Journal of Toxicology and Environmental Health Sciences Vol. 3(6) pp. 155-163, June 2011
Available online at http://www.academicjournals.org/JTEHS
ISSN 2006-9820 ©2011 Academic Journals

Full Length Research Paper

Micronucleus test in post metamorphic *Odontophrynus cordobae* and *Rhinella arenarum* (Amphibia: Anura) for environmental monitoring

Beatriz Bosch[1]*, Fernando Mañas[2,3], Nora Gorla [2,3] and Delia Aiassa[1]

[1]Departamento de Ciencias Naturales, Facultad de Ciencias Exactas Físico-Químicas y Naturales (FCEFQN), Universidad Nacional de Río Cuarto (UNRC), Argentina.
[2]Facultad de Agronomía y Veterinaria (FAV) Universidad Nacional de Río Cuarto (UNRC), Argentina.
[3]CONICET, Argentina.

Accepted 28 March 2011

The genotoxic effect of cyclophosphamide and glyphosate in a commercial formulation were determined using the micronucleus test in peripheral blood erythrocytes of *Odontophrynus cordobae* and *Rhinella arenarum*, amphibians widely distributed in the Province of Córdoba, Argentina. For this, the basal frequency of the micronucleated erythrocytes (MNE) was determined by: 0.40±0.18 MNE/1000 erythrocytes in *Odontophrynus cordobae* and 0.30±0.09 MNE/1000 erythrocytes in *Rhinella arenarum*. The frequency of MNE in *Odontophrynus cordobae* increased after 5 days of exposure to glyphosate (100 mg ai/L) and cyclophosphamide. After 2 and 5 days of exposure to glyphosate (200, 400 and 800 mg ai/L), the MNE frequency in *Rhinella arenarum* was higher than the basal frequency, as it occurred in the group exposed to cyclophosphamide. Regarding acute toxicity and genotoxicity, the results show that *Odontophrynus cordobae* is more sensitive to cyclophosphamide and glyphosate exposure than *Rhinella arenarum*. A correlation was detected between exposure concentration and MNE frequency in *Rhinella arenarum*.

Key words: Genotoxicity, micronucleus test, roundup®, glyphosate, amphibians.

INTRODUCTION

Many authors have reported that in the past 30 years there has been a significant decline in amphibian populations in diverse parts of the world (Hayes et al., 2010). Multiple causes have been suggested to explain this decline and among them, environmental pollution due to chemicals is gaining attention. Amphibians, like other organisms inhabiting agroecosystems, are highly exposed to agrochemicals and a correlation has been reported between the use of pesticides and the decline of amphibian populations (Beebee and Griffiths, 2005; Jones et al., 2010). Pesticides, including insecticides and herbicides, are particularly detrimental to amphibians due to its aquatic habitat, sensitive skin and unprotected eggs (Govindarajulu, 2008; Bouhafs et al., 2009).

Glyphosate based herbicides are the most widely non-selective, broad-spectrum herbicides used in the world. In Argentina, glyphosate use on its wide variety of commercial formulations has increased dramatically. With the increased use of glyphosate-based herbicides containing the surfactant polyethoxylated tallowamine (POEA) increased concern about the potential impact that the formulations may have on amphibians' populations. Some studies indicate that the toxic effect of glyphosate herbicides containing surfactants is higher than the active ingredient (ai) per se. Thus, the toxicity of

*Corresponding author. E-mail: betinabosch@gmail.com. Tel/Fax: 54-358-4676230.

some formulated glyphosate products to amphibians is greater than that caused by the active ingredient (Relyea, 2005a; Mann et al., 2009; Bernal et al., 2009; Modesto and Martinez, 2010). Furthermore, there is a global concern regarding the adverse genetic response that the formulated herbicide may have on non-target organisms (Clements et al., 1997; Holečková, 2006; Çavaş and Könen, 2007; Cavalcante et al., 2008; Andreikėnaitė et al., 2007; Poletta et al., 2007; Baršienė et al., 2008; Poletta et al., 2009; Vera et al., 2010a). Recent studies have shown that amphibians are one of the most sensitive vertebrate groups to the toxicological effects of this herbicide (Govindarajulu, 2008).

Several end-points have been used to assess genotoxicity in aquatic and terrestrial organisms. Among them, the micronucleus test (MN) has been widely used in various species to detect clastogenic or aneugenic effects, both under laboratory conditions or *in situ* studies (Udroiu, 2008). Micronuclei are chromosome fragments or whole chromosomes which were not incorporated into the main nucleus, in cells of any actively dividing tissue. Increasing frequency of MN is considered as a biomarker of genotoxic effect at subcellular level and an early response of chromosomal damage (Fenech, 2000; (Cuenca and Ramírez, 2004; Garaj-Vrhovac et al., 2008). This biomarker has been measured in amphibian species that inhabit environments where large quantities of pesticides and other chemical substances are periodically poured (Lajmanovich et al., 2005; Cabagna et al., 2006; Vera Candiotti et al., 2010b).

The micronucleus test has been frequently used to detect genotoxicity induced by clastogenic and aneugenic agents in pre-metamorphic anuran amphibians and urodeles (Jaylet et al., 1986; Marty et al., 1989; Cabagna et al., 2006; Mouchet et al., 2006; Vera et al., 2010b), but few used in post-metamorphic amphibians (Zhuleva and Dubinin, 1994; Matson et al., 2005). The interest of the study of post-metamorphic anuran amphibians is related to the temporal and spatial preponderance of this stage in agroecosystems, which added to the characteristics of this group and turned them into the organisms that are recommended for developing of biomarkers for environmental monitoring (Burggren et al., 2007).

The objectives of this study were:

i) to define the basal MN frequency in peripheral blood erythrocytes of adult *Odontophrynus cordobae* (Anura: Cyclorhamphidae) and *Rhinella arenarum* Hensel, 1867 (Anura: Bufonidae), amphibians widely distributed in the agroecosystems of the Province of Córdoba, Argentina.
ii) propose the variation of these species in MN frequency as a biomarker of genotoxic effect using cyclophosphamide as positive control.
iii) to test the MN frequency with four different concentrations of glyphosate herbicide in a commercial formulation.
iv) to compare the sensitivity of both amphibians to these compounds and propose the micronucleus test in these species as a diagnostic tool and monitoring of environmental quality.

MATERIALS AND METHODS

Animals

Two species of anuran amphibians in post-metamorphic stage, *O. cordobae* and *R. arenarum* were selected as bioassay organisms. These anurans have an extensive Neotropical distribution, and inhabit natural areas, agricultural land and urban territories with availability of temporary ponds (Leynaud et al., 2006). Both species are easily collected by hand and acclimated to laboratory conditions.

Specimens of *O. cordobae* were manually collected in temporary ponds in a pristine zone (Villa de Las Rosas, Province of Córdoba, lat. S 31° 56'; leng. W 65° 4'; alt. 713 m.a.s.l.) during September and October, 2005. Specimens of *R. arenarum* were manually collected in temporary ponds in a suburban area, in the east of Río Cuarto, (Province of Córdoba, 33°06' lat. S, 64°25' leng. O; alt. 438, 62 m.a.s.l.) during November and December, 2008 (Figure 1).

The average total size (snout-vent) was 10.45±0.15 cm for *R. arenarum* and 6.20± 0.35 cm for *O. cordobae*. The animals collected were those considered young adults in reproductive age, according to their body size. The animals were kept under laboratory conditions at ambient temperature and natural photoperiod, according to the time of the year in which each test was performed, for acclimatization before starting the assays. They were placed in 10 L plastic containers that were lined with damp paper towels to keep the animals hydrated. During this time, the animals were fed on larvae of *Tenebrio molitor* (Coleoptera: Tenebrionidae) bred in the laboratory.

Chemicals

The following substances were used: lyophilized cyclophosphamide monohydrate (CAS Nº 6055-19-2), Microsules® and isopropilamine salt of N-phosphomethyl-glycine, glyphosate, (CAS Nº 38641-94-0) Roundup® (glyphosate 48%). All test solutions were freshly prepared before use and renewed every 48 h.

Treatments

Hayashi (2007) recommendations for the treatments were followed regarding the number of animals per treatment group, the route of exposure to the substances tested, the animal preparation and the test conditions. After acclimatization the animals were assigned to groups of equal size (n = 5) as follows:

(I) Negative control group (C).
(ii) Positive control group (PC).
(iii) Groups exposed to glyphosate (G).

Each experimental group consisted of three males and two females; sex was determined by the presence/absence of vocalization activity and position in amplexus, observed in the field.

The negative control group was kept in water. Cyclophosphamide, well known as a genotoxic substance (clastogenic effect), was used as a positive control. The positive control group was kept in 40 mg/L cyclophosphamide during 5 days. The groups exposed to the herbicide were kept in Roundup ® solutions prepared in water, at the following concentrations: glyphosate 100 mg (G100), 200 mg (G200), 400 mg (G400) and 800 mg (G800) active ingredient (a.i.)/ L. Dermal exposure was achieved by keeping the animals in the corresponding solutions in a volume sufficient to cover half of the distal humerus.

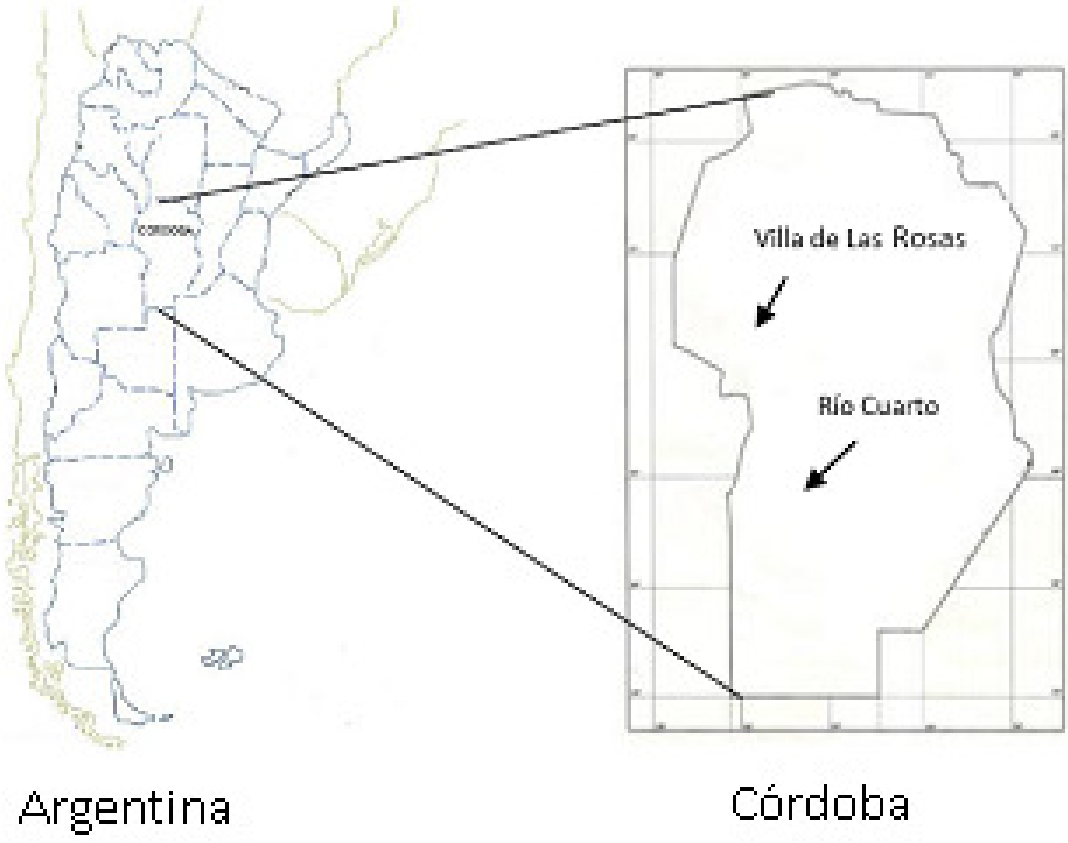

Figure 1. Study localities.

Blood samples were obtained from Days 2 and 5 of treatment, through a small incision in the angularis vein. A drop of blood was immediately smeared on a clean slide which was dried at room temperature for 24 h. After fixation with absolute methanol for 15 min, the slides were stained with May Grünwald-Giemsa, washed with distilled water and air dried.

Protocols described for anuran amphibian larvae (Campana et al., 2003; Feng et al., 2004) were used for the MN test and adapted to post-metamorphic stages.

The slides were coded and blind-scored by one researcher at 1000× magnification with an optic microscope (Nikon Labophot 2), and were photographed using a digital camera (HP Photosmart 935). The number of erythrocytes with MN was determined by analyzing 3000 erythrocytes per animal. Only non-overlapping cells with intact cellular and nuclear membrane were counted.

For the MN identification, the following criteria were used:

(a) The diameter of MN usually varies between $1/16^{th}$ and $1/3^{rd}$ of the mean diameter of the main nuclei.
(b) MN are non-refractive and, therefore, they can be readily distinguished from artifacts such as staining particles.
(c) MN are not linked or connected to the main nuclei.
(d) MN may touch but not overlap with the main nuclei, and the micronuclear boundary should be distinguished from the nuclear limit.
(e) MN usually has the same staining intensity as the main nuclei but, occasionally, it may be more intense (Fenech et al., 2003). The frequency of micronucleated erythrocytes was expressed as the number of micronucleated erythrocytes (MNE)/1000 analyzed erythrocytes.

Statistical analysis

The Kolmogorov–Smirnov test was performed to verify whether the results follow a normal distribution. Student´s t test was used to analyze the data from cyclophosphamide assays. The data from the Roundup® experiments were analyzed by one-way ANOVA with Dunnett's post-test. The statistical analysis was performed using GraphPad Prism version 5.00. The relationship between the Roundup® concentrations (mg a.i. /L) tested in *R. arenarum* and the observed frequency of MNE was determined by a correlation and regression analysis.

RESULTS, DISCUSSION AND CONCLUSION

The erythrocytes from both species showed a characteristic elliptical shape, with an approximate size of 22 ×15 µm, and an elliptical nucleus in a central position. We found one MN per cell which was located close to the main nucleus (Figures 2 and 3).

The basal frequency of MNE in *O. cordobae* was higher, 0.40 ± 0.18 MNE/1000 analyzed erythrocytes, than that found in *R. arenarum,* 0.30 ± 0.09 MNE/1000 analyzed erythrocytes (Figures 4 and 5). These parameters have not been previously reported and could

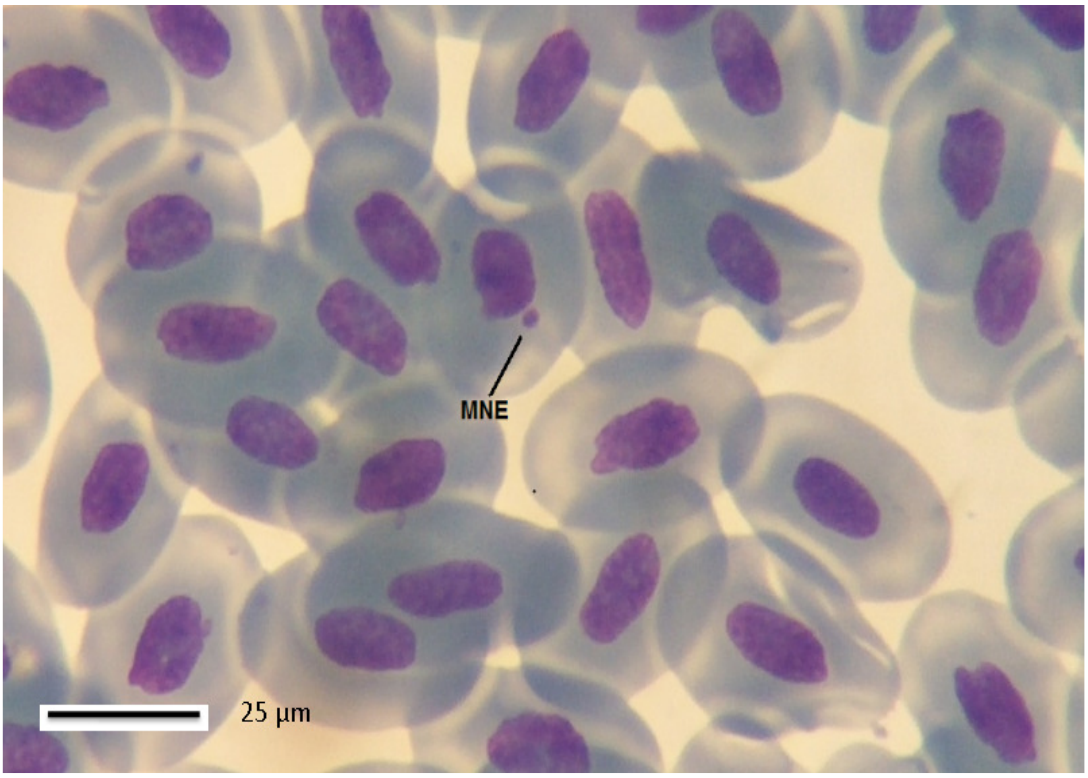

Figure 2. Micronucleated erythrocyte (MNE) in peripheral blood smear from *Odontophrynus* cordobae.

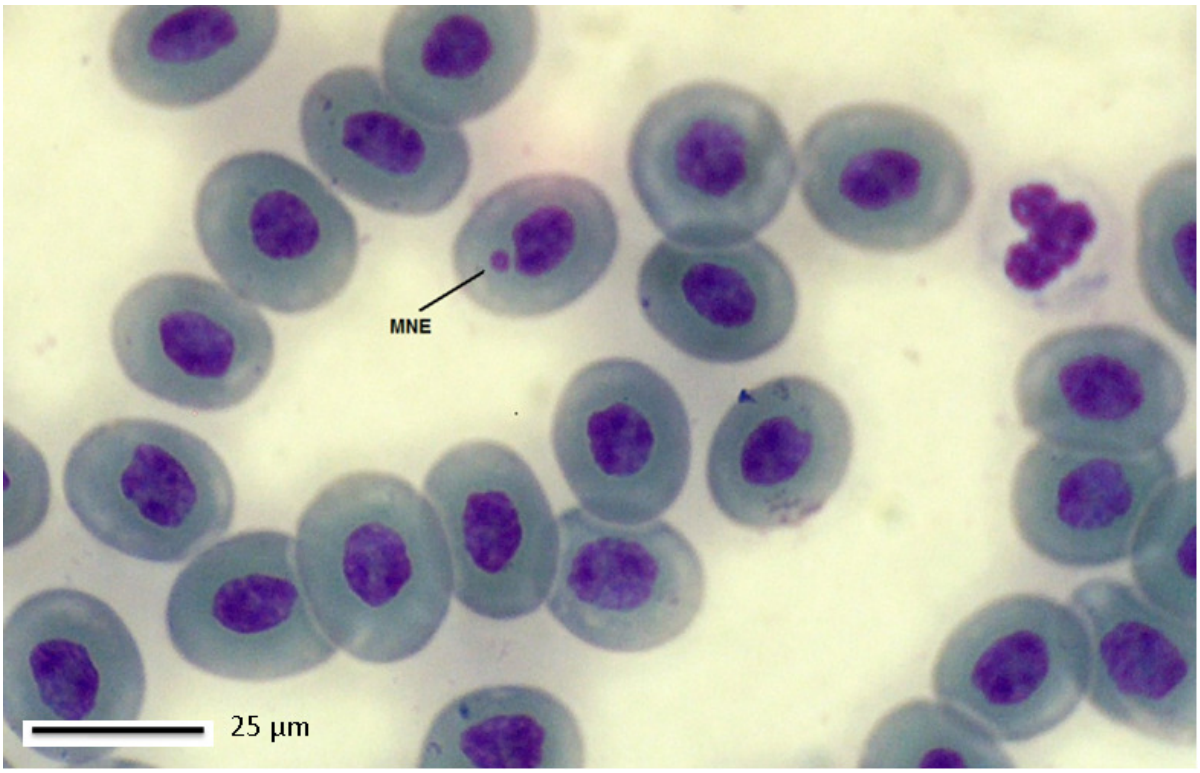

Figure 3. Micronucleated erythrocyte (MNE) in peripheral blood smear from *Rhinella arenarum.*

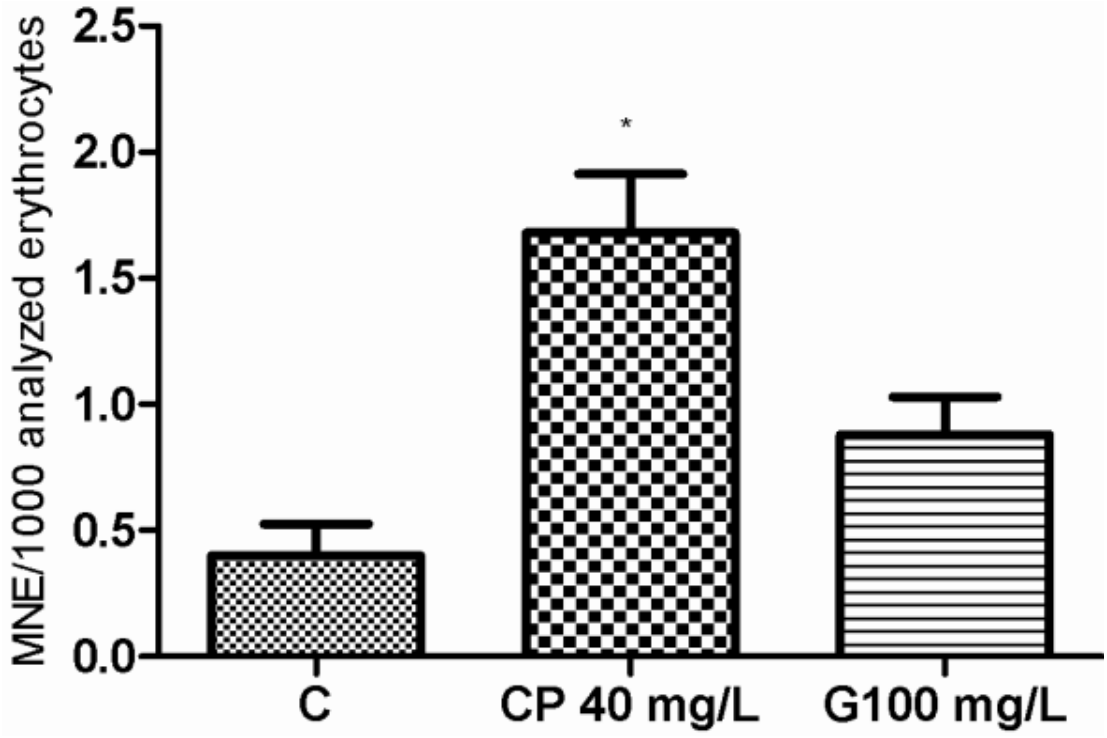

Figure 4. Mean frequencies of micronucleated erythrocytes per 1000 analyzed erythrocytes (MNE/1000 analyzed erythrocytes) in *O. cordobae*. for the negative control group (C), cyclophosphamide positive control group (CP) and groups exposed to Roundup® at 100 mg a.i./L (G100) at day 5 of treatment (*statistically significant difference $p<0.05$ as compared to the negative control), n= 5 animals per group.

be used as a reference for the sampling of both species in other agricultural areas of the province of Córdoba. In Argentina, other research groups have selected other species of the genus *Odontophrynus* (*O. americanus*) and *R. arenarum* in pro-metamorphic stage as bioassay organisms to perform the MN test (Cabagna et al., 2006; Vera et al., 2010a). Cabagna et al. (2006) reported lower basal MNE frequencies in tadpoles of the species *Odontophrynus americanus* than those observed in post-metamorphic stages of *O. cordobae* in this study. In contrast, the basal frequencies reported for larvae of the species *R. arenarum* (Vera et al., 2010a) are higher than those found in this study, suggesting a greater sensitivity of larvae in *R. arenarum*.

Concentrations of glyphosate G200, G400 and G800 were lethal to *O. cordobae.* The group exposed to cyclophosphamide for 5 days showed significant differences in MNE frequency when compared to the control group (1.68 ± 0.52 MNE/1000 analyzed erythrocytes, $p < 0.05$; $t = 4{,}824$; Figure 4). In *R. arenarum*, the basal frequency of MNE was duplicated in the group of amphibians that received dermal exposure to cyclophosphamide, with a statistically significant difference ($p<0.05$) for 2 ($t=2.33$) and 5 ($t= 3.77$) days. After the application of the test with this known clastogenic as a positive control, we can ensure that micronucleus test in post-metamorphic *O. cordobae* and *R. arenarum* can be used as a biomarker of genotoxic effect for environmental monitoring as it has been assayed for pro-metamorphic stages (Cabagna et al., 2006; Vera et al., 2010a).

Originally, blood samples were planned to be obtained at Days 5 and 9 for *O. cordobae* and *R. arenarum*. During the first day of treatment, the groups of *O. cordobae* exposed to glyphosate (G200, G400 and G800) showed signs associated with acute toxicity (massive blood shedding and changes in the color of the skin), and in the second day, the effects were lethal in the three groups. At Day 5 of the treatment, only samples from the negative control, positive control, and G100 group could be obtained (Figure 4). Govindarajulu (2008) reported a direct effect (lysis) on the skin of amphibians in post-metamorphic stages by glyphosate based formulations. As a consequence of these results, sampling for the tests in *R. arenarum* were brought forward to Days 2 and 5 of treatment (Figure 5). Our results, however, show that *O. cordobae* presented a higher acute toxicity than *R. arenarum*. Concentrations of exposure which were lethal for *O. cordobae*. (G200, G400 and G800 mg a.i./L) did not affect *R. arenarum*. The latter only showed mild signs

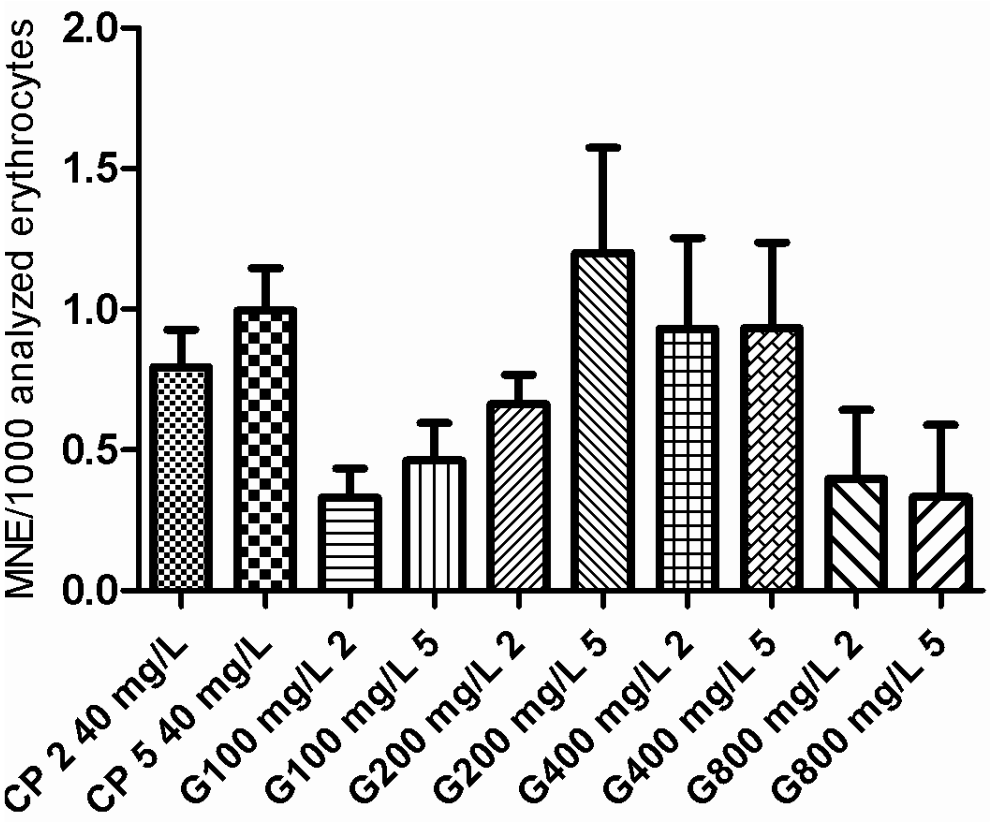

Figure 5. Mean frequencies of micronucleated erythrocytes (MNE/1000 analyzed erythrocytes) in the treatment groups of *R. arenarum* with cyclophosphamide at day 2 (CP 2) and at day 5 (CP 5); with Roundup® 100 mg a.i /L at day 2 (G100 2) and at day 5 (G100 5); 200 mg a.i./L at day 2 (G200 2) and at day 5 (G200 5); 400 mg a.i./L at day 2 (G400 2) and at day 5 (G400 5); and 800 mg a.i../L at day 2 (G800 2) and at day 5 (G800 5), n= 5 animals per group.

of toxicity.

Besides, at a concentration of 100 mg a.i /L (G100), *O. cordobae* had a higher frequency of MNE (0.88 ± 0.33 MNE / 1000 analyzed erythrocytes) than *R. arenarum* (0.46 ± 0.16 MNE/1000 analyzed erythrocytes). Some studies have examined whether it is the active ingredient (glyphosate) or other components in the commercial formulations of the herbicides the cause of toxicity in anurans and other amphibians (Govindarajulu, 2008). There are a number of formulations of glyphosate-based herbicides all of which have the same basic ingredients: isopropylamine salt of glyphosate, a surfactant often undisclosed, unspecified inert substances and water. Polyethoxylated tallow amine (POEA) is a surfactant derived from animal fat, mainly used in glyphosate-based products (Giesy et al., 2000, Modesto and Martinez, 2010). It is worth noting that laboratory studies have shown that POEA is a substance that causes high mortality in fish and amphibians, even though it is legally classified as an inert ingredient (Jones et al., 2010).

According to the results, we could assume that the commercial formulation of glyphosate used in the present work has a clastogenic effect in the studied anurans, agreeing with the report of Clements et al. (1997), who applied the comet assay in tadpoles of *Lithobates catesbeianus* (bullfrog) exposed to a glyphosate-based formulation.

The concentrations used in this work were approximately 10 times higher than those utilized in previous studies with glyphosate in pro-metamorphic amphibians. We considered the observations of other authors who suggested that the post-metamorphic stages of the Australian Sign-bearing Froglet (*Crinia insignifera*) were 14 times less sensitive to glyphosate than the tadpoles of the same species (Mann and Bidwell, 1999). It is important to note that the concentrations recommended by the agrochemical industry, ranges from 1 to 2% (that is, 10 to 20 ml/L of the solution) which is equivalent to 4800 to 9600 mg a.i./L, a concentration 10 to 20 times higher than those tested in the present work.

Previous studies in amphibians using glyphosate report acute toxicity in tadpoles (Smith, 2001; Lajmanovich et al., 2003, Howe et al., 2004; Cauble and Wagner, 2005, Relyea, 2005a, 2005b). However, many amphibians

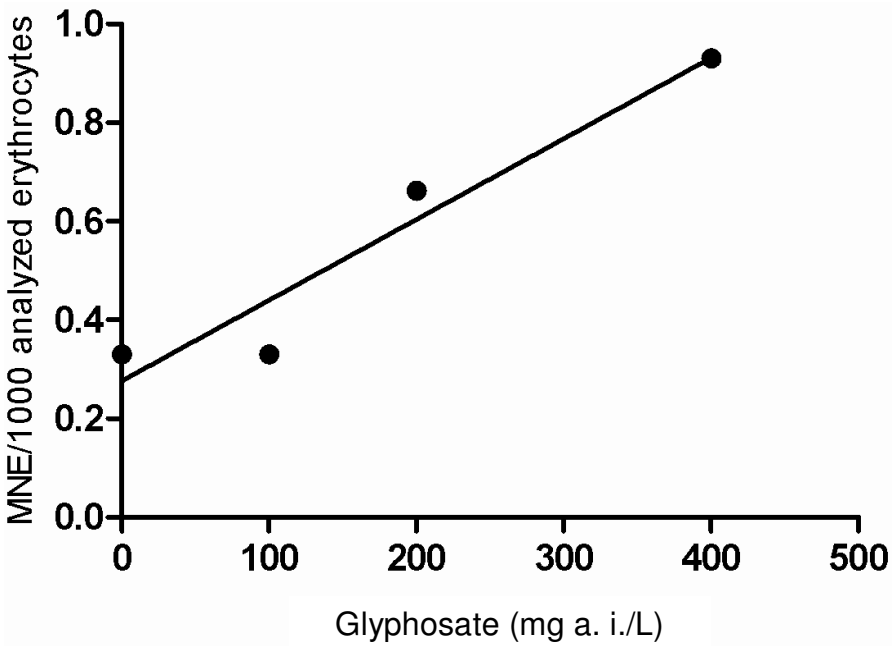

Figure 6. Regression analysis between Roundup® concentration (100, 200 and 400 mg a.i./L) and the frequency of micronucleated erythrocytes per 1000 analyzed erythrocytes (MNE/1000 analyzed erythrocytes) in *R. arenarum*, at day 2 of treatment (r^2= 0.96, $p<0.05$), n= 5 animals per group.

spend a large fraction of their life in the post-metamorphic stage. References on the effect assessment of glyphosate in amphibians in post-metamorphic stages, as in the current genotoxicity study, appears to be restricted to only two Australian species (Mann and Bidwell, 1999). In Relyea (2005a) study, the maximum dosage of domestic use Roundup® caused a decrease on survival in three species of anurans within 24 h of spraying. It is suggested that herbicide exposure and its impact on the amphibian's post-metamorphic stages may be non-trivial and should not be ignored, and the frequency and magnitude of these effects need further investigation (Relyea, 2005b). Recently, the impact of environmental exposure to glyphosate on frog populations in Colombia's coca fields has also been discussed (Solomon et al., 2007).

To our knowledge there is only one study that evaluated genotoxicity of glyphosate in amphibians using another test. In that study, the comet assay was performed in erythrocytes from *L. catesbeianus* tadpoles to asses DNA damage following exposure to the herbicide Roundup® (Clements et al., 1997). All tadpoles treated with 108 mg a.i./L Roundup ® died within 24 h, and animals treated with 1.7 to 27 mg a.i./L Roundup ® showed significant DNA damage when compared with unexposed control animals.

No statistically significant differences were found in the mean frequency of MNE among the experimental groups in *R. arenarum* ($p>0.05$; ANOVA). Despite this, it is interesting to note that the herbicide seems to produce a variation in the frequency of MNE in a concentration response manner (Figures 6 and 7). At day 2 of treatment, increments in the average frequency of MNE were observed with increasing concentrations of the herbicide up to G400 included (r^2 = 0.96, α = 0.05) (Figure 6). At Day 5 of treatment, a decrease in the mean frequency of MNE was observed from G200 to G800 mg ai/L, (r^2 = 1, $p <0.05$) (Figure 7). This difference in the behavior of the average MNE frequencies observed at Days 2 and 5 may be due to a toxic effect of the herbicide on hematopoietic organs. Analysis of more replications and appropriated histological methods can reveal more information on such effects.

Given the biological and ecological characteristics of the studied species, it would be convenient to extend this evaluation to pure glyphosate to rule out the effect of other components present in the commercial formulation. In addition, future studies should look into the biological effects of AMPA, the main environmental breakdown product of glyphosate, often found as a pollutant. To this end, we have detected, by three different assays, the potential genotoxic effect of AMPA in mammalian cells *in vitro* and *in vivo* (Mañas et al., 2009).

The MN test is a valuable tool to monitor and to identify the genotoxic effect of environmental pollutants, as well to predict and, probably, to prevent the consequences of exposure to them. Since cytogenetic manifestations are considered an early genotoxic finding, the MN test allows

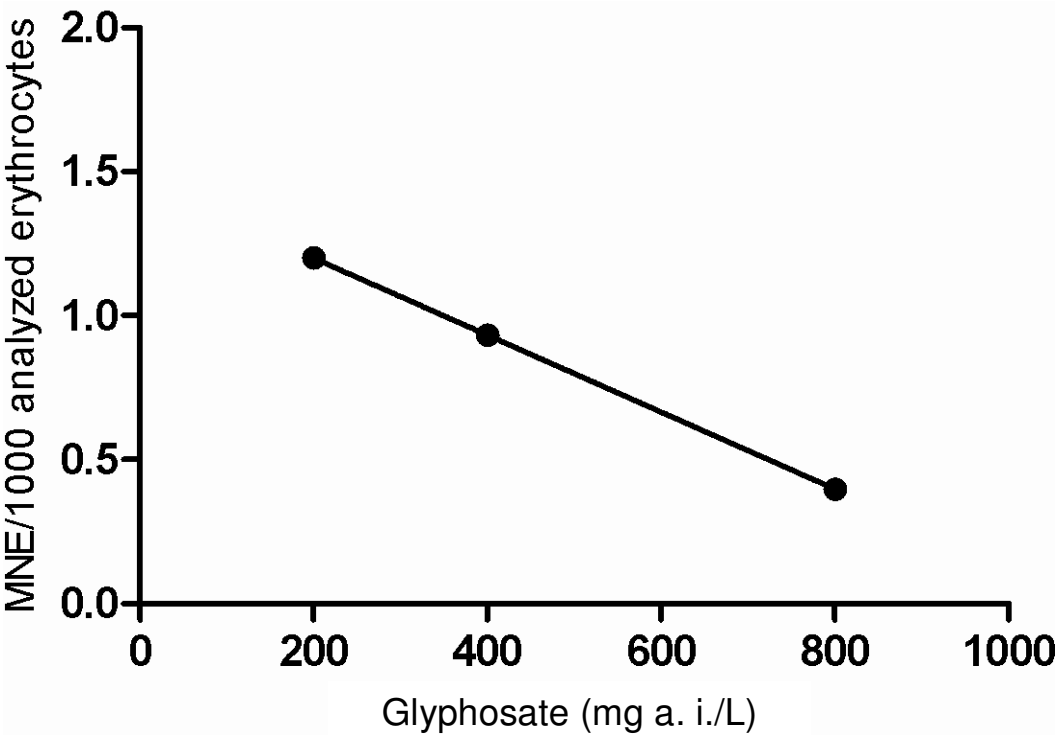

Figure 7. Regression analysis between the groups exposed to Roundup® (200, 400 and 800 mg a.i./L) and the frequency of micronucleated erythrocytes per 1000 analyzed erythrocytes (MNE/1000 analyzed erythrocytes) in *R. arenarum*, at day 5 of treatment (r^2=1, $p<0.05$), n= 5 animals per group.

detecting a level of genetic damage when it is still reversible (Cuenca and Ramírez, 2004; Zhu et al., 2005).

Most of the studies of genotoxicity in amphibian have focused on anuran larvae; therefore, little information is available on the impact of glyphosate on adult amphibians, as reported here. The major concern is related to the excessive amounts of glyphosate in commercial formulations which are incorporated to the soil and the ecosystems in Argentina, and to their possible negative effect on amphibian populations.

Our results show that both species in post-metamorphic stage have a basal spontaneous occurrence of MNE, which can also be induced by cyclophosphamide and a glyphosate-based formulation Therefore, the MN test offers a useful tool to evaluate the genotoxic effect of environmental polluents. However, as this is an exploratory work, it needs future investigation under other experimental conditions such as different exposure concentrations and duration of treatment. The development and validation of biomarkers *in vivo*, like the MN test in peripheral blood in *O. cordobae* and *R. arenarum,* provides a basis for the *in situ* assessment of the potential risk of environmental exposure to genotoxic agents, being this a present concern at ecological as well as public health level.

We concluded that both amphibians can be used for environmental monitoring of pesticide contamination.

REFERENCES

Andreikėnaitė L, Baršienė J, Vosylienė M (2007). Studies of micronuclei and other nuclear abnormalities in blood of rainbow trout (*Oncorhynchus mykiss*) treated with heavy metal mixture and road maintenance salts. Acta Zool. Lit., 17: 213-219.

Beebee T, Griffiths R (2005). The amphibian decline crisis: A watershed for conservation biology? Biol. Conserv., 125: 271-285.

Baršienė J, Rybakovas A, Förlin L, Šyvokienė J (2008). Environmental genotoxicity studies in mussels and fish from the Göteborg area of the North Sea. Acta Zool. Lit., 18: 240-247.

Bernal M, Solomon K, Carrasquilla G (2009). Toxicity of Formulated Glyphosate (Glyphos) and Cosmo-Flux to Larval Colombian Frogs. Lab. Acute Toxicity J. Toxicol. Environ. Health, 72: 961-965.

Burggren W, Warburton S (2007). Amphibians as animal models for laboratory research in physiology. ILAR J., 48: 260-269.

Bouhafs N, Berrebbah H, Devaux A, Rouabhi R, Djebar J (2009). Micronucleus Induction in Erythrocytes of Tadpole *Rana saharica* (Green Frog of North Africa) Exposed to Artea 330EC. Am. Eur. J. Toxicol. Sci., 1: 7-12.

Cabagna M, Lajmanovich R, Peltzer P, Attademo A, Ale A (2006). Induction of Micronucleus in Tadpoles of *Odontophrynus americanus* (Amphibia: Leptodactylidae) by the Pyrethroid Insecticide Cypermethrin. Toxicol. Environ. Chem., 88: 729-737.

Campana M, Panzeri A, Moreno V, Dulout F (2003). Micronuclei induction of *Rana catesbiana* tadpoles by the pyrethroid insecticide lambda-cyhalothrin. Genet. Mol. Biol., 26: 99-103.

Cauble K, Wagner R (2005). Sublethal effects of the herbicide Glyphosate on amphibian metamorphosis and development.

Bull. Environ. Contam. Toxicol., 75: 429-435.
Çavaş T, Könen S (2007). Detection of cytogenetic and DNA damage in peripheral erythrocytes of goldfish (*Carassius auratus*) exposed to a glyphosate formulation using the micronucleus test and the comet assay. Mutagenesis, 22: 263-268.
Cavalcante D, Martinez C, Sofia, S (2008). Genotoxic effects of Roundup® on the fish *Prochilodus lineatus*. Mutation Research/Genetic Toxicol. Environ. Mutagen., 655: 41-46.
Clements C, Ralph S, Petras M (1997). Genotoxicity of selected herbicide in *Rana catesbeiana* tadpoles using the alkaline single-cell gel DNA electrophoresis (comet) assay. Environ. Mol. Mutagen., 29: 277-288.
Cuenca P, Ramírez V (2004). Environmental mutagenesis and use of biomarkers to predict cancer risk. Rev. Biol. Trop., 52: 585-590.
Fenech M (2000). The *in vitro* micronucleus technique. Mutat. Res., 445: 81-95.
Fenech M, Chang W, Kirsch-Volders M, Hollandd N, Bonassi S, Zeigerf E (2003). HUMN project: Detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutat. Res., 534: 65-75.
Feng S, Kong Z, Wang X, Zhao L, Peng P (2004). Acute toxicity of two novel pesticides on amphibian, *Rana N. Hallowel*. Chemosphere, 56: 475-463.
Garaj-Vrhovac V, Gajski G, Ravlić S (2008). Efficacy of HUMN criteria for scoring the micronucleus assay in human lymphocytes exposed to a low concentration of p,p'-DDT. Braz. J. Med. Biol. Res., 41: 473-476.
Giesy J, Dobson S, Solomon K (2000). Ecotoxicological risk assessment for Roundup® herbicide. Environ. Contam. Toxicol., 167: 35–120.
Govindarajulu P (2008). Literature review of impacts of Glyphosate herbicide on amphibians: What risks can the silvicultural use of this herbicide pose for amphibians in B.C.? B.C. Ministry of Environment, Victoria, BC. Wildlife Report N° R-28.
Hayashi M (2007). Three Rs in mutation research- From *in vivo* to *in silico* evaluation - AATEX 14, 9-13. Proc. 6th World Congress on Alternatives and Animal Use in the Life Sciences.
Hayes TB, Falso P, Gallipeau S, Stice M (2010). The cause of global amphibian declines: a developmental endocrinologist's perspective. J. Exp. Biol., 213: 921-933.
Holečková B (2006). Evaluation of the *in vitro* effect of glyphosate-based herbicide on bovine lymphocytes using chromosome painting. Bull. Vet. Inst. Pulawy, 50: 533-536.
Howe CM, Berrill M, Pauli BD, Helbing CC, Werry, K, Veldhoen N (2004). Toxicity of Glyphosate-based pesticides to four North American frog species. Environ. Toxicol. Chem., 23: 1928-1938.
Jaylet A, Deparis P, Ferrier V, Grinfeld S, Siboulet R (1986). A new micronucleus test using peripheral blood erythrocytes of the newt *Pleurodeles waltl* to detect mutagens in fresh-water. Mutat. Res., 164: 245-257.
Jones D, Hammond J, Relyea R (2010). Roundup ® and amphibians: the importance of concentration, application time and stratification. Environ. Toxicol. Chem., 29: 2016-2025.
Lajmanovich R, Sandoval M, Peltzer P (2003). Induction of Mortality and Malformation in *Scinax nasicus* Tadpoles Exposed to Glyphosate Formulations. Bull. Environ. Contam. Toxicol., 70: 612–618.
Lajmanovich R, Cabagna M, Peltzer P, Gabriela A, Stringhini G, Attademo A (2005). Micronucleus induction in erythrocytes of the *Hyla pulchella* (Amphibia: Hylidae) tadpoles exposed to insecticide endosulfan. Mutat. Res., 587: 67-72.
Leynaud G, Pelegrin N, Lescano J (2006). Amphibians and Reptiles. In: Maeshlands of Río Dulce and lagoon Mar Chiquita (Córdoba, Argentina). (ed Bucher E.H.), National Academy of Sciences (Córdoba, Argentina), pp. 219-235.
Mañas F, Peralta L, Raviolo J, García Ovando H, Weyers A, Ugnia L, Gonzalez Cid M, Larripa I, Gorla N (2009). Genotoxicity of AMPA, environmental metabolite of glyphosate, assessed by the comet assay and cytogenetic tests, Ecotoxicol. Environ. Saf., 72: 834-883.
Mann RM, Bidwell JR (1999). The toxicity of Glyphosate and several Glyphosate formulations to four species of southwestern Australian frogs. Arch. Environ. Contam. Toxicol. 36: 193-199.
Mann RM, Hyne R, Choung C, Wilson S. (2009). Amphibians and agricultural chemicals: Review of the risks in a complex environment. Environ. Pollut., pp. 1–25.
Marty J, Lesca P, Jaylet A, Ardourel C, Rivière JL (1989). In vivo and in vitro metabolism of benzo(a)pyrene by the larva of the newt, *Pleurodeles waltl* Comp. Biochem. Physiol. C., 93: 213-219.
Matson C, Palatnikov G, Mc Donald T, Autenrieth R, Donnelly K, Anderson T, Canas J, Islamzadeh A, Bickham J (2005). Patterns of genotoxicity and contaminant exposure: Evidence of genomic instability in the marsh frogs (*Rana ridibunda*) of Sumgayit, Azerbaijan. Environ. Toxicol. Chem., 24: 2055-2064.
Modesto KA, Martinez C (2010). Effects of Roundup Transorb on fish: Hematology, antioxidant defenses and acetylcholinesterase activity. Chemosphere, pp. 781-787. doi:10.1016/j..2010.07.005.
Mouchet F, Gauthier L, Mailhes C, Jourdain M, Ferrier V, Triffault G, Devauxe A (2006). Biomonitoring of the genotoxic potential of aqueous extracts of soils and bottom ash resulting from municipal solid waste incineration, using the comet and micronucleus tests on amphibian (*Xenopus laevis*) larvae and bacterial assays (Mutatox® and Ames tests). Sci. Total Environ., 355: 232-246.
Poletta G, Larriera A, Kleinsorge E, Mudry M (2009). Genotoxicity of the herbicide formulation Roundup® (glyphosate) in broad-snouted caiman (*Caiman latirostris)* evidenced by the Comet assay and the Micronucleus test. Mutat. Res./Genet. Toxicol. Environ. Mutagen., 672: 5-102.
Poletta G, Larriera A, Kleinsorge E, Mudry M (2007). *Caiman latirostris* (broad-snouted caiman) as a sentinel organism for genotoxic monitoring: Basal values determination of micronucleus and comet assay. Mutat. Res./Genet. Toxicol. Environ. Mutagen., 650: 202-209.
Relyea R (2005a). The lethal impacts of Roundup and Predatory stress on Six Species of North American Tadpoles. Arch. Environ. Contam. Toxicol., 48. 351-357.
Relyea R (2005b). The lethal impact of Roundup on aquatic and terrestrial amphibians. Ecol. Appl., 15: 1118-1124.
Smith GR (2001). Effects of acute exposure to a commercial formulation of Glyphosate on the tadpoles of two species of anurans. Bull. Environ. Contam. Toxicol., 67: 483-488.
Solomon K, Anadon A, Carrasquilla G, Cerdeira AL, Marshall J, Sanin LH (2007). Coca and poppy eradication in Colombia: environmental and human health assessment of aerially applied Glyphosate. Rev. Environ. Contam. Toxicol., 190: 43-125.
Udroiu I (2008). The micronucleus test for aquatic toxicology. En: Aquatic Toxicology Research Focus. Elías Svensson (ed). Nova Science Publishers, Inc., pp. 145-160.
Vera Candioti JV, Soloneski S, Larramendy ML (2010a). Genotoxic and cytotoxic effects of the formulated insecticide Aficida® on *Cnesterodon decemmaculatus* (Jenyns, 1842) (Pisces: Poeciliidae). Mutat. Res./Genet.Toxicol. Environ. Mutagen., 703: 180-186.
Vera CJV, Natale GS, Soloneski S, Ronco AE, Larramendy ML (2010b). Sublethal and lethal effects on *Rhinella arenarum* (Anura, Bufonidae) tadpoles exerted by the pirimicarb-containing technical formulation insecticide Aficida®. Chemosphere, 78: 249-255.
Zhu L, Huang Y, Liu G (2005). Using DNA damage to monitor water environment. Chin. J. Ocean. Limn., 3: 340-348.
Zhuleva L, Dubinin N (1994). Use of the micronucleus test for assessing the ecological situation in regions of the Astrakhan district. Genetika, pp. 999-1004.