Environmental Assessment and Experimental Trial on the Effects of BTX Exposure on Adults and Embryos of *Bufo regularis*

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ABSTRACT

One of the causes of ecological deterioration is the increase of existing and emerging contaminants. Therefore, investigating the effects of daily-used product residues on river ecosystems, animal health, water quality, and biotic integrity is crucial. Field investigations were conducted to examine daily-used product residues along the Nile River in four cities of Assiut governate. Benzene, toluene, and xylene (BTX) are present in gasoline and are used as antiknock agents in petrol. They are also used in the production of daily-used products, paints, polymers, pharmaceuticals, pesticides, and so forth. Given their widespread use and exposure, the current study aims to determine the effect of BTX on *Bufo regularis*. The toxicity of BTX in *Bufo regularis* was assessed using erythrocyte alterations, nuclear abnormalities, and hematobiochemical parameters. A seven-day acute exposure to each chemical resulted in morphological alterations in erythrocytes. The group exposed to benzene and toluene had a decrease in red blood cells and hemoglobin levels with an increase in erythrocyte sedimentation rate. Hepatic enzymes, including alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase, increased significantly in BTX groups. Similarly, total protein and total bilirubin levels increased dramatically in groups exposed to BTX. Results of the present study showed that benzene and toluene have a more significant effect on the

blood biochemistry and hematology of *Bufo regularis* compared with xylene. This research will provide fundamental information on the toxicological effects of these chemicals on amphibians, which can be utilized to develop policies and management strategies to address the decline of amphibian populations.

INTRODUCTION

The accelerated civilizational race is associated with the contamination of water bodies especially rivers with various pollutants, including aromatic monocyclic hydrocarbon compounds that enter the environment through both anthropogenic and natural processes (Abdelhamed et al., 2023; Bu et al., 2014; Mohamed et al., 2023; Wu et al., 2022). The latter compounds are widely used as chemical intermediates, solvents, fuel additives, and cleaners in petrochemical industries and household activities (Davidson et al., 2021). They have access to aquatic ecosystems through natural seeps, atmospheric deposition, urban runoff, sewage disposal, coastal refineries, transport losses, and combustion of fossil fuel (Asejeje et al., 2020; Coatu et al., 2016). Because of their hydrophobicity, their presence poses serious safety concerns to aquatic creatures, which also can be extended to consumers through the food chain (Akinsanya et al., 2020).

Benzene, toluene, and xylene (BTX) are monocyclic aromatic hydrocarbons found in oil and gasoline (Potter, 1992) that can be produced by natural gas and petroleum condensate. The primary source of BTX includes gas spills, leaky service station tanks, and gases from vehicles (Barbee and Brown, 1986; Fedato et al., 2010; Stockman, 1987). A large quantity of BTX is released into the environment during the transport and processing of gas and petroleum products and from industry during the production of thinners, paints, adhesives, inks, paints, lacquers, cosmetics, and pharmaceutical products. These compounds are used individually and in mixtures as solvents in industry and household products (Reisch, 1992). Compared with other hydrocarbons, BTX are relatively more water soluble and pose a serious threat to aquatic life. Anthropogenic activities could increase the release of BTX in the environment, and the toxic effect of gasoline is due to its water-soluble fraction that contains high amount of BTX (Fedato et al., 2010). Therefore, the U.S. Environmental Protection Agency classified BTX as a priority pollutant (USEPA., 1997).

Exposure to BTX is more common through air and food than water (Leusch and Bartkow, 2010). Among the three chemicals, benzene is the most toxic and is classified as carcinogenic compared with xylene and toluene (Singh et al., 2010). Alterations in the gene encoding proteins in the liver were found following exposure of *Danio rerio* embryo to benzene (Wu et al., 2022), hematobiochemical, mutagenic, and histopathological changes in *Oreochromis niloticus* exposed to BTX (Sayed et al., 2023a), and oxidative stress and immunopathological alterations of *Clarias gariepinus* exposed to BTX (Sayed et al., 2023b).

Amphibians are excellent bioindicators and the most threatened vertebrate species (Saber et al., 2017). They play an important role in the environment as prey or predator, as well as in the energy transfer between terrestrial and aquatic habitats (Gillilland et al.,

2001). Various environmental stressors, such as disease, habitat destruction, climate change, and pollution, are all potential threats to amphibian diversity (Blaustein et al., 2011).

According to the International Union for Conservation of Nature (IUCN), amphibians are declared as the most threatened vertebrate species worldwide (Saber et al., 2017). A decline in the amphibian population can seriously affect the community, including loss of species diversity and richness and unbalanced ecosystem energy flow. Amphibians have a porous skin that serves as an organ of respiration; therefore, amphibians are more prone to airborne toxicants (Vitt and Caldwell, 2014). Therefore, determining the individual effect of pollutants on amphibian physiology is necessary to devise proper conservation and management strategies.

Most toxicological studies on amphibians focus on developmental and growth-related abnormalities, behavior alterations, and disease susceptibility (Karraker et al., 2008; Mahaney, 1994; Relyea and Jones, 2009; Shinn et al., 2008; Snodgrass et al., 2008). On the other hand, the effect of various toxicants on blood biochemistry is not highlighted. Fish and amphibian's hematological and biochemical biomarkers are used in toxicological studies as a reliable tool to assess the physiological status of organisms after exposure to a toxicant (Mekkawy et al., 2011; Sayed and Moneeb, 2015; Sayed, 2016c). In the present study, the daily-used water product residue at the Nile River was assessed, and various physiological and hematological biomarkers were used to examine the individual toxicity of BTX on amphibians, particularly *Bufo regularis*.

MATERIALS AND METHODS

1. Study area

A field survey of the water collected from the Nile River was conducted to assess the physicochemical analysis and assessment of harmful chemicals using a 1:5,000 scale map (Fig. 1).
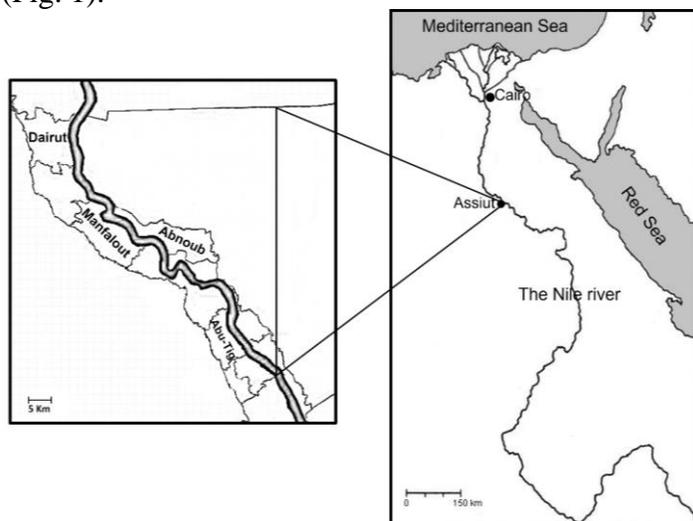
Fig. 1. Map of the study area showing the sampling sites and boundaries of the human communities along the Nile River**.**

2. Physicochemical analysis and assessment of harmful chemicals in water

In this study, water quality was evaluated, including temperature, pH, electrical conductivity, biological oxygen demand, turbidity, diethylene, chemical oxygen demand, total dissolved solids, total suspended solids, total solids, oils and greases, nitrate, nitrite, total alkalinity, total organic carbon, fluoride, sulfate, and orthophosphate. Water samples were collected (three samples from each site) in standard sampling water bottles (without head space) from a depth of 50 cm (with limited exposure to sunlight) and then stored in an icebox. The sampling time was fixed for all collection days within only one week. Some parameters, such as formaldehyde, ammonia, diethylene glycol, and BTX, were assessed using multiprobes (Lovibond water testing, Italy).

Moreover, we evaluated 11 harmful chemicals, including five heavy metals (Cd, Cu, Zn, Pb, and Cr) and six organic residues (formaldehyde, ammonia, diethylene glycol, and BTX) using gas chromatography with an electron capture detector.

3. Adult toad (*Bufo regularis*)

In total, 96 adult male Egyptian toads, Bufo regularis (25 to 30 g), were obtained from the educational farm of the Egyptian toad at Assiut University. They were kept in large glass tanks with water and mud from the normal environment and fed on grasses and earthworms.

For at least four weeks before the experiment, the toads had been adapted to laboratory conditions at 25°C–28°C and reduced humidity by 50%. Then, toads were classified into four groups (24 in each group in triplicate). The first group was treated with water mixed with BTX, where the toads were not exposed to any toxicant and were considered the control group; the second group of toads was exposed to benzene (0.762 ng/L); the third group of toads was exposed to toluene (26.614 ng/L); and the fourth group of toads was exposed to xylene (89.403 ng/L).

The exposure concentration of the BTX was environmentally relevant concentrations according to data presented in Table 1 and continued for seven days.

4. Chemicals sources

Benzene (99.8%), toluene (99.8%), and xylene (98.5%) were purchased from Sigma-Aldrich (Cairo, Egypt).

5. Sampling

After seven days of exposure to BTX, six samples from each group (two from each replicate) were sedated by immersing their head in ice for analysis (Hamed et al., 2019b). Blood was collected from posterior vena cava and stored in labeled heparinized and nonheparinized tubes for further analysis.

6. Erythrocyte alterations

Blood smears were prepared and fixed in absolute methanol for 10 sec. after drying at room temperature. Slides were stained with hematoxylin and eosin and then dehydrated

in ascending grades of alcohol (70, 80, 90, 95, and absolute). Finally, the slides were xylene-cleared and mounted by DPX (Hamed et al., 2021; Hamed et al., 2019a; Pascoe and Gatehouse, 1986).

7. Hematological and biochemical analyses

Immediately after blood collection, red blood cell (RBC) count, white blood cell (WBC) count, and hemoglobin concentration (Hb), hematocrit (Ht) were determined using an automated technical analyzer (Mindray Bc-2800). Other indices were calculated using the following formulae by (Dacie and Lewis, 1991):
MCHC (%) = Hb/Ht × 100, MCV (pg) = Hb/RBC × 10, MCV (µ3) = Ht/RBC × 10.
The erythrocyte sedimentation rate (ESR) was measured as follows (Murachi, 1959):
The serum was isolated for biochemical analysis. Serum samples were analyzed for creatinine (Cr), aspartic aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), glucose, total bilirubin, LDH, G6PDH, and total protein using the kits manufactured by SG Mitalia Company, USA.

8. Tadpole's exposure trail

Tadpoles of Egyptian Bufo regularis (Stage 52) were obtained from the Educational Toad Aquaponic Unit at Assiut University. Tadpoles were faded on brine shrimp (Artemia franciscana) three times a day and kept together in 50 L rectangular tanks containing decolorized tap water (conductivity, 2000 ls/cm; pH 7.4; oxygen, 89%–96% saturation; temperature, 26ºC-27ºC; photoperiod, 12:12 light:dark).
The adapted embryos (Stage 55) were subdivided into four large tanks (200 embryos in each tank). The experiment conditions were acclimatization with daily changing of all the decolorized tap water. The first group of embryos was treated with water mixed with BTX, where the tadpoles were not exposed to any toxicants and were considered the control group; the second group of tadpoles was exposed to benzene (0.762 ng/L); the third group of tadpoles was exposed to toluene (26.614 ng/L); the fourth group of tadpoles was exposed to xylene (89.403 ng/L). The experiment continued for seven days, and then five samples (Stage 64) were taken and fixed in Davidson solution for histological and histochemical preparation.

9. Histological and histochemical preparations

Fixed specimens were dehydrated and subsequently embedded in paraffin. Transverse serial sections were cut at 5–7 µm, stained by Harris's Hematoxylin and Eosin stain (H&E) (Bancroft and Steven, 1982) and Milligan's Trichrome stain (1946) staining of muscle and connective tissue. Stained sections were studied using a Zeiss microscope and a digital-colored video camera (SONY, DSC-TX200/TX200V).

10. Statistical analysis

Statistics were calculated using SPSS Statistics 20, data were checked for normality using the Kolmogorov–Smirnov test, and homogeneity was analyzed using Levene's tests. The statistical difference among groups was calculated using ANOVA followed by Tukey's post hoc test.

11. Ethical statement

The committee of the Molecular Biology Research and Studies Institute at Assiut University approved the ethical code (MB -21-27-R) for this study.

RESULTS

1. Presence of harmful chemical substances in water

In this study, we detected 11 harmful chemical substances among the investigated harmful chemicals. The chemical substances included five heavy metals (Cd, Cu, Zn, Pb, and Cr) and six organic residues (formaldehyde, ammonia, diethylene glycol, and BTX). The presence of harmful chemicals in water is illustrated in Table 1.

These results indicate that BTX was present at all sites among the harmful chemical substances. All three chemicals were present at levels above the critical screening values, reaching permissible limits (Masih et al., 2018); therefore, they seriously threatened aquatic animals.

2. Blood smear of *Bufo regularis* exposed to BTX

The blood smear of Bufo regularis exposed to BTX is shown in Figure 2. Blood smear from the control group showed a normal morphology with a centrally located nucleus. Various levels of morphological changes were observed in the blood smear of Bufo exposed to BTX. The observed morphological changes are characterized by the presence of teardrop-shaped cells, crenated cells (Cr), acanthocytes (Ac), spinocytes (Sp), eccentric nuclei (Eco), kidney nuclei (Kn), bionuclei (Bin), monocytes (Monc), basophils (Baph), eosinophils (Eoph), notched nuclei (Nn), bilobed nuclei (Bl); swollen nuclei (So); lobed nuclei (LO); microcytes (Mic); schistocytes (Sh), and sickle cells (Sk). A significant difference was observed among the BTX-exposed groups compared with the control group at $P < 0.05$.
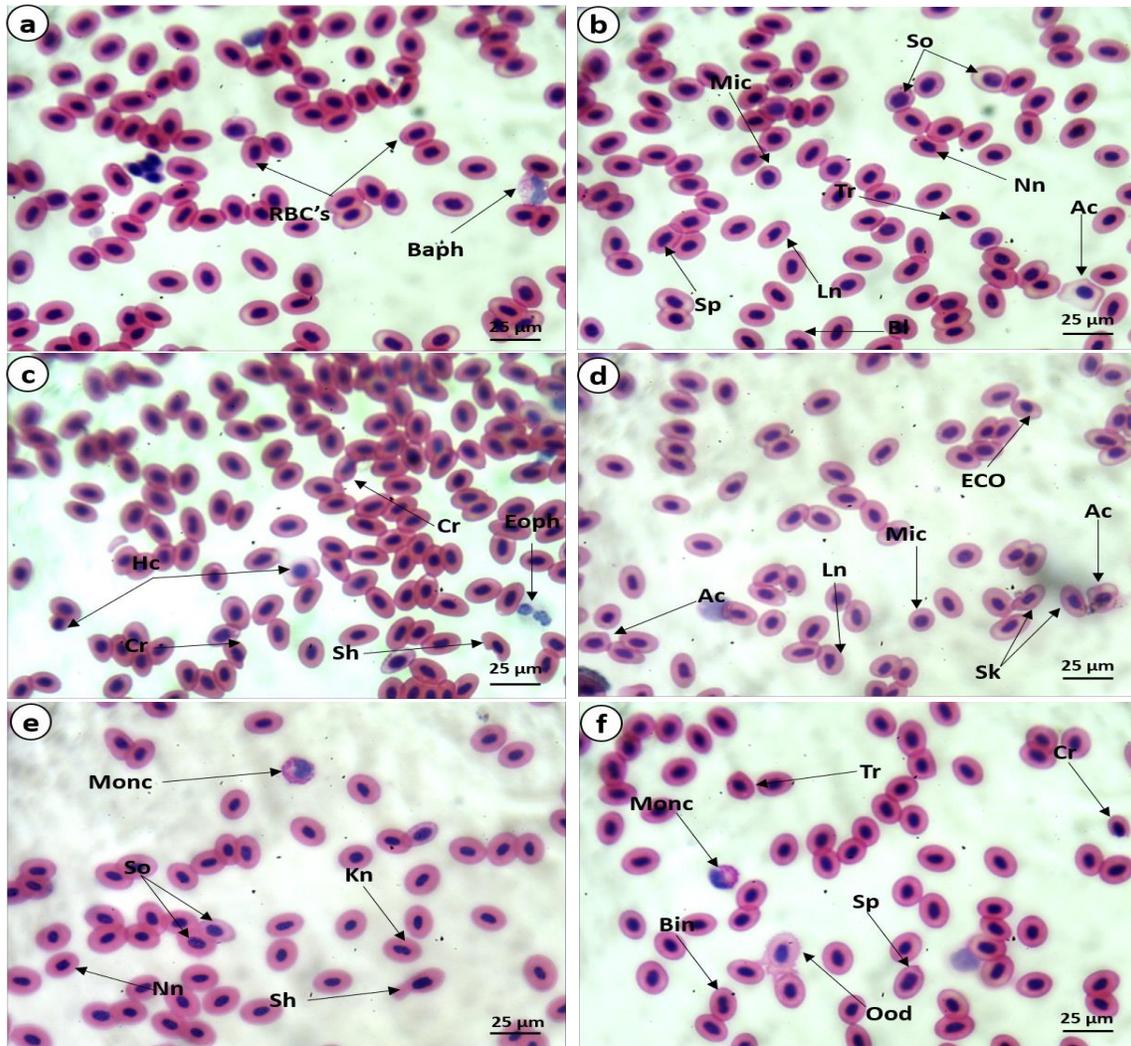
Fig. 2. Blood film of Bufo regularis: (a) control, (b) exposed to toluene (26.96 mg/L) for seven days, (c, d) exposed to benzene (0.81 mg/L) for seven days, and (e, f) exposed to xylene (25.11 mg/L) for seven days. Red blood cells (RBCs), teardrop-shaped cell (Tr), crenated cell (Cr), acanthocyte (Ac), spinocyte (Sp), eccentric nucleus (Eco), kidney nucleus (Kn), bionucleus(Bin), monocyte(Monc), basophil (Baph), eosinophil (Eoph), notched nuclei (Nn), bilobed nuclei (Bl), swollen nuclei (So), lobed nuclei (LO), microcyte (Mic), schistocyte (Sh), and sickle cells (Sk), H&E staining, and scale bar: 25 µm.

3. Effect of BTX on hematological parameters of *Bufo regularis*

The RBC count was significantly reduced in amphibians exposed to BTX compared to the control group. However, the exposure of benzene and xylene produced more reduction in RBC count. Hemoglobin, hematocrit, and packed cell volume also decreased in the exposed groups compared to the control group. In contrast, the mean corpuscular volume decreased in groups exposed to benzene and toluene compared to the control group (Table 2).

No statistically significant difference was recorded in mean corpuscular hemoglobin among the control and exposed groups, whereas the mean corpuscular hemoglobin concentration decreased significantly between the control and treatment groups. In addition, ESR increased significantly only in groups exposed to benzene and toluene (Table 2). The WBC count decreased substantially in groups exposed to benzene and toluene compared to the control group. In contrast, the group exposed to xylene showed no significant alterations in WBC count compared to the control group. Therefore, reduced RBC, low hemoglobin, and MCHC can lead to macrocytic hypochromic anemia among exposed organisms.

4. Effect of BTX on the physiological parameters of *Bufo regularis*

Serum hepatic biomarkers such as AST, ALP, and ALT were evaluated after seven-day exposure to BTX. A significant increase in AST, ALT, and LDH levels was recorded in all exposed groups compared to the control group. In contrast, the ALP level decreased significantly in groups exposed to BTX compared to the control group. Similar to hematological parameters, the effect was more pronounced in benzene- and toluene-exposed groups. Moreover, total protein showed a significant increase in benzene- and toluene-exposed groups, whereas total bilirubin increased significantly in all exposed groups compared with control (Table 3).

5. Histological changes of *Bufo regularis* tadpole's

Pronephrotic tubules appear as elongated or rounded in their shape. Their lumen was constricted or very narrow. Some pronephros showed deeply stained basophilic by H&E and contained open-phase nuclei; some nuclei have deeply stained nucleoli in the center of nuclei or dense chromatin at the nuclei in the control group. Few pronephrotic tubules completely degenerated; they lost their basal membrane, and necrotic regions were noticed. Luminal casts were detected at the center of the pronephros, as well as a widening of intrapronephrotic space, which contains the degenerated and hemopoietic granulated cells. Many granulated cells contained deeply stained nuclei and showed signs of degeneration. Apoptotic bodies were seen in intrapronephrotic space in the tadpoles exposed to benzene (0.762 ng/L), as shown in (Fig. 3a).
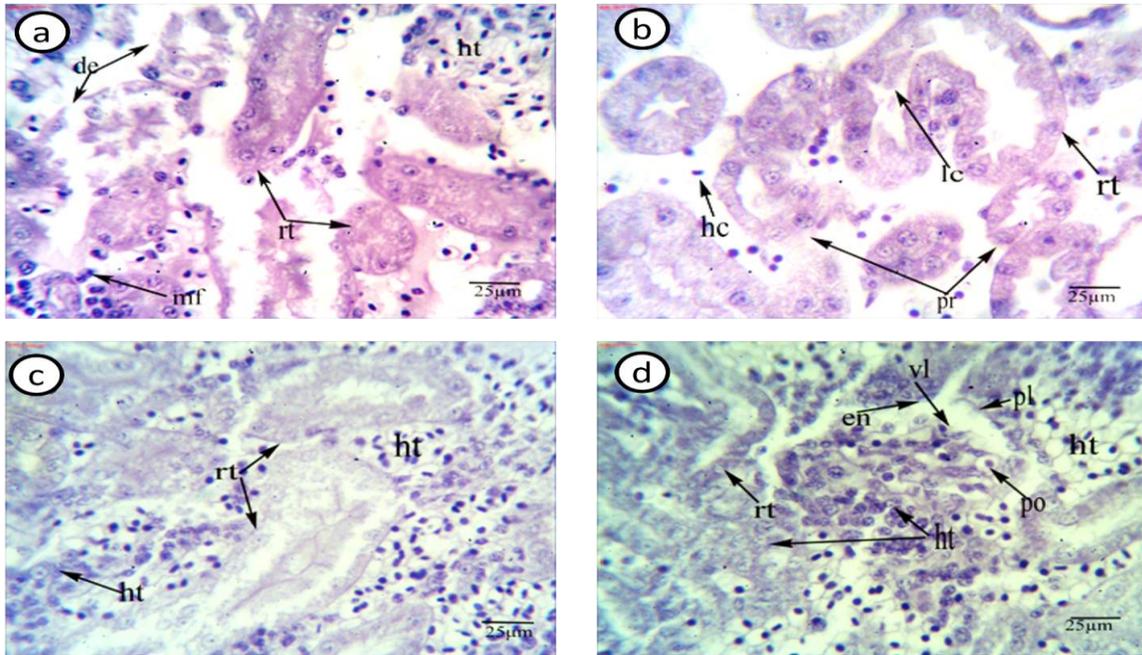
Fig. 3. Sections of the kidney of Bufo regularis tadpoles (Stage 64) showing (a) control, (b) tadpoles exposed to benzene (0.762 ng/L), (c) tadpoles exposed to toluene (26.614 ng/L), and (d) tadpoles exposed to xylene (89.403 ng/L), apoptotic bodies (ap), degeneration (de), endothelial cells (en), hemopoietic cells (hc), hemopoietic tissue (ht), lumen casts (lc), melanomacrophage (m), mitotic figure (mf), partial layer (pl), podocyte (po), proximal convoluted tubule (pr), renal tube (rt), visceral layer (vl), staining with H&E stain (x 400), and scale bar= 25µm.

Pronephrotic tubules are rounded or kidney-shaped. They were lined by cubic cells with basophilic cytoplasm. The nuclei are of the open-phase types, which contain two or three vesicular nucleoli. Widening of the intrapronephrotic space was encouraged by casts of degenerated tubules and hematopoietic cells. Large ducts lined by tall cubic cells were noticed in tadpoles exposed to toluene (26.614 ng/L), as shown in Fig. 3b.

In tadpoles exposed to xylene (89.403 ng/L), the section showed pronephrotic tubules rounded and rod shapes lined by cubic cells with open-phase type. Their lumens were narrow and stained by H & E stain. The appearance of a glomerulus was noticed in this stage, which was delimited by collagen fibers of a partial layer outside and visceral layer inside and a space between the glomerulus and the capsule called the Bowman's space. Hemopiotic tissues were more distinct in this stage with different types of cells.

The tadpoles exposed to xylene (89.403 ng/L) showed pronephrotic tubules, which take different shapes, elongated, rounded, and ovoid and consisting of a double membraned capsule (Bowman's capsule) enclosing a tuft of blood capillaries (glomerulus), some of these tubules are degeneration, and they lined by cubic cells with open-phase nuclei with three nucleoli. Widening of intrapronephrotic tubules was encouraged by hemopoietic granulated cells. Few pigment granules were noticed. The last section of nephrons contains a glomerulus lined by a parietal layer from the outside and a visceral layer from the inside; the podocyte is more distinct in this structure. Walfian ducts were noticed.

5. Histchemical changes of *Bufo regularis* tadpole's

In the control tadpoles, the section showed rounded and rod-shaped pronephrotic tubules lined by open-phase cubic cells. The collagenous fiber was noticed around the pronephros at the basal membrane and the brush border due to their high glycoprotein content as the basement membrane's main structures, which were rich in collagen glycoproteins. Their lumen was narrow and stained green by Milligan stain (Fig. 4a). The appearance of glomerulus was noticed in this stage, which was delimited by collagen fibers of partiwasayer outside and visceral layer inside. Few collagens were localized around the capillaries in the glomerulus tufts in the center of the glomerulus in the tadpoles exposed to benzene (0.762 ng/L), as shown in Fig. 4b. Widening of the intera-pronephrotic tubular space was observed in faint staining collagenous fiber and encouraged by a huge amount of hemopoietic granulated cells in the tadpoles exposed to toluene (26.614 ng/L), as shown in Fig. 4c.
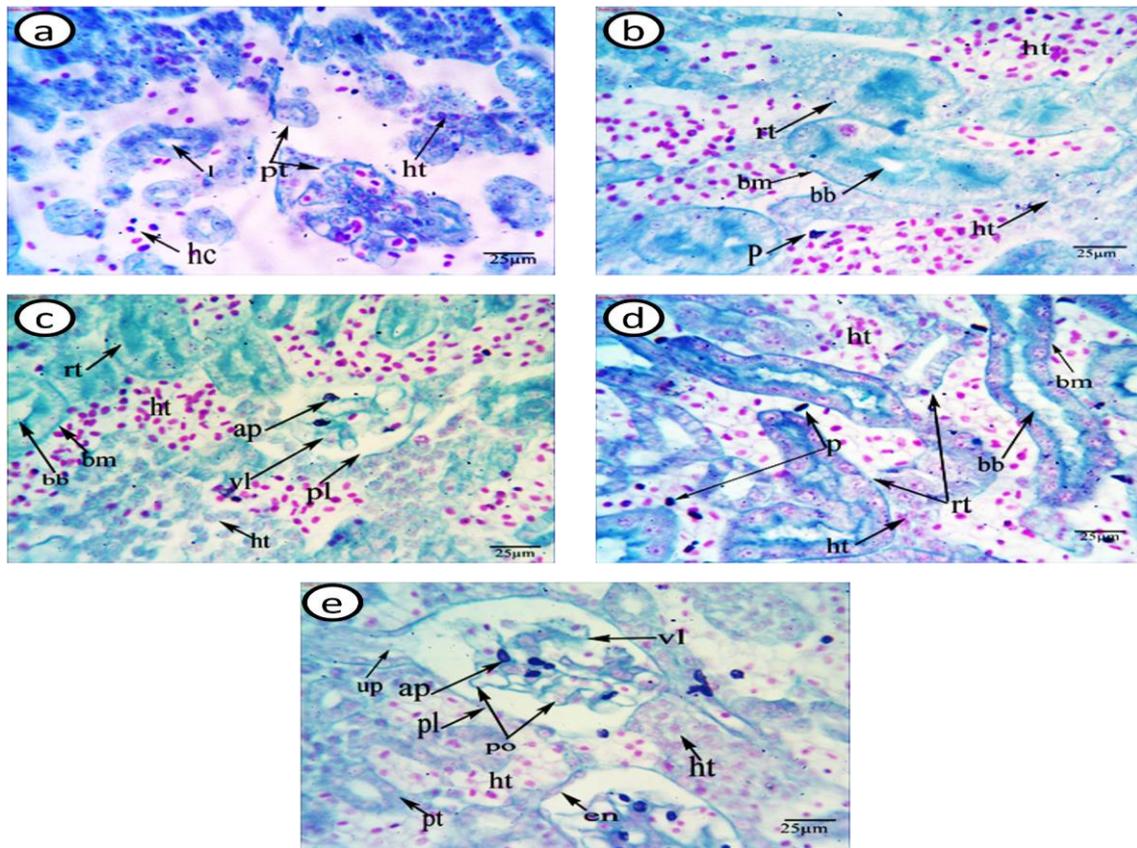
Fig. 4. Sections of the kidney of *Bufo regularis* tadpoles (Stage 64) showing (a) control, (b) tadpoles exposed to benzene (0.762 ng/L), (c) tadpoles exposed to toluene (26.614 ng/L), and (d, e) tadpoles exposed to xylene (89.403 ng/L), apoptotic bodies **(ap), bruch** border (bb), basement membrane (bm), endothelial cells (en), hemopiotic cells (hc), hemopiotic tissue (ht), liver (l), lumen (l), mesengial cells (mc), pigments (p), parital layer (pl), podocyte (po), proximal convoluted tubule (pt), renal tube (rt), visceral layer (vl), urinary pore (up), walfian duct (wd), staining with Milligan stain (x 400), and scale bar= 25μm.

The tadpoles exposed to xylene (89.403 ng/L) showed pronephrotic tubules, which take different shapes: elongated, rounded, and ovoid. Stain-positive materials with Milligan are localized at the basement membrane and in the brush border of epithelium. They were lined by cubic cells with open-phase nuclei with three nucleoli. The lumen of pronepherons stains faint green with Milligan. Widening of intrapronephrotic tubules was encouraged by hemopoietic granulated cells. Few pigment granules were noticed, and a large number of apoptotic bodies were noticed.

DISCUSSION

The global nature of petroleum-derived hydrocarbons, their extensive disposal in the aquatic environment, and their multifaceted toxicological targets add fuel to the scientific community in its persistent pursuit of practical solutions to their toxicity. This study primarily aims to conduct field screening of commonly used products, with a specific focus on hydrocarbons (BTX). Hydrocarbons are crucial in carrying conjugated derivatives of contaminants for their subsequent eliminatio (Akaishi et al., 2004).

The estimated level of benzene in rural areas is 0.06 ppb; however, in areas with dense population and industrial estates, it increases up to 107 ppb. High levels of benzene, up to 3000 ppb, are recorded in the air of petrol stations. Many household products, such as adhesives, nail polish, and paints, contain toluene. It is also released during the production and transport of petroleum and petroleum products. The average air level of toluene in suburban areas ranges from 1.3 to 6.6 ppb (ATSDR, 2000). However, the detected level of toluene in areas with high traffic reaches 350 ppb. Industries and motor vehicles are considered the main source of xylene emissions. Ambient air contains 0.23 ppb of xylenes, but the level of xylene in the air increases to 178 ppb in industrial areas and areas with heavy traffic (Singh et al., 2010).

It is also essential to study their loads, changes, origins, and bioaccumulative tendencies in aquatic organisms, especially fish, in order to understand their health risks, stability, and origins. (Xu et al., 2017). Among the harmful chemical substances, BTX were frequently observed in this investigation. The environmental effects of harmful chemicals range from cell damage to death in humans and animals (Atique and An, 2018; Gao et al., 2008; Sayed et al., 2023a; Sayed et al., 2023b).

This study investigated eleven harmful chemical substances, including five heavy metals (Cd, Cu, Zn, Pb, and Cr) and six organic residues (formaldehyde, ammonia, diethylene glycol, and BTX). Nearly all study sites detected high levels of PAHs, and some sites consistently detected high levels of PAHs (Table 1). Heavy metals can remain in sediments for many decades before becoming accessible again through specific processes in flowing water bodies (Guo et al., 2015; Moon et al., 2020). Research has shown that nutrient levels, organic matter, and nonalgal turbidity decrease the abundance of native, riffle benthic, amphibian, and sensitive fish species (Atique and An, 2018; Kim and An, 2015).

The hematological indices for amphibians exposed to toxicants are often associated with health status (Sayed, 2016a), often reflecting the changes in physiological state

(Mekkawy et al., 2011). In the present study, Bufo regularis was exposed to BTX individually, and the effect of these chemicals on erythrocyte morphology, blood hematology, and biochemistry was evaluated. The morphological evaluation of erythrocytes is rapid, and it is a reliable tool for estimating the toxicological effect of a pollutant (Sayed et al., 2014). Many studies have used morphological alteration and nuclear abnormalities of erythrocytes as a biomarker of genotoxicity after exposure to a toxicant (Gomes et al., 2015; Sayed and Moneeb, 2015; Sayed and Hamed, 2017). A close relationship is observed between DNA damage and erythrocyte abnormalities. Therefore, morphological alterations in erythrocytes are considered a reliable, simple, and robust biomarker for studying genotoxicity and cytotoxicity in eukaryotic organisms (Gomes et al., 2015; Mekkawy et al., 2011; Sayed, 2016b). In the present study, general erythrocytes' alteration after exposure to BTX includes schistocytes, sickle cells, crenated cells, teardrop-shaped cells, acanthocyte, and spinocytes, whereas the nuclear abnormalities include bilobed, lobed, swollen, eccentric, and kidney-shaped nucleus. These results indicate that BTX may cause genetic instability that leads to erythrocyte abnormalities. The Hb decreased in B. regularis exposed to BTX. The decrease in hemoglobin may be due to the lysis of erythrocytes after exposure. Exposure of benzene to human also caused a decrease Hb and RBC count (Ibrahim et al., 2014).

There was a noticeable adverse impact on hematological and biochemical changes in the toad after exposure to organic solvents (BTX). Similar results were reported in toads after exposure to a variety of toxicants (El-Mofty et al., 1992; Sayed, 2016a), where RBCs, HB, Hct, platelets, and MCHC levels were decreased and MCV and WBCs were increased in Clarias gariepinus when exposed to different doses of xylene and in O. niloticus after exposure to BTX (Sayed et al., 2023a)(Sayed et al., 2023b). One reason for this hematological damage is anemia and leucopenia (Chris et al., 2022; Luskova et al., 2002). Hepatic enzymes such as ALT, ALP, and AST are abundant in the liver and used for protein metabolism. Hepatic biomarkers (ALT, ALP, and AST) increased in groups exposed to benzene and toluene. The increase in these biomarkers may reflect liver dysfunction and injury after exposure to these toxicants (El-Sayed et al., 2007).

CONCLUSION

The study examined the impact of environmentally significant levels of BTX on Bufo regularis, an Egyptian toad species. The investigation revealed significant changes in the hematology and biochemistry profiles of the toads. The findings indicate that the well-being of the largest river in Egypt and its water quality are under significant threat due to the presence of hazardous chemicals. To address this issue, it is imperative to enforce stringent measures that can effectively reduce their inflow into riverine ecosystems.

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Table 1. The presence of harmful chemicals in water (means ± SE) in the sampling sites

Sites Parameters	Units	Dairut	Manfalut	Abnub	Abu-Tig
Temperature	C°	20.83 ± 0.44 a (21.5-20)	21.266 ±0.145a (21.5-21)	20.76±0.39 a(21.3-20)	21.166±0.166a (21.5-21)
pH	Unit	8.366±0.088a (8.5-8.2)	8.366±0.088a (8.5-8.2)	8.43±0.03a (8.5-8.4)	8.9±0.208b (9.2-8.5)
Conductivity	ms/cm	0.466±0.044a (0.55-0.4)	0.423±0.0819a (0.55-0.27)	0.326±0.0385 a(0.4-0.27)	0.31±0.010a (0.33-0.292)
DO	ppm	5.766±0.145a (6-5.5)	6.1±0.208 a(6.5-5.8)	6.133±0.3179 a(6.5-5.5)	6.066±0.176a (6.4-5.8)
Turbidity	NTU	0.6±0.057a (0.7-0.5)	0.5±0.0577a (0.6-0.4)	0.53±0.088a (0.7-0.4)	0.533±0.033a (0.6-0.5)
Diethylene	ppm	5±0.416 a(5.8-4.4)	5±0.577a (6-4)	4.33±0.33a (5-4)	5.43±0.27a (5.8-4.9)
Chemically consumed oxygen	ppm	8.66±0.726a (10-7.5)	8.5±0.76 a(10-7.5)	8.23±0.145 a(8.5-8)	8.066±0.296 a(8.5-7.5)
Total dissolved solids	ppm	290.66±26.339 a(340-250)	267±47.077a (340-179)	208.66±21.309a (250-179)	273±41.525a (340-197)
Total suspended solids	ppm	14.66±2.40 a(18-10)	15.66±2.848a (19-10)	16.66±1.20 a(19-15)	14.33±2.33 a(18-10)
Total solids	ppm	305.33±24.39a (350-266)	282.66±44.726a (350-198)	225.33±20.73a (266-198)	287.333±40.337 a(350-212)
Oils and grease	ppm	0.2±0.057 a(0.3-0.1)	0.1833±0.044 a(0.25-0.1)	0.1±0 a(0.1-0.1)	2.3±1.153b (4-0.1)
Nitrates	ppm	0.97±0.096 a(1.12-0.79)	0.996±0.1183 a(1.2-0.79)	1.073±0.0896 a(1.2-0.9)	0.8966±0.0606 a(1-0.79)
Nitrite	ppm	0.019±0.0039 a(0.027-0.014)	0.0123±0.00328 a(0.017-0.006)	0.015±0.00624a (0.027-0.006)	0.0143±0.00145a (0.017-0.012)

Table 1. (Continued)

Total alkalinity	ppm	133.33±15.376 a(164-116)	118.66±1.33 a(120-116)	133.33±15.376 a(164-116)	117.33±1.33 a(120-116)
Total organic carbon	ppm	36.66±3.898 a(43.5-30)	35.13±4.219 a(43.5-30)	33.8±1.386 a(36.5-31.9)	27.33±11.609a (44-5)
Fluoride	ppm	0.406±0.037a (0.47-0.34)	0.436±0.049 a(0.5-0.34)	0.47±0.03a (0.5-0.41)	0.4366±0.049a (0.5-0.34)
Sulfate	ppm	59.66±10.17 a(74-40)	50.66±7.446 a(65-40)	56.33±8.838 a(74-47)	51±7.37a (65-40)
Orthophosphate	ppm	0.35±0.06 a(0.47-0.27)	0.3166±0.029 a(0.37-0.27)	0.283±0.139a (0.47-0.01)	0.196±0.094 a(0.31-0.01)
Cadmium	ppm	0.0004±0.0002 a(0.0008.-0.0002)	0.00013±0.00006a (0.0002-0)	0.000266±0.000266 a(0.0008-0)	ND
Lead	ppm	0.001±0.001 (0.003-0)	ND	0.001±0.001 (0.003-0)	ND
Copper	ppm	ND	ND	ND	ND
Chromium	ppm	0.009±0.0085 a(0.026-0)	0.0023±0.00185 a(0.006-0)	0.0106±0.0078 a(0.026-0)	ND
Zinc	ppm	0.0009±0.00029 a(0.0014-0.0004)	0.00096±0.00026 a(0.0014-0.0005)	0.00043±0.00003a (0.0005-0.0004)	0.0006±0.000185a (0.001-0.0004)
Formaldehyde	μ/l	0a	0a	5.33±2.905 b(10-0)	0a
Ammonia	mg/L	1257.33±35.408 a(1322-1200)	1221.66±100.0138 a(1420-1100)	1566.66±29.059b (1620-1520)	1346.66±73.33 a(1420-1200)
Diethylene glycol	μ/l	1.33±0.66 a(2-0)	0.66±0.66 a(2-0)	1.33±1.33 a(4-0)	0.66±0.66 a(2-0)
Benzene	ppt	567.33±21.34a (610-545)	604±45.0148a (651-514)	762.33±27.424 b(810-715)	542±46.9 a(610-452)
Toluene	ppt	24387±63.237 b(24510-24300)	23482.66±29.202 a(23541-23451)	26614±182.379 c(26960-26341)	23642±350.158a (24321-23154)
Xylene	ppt	89403.66±66907.87 a(223214-21456)	88647.66±66404.699a (221453-21345)	24403.33±673.936 a(25110-23056)	16296.715±8136.9a (24516-23.145)

Different letters indicate significance at p < .05 (one-way ANOVA followed by Tukey's posttest), N: 3. parts per million (ppm), parts per trillion (ppt)

Table 2. Effect of benzene, toluene, and xylene on Hematological parameters of Bufo regularis.

Hematological parameters	Units	Control	Benzene	Toluene	Xylene
RBC's	105/mm3	9.535±0.15	8.333±0.071****	8.800±0.14**	8.467±0.12****
Hb	g/dl	10.42±0.14	8.983±0.10****	9.567±0.12**	9.183±0.21****
Ht (PCV)	%	25.50±0.42	20.17±0.40****	21.83±0.30****	24.50±0.61
MCV	μm^3	26.78±0.63	24.21±0.50*	24.83±0.35	28.93±0.53*
MCH	Pg	10.93±0.17	10.79±0.18	10.88±0.18	10.84±0.1403
MCHC	%	40.87±0.40	44.65±1.16*	43.87±0.92	37.52±0.60*
ESR	mm/hr	5.600±0.073	8.550±0.21****	7.017±0.12****	5.983±0.070
WBC's	Thousands/mm3	10.16±0.4729	8.033±0.15****	8.967±0.16*	9.383±0.098

RBC, red blood cell; Hb; hemoglobin; PCV, packed cell volume; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; WBCs, white blood cells; ESR, erythrocyte sedimentation rate; MN, micronuclei.

Results are expressed as means ± SEM of 6 toads per group.

Stars indicate significance at $p < .05$ (one-way ANOVA followed by Tukey's posttest).

Table 3. Effect of benzene, toluene, and xylene on blood biochemical parameters of *Bufo regularis.*

Hematological parameters	Units	Control	Benzene	Toluene	Xylene
AST	µ/ml	172.7±1.174	190.8±2.651****	186.7±1.820***	183.0±2.066*
ALT	µ/ml	30.00±0.632	37.00±1.125***	35.83±1.078**	32.50±0.670
ALP	µ/ml	16.42±0.359	12.20±0.148****	13.47±0.135****	15.43±0.453
LDH	µ/ml	280.2±2.247	303.4±2.327****	295.4±1.051****	287.0±0.962
G6PDH	µ/ml	256.6±4.520	238.2±2.618**	243.2±2.982	250.0±3.895
Glucose	mg/dl	80.53±2.058	73.70±1.325*	75.40±1.413	76.03±0.958
Total protein	g/dl	3.50±0.1438	4.817±0.153***	4.817±0.207***	4.167±0.164
Total bilirubin	mg/dl	0.1400±0.004472	0.2000±0.010****	0.1817±0.0065**	0.1767±0.0042**

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), glucose-6-phosphate dehydrogenase (G6PDH).

Results are expressed as means ± SEM of 6 toads per group.

Stars indicate significance at $p < .05$ (one-way ANOVA followed by Tukey's posttest).