



## *In situ* assessment of genotoxicity in tadpoles (Amphibia: Anura) in impacted and protected areas of Chapada Diamantina, Brazil

Avaliação *in situ* da genotoxicidade em girinos (Amphibia: Anura) em áreas impactadas e protegidas na Chapada Diamantina, Brasil

M. B. Silva<sup>1\*</sup>; R. E. Fraga<sup>1</sup>; P. B. Nishiyama<sup>1</sup>; N. L. B. Costa<sup>1</sup>; I. S. S. Silva<sup>1</sup>; D. A. Brandão<sup>1</sup>; T. S. Queiroz<sup>1</sup>; M. A. Rocha<sup>1</sup>; F. A. Juncá<sup>2</sup>

<sup>1</sup>Laboratório de Zoologia, Universidade Federal da Bahia, Campus Anísio Teixeira, Instituto Multidisciplinar em Saúde, 45029-094, Vitória da Conquista-Bahia, Brasil

<sup>2</sup>Museu de Zoologia, Universidade Estadual de Feira de Santana, Departamento de Ciências Biológicas, Museu de Zoologia, 44036-900, Feira de Santana-Bahia, Brasil

\*biolmarcioborba@gmail.com

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We evaluated the genotoxic potential of surface water bodies in an area impacted by agrochemicals, in the Ibicoara-Mucugê agropole, Bahia and those within protected areas in Chapada Diamantina, Bahia, Brazil, using the micronucleus test as a biomarker in tadpole species native neotropicals. Our hypothesis is that the closer to agricultural farms, the more affected by mutagenic and genotoxic substances will be the environment and resident organisms. The micronucleus frequencies were determined in tadpoles of 11 species collected in 11 surface water bodies in coffee, corn, vegetable, and pumpkin plantations, as well as in adjacent sites within a protection area. Tadpoles of seven species found in agricultural sites exhibited higher frequencies of micronuclei compared to the six species from the protected locations. The species *Bokermannohyla oxente* and *Leptodactylus latrans* collected in agricultural areas exhibited the highest frequencies of micronucleated erythrocytes compared to the populations from the protected areas ( $p < 0.05$ ). Our results indicate that compounds present in surface water bodies in agricultural areas of the Ibicoara-Mucugê farm have genotoxic effects on anurans, showing the stress responses caused by agricultural activities associated with the use of complex mixtures of pesticides. Thus, anurans are excellent sentinel species in environmental monitoring, previously indicating the effect of xenobiotics on the environment.

Keywords: Anura, biomonitoring, micronuclei

Avaliamos o potencial genotóxico de corpos d'água superficiais em uma área impactada por agroquímicos, no agropólo Ibicoara-Mucugê, Bahia e aqueles dentro de áreas protegidas na Chapada Diamantina, Bahia, Brasil, por meio do teste de micronúcleos como biomarcador em espécies de girinos neotropicais nativos. Nossa hipótese é que quanto mais perto das fazendas agrícolas, mais afetados por substâncias mutagênicas e genotóxicas serão o meio ambiente e os organismos residentes. As frequências de micronúcleos foram determinadas em girinos de 11 espécies coletadas em 11 corpos d'água superficiais em plantações de café, milho, hortaliças e abóbora, bem como em sítios adjacentes dentro de uma área de proteção. Os girinos de sete espécies encontradas em locais agrícolas exibiram altas frequências de micronúcleos em comparação com os girinos de seis espécies coletadas em locais protegidos. Girinos de *Bokermannohyla oxente* e *Leptodactylus latrans* coletados em áreas agrícolas exibiram maiores frequências de eritrócitos micronucleados quando comparados as populações coletadas nas áreas protegidas ( $p < 0,05$ ). Nossos resultados indicam que compostos presentes em corpos d'água superficiais em áreas agrícolas do agropólo Ibicoara-Mucugê possuem efeitos genotóxicos para os anuros evidenciando o estresse causado pelas atividades agrícolas associadas ao uso de misturas complexas de agrotóxicos. Assim, anuros são excelentes espécies sentinelas em monitoramento ambiental, indicando previamente o efeito de xenobióticos no ambiente.

Palavras-chave: Anura, biomonitoramento, micronúcleos

### 1. INTRODUCTION

Agricultural activities are one of the principal causes of environmental degradation, either by habitat fragmentation or by the utilization of agricultural chemicals that contaminate the soil, water,

and local biota [1]. According to Schiesari and Grillitsch (2010) [2] and Moreira et al (2019) [3], Brazil is the world's largest consumer of pesticides, resulting in an enormous input of environmental contaminants to soil and surface and subterranean waters, generating chemical pollution that, by its magnitude and omnipresence, threatens all forms of biodiversity.

Many authors have reported significant decreases in amphibian populations in many parts of the world during the last thirty years [4, 5, 6]. Among the possible causes proposed to explain those declines, environmental pollution has emerged near the top of the list, especially among populations living near centers of agricultural production [6-10].

The morphophysiological, ecological, reproductive, and behavioral characteristics of amphibians have been found to be quite sensitive to environmental pollutants. Their skins are highly permeable and offer little resistance to the absorption of many substances, including contaminants that can increase their susceptibility to illnesses and predators, incapacitate their reproduction, or negatively impact biochemical functions, thus compromising their survival [2, 11].

Micronuclei are an important genotoxic biomarker, that can indicate irreversible genetic damage in amphibian populations. They are defined as fragments of chromosomes or whole chromosomes that have not been incorporated into the main nucleus in cells that actively divide from any tissue [6, 12]. The examination of micronuclei represents one of the best *in vivo* investigative cytogenetic approaches in the field of toxicology [13], as it involves quantifying micronucleus frequencies as a measure of integrated responses to accumulated stress caused by exposure to complex mixtures of environmental contaminants [14].

Micronucleus quantification in erythrocytes has been used with various fish and amphibian species to monitor potentially genotoxic aquatic pollutants [15-21]. Numerous investigations examining amphibian species inhabiting environments contaminated by large quantities of pesticides and other chemical substances have been undertaken and evidenced the efficiency of monitoring that biomarker [12, 22-27].

The Chapada Diamantina highlands are situated in the central portion of Bahia State in northeastern Brazil, within the "caatinga" dryland domain. The region is mountainous, with elevations up to 2000 m a.s.l., and represents the northern portion of the Espinhaço Range that lies slightly inland from the Atlantic coast. The region is famous for its natural beauty and comprises several different ecosystems (dryland caatinga vegetation, neotropical savanna ["cerrado"], rock outcrops, gallery forests, etc.) [28] demonstrating high degrees of endemism. That region has experienced intense agricultural development, however, especially along the Ibicoara-Mucugê plateau, with extensive plantations of coffee, potatoes, rice, vegetables, tomatoes, corn, and others. The agricultural model developed in the region is large-scale and directed toward exportation, with intensive vegetation suppression and significant applications of agrochemicals (herbicides, fungicides, and pesticides) [29]. Therefore, we seek to evaluate the genotoxic potential of agribusiness activities by assessing micronuclei biomarkers' responses of native neotropical tadpoles species (Amphibia: Anura) at different surface water bodies from areas impacted by agrochemicals used in large-scale agriculture compared to preserved waters at the neighboring Chapada Diamantina National Park. Our hypothesis is that the closer the agricultural farms, the more affected by mutagenic and genotoxic substances will be the environment and resident organisms. Such results can contribute to the monitoring of the toxic effects of xenobiotics in contaminated areas, through these sentinel organisms since our data refer to the real conditions of contamination and reveal the consequences of these stressors in the local biological community.

## 2. MATERIALS AND METHODS

This study was conducted with the formal approval of Ethics Committee on the Use of Animals of the Universidade Estadual de Feira de Santana (CEUA-UEFS), under registry number 02/2012. Animal collection was authorized by the Ministério do Meio Ambiente do Brasil (ICMBio license No. 27305-1). Tadpoles were sampled between November 2012 and February 2013 (corresponding to the rainy period) in six surface water bodies presumably contaminated by agro-defensive chemicals, and in five sites not subjected to that chemical input in the Chapada Diamantina National Park, Bahia State, Brazil (Figure 1).

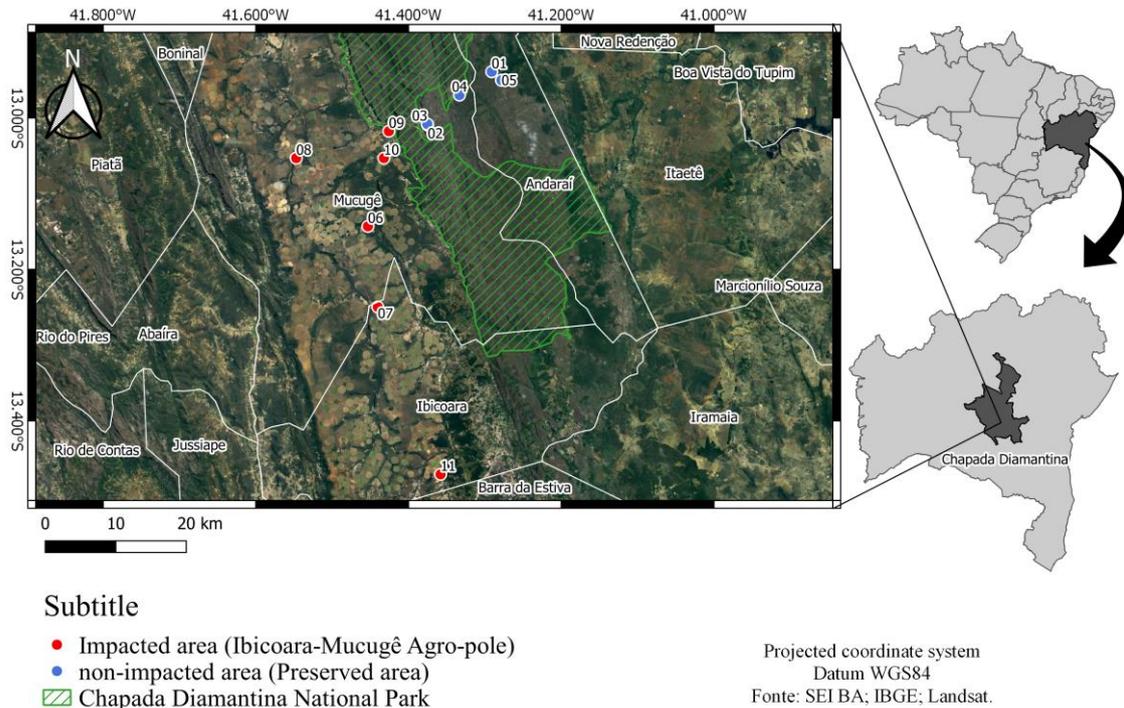


Figure 1: Localization of the sampling sites in the Chapada Diamantina – Bahia State, Brazil. Sampling points 1-5 in preserved areas within and/or around the Chapada Diamantina National Park, sampling points 6-11 in areas impacted by agrochemicals in agricultural areas within the Ibicara-Mucugê Agro-pole. The Paraguaçu river is highlighted.

Farms' plantations were coffee (reservoir and two streams – sites 06, 07 and 11 respectively), corn (stream - site 08), vegetables (puddle- site 09), and pumpkin (swamp – site 10); the tadpoles were collected near dams (n = 1), streams (n = 3), pools and swamps (n = 3). Farms are located in the municipalities of Ibicara and Mucugê in a densely agricultural region known as the Ibicara-Mucugê Agro-pole. During the fieldwork, we observed severe environmental degradation resulted from vegetation suppression and pesticide use linked to the agricultural activities.

The samples at the non-impacted areas were taken in from the aquatic environments within the borders of the well-protected Chapada Diamantina National Park, in the municipalities of Mucugê and Palmeiras, including streams (n = 2) and rocky pools (n = 3). They were swamps (sites 01 and 04), streams (02 and 03), and puddles between rocks of the Piaba river (site 05).

The tadpoles of 11 frog species were collected using nets and held while in the field in 5 L plastic sacks containing water from their respective collection sites. The specimens were subsequently transported to the Zoology Laboratory at the Federal University of Bahia, Campi Anísio Teixeira, in the municipality of Vitória da Conquista, for the analysis. A total of 154 tadpoles were analyzed, being 73 from the environmentally preserved sites (Chapada Diamantina National Park) and 81 from sites impacted by agrochemicals (agricultural areas).

The tadpoles collected demonstrated development stages 26 through 43, following Gosner (1960) [30]. In the preserved areas, we analyzed the tadpoles of: *Bokermannohyla oxente*, stages 26-41 (point 4, n = 12 tadpoles), *Leptodactylus latrans*, stages 28-30 (point 3, n = 12); *Leptodactylus mystacinus*, stages 39-41 (point 1, n= 21); *Leptodactylus vastus*, stages 36-43 (point 1, n= 4); *Physalaemus cuvieri*, stages 26–36 (points 2 and 3, n = 11); and *Rupirana cardosoi*, stages 34–40 (point 5, n = 13).

In the farming areas, we analyzed the tadpoles of: *Bokermannohyla* sp. stages 26–31 (point 7, n = 30 ); *B. oxente*, stages 26–41 (point 11, n = 07); *Boana albopunctata*, stages 30–36 (point 6, n = 15); *L. latrans*, stages 30–40 (point 8, n = 17); *Odontophrynus carvalhoi*, stages 26 -36 (point 6, n = 4); *Scinax x-signatus*, stages 26-36 (point 9, n = 4); and *Scinax montivagus*, stages 26-39 (point 9, n = 4).

Immediately after arriving in the laboratory, we acclimatized the captured animals for six hours in aquariums containing water from their respective collectionsites. The tadpoles were then anesthetized with 2% lidocaine chlorite gel and blood samples collected by cardiac puncture. A 10  $\mu$ L aliquot of each blood sample was deposited in a micro-tube containing 500  $\mu$ l of physiological solution and 10  $\mu$ l of EDTA. Blood smears were then prepared (two slides per tadpole) and fixed with methanol for 10 min., dried at room temperature, and finally stained with 6% May Grunwald-Giemsa in Sorensen buffer (pH 6.8) for 20 min [31].

Micronucleus frequencies were determined by examining 1000 erythrocytes on each slide for each tadpole, using an oil immersion light microscope (at 1000X magnification) [31]. The coded and randomized slides were evaluated by a single observer. The criteria adopted for determining the presence of micronuclei follow Silva et al. (2020) [31] being: non-refractory particles with shapes, colors, and intensities similar to the principal nucleus, although not connected to it; their sizes would be smaller, up to one third of the size of the principal nucleus.

We determined the basal frequencies of micronucleated cells for each species as the mean number and standard deviation of erythrocytes that demonstrated that anomaly, expressed in per 1000 cells (%). The Kruskal Wallis test was used to determine whether there were statistically significant differences between the micronucleus frequencies recorded at the different sampling points. The Dunnett's test was used to examine the main significant effects even further. The data of micronucleus frequencies observed in the two species occurring in both types of environments (impacted by agrochemicals and preserved) were analyzed using the Mann Whitney test;  $p \leq 0.05$  was considered indicative of statistical significance. All the statistical tests were performed using Graph Pad Prism 5.00 software (GraphPad Software Inc., San Diego, CA, EUA).

### 3. RESULTS AND DISCUSSION

A single micronucleus was predominant in the erythrocytes analyzed. Other erythrocytes, however, demonstrated nuclear abnormalities, such as having a lobed nucleus or being bi-nucleated (arrowheads in Figures 2a and 2b respectively). Those abnormalities were considered in our analysis as they are related to errors of cell division, processes of cellular death, as well as genotoxicity and/or mutagenicity [17] and have origins similar to micronuclei [32] as they occur at greater frequencies in situations of dramatic alterations in diet, pathology, or metabolic stress [33].

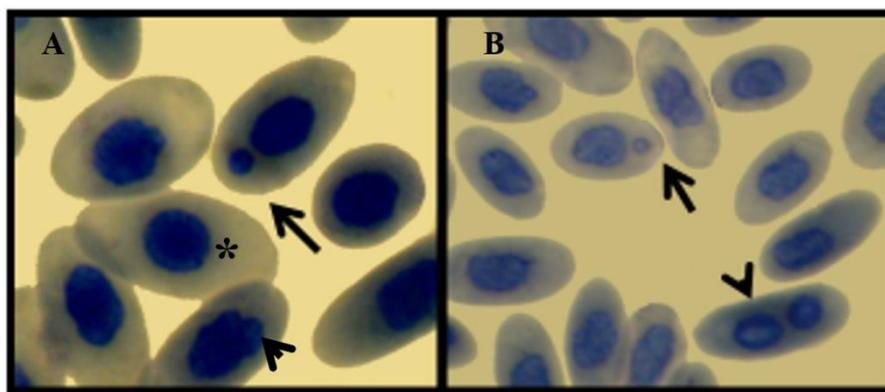


Figure 2: Nuclear alterations of erythrocytes in tadpoles collected from agricultural areas within the Ibicoara-Mucugê Agro-pole, Chapada Diamantina – Bahia State, Brazil. Blood smears stained using the Giemsa method, at 1000X magnification. A: Micronucleated erythrocytes (arrow), a normal nucleus (\*) and a nucleus with lobes (arrowhead). B: Binucleated erythrocyte (arrowhead) and a micronucleus (arrow).

The frequencies of micronucleated erythrocytes were greater in tadpoles collected in impacted areas than in preserved sites (Table 01) (Figure 3) ( $U = 1561$ ,  $p < 0,0001$ ). Several studies have indicated that the runoff of agricultural contaminants into aquatic environments can directly affect

the survival, growth, and developmental rates of tadpoles, compromising their health, increasing their vulnerability to opportunistic parasites, and increasing the frequencies of nuclear erythrocytes aberrations or cell hemolysis [4, 6, 9, 10, 24, 26], thus altering community structures [34].

Table 1: Basal frequencies (mean  $\pm$  standard deviation) of micronucleated erythrocytes (per 1000 cells) observed in tadpoles in agricultural areas in the Ibicoara – Mucugê Agro-pole and in preserved environments in the Chapada Diamantina National Park – Bahia State, Brazil.

Species	Preserved environments	Agricultural areas
Hylidae		
<i>Bokermannohyla</i> sp.	-	2.40 $\pm$ 1.81
<i>B. oxente</i>	1.25 $\pm$ 1.48	2.75 $\pm$ 0.96
<i>Boana albopunctata</i>	-	2.33 $\pm$ 1.99
<i>Scinax montivagus</i>	-	0.75 $\pm$ 1.50
<i>S. x-signatus</i>	-	1.00 $\pm$ 1.00
Leptodactylidae		
<i>Leptodactylus latrans</i>	0.42 $\pm$ 0.67	1.47 $\pm$ 1.12
<i>L. mystacinus</i>	1.43 $\pm$ 1.08	
<i>L. vastus</i>	0.33 $\pm$ 0.58	
<i>Physalaemus</i> cf. <i>curvieri</i>	0.36 $\pm$ 0.67	
<i>Rupirana cardosoi</i>	0.69 $\pm$ 0.63	
Odontophrynidae		
<i>Odontophrynus carvalhoi</i>	-	3.75 $\pm$ 0.87
Total	1.01 $\pm$ 1.09	2.18 $\pm$ 1.79

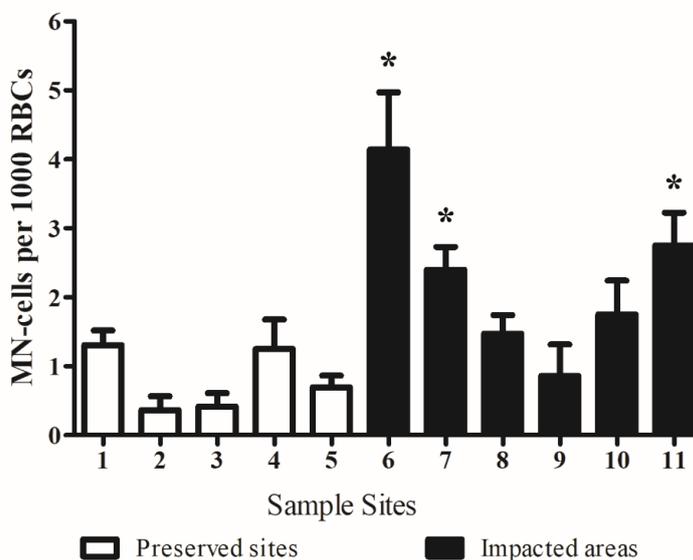


Figure 3: Mean micronucleus frequencies in tadpoles per collection site (points 1 to 11) in the Ibicoara – Mucugê Agro-pole and the Chapada Diamantina National Park, Bahia State, Brazil . \* Indicates significant differences ( $p < 0.05$ ).

Among the sites analyzed, impacted environments 06, 07 and 11 (coffee plantations) exhibited the highest micronucleus frequencies observed in the present study (Figure 3), statistically significant ( $K = 43.96$ ;  $p < 0.001$ ). Those plantations have taller and denser vegetations than the other cultures (corn, vegetables, and squash), and therefore likely used greater quantities of agro-

defensive chemicals. Carneiro et al. (2012) [35] reported that just a single hectare of coffee plants is treated with an average of 10 L of pesticides (herbicides, insecticides, fungicides). It is also important to note that surface water bodies serve as collection basins for complex chemical mixtures of diverse origins, including a variety of toxic products from industrial effluents and urban residences [13].

Preserved environments 02 and 03, on the other hand, demonstrated the lowest frequencies of tadpole erythrocyte micronuclei as compared to the impacted sites, except point 09 (Figure 3). The basal micronucleus frequencies seen in preserved areas between the towns of Mucugê and Palmeiras may reflect spontaneous events [13] and are therefore treated as comparative references for other populations of the same species present in agroecosystems.

Although we have not measured the concentration of pesticides in aquatic bodies, we collect tadpoles in places where we have identified the use of pesticides, such as the identification of inadequately disposed containers in the areas or the identification of the application *in loco*. Our study does not aim to classify pesticides and / or define they are responsible for genetic changes, but to highlight the stress caused by agricultural activities associated with the use of complex mixtures of pesticides, such as those we observed, including herbicides, insecticides, and fungicides. Thus, different from experimental studies, this work verifies the genomic damage in animals exposed in their natural habitat, under real conditions of contamination. However, further studies are in need to give further support of our findings as well as the isolation and characterization the pesticides that may be present in the surface water bodies.

Among the species analyzed, the tadpoles of *Odontophrynus carvalhoi* demonstrated the highest micronucleus frequencies. There were statistically significant differences in micronuclei frequencies between populations of *L. latrans* ( $U=45.50$ ;  $p=0.0088$ ) and *B. oxente* ( $U=8.000$ ;  $p=0.0497$ ) occurring in impacted vs. preserved areas. That observation reinforces the apparent genotoxic (clastogenic) impacts of agricultural activities on anuran species, as they can induce, or increase genetic damage in tadpoles, decreasing the adaptive potentials of those species through the elimination of tadpoles with genotypes susceptible to chemical impacts [36, 37].

Ecotoxicological studies involving these neotropical species are scarce. Tadpoles of *Odontophrynus carvalhoi* exposed to concentrations of the commercial formula of the organophosphate chlorpyrifos (Klorpan®) showed significant genetic changes in erythrocytes [31], immunosuppression in leukocytes [38], in addition to decreased survival and tail changes, making tadpoles slower and, therefore, perhaps more susceptible to predation and/or less efficient in foraging [39]. Such findings show the sensitivity of this specie to agrochemicals.

The anuran species analyzed in this work showed the presence of mutagenic agents in aquatic bodies from agricultural farms sampled in the Ibiçara-Mucugê agropole when comparing the specimens present in the preserved area of the Chapada Diamantina National Park. Similar results were found by Gonçalves et al. (2017) [40], for the species of *Physalaemus curvieri*, where populations living in agricultural areas (soybeans and corn) exhibited higher frequencies of DNA damage than those belonging to non-agricultural areas in the state of Goiás, Brazil. In this way, we can consider that anuran amphibians are excellent sentinels in environmental monitoring processes, showing previously impacts of xenobiotics to biota.

*Odontophrynus carvalhoi*, *B. oxente*, *B. albopunctata*, and *L. latrans* exhibited the greatest frequencies of micronucleated cells in the impacted environments. The variability of micronucleus frequencies observed in the different species investigated here could be related to differences in their metabolic competence and mechanisms of DNA repair. Those frequencies, however, were greater than those reported: by Gonçalves et al. (2012) [27] for adult individuals of *Proceratophrys goyana*, *Dendropsophus soaresi*, and *Leptodactylus leptodactyloides* near nickel mines in the region around Niquelândia-Barro Alto, Goiás State; by Babini et al. (2015) [24] for *Rhinella arenarum* in agroecosystems in Córdoba, Argentina; and by Pollo et al. (2016) [26] for tadpole and adult specimens of *Hypsiboas cordobae* near fluoride mines in Cerros Negros, Córdoba, Argentina. Those differences could be related to the greater sensibility of larval stage anurans, as they continually inhabit aquatic environments exposed to xenobiotics, thus being even more consistently exposed to aquatic contaminants than adults [26], in addition to interspecific differences in terms of their sensibilities to contaminants.

#### 4. CONCLUSION

In synthesis, the results of *in vivo* micronucleus tests indicated that chemical compounds present in the surface waters of agricultural areas surveyed in the Chapada Diamantina contain genotoxic substances that constitute potential dangers to local animal populations, especially anuran amphibians, and therefore cause significant damage to the environment and human health. Additionally, we were able to demonstrate that the neotropical anuran species *O. carvalhoi*, *B. oxente*, *B. albopunctata*, and *L. latrans* are competent sentinels for evaluating contamination in surface water bodies.

#### 5. ACKNOWLEDGEMENTS

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