Pollution 2022, 8(4): 1387-1397
DOI: 10.22059/POLL.2022.339894.1386

RESEARCH PAPER

Biomarker Responses in *Sclerophrys regularis* (Anura: Bufonidae) Exposed to Atrazine and Nitrate

Rashad ElSayed Mohammed Said[1*], Aml Sayed Said[2], Samy Abdel Latif Saber[3] and Boshra Abd ElAziz ElSalkh[2]

[1] Department of Zoology, Faculty of Science, Al-Azhar University, Assiut, Egypt.
[2] Department of Zoology, Faculty of Science (Girls), Al-Azhar University, Cairo, Egypt.
[3] Department of Zoology, Faculty of Science, Al-Azhar University, Cairo, Egypt.

Received: 02.03.2022, Revised: 20.06.2022, Accepted: 20.07.2022

Abstract
The impact of atrazine and nitrate, the most used pesticide and fertilizer, on the health of *Sclerophrys regularis* was investigated in this study. We exposed the tadpole of *Sclerophrys regularis* to atrazine and nitrates to assess abnormalities and genotoxicity as biomarkers for environmental impacts. The proportional malformed toads were shown to be high when treated with nitrate alone (17%) or in combination (13%) or even treated with atrazine alone (the minimal, 5%), limbs and tail deformities are the most observed. Also, abnormal activity and movement were detected at all treated groups. In addition, Genotoxicity was measured by micronucleus test (MN) in detection of nuclear abnormalities in given species. The results indicate that individuals exposed to atrazine or nitrate or that exposed to both, exhibited a significantly higher degree ($p < 0.01$) of nuclear lesions. These results constitute key assessment of developing abnormalities and MN test in this species in Egypt and suggest that developing abnormalities and /or MN test and the degree of nuclear lesions of RBCs can be a useful indicator of the degree of environmental stress and ecosystem disrupting experienced by amphibian populations.

Keywords: Genotoxicity, Micronucleus test, Abnormality, Behavioral activity

INTRODUCTION

Amphibians, despite being a diverse group of species, are under danger all around the world (Brühl et al., 2013; AmphibiaWeb, 2020) and thereby, the rate of disappearance rises (Houlahan et al., 2000; Sparling et al., 2001; Stuart et al., 2004; Becker et al., 2007). Multiple factors such as increased ultraviolet radiation, fungal and bacterial epidemics, droughts, acid precipitation, climate change, habitat destruction and fragmentation, exotic species, heavy metals, acid rain, pesticides, and fertilizers can all work together to cause mortality or sublethal effects. Agricultural contaminants have been linked to the demise of several amphibian populations (e.g., Davidson,2004; Davidson and Knapp, 2007; Hamer et al., 2004; Sparling and Fellers, 2009; Baker et al., 2013). Pollutants not only influence the health and survival of species, but they can also interact with their DNA (Rajaguru et al., 2001). Many toxicity tests focus on the steady concentrations of a substance (Earl and Whiteman 2009). The pesticide atrazine, which is commonly used, can travel far from its application site (Karlsson et al., 2020) even in remote areas where it is not used. An ecological risk assessment of atrazine in North America has revealed that many ecosystems including streams, rivers, and reservoirs are facing greatest risk (Solomon et al., 1996).

* Corresponding author Email: banjawy@yahoo.com

On the other hand, adjacent habitats could be exposed to high quantities of atrazine on a regular basis, affecting amphibians during the breeding season (Allran and Karasov, 2000). After being exposed to toxicants, animals' behavior may change, which could be attributed to a change in physiological processes (Nkontcheu et al., 2017). Male African clawed frogs *Xenopus laevis* exposed to atrazine become completely feminized (Hayes et al., 2010). Furthermore, atrazine has been shown to diminish the quantity of red blood cells in a variety of species, including mice (Chang et al., 2021) and fish (Akhtar et al., 2021). Over the last 50 years, global nitrate contamination has increased, owing to increased fertilizer use and sewage generation (Edwards et al. 2006). Through the widespread use of fertilizers, the creation of human and livestock waste, and the combustion of fossil fuels, agricultural use of nitrogen-based fertilisers, human activities significantly increased the amount of reactive nitrogen in the environment (Rouse et al., 1999; Ilha and Schiesari 2014). The goal of this study is to assess the possibility of genotoxicity and malformations in the anuran *Sclerophrys regularis* subjected to atrazine and nitrate to assess their hazardous potential.

MATERIALS AND METHODS

To minimize further harmful repercussions, tadpoles of the Egyptian toad *Sclerophrys regularis* were gathered from Al-Wahat region, which is a natural ecosystem with low contamination. Tadpoles were caught at the Gosner stage (24-27), when their bodies resembled small pin heads, roughly 2-3 weeks after hatching (Gosner, 1960). Hundreds of larval tadpoles were gathered in aerated plastic bags immersed in their ground water and transported to the laboratory, where they were evenly dispersed into four plastic containers with a width of 37-40 cm and a height of 25 cm, each containing 8 L of dechlorinated tap water, the tadpoles were then allowed to acclimate in the holding containers for seven days before the bioassay (Cardwell et al., 2011). Tadpoles were fed dry algae and commercially available fish feeding grains. The larvae were grown in natural settings, with a 12:12 hours light: dark cycle and a room temperature of 30-35 degrees Celsius throughout the experiment. The water in each container was replaced every three days (Ezemonye & Tongo, 2009).

The herbicide organochlorine was used as an atrazine source, while sodium nitrate was used as a fertilizer source in the toxicity tests. Dechlorinated water was utilized for acclimatization, control tests, and the preparation of various test chemical concentrations. Toxicity test stock solutions of 300 μg/L pure commercially available atrazine and 200 mg/L sodium nitrate (Ryder et al., 2009). Toxicity tests were carried out by filling 4 containers, each contains 8 liters of dechlorinated tap water and constantly inserted air pump in each with concentrations of 1) 300 μg/l of atrazine 2) 200 mg/l of sodium nitrate 3) combination of 300 μg/l of atrazine and 200 mg/l of sodium nitrate and 4) 0 concentration of both as control. These concentrations are chosen as sublethal doses according to pre-study test on many concentrations which resulted in a sublethal dose of atrazine at 300 μg/l and of nitrate at 200 mg/l. Larval tadpoles were distributed into the containers as 100 larvae in each to make four groups which were labeled atrazine (A), nitrate (N), combination (AN) and control C groups, respectively. Since atrazine has a minimum half-life of 48 hours in water, a new solution of water and chemicals was made every 3 days in each container (Solomon et al., 1996), and this work had been done until metamorphosis (complete tail reabsorption—Niewkwoop–Faber Stage 66) was reached.

Animal behavior was rated based on two observations. 1) movement activity, which was observed based on weak and normal movement or swimming, as well as their position in the water column within their tanks. 2) Feeding activity, which is determined by their demand for food and the amount of food grains left at the bottom of the tank. The observation was done as an evaluating or estimating ratio for activity of each group during the entire length of the experiment, and the behavior study was set as minor support to the other outcomes.

Animals were observed and examined in order to characterize anomalies and catalogue them using standardized nomenclature and morphologic diagrams, as well as malformation terminology (Meteyer, 2000; Richard 2003). The percentage of animals having each type of abnormality in comparison to the total number of individuals investigated was computed for each type of abnormality. Using a digital camera, abnormal individuals were photographed.

MN test was performed to assess DNA damage according to Feng et al. (2004) and Güner et al. (2011). Blood samples were collected via a heart puncture and smeared on cleaned glass microscope slides. The slides were then fixed with methanol, air-dried, and stained with a 10% Giemsa solution. A total of 2000 erythrocytes with one micronucleus (MN) or two binucleated nuclei (BN), heart shaped, kidney shaped, irregular shaped, and notched nuclei were scored for each animal. Five animals were chosen at random from each group and their RBCs were scored at 1000 times magnification to determine the prevalence of micronucleated RBCs and nuclear lesions (Osman et al., 2011; Gürcü et al. 2022).

$$\textbf{RBCs (MN/NL)}\ \text{‰} = \frac{\text{Number of cells containing micronuclei}}{\text{Total number of cells counted}} \times 1000$$

Analysis of variance on SPSS (Version 20) and LSD post hoc test was used to test the current data. Probability values ≤0.05 and ≤0.01 were defined as significant throughout the current work.

RESULTS AND DISCUSSION

The movement and activity were detected along the whole period of the experiment. The abnormal activity was represented by (abnormal slow movement, low feeding activity, always taking the lower position in the water column). The control group showed normal activity along the whole period, only 2 animals had little abnormal movement (Fig.1). The abnormal activity gave the highest rate (about 90% of the total animals) at the combination (AN) treatment, whereas nitrate treatment gave (about 75%). The abnormal activity also was common at atrazine treatment (about 60%).

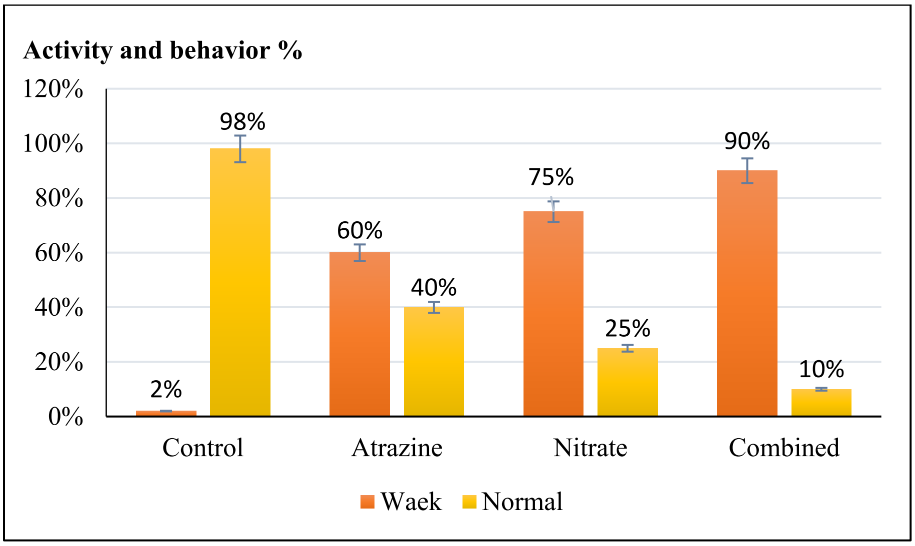

Fig 1. Activity of *Sclerophrys regularis* exposed to all treatments.

The study revealed the presence of missing and misshapen limbs as like as tail deformities (Table 1 and Figs. 3-5). The common types of malformations observed here were misshapen limbs and misshapen tail with a total number 17 and 16 respectively, whereas Amelia was rare with total

Table 1. Abnormality types and abnormality percentages for each treatment.

		Amelia	Misshapen hind limbs	Misshapen tail	Total
Treatment	C	0	0	0	0%
	A	0	1	4	5%
	N	0	7	6	13%
	AN	2	9	6	17%
Total		2	17	16	

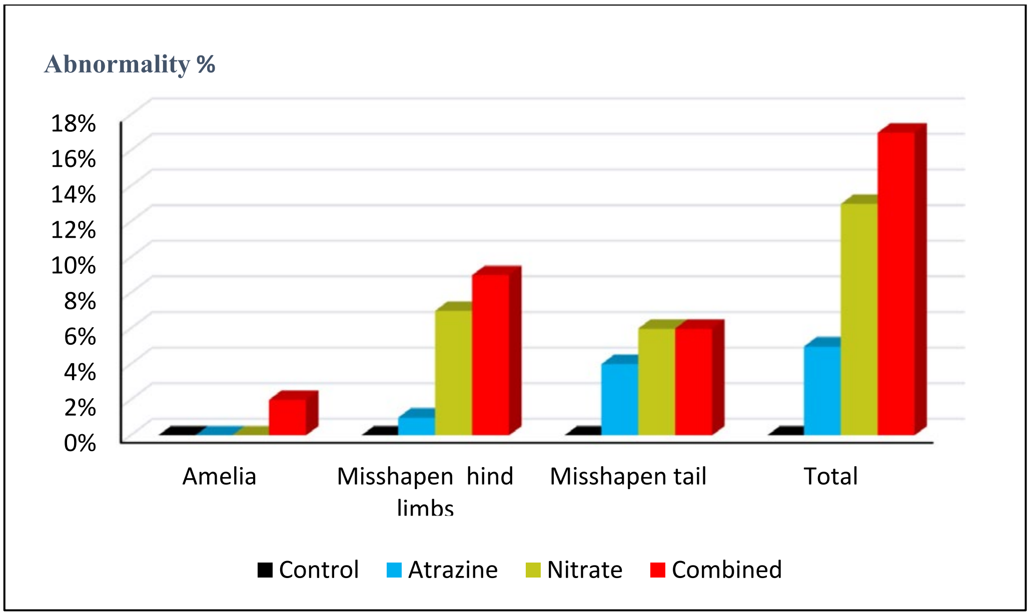

Fig. 2. Different abnormalities of *Sclerophrys regularis* after exposure to different treatments.

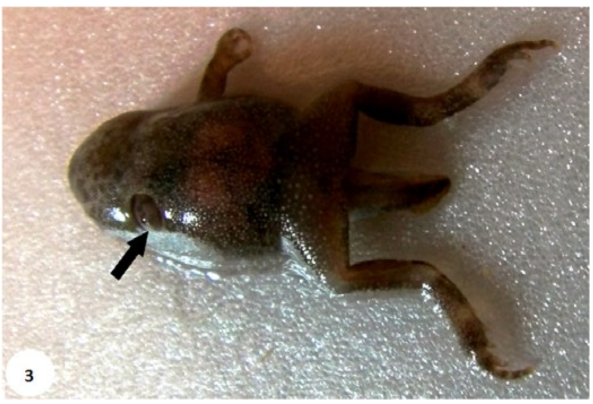

Fig. 3. Amelia of *Sclerophrys regularis* detected after exposure to treatment.

Fig. 4. Misshapen tai l of *Sclerophrys regularis* detected after exposure to treatment.

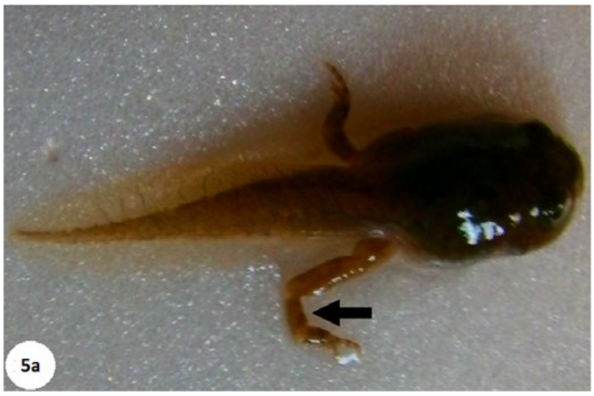

Fig. 5a. Misshapen right hind limb of *Sclerophrys regularis* detected after exposure to treatment

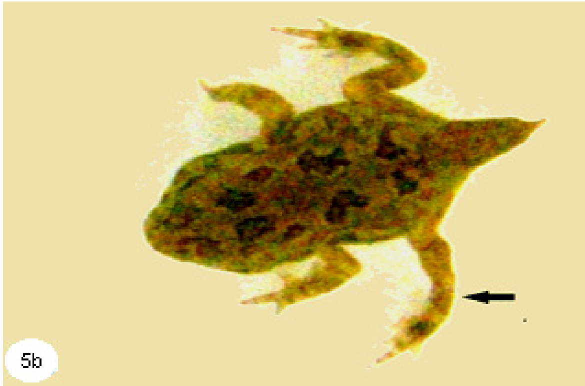

Fig. 5b. Misshapen left hind limb of *Sclerophrys regularis* detected after exposure to treatment.

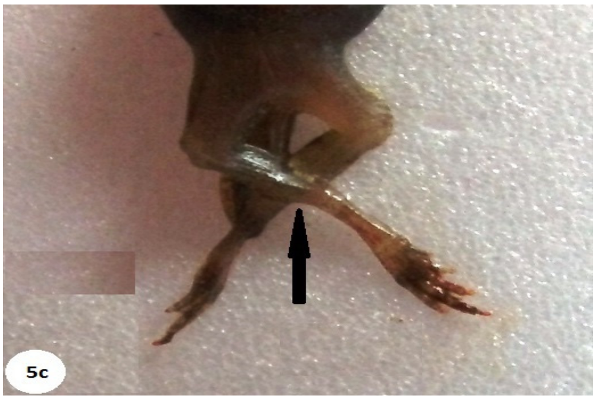

Fig. 5c. Misshapen right and left hind limb of *Sclerophrys regularis*.

Table 2. Means ± SD of micronucleated and nuclear lesions (‰) of toads exposed to nitrate and atrazine in relevant to control groups.

Parameter (‰)	Control	Nitrate	Atrazine	Combination
MN	2.120 ± 1.213	14.121 ± 4.322	18.829 ± 2.622	20.889 ± 3.622
BN	1.333 ± 0.528	7.351 ± 2.241	17.367 ± 2.520	19.667 ± 2.500
Lobe-shaped	0.700 ± 0.140	3.300 ± 1.236	11.556 ± 3.388	14.556 ± 3.358
Heart-shaped	0.355 ± 0.177	2.657 ± 0.144	7.222 ± 0.202	9.222 ± 1.202
Kidney-shaped	0.233 ± 0.077	1.111 ± 0.928	3.356 ± 1.036	5.536 ± 1.236
Irregular- shaped	000	1.252 ± 1.093	3.431 ± 1.554	5.222 ± 1.564
Notched nucleus	000	0.586 ± 0.327	2.434 ± 1.336	3.444 ± 1.236

number (2). The proportional malformed toads were presented as, control (0%), atrazine (5%) nitrate (13%), and combination (17%) of the total investigated numbers 100 for each group (Table1 and Fig.2). The types and rates of abnormalities obtained in the tested animals were calculated (Fig. 2). Amelia exhibited the highest (2%) at AN group. Misshapen limbs exhibited the highest (9%) at AN group. Misshapen tail exhibited the highest (6%) at both N &AN group. Total rate of abnormalities exhibited the highest (17%) at AN group and was the minimum at C group (0%).

The Induction of MN and NL was increased significantly in the RBCs of toads exposed to combined treatment (20.889±3.622‰ MN) compared to control (2.120±1.213‰ MN), the

Table 3. LSD multiple comparison testing the different significances of micronucleated RBCs and nuclear lesions in blood of toads exposed to nitrate and atrazine in relevant to control groups.

Parameters	Control & Nitrate	Control & Combined	Control & Atrazine	Nitrate & Combined	Nitrate & Atrazine	Atrazine & Combined
MN	12.001**	18.769**	16.709**	6.768**	4.708**	2.060*
BN	6.018**	18.334**	16.034**	12.309**	10.016**	2.300*
Lobe-shaped	2.600**	13.856**	10.856**	11.256**	8.256**	3.000**
Heart-shaped	2.333**	8.867**	6.887**	6.565**	4.565**	2.000*
Kidney-shaped	1.219*	3.203**	3.123**	4.425**	2.245**	2.174*
Irregular-shaped	1.252*	5.222**	3.431**	5.222**	2.178**	1.791*
Notched nucleus	0.586NS	3.444**	2.434**	3.444**	1.848**	1.010*

*: The mean difference is significant at the 0.05 levels

**: The mean difference is significant at the 0.01 levels

NS: The mean difference is not significant

increase was less at atrazine treatment (18.829±2.622‰ MN). The lower mean of MN and NL was in nitrate alone (14.121±4.322‰ MN). To compare the different means of the weight, LSD was done (Table 3). Between control and atrazine, MN, BN, and all shapes gave highly significant differences ($p \leq 0.01$). Between control and nitrate, MN, BN, and all shapes gave highly significant differences ($p \leq 0.01$). Between control and combination treatment, MN, BN, Lobed-shaped, heart-shaped, kidney shape and irregular shapes gave significant differences ($0.05 \geq p \leq 0.01$) except for notched shape which gave non-significant difference ($p < 0.05$). Between atrazine and nitrate, MN, BN, and all shapes gave highly significant differences ($p \leq 0.01$).

The movement and feeding activity in this study was lower than control which were observed throughout the experiment period at all the three treatments with the least activity at the atrazine-nitrate treatment, also changing their behavior by locating mostly at lower position in water column and unbalanced movement were common. Other research found a variety of anomalous behaviors in response to various treatments; for example, larvae of the African clawed frog *Xenopus laevis* showed a substantial decrease in activity when exposed to 200 µg/l atrazine (Heckmann, 2009), feeding time, lateral and vertical swimming, wiggling motions, restlessness, and paralysis were all reduced (Krishnamurthy, 2006), reduced feeding activity , swimming was less vigorous, and there was disorientation and paralysis (Marco et al., 1999), change activity level and location in the water column (Egea-Serrano et al., 2011) , reduced activity and physical abnormalities on exposure to acute and chronic doses of nitrate (Hecnar, 1995). Larval defensive behavior is affected by weakened movement, reduced activity, reduced eating behavior, and growth retardation, making them more vulnerable to predation. Toxicants can decrease sensory capacities, making it harder for tadpoles to notice predators or other hazards. Toxicants can also impair locomotor abilities, making it difficult for tadpoles to escape from enemies. Such chemicals were reported to impact larval protective behavior, induce hyperactivity, makes it difficult for them to recognize predator chemical cues. (Ortiz-Santaliestra et al., 2010; Ehrsam et al., 2016).

Decreases in faunal population, reproductive failure, survival success, and an increase in the prevalence of malformations could all be signs of serious environmental degradation (Mahdy et al., 2020; Abdel-Wahab et al., 2022; Farrag et al., 2022). The emergence of extra limbs in frogs, toads, and salamanders has piqued biologists' interest for years (Ouellet, 2000). Some anomalies have been documented in this study, including Amelia, malformed limbs, and deformed tails. Bionda et al. (2012) recoded comparable abnormalities in the anuran *Rhinella arenarum* obtained from agricultural lands around an Argentina lake, such as ectromelia, ectrodactyly, and

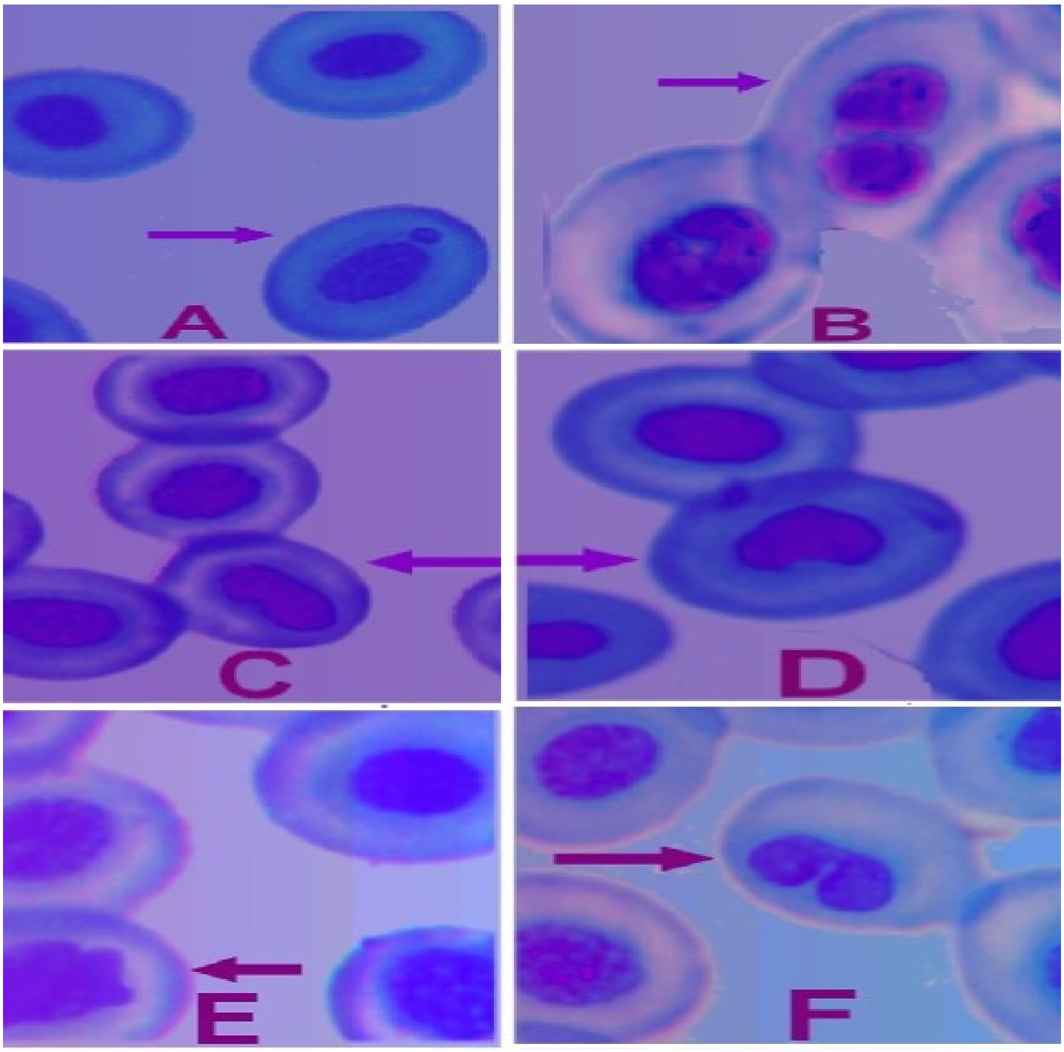

Fig. 6. Photomicrographs of erythrocytes of *Sclerophrys regularis* exposed to a treatment showing nuclear abnormalities: micronucleus (A), binoculus (B), kidney shaped nucleus (C), heart shaped nucleus (D), irregular shaped nucleus (E) and notched nucleus (F).

hemimelia, and linked these abnormalities to urban effect and water deterioration. Moreover, phocomelia (absence of the proximal portion of a limb with the foot attached very close to the body), absence of horny covering in eyes, missing tympanum, hemimelia, ectrodactyly, brachydactyly (short toe), forelimb remaining under skin, polydactyly (extra digit), polymelia (complete extralimb), syndactyly (fusion of digits), alterations in the back skin and ulcerated skin combined represented fewer than 28.2% of all abnormal anurans. Most cases of abnormalities that reported by Paola et al. (2011) particularly ectromelia, were detected in agricultural sites. Malformations such as edemas and spinal curvatures were detected in >90 % of individuals exposed to ammonium nitrate (Ortiz-Santaliestra & Marco, 2015). These abnormalities may be due to the ability of chemicals to be highly toxic to induce gene deformation during their so early larval stages (Hayes et al., 2010).

The occurrence of MN and NL is thought to be a biomarker of genotoxic impact at the subcellular level, as well as an early reaction to chromosomal damage (Garaj-Vrhovac et al., 2008; Brooks et al. 2015). Even though the cause of nuclear abnormalities is unknown, they are assumed to be markers of genotoxic damage (Güner et al., 2011). The torsional strain created by the movement of the chromatids towards opposing poles during anaphase was thought to be the cause of chromosomal breakage (Smith et al., 1998). Considering the degrees of impact of the investigated animals in relevant to control processed animals, the statistical means and LDS test have demonstrated the presence of significant differences in the induction of erythrocytic abnormalities between all treatments and control group and between all three treatments and each other. The induction was higher at combined treatment (MN=20.889±3.622‰) and lower at nitrate treatment (MN=14.121±4.322‰) between all three treatments. In addition, all treatments gave highly significant increase in the means of all micronucleated (MN), binucleated (BN) and nuclear lesions (NL) of *Sclerophrys regularis* erythrocytes compared to control. These alterations in gene level may be the cause of resulting fluctuation asymmetry and morphological abnormalities in addition to the growth alterations and the developmental instability discussed previously. Also, the highest damage in combined treatment was correlated to the increased rate of abnormal growth, morphological abnormalities, abnormal behavior and increased mortality. In other words, a positive correlation was demonstrated between the most investigated abnormalities and the induction of micronucleated RBCs and nuclear abnormalities in *Sclerophrys regularis* indicating the degree of nuclear damage measured by MN test to be key evidence measuring the degree of pollution. These findings agree with many field and laboratory studies. Ahmad and Saleh (2010) attributed the increased number of micronucleus in erythrocytes *of Rana ridibunda* to agricultural pollution. Said (2013) investigated increase in MN with different degrees in three sites of different levels of pollution along the river Nile in Egypt. Huang et al. (2007) scored higher micronucleated RBCs of toad *Bufo raddei* in polluted areas compared to those collected from relatively clean areas. Similar results were obtained when Feng et al. (2004) tested other pesticides on amphibians, all these findings together with our results suggest the appearance of micronuclei to be very sensitive to less stress in the environment. Giant toads (*Rhinella marina*) from industrial zones exhibited greater micronuclei diversity and more nuclear lesions in their erythrocytes (Cruz-Santiago et al., 2022).

CONCLUSION

It is possible that atrazine and nitrate cause amphibian deformities and have genotoxic potential. Throughout the testing period, the atrazine-nitrate treatment had the least movement and eating activity, with the least activity at the atrazine-nitrate treatment. Furthermore, uneven movement was prevalent. This study has also documented several malformations, such as Amelia, misshapen limbs, and distorted tails. To detect nuclear abnormalities in erythrocytes, the micronucleus test is used to assess toxicity. Individuals exposed to atrazine or nitrate, or both, exhibited higher degree ($p < 0.01$) of nuclear lesions. As a result, we advise against applying pesticides and soil fertilizers more than the World Health Organization's recommendations due to their detrimental effects on living organisms and human health. All these successive findings could be an important contributor to reducing amphibian populations within their habitats. By that way, the altered behavior is a reflecting mirror to the toxic stress that body organs can undergo on exposure to both chemicals.

GRANT SUPPORT DETAILS

The present research did not receive any financial support.

CONFLICT OF INTEREST

The authors declare that there is not any conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/ or falsification, double publication and/or submission, and redundancy has been completely observed by the authors.

LIFE SCIENCE REPORTING

No life science threat was practiced in this research

REFERENCES

Abdel-Wahab, M., Yassien, M. H., Thabet, A, A., Said, R.E.M., Mahdy, A., Amer. O.S.O. and Saber, S. A. (2022). Seasonal variations of some heavy metal concentrations in seawater, sediment, and the surf clam, Mactra olorina (Philippi, 1846) in the Great Bitter Lake, Suez Canal, Egypt. *Egyptian Journal of Aquatic Biology and Fisheries* 26(1), 83-98.

Ahmad, K. and Saleh, K. (2010). Clastogenic studies on Tandaha Dam water in Asser. 16, 33-42.

Akhtar, N., Fiaz Khan, M., Tabassum, S. and Zahran, E. (2021) Adverse effects of atrazine on blood parameters, biochemical profile and genotoxicity of snow trout (*Schizothorax plagiostomus*). *Saudi journal of biological sciences* 28(3), 1999-2003.

Allran, J. W. and Karasov, W. H. (2000). Effects of atrazine and nitrate on northern leopard frog (*Rana pipiens*) larvae exposed in the laboratory from posthatch through metamorphosis. *Environmental Toxicology and Chemistry* 19, 2850-2855.

AmphibiaWeb (2020). Worldwide Amphibian Declines: What is the scope of the problem, what are the causes, and what can be done?

Baker, N.J., Bancroft, B.A. and Garcia, T.S. (2013). A meta-analysis of the effects of pesticides and fertilizers on survival and growth of amphibians. Science of The Total Environment 449, 150-156.

Becker, C.G., Fonseca, C.R., Haddad, C.F.B., Batista, R.F. and Prado, P.I. (2007). Habitat split and the global decline of amphibians. *Science* 318(5857), 1775-1777.

Bionda, C., Salas, N., Caraffa, E., Baraquet, M. and Martino, A. (2012). On abnormalities recorded in an urban population of *Rhinella arenar*um from central Argentina.

Brühl, C.A., Schmidt, T., Pieper, S. and Alscher, A. (2013). Terrestrial pesticide exposure of amphibians: An underestimated cause of global decline? Scientific Reports 3(1), 1135

Brooks, S.J., Harman, C., Hultman, M.T. and Berge, J.A. (2015). Integrated biomarker assessment of the effects of tailing discharges from an iron ore mine using blue mussels (*Mytilus spp*.). *Science of The Total Environment* 524-525, 104-114.

Cardwell, R. D., Purdy, R. and Bahner, R. C. (2011). Aquatic toxicology and hazard assessment : Seventh symposium.

Chang, J., Liang, C., Wang, Y., Yong, L., Mao, W., Yang, H., Jia, X., Liu, Z. and Song, Y. (2021). Toxic effects of atrazine on immune function in BALB/c mice. *Environmental Science and Pollution Research* 28, 1-17.

Cruz-Santiago, O., Castillo, C.G., Espinosa-Reyes, G., Pérez-Maldonado, I.N., González-Mille, D.J., Cuevas-Díaz, M.d.C. and Ilizaliturri-Hernández, C.A. (2022). Giant Toads (*Rhinella marina*) From the Industrial Zones of Low Basin of the Coatzacoalcos River (Veracruz, MX) Presents Genotoxicity in Erythrocytes. *Bulletin of Environmental Contamination and Toxicology* 108(1), 64-70.

Davidson, C. (2004). Declining downwind: amphibian population declines in California and historical pesticide use. Ecological Applications 14(6), 1892-1902.

Davidson, C. and Knapp, R.A. (2007). Multiple stressors and amphibian declines: dual impacts of pesticides and fish on yellow-legged frogs. Ecological Applications 17(2), 587-597.

Earl, J. and Whiteman, H. (2009). Effects of pulsed nitrate exposure on amphibian development. Environmental toxicology and chemistry / SETAC 28, 1331-1337.

Edwards, T.M., McCoy, K.A., Barbeau, T., McCoy, M.W., Thro, J.M. and Guillette, L.J. (2006). Environmental context determines nitrate toxicity in Southern toad (*Bufo terrestris*) tadpoles. Aquatic Toxicology 78(1), 50-58.

Egea-Serrano, A., Tejedo, M. and Torralva, M. (2011). Behavioral responses of the Iberian waterfrog, *Pelophylax perezi* (Seoane, 1885), to three nitrogenous compounds in laboratory conditions. *Ecotoxicology Ecotoxicology* 20, 1246-1257.

Ehrsam, M., Knutie, S.A. and Rohr, J.R. (2016). The herbicide atrazine induces hyperactivity and compromises tadpole detection of predator chemical cues. *Environmental Toxicology and Chemistry* 35(9), 2239-2244.

Ezemonye, L. I. N. and Tongo, I. (2009). Lethal and sublethal effects of atrazine to amphibian larvae.

Jordan Journal of Biological Sciences : an International Peer-reviewed Scientific Journal. 2, 29-36.

Farrag, M.M., Said, R.E., Elmileegy, I.M., Abou Khalil, N.S., El-Sawy, M.F. and Osman, A.G. (2022). Effects of penconazole and copper nanoparticle fungicides on redbelly tilapia, *Coptodon zillii* (Gervais, 1848): Reproductive outcomes. *International Journal of Aquatic Biology* 9(6), 370-382.

Feng, S., Kong, Z., Wang, X., Zhao, L. and Peng, P. (2004). Acute toxicity and genotoxicity of two novel pesticides on amphibian, *Rana N. Hallowell. Chemosphere* 56, 457-63.

Garaj-Vrhovac, V., Gajski, G. and Ravli , S. (2008). Efficacy of HUMN criteria for scoring the micronucleus assay in human lymphocytes exposed to a low concentration of p,p'-DDT. *Brazilian Journal of Medical and Biological Research* 41, 473-476.

Gosner, K. L. (1960). A Simplified table for staging anuran embryos andlarvae with notes on Identification. *Herpetologica* 16, 183-190.

Güner, U., Dilek, F. and Muranm1, G. (2011). Micronucleus Test, Nuclear abnormalities and accumulation of Cu and Cd on *Gambusia affinis* (Baird & Girard, 1853). *Turkish Journal of Fisheries and Aquatic Sciences* 11, 615-622.

Gürcü, B., Koca, S., Başimoğlu Koca, Y., Çöllü, F. and Tuğlu, M.I. (2022). Genotoxic Effect, Oxidative Stress and Cell Death due to Metronidazole Application in Gills and Liver Tissues of Rainbow Trout (*Oncorhynchus mykiss*). *Pollution* 8(3), 804-819.

Hamer, A.J., Makings, J.A., Lane, S.J. and Mahony, M.J. (2004). Amphibian decline and fertilizers used on agricultural land in south-eastern Australia. Agriculture, Ecosystems & Environment 102(3), 299-305.

Hayes, T. B., Khoury, V., Narayan, A., Nazir, M., Park, A., Brown, T., Adame, L., Chan, E., Buchholz, D., Stueve, T. and Gallipeau, S. (2010). Atrazine induces complete feminization and chemical castration in male African clawed frogs (*Xenopus laevis*). *Proceedings of the National Academy of Sciences* 107, 4612-4617.

Heckmann, J. G. (2009). Effects of atrazine, chlorpyrifos, and their interactions on anti-predator behavior and activity in African clawed frogs, *Xenopus laevis*.

Hecnar, S. J. (1995). Acute and chronic toxicity of ammonium nitrate fertilizer to amphibians from southern ontario. *Environmental Toxicology and Chemistry* 14, 2131-2137.

Huang, D., Zhang, Y., Wang, Y., Xie, Z. and Ji, W. (2007). Assessment of the genotoxicity in toad Bufo raddei exposed to petrochemical contaminants in Lanzhou Region, China. *Mutation Research/ Genetic Toxicology and Environmental Mutagenesis* 629, 81-88.

Houlahan, J., Findlay, C.S., Schmidt, B.R., Meyer, A.H. and Kuzmin, S.L. (2000). Quantitative evidence for global amphibian population declines. Nature 404(6779), 752-755.

Ilha, P. and Schiesari, L. (2014). Lethal and Sublethal Effects of Inorganic Nitrogen on Gladiator Frog Tadpoles (*Hypsiboas faber*, Hylidae). Copeia 2014(2), 221-230.

Karlsson, A.S., Lesch, M., Weihermüller, L., Thiele, B., Disko, U., Hofmann, D., Vereecken, H. and Spielvogel, S. (2020). Pesticide contamination of the upper Elbe River and an adjacent floodplain area. *Journal of Soils and Sediments* 20(4), 2067-2081.

Krishnamurthy, S. (2006). Effects of nitrate on feeding and resting of tadpoles of *Nyctibatrachus major* (anura: Ranidae).

Mahdy, A., F. A. Ahmed, A., Y. M. Idris, M., M. A. Mohamed, I., A. A. Samie, M., A. M. Mayof, A., A. B. Emgower, S. and Said, R. E.M. (2020). The effects of nesting ground temperatures on incubation and hatchability of loggerhead turtle *Caretta caretta* inhabiting the Mediterranean Sea Coast, Libya. Egyptian *Journal of Aquatic Biology and Fisheries* 24(5), 111-124.

Marco, A., Quilchano, C. and Blaustein, A. R. (1999). Sensitivity to nitrate and nitrite in pond-breeding amphibians from the Pacific Northwest, USA. *Environmental Toxicology and Chemistry* 18, 2836-2839.

Meteyer, C. U. (2000). "Field guide to malformations of frogs and toads: with radiographic interpretations," Rep. No. 2000-0005, Reston, VA.

Nkontcheu, D.B.K., Tchamadeu, N.N., Ngealekeleoh, F. and Nchase, S. (2017). Ecotoxicological effects of imidacloprid and lambda-cyhalothrin (insecticide) on tadpoles of the African common toad, *Amietophrynus regularis* (Reuss, 1833)(Amphibia: Bufonidae). Emerging Science Journal 1(2), 49-53.

Ortiz-Santaliestra, M. E., Fernández-Benéitez, M. J., Marco, A. and Lizana, M. (2010). Influence of ammonium nitrate on larval anti-predatory responses of two amphibian species. *Aquatic Toxicology* 99, 198-204.

Ortiz-Santaliestra, M. E. and Marco, A. (2015). Influence of dissolved oxygen conditions on toxicity of ammonium nitrate to larval natterjack toads. *Arch Environ Contam Toxicol* 69, 95-103.

Osman, A. G. M., Abd El Reheem, A.-E.-B. M., Moustafa, M. A., Mahmoud, U. M., Abuel-Fadl, K. Y. and Kloas, W. (2011). In situ evaluation of the genotoxic potential of the river Nile: I. Micronucleus and nuclear lesion tests of erythrocytes of *Oreochromis niloticus niloticus* (Linnaeus, 1758) and *Clarias gariepinus* (Burchell, 1822). *Toxicological & Environmental Chemistry* 93, 1002-1017.

Ouellet, M. (2000). Amphibian deformities: current state of knowledge. *Ecotoxicology of amphibians and reptiles*, 617-661.

Paola, M., Peltzer, Rafael, C., LAJMANOVICH, Laura, Sánchez, Andrés, ATTADEMO, Celina, JUNGES, Clarisa, !=., BIONDA, Adolfo, Martino, and Bassó, A. (2011). Morphological abnormalities in amphibian populations from the mid-eastern region of Argentina.

Rajaguru, P., Kalpana, R., Hema, A., Suba, S., Baskarasethupathi, B., Kumar, P. and Kalaiselvi, K. (2001). Genotoxicity of some sulfur dyes on tadpoles (*Rana hexadactyla*) measured using the comet assay. Environmental and molecular mutagenesis 38, 316-322.

Richard , L. (2003). "Investigations into the causes of amphibian malformations in the lake Champlain basin of New England ".

Rouse, J. D., Bishop, C. A. and Struger, J. (1999). Nitrogen pollution: an assessment of its threat to amphibian survival. *Environ Health Perspect* 107, 799-803.

Ryder, T. B., Parker, P. G., Blake, J. G. and Loiselle, B. A. (2009). It takes two to tango: reproductive skew and social correlates of male mating success in a lek-breeding bird. *Proceedings. Biological sciences* 276, 2377-2384.

Said, R. E. M. (2013). Using of some amphibians as bioindicator of aquatic pollution of Egyptian river Nile. PhD thesis, Al–Azhar university , Faculty of science, Assiut branch. Egypt, pp 284.

Smith, L. E., Parks, K. K., Hasegawa, L. S., Eastmond, D. A. and Grosovsky, A. J. (1998). Targeted breakage of paracentromeric heterochromatin induces chromosomal instability. *Mutagenesis* 13, 435-444.

Solomon, K. R., Baker, D. B., Richards, R. P., Dixon, K. R., Klaine, S. J., La Point, T. W., Kendall, R. J., Weisskopf, C. P., Giddings, J. M., Giesy, J. P., Hall Jr., L. W. and Williams, W. M. (1996). Ecological risk assessment of atrazine in North American surface waters. *Environmental Toxicology and Chemistry* 15, 31-76.

Stuart, S. N., Chanson, J. S., Cox, N. A., Young, B. E., Rodrigues, A. S., Fischman, D. L. and Waller, R. W. (2004). Status and trends of amphibian declines and extinctions worldwide. *Science* 306, 1783-6.

Sparling, D.W. and Fellers, G.M. (2009). Toxicity of two insecticides to California, USA, anurans and its relevance to declining amphibian populations. Environmental Toxicology and Chemistry: An International Journal 28(8), 1696-1703.

Sparling, D.W., Fellers, G.M. and McConnell, L.L. (2001). Pesticides and amphibian population declines in California, USA. Environmental Toxicology and Chemistry 20(7), 1591-1595.