisol (μg/dl)</td>
<td>6.14 ± 0.16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>16.4 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24.22 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
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</table>

Means within the same row bearing different letters (a, b and c) are significantly different (p < 0.05).

**Fig. 4.** (A) Photomicrographs of control juvenile *O. niloticus* liver showing normal hepatocyte and sinusoidal architecture, HE (Bar = 50 μm). (B–E) Photomicrographs of liver histopathological changes of juvenile *O. niloticus* exposed to 1/2 96-h LC50 value of DBP throughout a 96-h exposure period showing focal area of coagulative necrosis (B), necrosis in the pancreatic acini (C), severe diffuse vacuolations and hydropic degeneration in the hepatocytes (arrow), severe congestion of portal blood vessels (arrow head) (D), diffuse vacuolation of hepatocytes (arrow head) and interstitial aggregation of lymphocytes (arrow) (E), HE (Bar = 100 μm).
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DBP-associated damage to the gills manifested a severe congestion of the lamellar blood vessels and capillaries along with focal aneurysms in the secondary lamellae and extensive hemorrhaging (Figs. 5E and F). Hyperplasia of the lamellar epithelium was the most frequently observed gill lesion. Increase in the cellular layers of the lamellar epithelium was found to be associated with an increase in the number of goblet cells, fusion of the base of gill filaments and leukocytic infiltration of predominantly round cells. Extensive edema was observed to lift and separate the lining epithelium of the secondary lamellae from its basement membrane. Focal necrosis and desquamation of the lining epithelium were also visualized.

Discussion

Several studies have been published on DBP toxicity in early life stages of aquatic species, but reports with regard to its toxicity using LC50 fractions are relatively scarce. Also, the 96-h LC50 value in case of the O. niloticus fish is not absolutely estimated. In an attempt to fill this lacuna, this study was conceptualized in order to assess the 96-h LC50 of DBP in juvenile O. niloticus and also to measure the cellular and biochemical response of the fish to short-term exposure to DBP at sub-lethal concentrations.

The 96-h LC50 value is typically employed for the accurate assessment of acute toxicity. In the present study, the calculated LC50 of DBP for juvenile O. niloticus, as obtained based on a 96-h study, was 11.8 mg/l. The subsequent data indicated that DBP at the given concentration is moderately toxic for Nile tilapia. Several acute toxicity datasets have been reported for 96-h LC50 values in case of fish species other than Nile tilapia; the reported values were observed to range from 0.35 to 3.96 mg/l. Also, in a
previous study Zhao et al.\textsuperscript{59} reported that the DBP 96-h LC\textsubscript{50} in case of carp is 16.30 mg/l. The observed variation in the sensitivity of fish to DBP can be accounted for by differences in kinetic parameters, species, size, age, health as well as experimental conditions\textsuperscript{15}.

The valuable scientific data mined from acute toxicity studies was acquired from a combination of behavioral, clinical and postmortem observation of test animals in addition to the LC\textsubscript{50} value\textsuperscript{15}. The clinical alterations observed in the test subjects exhibited as perturbations in their respiratory and movement patterns and seemed to appear almost immediately after exposure to high DBP concentrations, where these behavioral deviations became more pronounced as DBP levels were increased. The altered respiratory pattern may be a byproduct of post-stress related excessive mucus secretion which results in the formation of a thick coat on the gill tissue which causes irritation to the gills\textsuperscript{17}. Behavior-related alterations observed in our study are hypothesized to be a strategy by which the animals adapt to changes in the surrounding environment upon exposure to pollutants.

The health of aquatic organisms is linked to the hyper generation of reactive oxygen species (ROS) and the antioxidant defense mounted by the body in order to eliminate them\textsuperscript{51}. Many studies have hypothesized that oxidative stress might be a key factor in PE-induced toxicity in an aquatic organisms such as C. elegans\textsuperscript{49}, zebrafish embryo\textsuperscript{54}, olive flounder\textsuperscript{27} and abalone\textsuperscript{60}.

In this study, exposure of juvenile O. niloticus to DBP elevated serum MDA content (indicative of lipid peroxidation); MDA is known to interact with free amines of macromolecules thereby inflicting cell injuries\textsuperscript{42}. This is suggestive of the fact that DBP exposure is capable of eliciting oxidative cell injury. Our results also indicate that there is a direct relationship between the dose to which the test subjects were exposed and the resultant oxidative damage. This conclusion was drawn from observations where it was seen that there is significant decrease in the MDA level in the group exposed to $\frac{1}{3}$ 96-h LC\textsubscript{50} as compared with those exposed to $\frac{1}{2}$ 96-h LC\textsubscript{50}. This is also suggestive of the fact that the body is able to eliminate an oxidative insult by an appropriate response of the antioxidant systems when exposed to low doses of pollutants. Our results corroborate findings of Xu et al.\textsuperscript{54} who reported a significant elevation in ROS and MDA production, reflective of oxidative stress, in zebrafish embryos and larvae upon exposure to DBP and DEP, while results of Mankidy et al.,\textsuperscript{33} showed that neither oxidative stress nor embryo toxicity were recorded in fathead minnows following DBP exposure of 1 mg/l. In the later case, it was hypothesized the antioxidant defense is able to neutralize the oxidative stress.

Biodefense mechanisms against intracellular oxidative stress consist of an enzyme-mediated antioxidant system and non-enzymatic antioxidants\textsuperscript{16}. The SOD activity was observed to be elevated in $\frac{1}{3}$ 96-h LC\textsubscript{50} treated O. niloticus while it appeared to be repressed in the $\frac{1}{2}$ 96-h LC\textsubscript{50} group. This is indicative of the dosage effect wherein the induced SOD activity is useful in elimination of excessive O$_2^-$ in an attempt to reduce concentration of ROS to a low steady-state level where cells are shielded from oxidative damages\textsuperscript{41} as observed in $\frac{1}{3}$ 96-h LC\textsubscript{50} group but, once the O$_2^-$ elimination capacity of SOD is saturated, the excess O$_2^-$ accumulates resulting in restrained or inhibited SOD activity as reported in the $\frac{1}{2}$ 96-h LC\textsubscript{50} group. This observation might be a result of peroxidative injury to liver cells followed by cell damage as was recorded in the histopathological findings. In corroborations of the above finding, Mankidy and his colleagues\textsuperscript{33} stated that relatively low doses of DBP (1 mg/l) induce antioxidant enzymatic activities in an attempt to counter oxidative insult, whereas higher doses of DEP (10 mg/l) failed to elicit increased activity in fathead minnows embryos owing to the exhaustion of the enzymes due to increased peroxidation status.

The depletion in GSH content recorded in our study may be attributed to increase usage of
GSH in an attempt to reduce oxidative impact.\textsuperscript{48) GSH is an important defense mechanism because it scavenges free radicals emitted as a byproduct from the metabolism of oxidative radicals as well as those that escape from detoxification by antioxidant enzymes.\textsuperscript{44} GSH is considered extremely relevant because it is a substrate for GPx which is responsible for transforming hydroperoxides to hydroxyl ions. Hence, depletion of GSH levels can result in enhancement of oxidative stress.\textsuperscript{32} Previous studies have reported that GSH levels have a tendency to decrease in fish that have been exposed to DBP.\textsuperscript{54,60} Results of this study prompt us to hypothesize that exposure to DBP resulted not just in damage to cellular lipids but also to DNA as can be concluded from the observed increase in MDA production and DNA damage both of which are likely to be byproducts of oxidative stress. With respect to assessment of the genotoxic effects provoked by exposure to DBP as evidenced by the alkaline comet assay of gill tissue, it was observed that there were significant alterations in the number of DNA breaks in the gills of the $\frac{1}{2}$ 96-h LC\textsubscript{50} DBP- exposed fish while less DNA damage was observed for the $\frac{1}{3}$ 96-h LC\textsubscript{50} DBP- exposed group as represented by a significant increase in the recorded tail length of the comet assay. No published reports have presented data regarding geno/cytotoxic effects of DBP on \textit{O. niloticus}. Some \textit{in vitro} studies have examined human lymphocytes and mucosal cells of the upper aerodigestive tract following exposure to DBP and di-iso-butyl-phthalate.\textsuperscript{28,29,30} Also, Anderson et al.\textsuperscript{3} have reported an increase in the tail moments of human leukocytes that have been treated with DEHP. D’Errico and his colleagues\textsuperscript{13} have forwarded the explanation the PEs induce toxicity via ROS production which in turns attacks cellular DNA resulting in DNA breaks and DNA-protein cross-links. Also, Abdul-Ghani et al.\textsuperscript{1} have reported an elevation of the 8-hydroxydeoxyguanosine levels in blood samples of chicks exposed to PEs which is suggestive of genetic toxicity.

Assessment of biochemical alterations is attuned to monitoring the impact of exposure of fish to pollutants.\textsuperscript{47} In this study it was found that DBP significantly alters serum biochemical parameters that are used to monitor and assess hepatic and renal parenchymatous tissue damage. This finding was reinforced by data from histological observations. The elevated ALT enzyme levels observed in groups exposed to low as well as high doses of DBP may indicate degenerative changes in the liver resulting from toxic insults on the hepatocytes leading to cellular enzymes being released into the serum which can be quantified as an indicator of hepatotoxic impact.\textsuperscript{22} Studies conducted by Barse et al.\textsuperscript{6} wherein \textit{C. mirgala} was exposed to 25 ppm diethyl phthalate (DEP)\textsuperscript{19} showed that there is a decrease in ALT activity in fish that are exposed to 1, 5 and 20 ppm of DEP. Also, elevated serum creatinine level was deduced to reflect exposure to DBP in the study conducted by Weuve et al.\textsuperscript{53} who also reported that urinary creatinine measurements are concurrent with the urinary PEs levels recorded in the Neonates Intensive Care Unit. Additionally, acute exposure of \textit{O. niloticus} to DBP was observed to significantly elevate levels of the stress-related hormone cortisol in both groups; this elevation can be seen in light of an adaptive response to the release of pro-inflammatory mediators such as IL-8 via the anti-inflammatory effects of cortisol where the oxidative stress caused by PEs leads to tissue damage thereby inducing their release.\textsuperscript{22}

Histological analysis of the liver tissue indicated hydropic degeneration and vacuolation of hepatocytes because liver is the main organ responsible for xenobiotic metabolism and hence is considered as a primary target for damage.\textsuperscript{37} Also, kidney histopathological analysis evidenced presence of contracted glomerular tuft and cloudy swelling of the renal tubules. Elevated concentrations of ALT, creatinine and urea, induced by exposure to DBP, can be correlated to toxic insult of DBP especially because DBP is extremely lipophilic and is absorbed into the gills
even when present in minute concentrations as is evidenced by the diverse lesions reported in this study. Similar results were reported in zebrafish when exposed to 100 or 500 mg/l DBP.

Conclusion

Considering the results of this study, evidence of the biochemical and histological damage inflicted as a result of exposure to sublethal concentrations of DBP has proved that DBP is capable of interfering with the antioxidant defense mechanism as well as hepato-renal functions. Furthermore, it can also provoke oxidative impact and DNA damage in juvenile *O. niloticus* fish. Additional studies are needed in order to shed light on alterations and evidence of repair during the recovery period following a DBP exposure.

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References


57) Zeng, F., Cui, K., Xie, Z., Wu, L., Luo, D.,

