DEVELOPMENTAL TOXICITY OF BISPHENOL A DIGLYCIDYL ETHER (EPOXIDE RESIN BADGE) DURING THE EARLY LIFE CYCLE OF A NATIVE AMPHIBIAN SPECIES

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Abstract: Bisphenol A diglycidyl ether (BADGE) is used in packaging materials, in epoxy adhesives, and as an additive for plastics, but it is also a potential industrial wastewater contaminant. The aim of the present study was to evaluate the adverse effects of BADGE on Rhinella arenarum by means of standardized bioassays at embryo–larval development. The results showed that BADGE was more toxic to embryos than to larvae at all exposure times. At acute exposure, lethality rates of embryos exposed to concentrations of 0.0005 mg/L BADGE and greater were significantly higher than rates in the vehicle control, whereas lethality rates of larvae were significantly higher in concentrations of 10 mg/L BADGE and greater. The toxicity then increased significantly, with 96-h median lethal concentrations (LC50s) of 0.13 mg/L and 6.9 mg/L BADGE for embryos and larvae, respectively. By the end of the chronic period, the 336-h LC50s were 0.04 mg/L and 2.2 mg/L BADGE for embryos and larvae, respectively. This differential sensitivity was also ascertained by the 24-h pulse exposure experiments, in which embryos showed a stage-dependent toxicity, with blastula being the most sensitive stage and S.23 the most resistant. The most important sublethal effects in embryos were cell dissociation and delayed development, whereas the main abnormalities observed in larvae related to neurotoxicity, as scare response to stimuli and narcotic effect. Environ Toxicol Chem 2016;35:3031–3038. © 2016 SETAC

Keywords: Bisphenol A diglycidyl ether Standardized toxicity bioassays Stage-dependent toxicity Teratogenesis Neurotoxicity

INTRODUCTION

Bisphenol A diglycidyl ether (2,20-bis[4-hydroxyphenyl]propane bis[3-epoxypropyl] ether), commonly known as BADGE (CAS number 1675-54-3), is a synthetic chemical obtained by the reaction of 1 mol of bisphenol A (BPA) with 2 mol of epichlorohydrin (ECH) [1]. Bisphenol A diglycidyl ether is mainly used in packaging materials such as storage vessels and as the lacquer coatings on food cans, which protects the food from metal contamination and prevents metal corrosion. Levels of bisphenols and their diglycidyl ethers as BADGE were reported in wastewater influents at concentrations from 0.00096 mg/L to 0.0016 mg/L [2]. The total annual production of BPA-based epoxy resins grows annually, implying a concern for public health because of impurities present in faulty formulations that can migrate into canned food, representing a toxicity risk [1]. In fact, BADGE and its derivatives were found in concentrations between 1 mg/kg and 12.5 mg/kg in many canned foods [3–5]. It is also a concern that BPA was reported as an endocrine disruptor by reversing gonadal sex and altering gonadal histoarchitecture [6,7]. The estrogenic activity of BADGE, however, is 100 times lower than that of BPA [8].

Nevertheless, BADGE can increase the proliferation of MCF7 breast cancer cells [9]. Therefore, European legislation has established that the sum of the migration levels of BADGE, its hydrolysis, and chlorohydroxy derivatives to food or food simulants should not exceed 9 mg/kg [10]. The Shell Tunstall Toxicology Laboratories [11] reported an increase in the frequency of chromosomal aberrations of in vitro rat liver cells exposed to BADGE. In 1986, the Scientific Committee on Food from the European Union evaluated BADGE as a monomer used in the production of plastic food contact materials, and it was classified into List 4A [12]. In 2000, it was reported that BADGE and its hydrolysis products can induce micronuclei in cultured human lymphocytes from 0.0125 mg/mL [13].

Because of BADGE’s different uses and its high production worldwide, it is relevant to know the risk to wildlife after epoxy resins reach the environment. Toxicological bioassays can provide information about the potential hazards of synthetic products in living organisms. Amphibians are frequently used for toxicity screening because of their high sensitivity to physicochemical stressors [14]. Moreover, standardized tests employing amphibian embryos have been used successfully to evaluate the toxicity of hazardous substances and environmental samples [15]. In contrast to bioassays that only evaluate acute toxicity of chemicals by a unique endpoint, such as 48-h or 96-h median lethal concentration (LC50), an amphibian embryo toxicity test (AMPHIOTOX) assesses toxicity using different endpoints (exposure times and developmental stages), giving more complete information about the toxicity profile of the substance. This test employs Rhinella arenarum (Fam. Bufonidae) embryos and larvae as the appropriate biological material to perform toxicity tests [16,17]. In addition to its extensive neotropical distribution—which includes Argentina, Bolivia, Brazil, Uruguay, and Paraguay—this species is easy to handle, produces large clutches (up to 40,000 eggs) and has a short life cycle, reaching the prometamorphic stage in about 7 d to 8 d after egg laying [15].

The aim of the present study was to evaluate the toxic effects of BADGE on Rhinella arenarum development. This was
achieved by means of the standardized AMPHITOX test at different developmental stages and exposure times by characterizing lethal and sublethal effects involving teratogenesis and ethological disorders.

**MATERIALS AND METHODS**

*Obtaining R. arenarum embryos and larvae*

Healthy *R. arenarum* adults weighing approximately 200 g to 250 g were collected in Lobos (Buenos Aires Province, Argentina; 35°11′ S, 59°05′ W) from a local provider. Ovulation of *R. arenarum* females was induced by means of an intraperitoneal injection of 1 homologous hypophysis in 1 mL of AMPHITOX solution per female [18], plus 5000 international units of human chorionic gonadotropin [19]. The AMPHITOX solution composition was 14.75 mg/L Na⁺, 22.71 mg/L Cl⁻, 0.26 mg/L K⁺, 0.36 mg/L Ca²⁺, and 1.45 mg/L HCO₃⁻, prepared in distilled water. Oocytes were fertilized in vitro using a testicular macerate homogenate suspended in AMPHITOX solution, resulting in a 10% spermatozoid suspension. Embryos were kept in this physiological solution at 20 ± 2 °C until they reached the stage required for each experimental protocol. For early life stage studies (embryos up to S.17), the jelly coat was dissolved by a 2-min treatment with 2.5% thioglycolic acid solution neutralized at pH 7.2 to 7.4 with 1.35 mL of saturated NaOH solution every 100 mL in AMPHITOX solution, and then thoroughly washed with AMPHITOX solution. Although the jelly coats can provide protection against toxicants [20], in some cases it is not relevant for protection purposes [21]. In the present study, we dejellied the embryos to select a homogeneous, high-quality biological material (round shape embryos with noncellular dissociation) both for the control and experimental groups. The eggs were inspected for quality and fertility. This biological material was considered acceptable if the fertility rate was greater than 75% per female and embryo survival at the neurula stage was greater than 70%.

*Chemicals and test solutions*

Technical-grade BADGE (99.9%; CAS number 1675-54-3) was obtained from Sigma Chemical. Stock solutions were prepared in analytical grade acetone to a final concentration of 10 g/L, and experimental solutions were prepared by diluting it with AMPHITOX solution. Acetone concentrations were always lower than 1.1% [22]. Both AMPHITOX solution and acetone treatments were simultaneously maintained as controls, and they did not differ statistically; thus, both treatments were combined and reported as the control throughout the remainder of the present study.

*Toxicity bioassays*

*Rhinella arenarum* embryos and larvae were used in the standardized semistatic bioassays following the AMPHITOX protocol [16,17]. Ten embryos were placed randomly in triplicate 10 cm glass Petri dishes containing 40 mL of AMPHITOX solution with or without BADGE (controls). The toxicity bioassays were performed under the conditions summarized in Table 1. Embryos and larvae were maintained at 20 ± 2 °C. Experiments were replicated 3 times.

To evaluate the stage-dependent sensitivity, different experimental conditions were performed as follows: 1) continuous exposure of embryos from early blastula stage (S.3–4) and larvae from complete operculum stage (S.25) onwards for 336 h; and 2) 24-h pulse exposure of embryos starting at early blastula (S.3–S.4), gastrula (S.10–12), rotation (S.15), tail bud (S.17), muscular activity (S.18), gill circulation (S.20), open mouth (S.21), opercular folds (S.23), and complete operculum (S.25) stages. After exposure, embryos were washed thoroughly, kept in AMPHITOX solution, and evaluated up to 336 h. The developmental stages were defined according to Del Conte and Sirlin [23].

Lethal effects were evaluated, and dead individuals were removed every 24 h. Larvae were fed with 3 granules of balanced fish food TetraColor per Petri dish every other day. Sublethal effects were studied under a stereoscopic microscope (Zeiss Stem DVI-4). Teratogenic effects were identified according to the *Atlas of Abnormalities* [24], and organisms were photographed with an Olympus X-42 digital camera mounted on the microscope objective. A teratogenic index was calculated as LC50/median effect concentration (EC50) at 96 h in embryos exposed from blastula. This index reflects the hazard of a test agent to produce malformations during embryonic development without significant lethality [22]. Behavioral alterations—including narcosis; spasmodic contractions; abnormal fast rotations; lateral or dorsal side lying; and abnormal swimming patterns—were evaluated because they are typical signs of neurotoxic stress [25]. General weakness was defined as lower and slower movements than control larvae. Narcosis was evaluated as the lack of sudden swimming response to gentle touching with a glass rod compared with control organisms, and finally, heartbeat was checked under the microscope. Starvation was determined by observing the granules after 24 h, whereas in control larvae, we found feces instead. Abnormal skin pigmentation was defined as irregular distribution of somatic pigmentation compared with normal pigmentation found in the control organism’s skin. Cellular dissociation was determined by observing detached cells floating in the perivitelline fluid and in the maintaining media. Delayed development was determined when the developmental stage of each embryo differed from the control in each concentration group. Larvae snout–vent lengths were recorded as a measure of the body size.

| Table 1. Conditions of bisphenol A diglycidyl ether (BADGE) bioassays |
|---------------------|------------------|------------------|
| Developmental stage | Treatment         | Exposure concentrations (mg/L BADGE) |
| Blasto (S.3–S.4)    | Continuous exposure | 0.0001, 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5 |
| Complete operculum (S.25) | Continuous exposure | 1, 5, 7.5, 10, 12.5, 15 |
| Blasto (S.3–S.4)    | 24-h pulse exposure | 0.0001–10 |
| Gastrula (S.10–S.12) | 24-h pulse exposure | 0.5–10 |
| Rotation (S.15)     | 24-h pulse exposure | 1–15 |
| Tail bud (S.17)     | 24-h pulse exposure | 1–15 |
| Muscular activity (S.18) | 24-h pulse exposure | 0.1–15 |
| Gill circulation (S.20) | 24-h pulse exposure | 0.5–15 |
| Open mouth (S.21)   | 24-h pulse exposure | 0.5–15 |
| Opercular folds (S.23) | 24-h pulse exposure | 5–17.5 |
| Complete operculum (S.25) | 24-h pulse exposure | 10–25 |
Data analysis

Lethal and sublethal effects were analyzed as LC50 and EC50, respectively, with upper and lower 95% confidence limits, using the US Environmental Protection Agency’s Spearman–Karber program Ver 1.5 [26]. The toxicity profile or isotoxicity curves [27] were plotted based on LC50 values at different times. The LC50 values were considered to be substantially different when the higher:lower ratio exceeded the corresponding critical value established by the American Public Health Association [28]. We conducted generalized linear mixed models assuming a binomial distribution of the error to evaluate the effect of concentration and exposure time on lethality and on the frequency of sublethal endpoints. The test described by Di Rienzo et al. [29] was used to compare treatment means at a significance level of \( p < 0.05 \). This analysis was conducted using InfoStat statistical software [30]. The lowest-observed-adverse-effect level (LOAEL) values were determined by the lowest concentration that has a statistically significant deleterious effect compared to the control group.

### RESULTS

**Lethal effects**

Continuous exposure of embryos from early blastula stage (S.3–4) and larvae from complete operculum stage (S.25) onward for 336 h. After BADGE treatment, the lethality rates of embryos and larvae gradually increased with the concentration and the exposure time (Table 2). Lethality rates of embryos exposed from 0.0005 mg/L BADGE and larvae exposed from 10 mg/L were significantly higher than vehicle control from 96 h onward. At 2.5 mg/L BADGE, larvae lethality was significantly

### Table 2. Mortality rates (%) of *Rhinella arenarum* embryos and larvae continuously exposed to bisphenol A diglycidyl ether (BADGE)*

| Developmental stage | Concentration (mg/L BADGE) | Exposure time (h) |
|---------------------|---------------------------|------------------|
|                     |                           | 96               | 168              | 336              |
| Embryos (S.3–4)     |                           |                 |                  |
| 0                   | 3.33 ± 3.33               | 6.67 ± 6.67      | 6.67 ± 6.67      |
| 0.0001              | 3.33 ± 3.33               | 3.33 ± 3.33      | 13.33 ± 3.33     |
| 0.0005              | 23.33 ± 8.82*             | 26.67 ± 6.67*    | 30.00 ± 5.77*    |
| 0.001               | 23.33 ± 8.82*             | 23.33 ± 8.82*    | 30.00 ± 10.00*   |
| 0.005               | 20.00 ± 10.00*            | 23.33 ± 8.82*    | 30.00 ± 5.77*    |
| 0.04                | 16.67 ± 6.67*             | 16.67 ± 6.67*    | 30.00 ± 0.00*    |
| 0.05                | 30.00 ± 10.00*            | 36.67 ± 6.67*    | 43.33 ± 8.82*    |
| 0.10                | 26.67 ± 3.33*             | 26.67 ± 3.33*    | 83.33 ± 12.02*   |
| 0.50                | 100.00 ± 0.00*            | 100.00 ± 0.00*   | 100.00 ± 0.00*   |
| 1.00                | 100.00 ± 0.00*            | 100.00 ± 0.00*   | 100.00 ± 0.00*   |
| 5.00                | 100.00 ± 0.00*            | 100.00 ± 0.00*   | 100.00 ± 0.00*   |
| Larvae (S.25)       |                           |                 |                  |
| 0                   | 0.00 ± 0.00               | 0.00 ± 0.00      | 0.00 ± 0.00      |
| 1.00                | 0.00 ± 0.00               | 3.33 ± 3.33      | 3.33 ± 3.33      |
| 2.50                | 3.33 ± 3.33               | 10.00 ± 10.00*   | 10.00 ± 10.00*   |
| 5.00                | 0.00 ± 0.00               | 93.33 ± 6.67*    | 100.00 ± 0.00*   |
| 7.50                | 0.00 ± 0.00               | 100.00 ± 0.00*   | 100.00 ± 0.00*   |
| 10.00               | 100.00 ± 0.00*            | 100.00 ± 0.00*   | 100.00 ± 0.00*   |
| 12.20               | 100.00 ± 0.00*            | 100.00 ± 0.00*   | 100.00 ± 0.00*   |
| 15.00               | 100.00 ± 0.00*            | 100.00 ± 0.00*   | 100.00 ± 0.00*   |

aData represent the percentage of mortality (mean ± standard error of the mean), \( n = 3 \).

*Significantly different from vehicle control by test described by Di Rienzo et al. [29] \( (p < 0.05) \).
increased compared with vehicle control at 168 h onward. The toxicity of BADGE was substantially higher in embryos than larvae at all exposure times (Table 2). Furthermore, BADGE toxicity in embryos exposed from the blastula stage increased considerably from 24 h to 48 h, with LC50 values of 0.35 mg/L BADGE (0.25–0.5 mg/L) and 0.15 mg/L BADGE (0.09–0.23 mg/L), respectively (Figure 1). From that time forward, however, toxicity did not substantially increase, with a 336-h LC50 value of 0.04 mg/L BADGE (0.02–0.08 mg/L). In contrast, the toxicity of BADGE to larvae did not vary considerably during acute exposure, with the LC50 values at 24 h and 48 h of 11.3 mg/L BADGE (11.1–12.5 mg/L) and 10.90 mg/L BADGE (10.70–11.10 mg/L), respectively. The 96-h LC50 decreased from 6.9 mg/L BADGE (6.6–7.1 mg/L) to 2.2 mg/L BADGE (2.1–2.4 mg/L) at the end of the chronic exposure.

24-h pulse exposure. No embryos were affected after being exposed to the lowest concentrations: 0.0001 mg/L to 0.001 mg/L. The toxicity profile obtained shows a clearly stage-dependent sensitivity to the epoxy resin, with early blastula (S.3–4) being the most sensitive stage, with a 24-h LC50 of 0.58 mg/L BADGE, and S.23 being the most resistant stage, with a 24-h LC50 of 14.9 mg/L BADGE (Figure 2). The remaining developmental stages had LC50s between 8 mg/L and 11.9 mg/L BADGE.

Sublethal effects

Continuous exposure of embryos from early blastula stage (S.3–4) and larvae from complete operculum stage (S.25) for 336 h. All embryos exposed at 0.5 mg/L BADGE (LOAEL value) and above showed cell dissociation and delayed development at 24 h (exposed embryos were in early gastrula stage, whereas controls were in late gastrula or rotation stage). At 96 h, the LOAEL remained constant, but other sublethal effects were also observed, such as reduced body size, hydropsy, acephaly, and axial flexure (Table 3). The 96-h EC50 was 0.17; therefore, the teratogenic index was 0.76. At chronic exposure, the LOAEL dropped to 0.1 mg/L BADGE, and behavioral alterations such as starvation, scare response to stimuli, and spasmodic contractions were recorded.

All early larvae exposed to concentrations of 10 mg/L BADGE and greater exhibited neurological alterations a few hours after exposure started. These effects were general weakness, spasmodic contractions, and shortening, erratic, or circular swimming. Moreover, all larvae exposed up to 15 mg/L BADGE developed narcosis after a few hours of exposure, followed by death. At 168 h, all larvae exposed to 5 mg/L showed starvation, abnormal skin pigmentation, scare response to stimuli, and tail/axial flexures, and then death (Figure 3). Those exposed to the lowest concentration (1 mg/L) developed hydropsy and abnormal skin pigmentation but no neurotoxic effects (Table 3). By the end of the bioassay, the LOAEL was 1 mg/L BADGE.

24-h pulse exposure. Table 4 summarizes the LOAEL values and the most conspicuous teratogenic and neurotoxic

### Table 3. Frequency (%) of embryos and larvae with sublethal effects after 96 h and 168 h bisphenol A diglycidyl ether (BADGE) treatment, respectively

| Sublethal effects                  | Concentration (mg/L BADGE) |
|-----------------------------------|-----------------------------|
|                                   | 0   | 0.0005 | 0.001 | 0.005 | 0.05 | 0.10 | 0.50 | 1   | 5   |
| **Embryos**                       |     |        |       |       |      |     |     |     |     |
| Acephaly                          | —   | 4.3 ± 4.3 | 3.8 ± 3.8 | 4.2 ± 4.2 | —   | —   | —   | —   | —   |
| Reduced body size                 | —   | —       | —       | —       | —   | —   | 100.0 ± 0.0* | —   | —   |
| Hydropsy                          | —   | —       | —       | 4.2 ± 4.2 | 8.0 ± 3.7 | 10.0 ± 4.2 | 100.0 ± 0.0* | —   | —   |
| Axial flexures                    | 6.9 ± 3.5 | 8.7 ± 4.9 | 7.7 ± 3.9 | —   | —   | 8.3 ± 4.9 | 8.0 ± 3.7 | 100.0 ± 0.0* | —   |
| Total abnormal embryos (%)        | 6.9 ± 3.5 | 8.7 ± 4.9 | 7.7 ± 3.9 | 8.3 ± 4.9 | 8.0 ± 3.7 | 100.0 ± 4.2 | 100.0 ± 0.0* | —   | —   |
| **Larvae**                         |     |        |       |       |      |     |     |     |     |
| Hydropsy                          | 14.3 ± 5.0 | —       | —       | 51.8 ± 5.3* | —   | —   | 100.0 ± 0.0* | —   | —   |
| Abnormal skin pigmentation        | —   | —       | —       | 51.8 ± 5.3* | 100.0 ± 0.0* | —   | —   | 100.0 ± 0.0* | —   |
| Tail/axial flexure                | —   | —       | —       | —       | —   | —   | 100.0 ± 0.0* | —   | —   |
| Scare response to stimuli         | —   | —       | —       | —       | —   | —   | 100.0 ± 0.0* | —   | —   |
| Starvation                        | —   | —       | —       | —       | —   | —   | 100.0 ± 0.0* | —   | —   |
| Total abnormal larvae (%)         | 14.3 ± 5.0 | —       | —       | 51.8 ± 5.3* | 100.0 ± 0.0* | —   | —   | 100.0 ± 0.0* | —   |

*Data represent the percentage of sublethal effects (mean ± standard error of the mean), n = 3.
* Significantly different from vehicle control by test described by Di Rienzo et al. [29] (p < 0.05).
Table 4. Lowest observed adverse effect level (LOAEL) values and most common sublethal effects produced by bisphenol A diglycidyl ether (BADGE) at different developmental stages of *Rhinella arenarum*.

| Developmental stage | Observation time (h) | Sublethal effects | LOAEL (mg/L BADGE) |
|---------------------|----------------------|-------------------|--------------------|
| Blastula (S.3–S.4)  | 24                   | Bifid spine       | 0.1                |
|                     | 24                   | Oral desquamation |                    |
|                     | 96–168               | Microcephaly/acephaly |                |
|                     | 96–168               | Tumors            | 0.1                |
|                     | 336                  | Delayed development|                    |
|                     | 336                  | Axial flexures    |                    |
|                     | 336                  | Reduced body size |                    |
|                     | 336                  | Scare response to stimuli|    |
| Gastrula (S.10–S.12)| 24                   | Delayed development| 1                  |
|                     | 24                   | Persistent yolk plug|                |
|                     | 96–168               | Cellular dissociation|                |
|                     | 336                  | Microcephaly/acephaly | 1                |
|                     | 336                  | Reduced body size |                    |
|                     | 336                  | Hydropsy          |                    |
|                     | 336                  | Axial flexures    |                    |
|                     | 336                  | Reduced body size |                    |
|                     | 336                  | Scare response to stimuli|    |
| Rotation (S.15)     | 24                   | Reduced body size | 1                  |
|                     | 24                   | Microcephaly      |                    |
|                     | 96–168               | Hydropsy          |                    |
|                     | 336                  | Axial flexures    |                    |
|                     | 336                  | Reduced body size |                    |
|                     | 336                  | Scare response to stimuli|    |
| Tail bud (S.17)     | 96–168               | Delayed development| 1                  |
|                     | 336                  | Reduced body size |                    |
|                     | 336                  | Hydropsy          |                    |
|                     | 336                  | Axial flexures    |                    |
|                     | 336                  | Reduced body size |                    |
|                     | 336                  | Scare response to stimuli|    |
| Muscular activity (S.18)| 24                  | Reduced body size | 5                  |
|                     | 96–168               | Axial flexures    |                    |
|                     | 336                  | Hydropsy          |                    |
|                     | 336                  | Abnormal skin pigmentation|   |
|                     | 336                  | Scare response to stimuli|    |
| Gill circulation (S.20)| 24                  | Delayed development| 0.5                |
|                     | 96–168               | Scare response to stimuli| 0.5 |
|                     | 336                  | Starvation        | 0.5                |
| Open mouth (S.21)   | 24                   | Abnormal skin pigmentation| 5               |
|                     | 96–168               | Scare response to stimuli| 5 |
|                     | 336                  | Hydropsy          | 5                  |
| Opercular folds (S.23)| 24                  | Reduced body size | 5                  |
|                     | 96–168               | Delayed development| 10                 |
|                     | 336                  | Scare response to stimuli| 10 |
|                     | 336                  | Axial flexures    | 10                 |
|                     | 336                  | Reduced body size | 15                 |

*A dash (—) indicates that there were no sublethal effects.*
effects caused by BADGE in embryos exposed at different developmental stages. Blastula was the most sensitive stage to BADGE, and S.23 was the most resistant (LOAEL values of 0.1 mg/L and 10 mg/L, respectively). The rate of malformations in control embryos was always less than 10% during all bioassays.

Abnormal embryos treated in blastula exhibited several teratogenic effects, such as bital spine, oral desquamation, tumors, delayed development, microcephaly, acephaly, and axial flexures, even at BADGE postexposure (Table 4 and Figure 4).

All embryos exposed in gastrula developed malformations and were delayed in their development 24 h after being washed, from concentrations of 1 mg/L and greater, highlighting cellular dissociation and persistent yolk plug, but only in 30% of the individuals.

The main sublethal effects observed in embryos exposed in rotation stage were microcephaly, hydropsy, axial flexures, and reduced body size, with a LOAEL of 1 mg/L BADGE at 24 h. These effects were also conspicuous in those embryos exposed in S.18 to concentrations of 5 mg/L and greater. These embryos also developed tail flexures.

Embryos at stages between S.20 and S.25 developed neurotoxic effects such as narcosis a few hours after the beginning of the exposure. This narcotic effect was irreversible for those embryos exposed to 7.5 mg/L BADGE in S.20 and S.21, and the embryos were dead after a few hours. On the other hand, the 24-h LOAEL value for embryos at S.23 and S.25 was 10 mg/L, but the 336-h LOAEL values increased to 17.5 mg/L and 15 mg/L, respectively. This fact points out the recovery capacity from malformations as well as from neurotoxic effects caused by BADGE, because larvae exposed to 10 mg/L BADGE at 336 h did not show any sublethal effect and were not significantly different from control. By the end of the bioassay, only larvae exposed at S.25 showed reduced body size in 20% of the individuals. No control larvae showed neurotoxicity at 336 h.

**DISCUSSION**

The results of the present study provide the first description of the lethal and sublethal effects of BADGE epoxy resin on the early life cycle of an amphibian species, *R. arenarum*. In continuous exposure bioassays, the beginning of the early development (blastula) was the most sensitive stage to the resin, with toxicity being highest during the acute period. In the chronic period (336 h), only embryos exposed to BADGE in concentrations lower than 0.5 mg/L survived, but they developed morphological alterations. On the other hand, the toxicity profile of BADGE in larvae was time-dependent, with LC50 values that decreased 5 times from the acute to the chronic exposure period. Our findings suggest there may be an increased susceptibility as the central nervous system matures, rather than a bioaccumulation, because it is known that BADGE is metabolized to nontoxic substances [31]. The low potency of BADGE to cause teratogenicity in larvae is coincident with the slowing rate of morphogenetic changes toward the latter developmental stages associated with the higher teratogenic index for embryos than that in larvae. It is well established that a teratogenic index higher than 1.5 implies a high risk for embryos to be malformed in the absence of significant lethality [22]. In the present study, embryo lethality occurred above 0.0005 mg/L BADGE exposure, concentrations lower than the reported values in wastewater influents (0.00096–0.0016 mg/L) [2].

Although the 24-h pulse exposure concentrations were relatively high, this experimental design allowed us to simulate environmental emergency conditions, such as accidental spills. This information has important value in risk assessment analysis of industrial contaminants, such as BADGE. Our experimental design also allows associating certain effects with characteristic morphogenetic events of the development. According to our findings, early blastula was the most susceptible stage, and S.23 was at least 25 times more resistant. Moreover, the highest incidence of malformations was at the beginning of the development, particularly blastula and gastrula stages. In this last stage, sublethal effects such as yolk plug persistence were observed just 24 h postexposure in embryos exposed to the highest concentrations. It is known that epoxy resins induce cytotoxic action, specifically in tissues with high cellular division rates [32]. Also, Steiner et al. [33] reported that glycidaldehyde, a BADGE metabolite, binds to adenine nucleotides. Therefore, all early developmental adverse effects might be related to the capacity of BADGE to alter DNA. This has also been identified in yeast, rat, and in vitro human studies, particularly in human workers [13,31,32,34].

It is noteworthy that morphological abnormalities also affected the swimming ability of larvae, which is likely to interfere with their general performance in the natural...
environment. These observations confirm the importance of reporting not only lethality but also developmental disorders, which make organisms more vulnerable to predation or other environmental stressors such as infectious agents, invasive species, and changes in physical and chemical parameters of the environment, influencing the physical condition of animals or their reproductive success [35].

There is only one previous study on the neurotoxic effects of BADGE in rodents [36], which demonstrated that exposed organisms significantly reduced water and food consumption. In the present study, embryos exposed to BADGE from S.20 expressed behavioral alterations such narcosis, just a few hours after the beginning of treatment. It is noteworthy that these behavioral markers have relevance as early warning systems when other toxicity parameters such as lethality are absent.

Narcosis is an interesting effect with potential ecotoxicological consequences that might be brought about by numerous structurally unrelated chemicals in relation to their high octanol–water partition coefficients [37]. Bisphenol A and epichlorohydrin, chemicals used to synthesize the BADGE epoxy resin can also cause induced narcosis on R. arenarum larvae [38,39].

Developmental effects of BADGE were only previously described in rats receiving oral doses [40]. Hence, the results obtained in the present study provide real, valuable ecotoxicological information about its toxicity, as well as details of the main malformations and behavioral alterations of this epoxy resin in nonmammal species.

The increase of industrial wastes is just one of the many factors that can contribute to the decline of many amphibian populations [41]. Industrial wastewaters containing BADGE, as well as its migration substances and metabolites, represent populations of this native amphibian.

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REFERENCES

1. Terasaki M, Kazama T, Shirashi F, Makino M. 2006. Identification and estrogenic characterization of impurities in commercial bisphenol A diglycidyl ether (BADGE). Chemosphere 65:573–881. 2. Ballesteros-Gómez A, Ruiz FJ, Rubio S, Pérez-Bendito D. 2007. Determination of bisphenols A and F and their diglycidyl ethers in wastewater and river water by coevative extraction and liquid chromatography–fluorimetry. Anal Chem Acta 603:51–59.
3. Hammarling L, Gustavsson H, Svensson K, Oskarsson A. 2000. Migration of bisphenol-A diglycidyl ether (BADGE) and its reaction products in canned foods. Food Addit Contam 17:937–943.
4. Biedermann M, Grob K. 1998. Food contamination from epoxy resins and organosols used as can coatings: Analysis by gradient NPLC. Food Addit Contam 15:609–618.
5. Uematsuy Hirata K, Suzuki K, Iida K, Saito K. 2001. Chlorohydrins of bisphenol A diglycidyl ether (BADGE) and of bisphenol F diglycidyl ether (BFDGE) in canned foods and ready-to-drink coffees from the Japanese market. Food Addit Contam 18:177–185.
6. Stoker C, Rey F, Rodriguez H, Ramos JS, Siroksy P, Larriera A, Luque EH, Muñoz-de-Toro M. 2003. Sex reversal effects on Caínán laintrois exposed to environmentally relevant doses of the xenoestrogen bisphenol A. Gen Comp Endocrinol 133:287–96.
7. Durando M, Cocito L, Rodríguez HA, Varayoud J, Rams JA, Luque EH, Muñoz-de-Toro M. 2013. Neonatal expression of amh, sox9 and sf-1 mRNA in Caimán laintrois and effects of in ovo exposure to endocrine disrupting chemicals. Gen Comp Endocrinol 15:31–38.
8. Lyons G. 2000. Bisphenol A: A known endocrine disruptor. World Wildlife Federation European Toxics Programme, Godalming, Surrey, UK.
9. Olea N, Pulgar R, Pérez P, Olea-Serrano F, Rivas A, Novillo-Ferraz A, Sonnenschein C. 1996. Estrogenicity of resin-based composites and sealants used in dentistry. Environ Health Persp 104:298–305.
10. European Commission. 2002. Commission Directive 2002/16/EC of 20 February 2002 on the use of certain epoxy derivatives in materials and articles intended to come into contact with foods. Offic J Eur Union L51:27–31.
11. Shell Tunstall Toxicology Laboratory. 1981. Toxicity studies with diglycidyl ether of Bisphenol A, EPITOKE 828, EPITOKE 1001, EPITOKE 1007. EPA Document No. 782010037. Houston, Texas, USA.
12. Commission of the European Communities. 1988. Reports of the Scientific Committee on Food: Nineteenth series. EUR 11322. Office for Official Publications of the European Communities, Luxembourg.
13. Suárez S, Sueiro RA, Garrido J. 2000. Genotoxicity of the coating lacquer on food cans, bisphenol A diglycidyl ether (BADGE), its hydrolysis products and a chlorohydrin of BADGE. Mutat Res 470:221–228.
14. Pérez-Coll C, Herkovits J. 1990. Stage dependent susceptibility to lead in Bufo arenarum embryos. Environ Poll 63:239–245.
15. Ferrari A, Angiulino L, Lascano C, Sotomayor V, Rosenbaum E, Venturino A. 2008. Changes in the antioxidant metabolism in the embryonic development of the common South American toad Bufo arenarum: Differential responses to pesticide in early embryos and autonomous-feeding larvae. J Biochem Mol Toxicol 22:259–267.
16. Herkovits J, Pérez-Coll CS, Herkovits FD. 2002. Ecotoxicological studies of environmental samples from Buenos Aires area using a standardized amphibian embryo toxicity test (AMPHITOX). Environ Pollut 116:177–183.
17. Herkovits J, Pérez-Coll CS. 2003. AMPHITOX: A customized set of toxicity tests employing amphibian embryos. In Linder GL, Krest S, Sparling D, Little EE, eds, Multiple Stressor Effects in Relation to Declining Amphibian Populations. STP111745. ASTM International, West Conshohocken, PA, USA, pp 46–60.
18. Pisanó A. 1957. Eficiencia funcional y estructura delle’ipoofisideanfibio. Arch Zool Ital 42:221–227 (in Italian).
19. Mann RM, Bidwell JR. 2000. Application of the FETAX protocol to assess the developmental toxicity of nonylphenol ethoxylate to Xenopus laevis and two Australian frogs. Aquat Toxicol 51:19–29.
20. Knapton AN, Roulston C, Stepenson GR, Boermans HJ. 2007. 2,4-D butoxyethyl ester kinetinics in embryos of Xenopus laevis: The role of the embryonic jelly coat in reducing chemical absorption. Arch Environ Contam Toxicol 52:113–120.
21. Räsänen K, Palkala M, Laurila A, Merilä J. 2003. Does jelly envelope protect the common frog Rana temporaria embryos from UV-B radiation? Herpetologica 59:293–300.
22. ASTM International. 1993. Standard guide for conducting the frog embryo teratogenesis assay-Xenopus (FETAX). E1439-12. In Annual Book of ASTM Standards, Vol 11.06. West Conshohocken, PA, USA pp 1199–1209.
23. Del Conte E, Sirlin L. 1951. The first stages of Bufo arenarum development. Acta Zool Lillo 12:495–499.
24. Bantle JA, Dumont JN, Finch RA, Linder G, Fort DJ. 1998. 2,4-D butoxyethyl ester kinetics in embryos of Xenopus laevis: A study with endosulfan on frog tadpoles. Ecotoxicology 12:1400–1250.
25. Denoël M, D’hooghe B, Ficetola GF, Brasseur C, De Pauw E, Thomé JP, Kestemont P. 2012. Using sets of behavioural biomarkers to assess short-term effects of pesticide: A study case with endosulfan on frog tadpoles. Environ Health Persp 31:1209.
26. US Environmental Protection Agency. 1999. Trimmmed Spearman– Karber Estimation of LC50 Values Users’ Manual. Office of Research and Development, Athens, Georgia.
27. Herkovits J, Helguero LA. 1998. Copper toxicity and copper-zinc interactions in amphibian embryos. Sci Total Environ 221:1–10.
28. American Public Health Association, American Water Works Association, Water Environment Federation. 2005. Standard Methods for the Examination of Water and Wastewater, 21st ed. Washington, DC.
29. Di Rienzo JA, Guzmán AW, Casanoves F. 2002. A multiple comparisons method based on the distribution of the root node distance of a binary tree. J Agric Biol Environ Stat 7:1–14.
30. Di Rienzo JA, Casanoves F, Balzarini MG, Gonzalez L, Tablada M, Robledo CW. 2015. Grupo InfoStat. Universidad Nacional de Córdoba, Argentina. [cited 2016 April 19] Available from: http://www.infostat.com.ar

31. Poole A, Van Herwijnen P, Weideli H, Thomas MC, Ransbotyn G, Vance C. 2004. Review of the toxicology, human exposure and safety assessment for bisphenol A diglycidyl ether (BADGE). Food Addit Contam 21:905–919.

32. Andersen M, Kiel P, Larsen H, Haxild J. 1978. Mutagenic action of aromatic epoxy resins. Nature 276:391–392.

33. Steiner S, Crane AE, Watson WP. 1992. Molecular dosimetry of DNA adducts in C3H mice treated with glycidaldehyde. Carcinogenesis 13:119–124.

34. Scientific Committee on Food. 1999. Opinion on bisphenol A diglycidyl ether (BADGE). SCF/CS/PM 3243 Final. European Commission. [cited 2016 April 19]. Available from: http://ec.europa.eu/food/fs/sc/scf/out28_en.pdf

35. Semlitsch RD. 1990. Effects of body size, sibship, and tail injury on the susceptibility of tadpoles to dragonfly predation. Can J Zool 68:1027–1030.

36. Yang YJ, Lee SY, Kim KY, Hong YP. 2010. Acute testis toxicity of bisphenol A diglycidyl ether in Sprague-Dawley rats. J Prev Med Public Health 43:131–137.

37. Sandermann H. 2008. Ecotoxicology of narcosis: Stereoselectivity and potential target sites. Chemosphere 72:1256–1259.

38. Hutler Wolkowicz IR, Aronzon CM, Perez Coll CS. 2013. Lethal and sublethal toxicity of the industrial chemical epichlorohydrin on Rhinella arenarum (Anura, Bufonidae) embryos and larvae. J Hazard Mater 263:784–791.

39. Hutler Wolkowicz I, Herkovits J, Perez-Coll CS. 2014. Stage-dependent toxicity of Bisphenol A on Rhinella arenarum (Anura, Bufonidae) embryos and larvae. Environ Toxicol 29:146–154.

40. Un-jun H, Yun-jung Y, Su-kyoung K, Jae-hyoung Y, Soon-chul M, Sae-chul K, Yeon-pyo H. 2007. Developmental toxicity by exposure to bisphenol A diglycidyl ether during gestation and lactation period in Sprague–Dawley male rats. J Prev Med Public Health 40:155–161.

41. Beebee TJC, Griffiths RA. 2005. The amphibian decline crisis: A watershed for conservation biology? Biol Conserv 125:271–285.