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Erythrocyte Recovery in *Oreochromis niloticus* Fish Exposed to Urban Effluents

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Abstract

Urban activities pollute aquatic ecosystems, and the integrity of organisms such as fish. The use of cytological techniques, such as the analysis of blood cellular integrity using the Micronucleus test, can help detect mutagenic damage as a result to urban effluents exposure. In this context, this study aimed to evaluate the frequency of micronucleus and other nuclear abnormalities in *Oreochromis niloticus* fish environmentally exposed to urban effluents in relation to their erythrocyte recovery capacity when exposed to clean water (30 and 45 days). The results indicated high copper, dissolved iron, nickel, and thermotolerant coliform levels in the urban stream. There was no difference in the frequency of micronuclei. In contrast, cells with nuclear nuclei, binucleates, kidney-shaped nuclei, notched nuclei, lobed nuclei, and segmented nuclei decreased according to the time the fish were exposed to clean water. When exposed to clean water, we conclude that urban fish recover from genotoxic and cytotoxic damage.

Keywords Ecotoxicology · Micronucleus test · Pollution · Urban stream

Anthropogenic activities in urban areas enhance the discharge of industrial and domestic effluents into water sources (Silva et al. 2020; D'Agostini and Maestra 2021; Sahani et al. 2022). These effluents, when discharged into water bodies, generate complex mixtures of biological and chemical agents, which, when interacting with organisms, can cause effects that are difficult to assess through a simple chemical water analysis (Vasanthi et al. 2013). The reactions and substances formed from these combinations can cause effects on aquatic biota and fish assemblages, in addition to increasing risks to human health (Sabino et al. 2021; Rani et al. 2022). Thus, the use of bioindicator organisms such as fish can generate a greater spectrum of health in aquatic ecosystems.

Fish, due to their strong dependence on water throughout their life cycle, are considered model organisms for assessing the aquatic environment quality (Silva et al. 2020). They have intimate interaction with different trophic levels and sensitivity to low concentrations of toxic substances (Rocha et al. 2009; Jesus et al. 2016). In this sense, fish become strong candidates for sentinels of the water resources quality. In this context, the micronucleus assay conducted in the peripheral blood of fish has been used in the genotoxicity assessment (De Silva and Pathiratne 2023). The technique is considered one of the least invasive and widely used in monitoring wildlife. It is based on the analysis of the blood cells' integrity that can inform about mutations and erythrocyte alterations (Arslan et al. 2015; Canedo et al. 2021). Micronuclei are small structures similar to main nuclei (Krupina et al. 2021) that arise from chromosomal fragments or entire chromosomes damaged during the process of cell division. Therefore, the use of this biomarker may indicate

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early responses on DNA damage, cytotoxicity and mitotic disturbances in species under environmental pressure.

Studies with fish exposed to pollutants indicate that the recovery of erythrocytes depends on the concentrations and duration of the experiment (Khan et al. 2018; Islam et al. 2019; Khatun et al. 2021). Considering this scenario, this study evaluated whether there is a difference in the frequency of micronuclei and other erythrocyte nuclear abnormalities in fish *Oreochromis niloticus* (Perciformes, Cichlidae) captured in polluted urban streams (domestic and industrial effluents) and the reanalysis of the animals after 30 and 45 days to see their ability to recover in clean water. The hypothesis of the study is that the frequency of DNA damage is higher in the animals in situ when compared to 30 and 40 days of recovery in clean water.

Materials and Methods

Rio Verde is the fourth most populous municipality in the Goiás State – Brazil, and the Sapo stream together with the Barrinha stream are the main water bodies that cross the city receiving domestic and industrial effluents. Consequently, urban, industrial, and agricultural advances bring risks to the health of these water bodies and their aquatic biodiversity. According to Parreira et al. (2017), due to excessive contamination and degradation of the Sapo stream, it cannot practice primary contact sports and the risk of using irrigation for horticulture and fruit growing.

To assess the contamination level present in the Sapo stream (17° 47′ 43.89″ S; 50° 56′ 17.94″ W) and the impact on the fish assemblage, fish of the species *O. niloticus*, known as Nile Tilapia were collected in October of 2018. These animals belonging to the cichlid family are bioindicators of poor environmental quality, given that they are opportunistic species that tolerate wide variations in habitat and degraded environments (Casatti et al. 2009; Cunico et al. 2011). They have an omnivorous feeding habit, which feed from insect larvae and detritus and are used as models in studies due to their easy handling in captivity, in addition to having a wide distribution in the sampled area. Specimens fishing (n = 54, 99.8 g ± 17.3 cm) was carried out using a net (30 mm mesh between nodes). Subsequently, the individuals were transported to the Laboratory of Ecotoxicology and Animal Systematics, Goiano Federal Institute in Rio Verde for analysis and inclusion in the scientific collection.

A total of 54 animals (sex not analyzed) were collected for this study. Of these, 18 underwent initial treatment (T0), which consisted of immediate extraction of blood samples (40 µL) as soon as they were removed from the stream (Fig. 1). The remaining 36 animals were placed in two different containers each one with a storage capacity of 500 L of water. The water used in these boxes came from an artesian well and underwent dechlorination. The two recovery groups, T1 and T2, remained for 30 and 45 days in clean water for further evaluation of the blood analysis. After the experimentation period end, blood samples were collected for each time and applied to the blood smear technique.

During the experimental period, the fish received commercial feed ad libitum and the leftovers were removed and the water renewed every 24 h. Water renewal occurred daily, with dechlorinated water after the siphoning procedure. The containers were covered with shade, allowing natural photoperiod. The physicochemical parameters

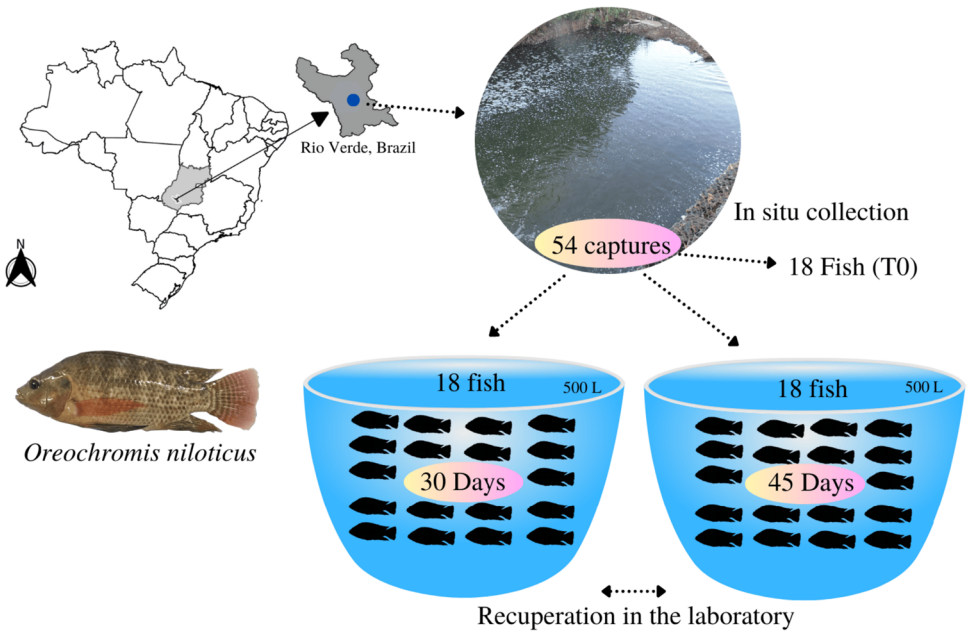

Fig. 1 Analysis of the frequency of micronuclei and other nuclear abnormalities in fish collected in an urban river after 30 and 40 days of recovery in clean water

variables of the water were measured once a day during all the experiment period using a Bante900P Portable Multiparameter Water Quality Meter (Bante instruments). At the end of the experiment, the fish were deposited at the IFGoiano, Rio Verde campus.

Blood samples were obtained by puncturing the caudal vein (Ishikawa et al. 2010) with the aid of a heparinized syringe (1 mL SR Insulin). Two slides were made per animal with approximately 40 µL of blood and applied to the blood smear technique. Subsequently, the slides were fixed in methanol for 20 min, stained with 5% Giemsa solution, washed with distilled water, and dried at room temperature. Cytological analysis was performed using an optical microscope (Laborana LAB-1001TB) with a coupled camera (Laborana 3.0Mp) at 1000× magnification. A single observer analyzed 2000 cells per animal to identify the micronucleus (MN). Data is presented in frequency (Obiakor et al. 2021).

The criteria used to identify the micronucleus (MN) were established as diameter less than 1/3 of the main nucleus (1); non-refringent, same color intensity as the main core (2); no connection to the main core (3); no overlap with the main core (4); and no more than four core-associated MNs (5) (Arcaute et al. 2016). In addition to MN, other erythrocyte nuclear alterations (ENAs) were also considered, such as cells with nuclear bud, binucleated cells, cells with beveled nuclei, cells with reniform nuclei, cells with lobed nuclei and cells with a segmented nucleus, according to Sula et al. (2019) and Singh et al. (2019).

All legal procedures were approved by the Ethics Committee for the Use of Animals of the Federal Institute of Goiano (CEUA/IFGoiano) and by the Chico Mendes Institute for Biodiversity Conservation (ICMBio/SISBIO), having the necessary licenses authorized by both institutions (CEUA, n. 6,548,100,418 and SISBIO, n.62687-1).

The physicochemical analyzes of water were conducted with a multiparameter for water analysis (Bante 900P) in situ and in the laboratory during the experiment to verify pH, conductivity, total dissolved solids (TDS), salinity, resistivity, dissolved oxygen (DO) and temperature. Then, water samples were collected at the specimen capture site, stored in proper bottles (−4°C) and sent to a specific laboratory for analysis of total coliforms (multiple-tube fermentation technique), metals and agrochemicals [inductively coupled plasma mass spectrometry by (ICP-MS)] which were based on CONAMA resolution nº 357 of March 2005 and DECREE nº 1745, of December 6, 1979, of Law 8544, of October 17, 1978.

MN and ENAs values are presented as mean ± standard deviation. The Kruskal–Wallis test followed by the Dunn test was performed as non-parametric data. Significant differences were considered when $p \leq 0.05$.

Results and Discussion

Water physicochemical analyzes (Table 1; Supplementary Material 1) were carried out on the samples in situ (Sapo stream) and ex situ (experimental water, animals kept in the laboratory). In the in situ sample, the anionic surfactants present values above the limit of quantification, but there is no minimum recommendation in the legislation. The metals, total copper (0.06 µg/L), dissolved iron (1.85 µg/L) and total nickel (0.05 µg/L), have quantification values above those allowed by Brazilian legislation (CONAMA 357/2005). The microbiological analysis revealed that the values of Thermotolerant Coliforms ($>1.6 \times 10^4$) drastically exceeded the quantification limits proposed by CONAMA, which is 1NMP/100 mL. As for the pesticides quantified in the water analysis, all were below the limits of quantification (Supplementary Material 1).

When analyzing the hydrological variables, in an in situ environment (stream) versus ex situ (recovery aquarium), attention is drawn to the ever lower values of dissolved solids, electrical conductivity and dissolved oxygen in the in situ environment. Considering that this environment is in an urbanized area, and that receives domestic and industrial effluents, these factors can increase the flow peaks of the stream due to surface runoff and illegal sewage release. Allied to this, it is worth noting that the specimens collect took place in October, the beginning of the rainy season (average of 173 mm/Clima-Data.org). However, highlighting that the values of dissolved solids are related to the presence of carbonates and bicarbonates in the environment, compounds that can modify the solubility of some metals (Da Silva et al. 2018). The values found in this work for the in situ environment can directly influence the increase in the abundance of species tolerant to these anthropized environments, given the receipt of urban and industrial effluents containing toxic compounds (Felipe and Súarez 2010; Sibanda et al. 2015).

Table 1 Mean values of the physical–chemical variables of the water collected in the Sapo stream (in situ) in the municipality of Rio Verde and of the water from the experiments (ex situ)

Hydrological variables	In situ environment	Ex situ environment
Temperature (°C)	25.1	24.2
pH	6.68	7.66
Dissolved oxygen (%)	3.76	13.97
Conductivity (µS/cm)	152.5	298
TDS (ppm)	75.8	145.6
Salinity (psu)	0.07	0.14
Resistivity (kΩ)	6.5	3.42

Furthermore, it should be noted that the lower rate of dissolved oxygen in this environment is related to factors such as salinity and temperature. The latter, combined with the pH evaluation, when altered can influence the toxicity of several pollutants (Hoffman et al. 2010; Pollo et al. 2015). From the evaluation of the chemical variables, it was observed that the metals concentrations in the water, such as Total copper, Total nickel, and Dissolved iron, exceeded the values adopted by the National Council for the Environment (CONAMA, 2005), leading to concern when any use for human supply occurs. One of the possible explanations for metals presence in urban streams is the discharge of sewage (Gagnon et al. 2006). These high levels of copper and nickel are indicative of toxicity for organisms that depend of the aquatic environment.

Micronucleus and other nuclear abnormalities have been reported in the erythrocytes from *O. niloticus* fish (Fig. 2; Table 2; Supplementary Material 2). There was no difference in the frequency of micronucleus in those animals obtained in situ when compared to animals that remained in the recovery period in the laboratory (Table 2). In contrast, in the analysis of other nuclear erythrocyte abnormalities, these were shown to be significantly greater in the in situ evaluation, in relation to the recovery period in clean water free of contaminants. In general, the change in water conditions in which the fish were subjected led to an average variation of erythrocyte damage from two to eight times more in animals in situ compared to animals that were between 30 and 45 days of recovery in the laboratory. Lobed nucleus, binucleated cells, kidney nuclei, and notched nuclei were the erythrocyte abnormalities with the highest mean frequencies

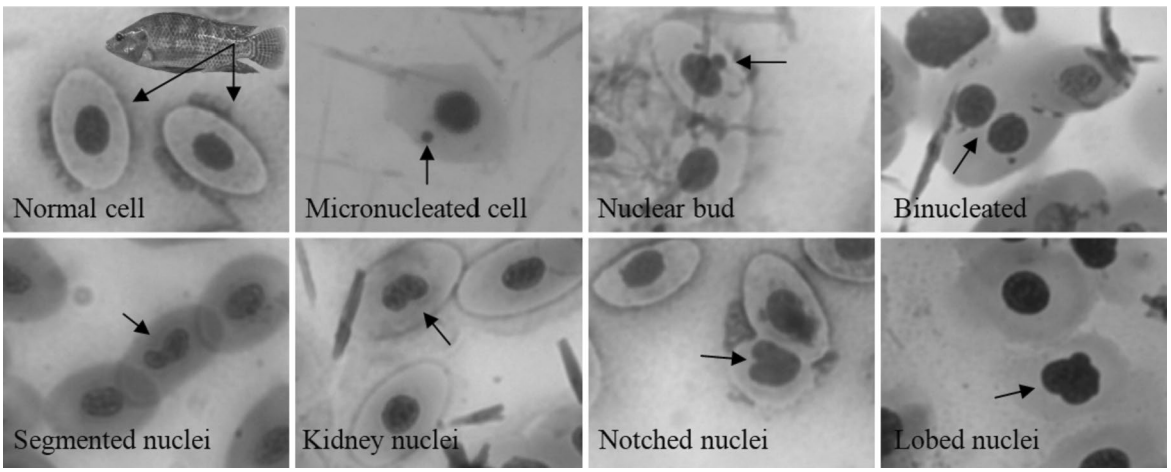

Fig. 2 Photomicrograph of micronucleated erythrocytes and other nuclear abnormalities in Nile Tilapia (*O. niloticus*). Magnification 1000×

Table 2 Frequency of micronuclei and other erythrocytic nuclear anomalies in erythrocytes of *O. niloticus* collected in the Sapo stream, Rio Verde municipality, Brazil

MN ENAs	Treatment						Statistics *p*
	T0		T1 30 days		T2 45 days		
	Mean ± SD	Median (range)	Mean ± SD	Median (range)	Mean ± SD	Median (range)	
MN	0.00 ± 0.00^{a}	0.00 (0.00)	0.00 ± 0.00^{a}	0.00 (0.00)	0.00 ± 0.01^{a}	0.00 (0.00)	0.367
NB	0.15 ± 0.10^{b}	0.13 (0.30)	0.09 ± 0.08^{ab}	0.05 (0.30)	0.04 ± 0.05^{a}	0.03 (0.15)	0.001
KN	0.16 ± 0.13^{b}	0.10 (0.35)	0.07 ± 0.08^{ab}	0.03 (0.20)	0.06 ± 0.08^{a}	0.00 (0.25)	0.016
NT	0.13 ± 0.10^{b}	0.15 (0.35)	0.03 ± 0.04^{a}	0.00 (0.15)	0.05 ± 0.06^{a}	0.05 (0.20)	0.000
LN	0.37 ± 0.21^{b}	0.45 (0.60)	0.22 ± 0.32^{ab}	0.13 (1.30)	0.06 ± 0.06^{a}	0.05 (0.20)	0.000
SN	0.07 ± 0.07^{b}	0.10 (0.20)	0.04 ± 0.05^{ab}	0.00 (0.15)	0.02 ± 0.04^{a}	0.00 (0.15)	0.039
BI	0.17 ± 0.17^{b}	0.15 (0.45)	0.03 ± 0.04^{a}	0.00 (0.10)	0.02 ± 0.03^{a}	0.00 (0.10)	0.000
Total ENAs	1.06 ± 0.57^{b}	0.90 (1.70)	0.46 ± 0.41^{a}	0.38 (1.60)	0.25 ± 0.24^{a}	0.20 (0.85)	0.000

A statistical difference between treatments is represented by different letters, while similar letters do not indicate a difference. Kruskal–Wallis test (post hoc Dunn's)

MN micronucleus, *NB* nuclear bud, *KN* kidney nuclei, *NT* notched nuclei, *LN* lobed nuclei, *SN* segmented nuclei, *BI* binucleated

in situ (Fig. 3). In addition to the individual analyzes of the ENAs, when added together, they also indicated a higher frequency of damage in animals exposed in the anthropized environment (Fig. 3).

Copper is a metal that is related to the inhibition of osmoregulatory mechanisms in fish, while nickel induces the MN formation (Grosell and Wood 2002; Okunola et al. 2015), and although they did not detect in this study the MN frequency, a high frequency of ENAs was evidenced, which are considered precursors of micronucleated cells formation, justifying the high frequency of these other abnormalities found in animals. Nuclear bud cells for example are potential indicators of DNA damage. Thus, these data corroborate that the contamination of water bodies by toxic pollutants, such as metals, generates biological effects on the health of populations of aquatic organisms (Lima et al. 2018). Additionally, heavy metals can gradually accumulate in fish. Given this capacity and considering fish as a source of food resources for humans, the presence of these ENAs, as morphological biomarkers and indicative of genotoxicity in fish, becomes worrying and creates an alert for food safety.

Although this study did not detect pesticide values above the limit of quantification, their low concentration can be explained by their dilution and solubility in water, however, it should be noted that there is no safe presence level of these products in the aquatic environment, considering that there are species that can accumulate and concentrate these compounds, leading to a magnification process (Ahmed et al. 2019; Xu et al. 2021). In addition, for wild fauna, few studies have evaluated the sublethal effects of these compounds, even at low concentrations.

This study found that the polluted site was responsible for the nuclear erythrocyte alterations observed, given that metal levels were above the established limit. In addition to the metallic elements, fecal coliforms exceeded the sanitary limits, confirming the pollution of the site. It is expected that in urban environments the disposal of untreated residential effluents will cause damage to the environment and consequently to the local ichthyofauna, since these effluents allow the bacteria proliferation (Ibrahim and Al-Khayat 2017). The same result evaluating the fecal coliforms presence was found in work carried out by Badr and EL-Dib (1978), using Tilapia as bioindicators and evaluating the impact on cell division, considering that these cell changes can have negative impacts, being transferred to the next generation.

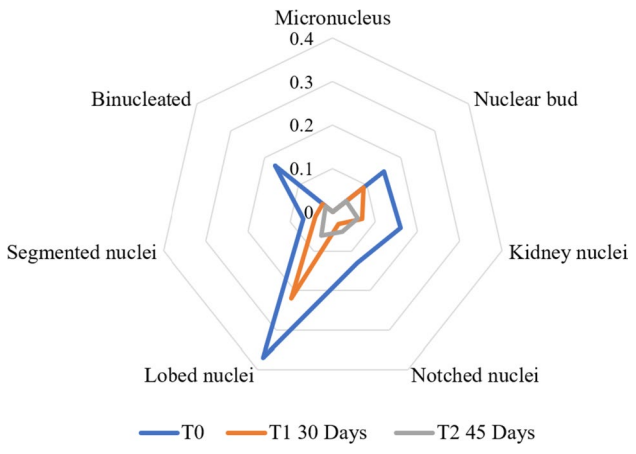

Fig. 3 Frequency response of micronucleus and other nuclear abnormalities in *O. niloticus* observed in urban river (T0) and 30 and 45 days later in recovery period in clean water tanks

Considering the characterization and genetic origin of the ENAs found in the present study, the cell with the shape of a lobed nucleus has a nucleus full of evaginations, while the cells with the shape of a nuclear bubble have a small slit entering the nucleus (Ghisi et al. 2014). For Çavas and Ergene-Gözükara (2005), these abnormalities formation may be related to failures in the segregation of entangled chromosomes, and to the amplification of genes through the break-fusion-bridge cycle during the elimination of the amplified DNA from the nucleus. The alterations that presented the notched nucleus present a conspicuous opening deepening to the center of the cell nucleus (Ghisi et al. 2014). This abnormality, according to Fernandes et al. (2007), can occur by the addition or loss of a chromosome and happens by the failure in the tubulin incorporation to form the spindle and cytokinesis under the aneugenic action of toxicants. This action can result in the binucleated cells formation. On the other hand, binucleated cells having two nuclei may indicate failures in the cytokinesis process (Ghisi et al. 2014). According to Çavas et al. (2005), this failure can result in genetic imbalance in cells, leading to carcinogenic effects. Cells with segmented nuclei seem to have the same origin as binucleated cells. Finally, the reniform nucleus is a precursor to the MNs formation or binucleation (Carrola et al. 2014; Harabawy and Mosleh 2014).

It appears that studies elucidating the contaminants impacts on fish assemblages based on the MN test have intensified in recent years. Del-Guercio et al. (2017) evaluated the impact of the domestic effluents (sewage) treatment on *O. niloticus*, revealing that its treatment was satisfactory in minimizing the MN formation. Batista et al. (2016) when evaluating the impact of anthropic activities (family farming, urban waste, among others) on the Corrente River, in Piauí, found that fish species had a mutagenic effect, alerting public agencies of the need to constantly monitor places with environmental degradation, mainly caused by discharges of urban sewage.

As observed in this study, only ENAs were significantly more frequent in fish initially collected in situ when compared to recovering animals kept in water free of contaminants. These findings indicate a significant decrease in genotoxic damage observed by the reduction in the

frequency of erythrocyte abnormalities in groups recovering from urban pollution. Thus, the potential recovery of the animals in 30 and 45 days in water free of contaminants is suggested. Some studies have shown that in fish exposed to a clastogen, the maximum induction of MN occurred between the first and fifth day after exposure (Al-Sabti and Metcalfe 1995). Grisolia and Cordeiro (2000) also observed an increase between the 2nd and 7th days and decreased on the 14th, remaining stable until the 30th day. The life expectancy of erythrocytes in fish is estimated at around 100 days but can be reduced as a consequence of exposure to contaminants (Guilherme et al. 2014). Although the erythrocyte cycle appears long, the decrease in genotoxic damage can be explained by the removal of damaged erythrocytes from circulation together with the production of new, undamaged cells (Guilherme et al. 2014). Thus, there appears to be resilience in animal health due to environmental conditions and the rearrangement of the animals' genetic repair system away from contaminants. Finally, although a significant frequency of MN was not observed, nuclear anomalies are a consequence of genotoxic agents in the urban stream.

Animals, when free from exposure to anthropized environments, can recover from genotoxic damage. Here, the micronucleus test was applied to the peripheral blood of *O. niloticus* obtained from an urban stream in the State of Goiás, Brazil. We did not find an increase in the frequency of micronuclei in animals collected in situ versus a recovery period of 30 and 45 days in clean water. Other nuclear abnormalities were significantly more frequent initially and decreased with recovery days. Considering elevated levels of copper, dissolved iron, nickel and thermotolerant coliforms may be the main environmental stressors for the increase in nuclear abnormalities. However, more research is needed on this subject, especially separating male and female animals, which was not considered here. This research can be used in the form of environmental education, demonstrating the effect of xenobiotics on aquatic organisms, and raising public health awareness, since animals, such as Nile Tilapia, are often collected by the population for consumption, in addition to direct contact with contaminated water.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00128-023-03833-2.

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Declarations

Conflict of interest We declare that there is no conflict of interest for this manuscript.

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