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Lethal and sublethal toxicity of the industrial chemical epichlorohydrin on *Rhinella arenarum* (Anura, Bufonidae) embryos and larvae

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HIGHLIGHTS

- ECH caused morphological abnormalities and neurotoxicity in *Rhinella arenarum*.
- ECH caused severe teratogenic effects in *Rhinella arenarum* early embryos.
- The most sensitive stage to ECH for lethality was early blastula (S.3–S.4).
- The most resistant stage was the gill circulation stage (S.20).
- The most sensitive stage for sublethal effects was also blastula.

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ABSTRACT

Lethal and sublethal toxicity of the major chemical used in epoxide compounds, epichlorohydrin (ECH) was evaluated on the early life cycle of the common South American toad, *Rhinella arenarum* (Anura, Bufonidae). The stages evaluated were (according to Del Conte and Sirlin): early blastula (S.3–S.4), gastrula (S.10–S.12), rotation (S.15), tail bud (S.17), muscular response (S.18), gill circulation (S.20), open mouth (S.21), opercular folds (S.23) and complete operculum (S.25). The LC50 and EC50 values for lethal and sublethal effects were calculated. The early blastula was the most sensitive stage to ECH both for continuously and pulse-exposures (LC50-24 h = 50.9 mg L^{-1}), while S.20 was the most resistant (LC50-24 h = 104.9 mg L^{-1}). Among sublethal effects, early blastula was also the most sensitive stage (LOEC-48 h = 20 mg L^{-1}) and it has a Teratogenic Index of 2.5, which indicates the teratogenic potential of the substance. The main abnormalities were persistent yolk plugs, cell dissociation, tumors, hydropsy, oral malformations, axial/tail flexures, delayed development and reduced body size. ECH also caused neurotoxicity including scarce response to stimuli, reduction in the food intake, general weakness, spasms and shortening, erratic or circular swimming. Industrial contamination is considered an important factor on the decline of amphibian populations. Considering the available information about ECH's toxicity and its potential hazard to the environment, this work shows the first results of its developmental toxicity on a native amphibian species, *Rhinella arenarum*.

1. Introduction

The effects of industrial contamination on wildlife represent a continuing restlessness. Synthetic chemicals pollute the natural environment during production processes, use and waste, and they can be taken up by living organisms. Among them, epichlorohydrin (ECH or 1-chloro-2.3-epoxypropane CAS Registry No.106-89-8) is an organic, volatile compound of the epoxide family. ECH is mainly used in the manufacture of glycerin, epoxy and phenoxy resins and, to a lesser extent, in the manufacture of elastomers, surfactants, ion exchange resins, plasticizers, dyestuffs, pharmaceutical products, oil emulsifiers, lubricants adhesives and water treatment resins [1], even knowing it is neither safe nor environmentally friendly [2]. It was estimated an annual production capacity of 100.000 million tons of ECH for a unique plant in Thailand which began operations in the first quarter of 2012 [3].

Thus, the increase of the use of ECH could threat the biodiversity representing a health risk for human and wild life organisms. As a result of its manufacture, use, storage or accidental spills

Abbreviations: ECH, epichlorohydrin; EPA, Environmental Protection Agency (USA); LD, lethal dose; LC, lethal concentration; LOEC, lowest observed effect concentration; EC, effective concentration; TI, Teratogenic Index; AS, AMPHITOX solution.

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and industrial wastewater discharges, ECH is released into the environment, both in air and liquid wastes. ECH has a small $\log K_{ow}$ (0.3–0.45) suggesting a low potential for bioaccumulation in aquatic organisms. However, as ECH has small $\log K_{oc}$ value, high solubility (66 g L^{-1} 20 °C) and half-life of 4–8 d in water, it is expected to be available for organisms [4].

In relation to human health, it is concerning the use of ECH in the reinforcement of paper for applications such as tea bags [3]. More importantly, a potential source of ECH is drinking-water due to its use as clarifier during water treatment. When added to water, ECH coagulates and traps suspended solids for easier removal. However, some free ECH may not coagulate and remain dissolved in water available as a contaminant [5]. ECH is readily absorbed and metabolized in glycidol and alpha-chlorohydrin (3-chloro-1,2-propanediol) [6–10]. EPA requires that the amount of uncoagulated ECH used in drinking water should be less than 2 μg L^{-1} [5].

There is very scarce information related to the toxicity of ECH and its metabolites in non-human organisms. For acute exposure, LD50s from 90 to 360 mg L^{-1} in rats [5,10] and LC 50 s from 18–35 mg L^{-1} in fishes and 10.6–72 mg L^{-1} in *Daphnia magna* were reported [4]. Few reports have determined the long term effects following ECH oral administration in rats, like gradual increase in lethality, weight losses, decrease in leukocytes and hyperplasia of the forestomach [11,12]. Moreover, there is an increasing concern on ECH as an antifertility agent mainly related to male reproductive organs. It was reported that 50 mg/kg ECH in male rats elicited apoptotic changes in epididymal tissue in 12 h [13] and 70 mg/kg ECH decreases sperm motility on day 7 [14]. With respect to alpha-chlorohydrin, it has been shown to be a potent male antifertility agent in rats [9].

Mutagenic effects of ECH were examined since 1978 by Bridges [15] in mammals and prokaryotes both *in vivo* and *in vitro* assays. Carcinogenicity in rats was reported as forestomach tumors, carcinomas, and sarcomas [10,16]. Moreover, ECH affects the G1/S progression or cell arrestment in human fibroblasts [17]. At subcellular level, it was revealed that ECH causes sister chromatid exchanges in exposed workers, DNA strand breaks cells, chromosomal aberrations in rodent cells, micronuclei and reactions with nucleophilic functionalities in DNA and proteins [6,18]. Based on bacterial mutagenicity and cytogenetic assays, ECH induces DNA damage directly and it is also a clastogen *in vitro* as well as *in vivo* models [19]. Both EPA [20] and the International Agency for Research on Cancer [18] classified ECH as probable carcinogen in groups 2A and B2 respectively. Moreover, the Maximum Contaminant Level Goals (MCLG) for ECH has been set at zero by EPA in order to avoid health risks [5].

The exposure of amphibians to an increased diversity of contaminants is considered as one of the most likely cause of the decline of their populations. The interference of physicochemical agents with the most sensitive periods of their life cycle, the embryo-larval development, could lead to failures in the dynamics of their populations, modifying their attributes as well as the structure and function of ecosystems and lastly the lost of biodiversity. This is a major concern for amphibian biodiversity protection worldwide since the 1960s [21]. There are many toxicological studies involving amphibians like *Rhinella* sp and *Xenopus* sp, because they are more sensitive than other vertebrate species to aquatic contaminants [22–26]. This is the reason they are useful for ecotoxicological studies and widely used to perform toxicity bioassays [27–32].

It is important to evaluate the effects of emergent pollutants in order to elucidate their possible toxicity on living organisms. To the best of our knowledge, no studies have investigated the toxic effects of ECH on amphibians. To this end, the current study was performed on early developmental stages of *Rhinella arenarum* (Anura, Bufonidae), a native toad from South America found from the coastal southern Brazil and from the east Bolivia to the south of Argentina [33]. This species has been widely used in ecotoxicological studies and hazard assessment due to its high sensitivity to a wide diversity of environmental contaminants mainly at embryonic and larval stages [34,35]. The knowledge of stage-dependent susceptibility to toxic agents contributes to basic scientific data in relation to differential sensitivity throughout the morphogenetic course. Moreover, these studies are relevant from an environmental perspective because they provide useful information in case of spills with concentrated chemicals.

In this context, the main aim of this work was to evaluate the lethal and sublethal effects of ECH on *R. arenarum* embryos and larvae by means of acute (for 96 h), short-term chronic (for 7 d), chronic (for 14 d) and 24 h pulse-toxicity bioassays. Sublethal toxicity was reported by performing the analysis of morphogenesis failures and behavioral alterations with optical and scanning electron microscopy.

2. Materials and methods

2.1. Obtaining R. arenarum embryos and larvae

Adult females and males were obtained in Lobos (Buenos Aires province, Argentina: 35°11′ S; 59°05′ W) from a local provider. Ovulation of the female toads was induced by means of intraperitoneal injections of a suspension of homologous hypophysis in AMPHITOX Solution (AS) per female preserved according to Pisanó [36] plus 5000 UI human chorionic gonadotropin (hCG). This combined procedure was performed in order to improve ovulation. Oocytes were fertilized *in vitro* using fresh sperm suspended in AS and embryos were kept in this physiological solution at 20 ± 2 °C until they reach the stage required for experimental exposures [37]. For studies with early life stages, embryos used before hatching (S.18) were previously dejellied by immersing them into a 2% thioglycolic acid solution at pH 7.2–7.4 for 2 min, followed by a thorough wash of the embryos. Larvae were fed with Tetra Color Fin Sinking Granules for Goldfish *ad libitum* every other day.

2.2. Chemicals and solutions

Technical-grade epichlorohydrin (ECH, CAS Registry No. 106-89-8) with a purity of 98% was obtained from Fluka Laboratories. A stock solution was prepared in analytical grade acetone to a final concentration of 1.16×10^5 mg L^{-1} ECH and experimental solutions were obtained by diluting the ECH stock solution with AS. Acetone concentrations were always lower than 1.1% [38]. Control embryos were simultaneously kept in AS and AS plus acetone at the highest concentration. The composition of AS was sodium chloride (NaCl) 36 mg L^{-1}, potassium chloride (KCl) 0.5 mg L^{-1}, calcium chloride ($CaCl_2$) 1 mg L^{-1} and sodium bicarbonate ($NaHCO_3$) 2 mg L^{-1}, prepared in distilled water.

2.3. Toxicity bioassays

Ten *R. arenarum* embryos/larvae were placed in 10 cm-diameter glass Petri dishes containing 40 mL of ECH solution (by triplicate) in at least five different concentrations according the following experimental conditions:

(1) Continuous exposure from early blastula (S.3–S.4) onwards: embryos were treated with concentrations ranging from 5 to 70 mg L^{-1} ECH for 14 d.
(2) Continuous exposure from complete operculum stage (S.25) onwards: early larvae were treated with concentrations ranging from 10 to 70 mg L^{-1} ECH during 14 d.
(3) Stage-dependent (pulse) exposure: embryos were treated during 24 h with concentrations from 10 to 90 mg L^{-1} ECH starting

at: early blastula (S.3–S.4), gastrula (S.10–S.12), rotation (S.15), tail bud (S.17), muscular response (S.18), gill circulation (S.20), open mouth (S.21), opercular folds (S.23) and complete operculum (S.25) stages. After exposure, embryos were thoroughly washed, kept in AS and evaluated up to 14 d.

Test media were renewed every 48 h throughout the development and dead organisms were removed every day.

In all experiments, survival and sublethal effects were evaluated each 24 h. Bioassays were performed with at least three batches from different parents.

Abnormalities were studied by means of stereoscopic microscopy (Zeiss Stemi DV4), identified according to the "Atlas of Abnormalities" [39] and photographed with an Olympus X-42 digital camera. In addition, other embryos were fixed in formol 4%, dehydrated in a gradient of ethanol, prepared for scanning electron microscopy (SEM) by means of the critical point technique and observed in a Philips XL-30 operated at 10 KW for ultrastructure evaluation.

2.4. Data analyses

Lethal and sublethal effects were statistically analyzed as LC50 and EC50 respectively with 5 [40]. To establish statistical differences between the LC50 values, a comparison was made considering the difference statistically significant when the higher LC50/lower LC50 exceeded the critical value (95% confidence interval) established by APHA [41]. The survival percentages were compared using Pearson's chi-square (χ^2) test to establish significant differences between exposed and control embryos. The Teratogenic Index (TI) was calculated as LC50/EC50 at 96 h of exposure [42].

3. Results

3.1. Lethal effects

3.1.1. Continuously exposed embryos from early blastula (S.3–S.4) onwards

No embryos exposed over 40 mg L^{-1} survived beyond 48 h (Fig. 1). Thereafter, lethality increased only 20% but reaching short-term chronic exposure (7 d), only survived 50% and 20% embryos exposed to 25 mg L^{-1} and 30 mg L^{-1} ECH respectively. Toward the end of the bioassay none embryos survived over 30 mg L^{-1} ECH. Moreover, 70% embryos exposed to the lower concentrations, 5 mg L^{-1} and 10 mg L^{-1} ECH and 13% embryos at 20 mg L^{-1} and 25 mg L^{-1} ECH survived up to 14 d, respectively.

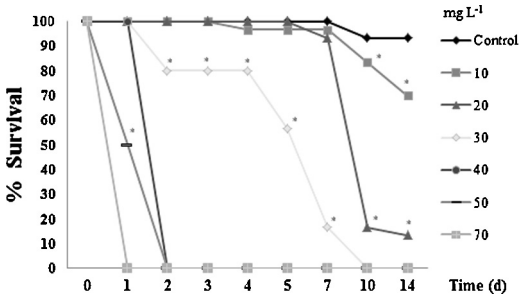

Fig. 1. Concentration–response relationship for *Rhinella arenarum* embryos continuously exposed to ECH from S.3–S.4 describing the rate of survival from the beginning of the exposure up to 14 d. *Significantly different with respect to control group ($P < 0.05$).

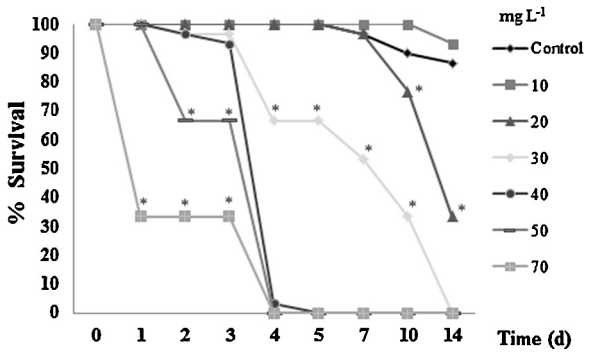

Fig. 2. Concentration–response relationship for *R. arenarum* larvae continuously exposed to ECH from S.25 describing the rate of survival from the beginning of the exposure up to 14 d. *Significantly different with respect to control group ($P < 0.05$).

3.1.2. Continuously exposed larvae from complete operculum stage (S.25) onwards

Only 30% larvae exposed to the highest concentration, 70 mg L^{-1} survived during the first 24 h. Concentration–response curves (Fig. 2) show an abrupt decline of survival after 72 h onwards, and non larvae survived over 30 mg L^{-1} ECH. Only 30% larvae exposed to 20 mg L^{-1} ECH survived up to the end of the bioassay, with non-significant differences between control and concentrations lower than 10 mg L^{-1}.

A comparison of LC50s for continuous exposures to ECH starting from S.3–S.4 to S.25 is plotted in Fig. 3. The LC50-24 h value was significantly higher in larvae than in embryos until 72 h, showing a greater initial resistance of larval stages. The toxicity profile curve based on LC50 values for embryos exposed from S.3–S.4 shows an early increase in toxicity that did not change significantly on time ($P > 0.05$) up to 5 d. In contrast, this curve for *R. arenarum* exposed from S.25 describes a greater initial resistance during the first 72 h followed by an abrupt mortality toward 96 h, reaching a LC50 up to half the initial value. At 14 d, the end of ECH exposure, LC50 for embryos and larvae reached similar values although statistically different from each other ($P < 0.05$).

3.1.3. Stage-dependent (pulse) exposure

The stage-dependent susceptibility of *R. arenarum* embryos and early larvae to ECH evaluated at nine developmental stages is represented in Fig. 4. The profile suggests that early developmental stages up to muscular response (S.18), are highly sensitive to ECH being early blastula the most susceptible stage with a LC50-24 h of 50.9 mg L^{-1} ECH. There were almost non-significant differences

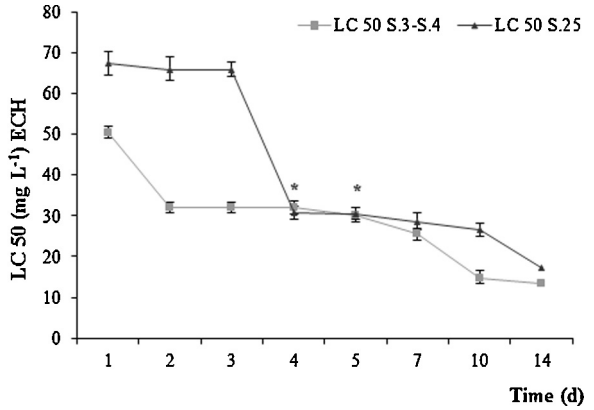

Fig. 3. Lethal concentration 50 (LC50) and their confidence limits of ECH on *R. arenarum* embryos and larvae continuously exposed for 14 d. *No significantly different between both developmental stages ($P > 0.05$).

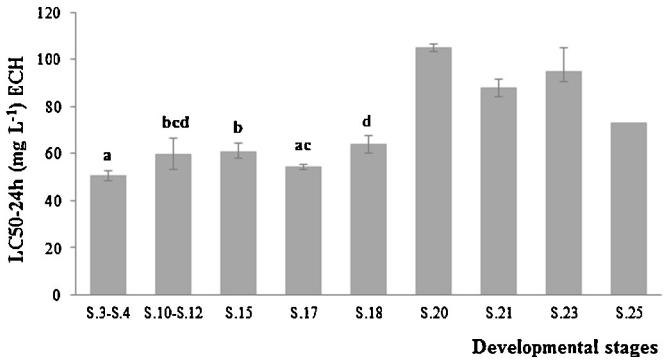

Fig. 4. Stage-dependent susceptibility of *R. arenarum* embryos and early larvae for 24 h pulse-exposure to ECH plotted as the LC50s and their corresponding confidence limits (95%). Same letters indicate non-significant differences ($P > 0.05$)

among the early developmental stages. Conversely, gill circulation (S.20) was the most resistant over the remainder of the stages, with a LC50-24 h of 104.9 mg L^{-1}. Fig. 5 compares the toxicity profile curves of pulse-exposed embryos corresponding to these extremely different responses to ECH after the end of exposure. Basically, S.20 was twice resistant than early blastula stage even up to the end of the evaluation.

In addition, lethality of control embryos and larvae in all the experimental conditions was always less than 10%.

3.2. Sublethal effects

For all experimental designs, sublethal effects were expressed as morphological abnormalities, which involve teratogenesis and reduced body size, and neurological alterations.

3.2.1. Continuously exposed embryos from early blastula (S.3–S.4) onwards

LOEC values for sublethal effects were 20 mg L^{-1} and 5 mg L^{-1} at 48 h and 7 d respectively. The main morphological effects were persistent yolk plugs, cell dissociation, tumors, hydropsy, oral region malformations (Fig. 6), skin surface irregularities, tail flexures, delayed development and reduced body size. Toward the end of acute exposure (96 h) the TI was 2.5.

Concerning development progress, almost 70% embryos exposed to 30 mg L^{-1} ECH showed delayed development. This sublethal effect was associated with reduced body size as exposure continued up to short-term chronic period (7 d) coincident with the end of embryonic development, affecting by this time those embryos exposed below 30 mg L^{-1} ECH.

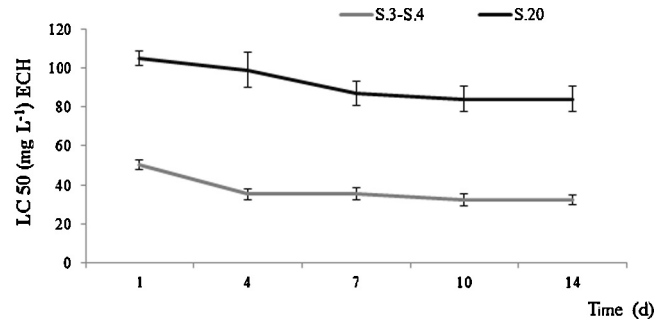

Fig. 5. ECH lethal concentration 50 (LC50) and their confidence limits of pulse-exposed early blastula (S.3–S.4) and gill circulation (S.20) *R. arenarum* embryos.

3.2.2. Continuously exposed larvae from complete operculum stage (S.25) onwards

LOEC values for sublethal effects were 50 mg L^{-1} and 20 mg L^{-1} ECH at 48 and 7 d, respectively. Ethological effects as scarce response to stimuli, food intake reduction, general weakness, spasmodic contractions and shortening, erratic or circular swimming related to neurotoxicity were observed.

3.2.3. Stage-dependent (pulse) exposure

LOEC values and the main sublethal effects, reported in 24 h pulse-exposed *R. arenarum* embryos, are shown in Fig. 7 and Table 1. As result of these short exposures, early blastula was the most sensitive stage, with a LOEC of 30 mg L^{-1} whereas S.20 was the most resistant with a LOEC of 70 mg L^{-1}.

4. Discussion

This work shows the toxic effects of the industrial compound epichlorohydrin (ECH), an organic chemical with increasing concern due to its high production which has steadily increased with 276, 330 and 640 million pounds in 1977, 1982 and 1990, respectively [19]. According to the Consulting's Directory of Chemical Producers [42], total ECH consumption will grow at an average annual rate of slightly over 6%.

In view of the scarce information about ECH toxicity on aquatic biota, this study provides the first results of its lethal and sublethal effects on the early life cycle of an amphibian species, the native South American toad, *R. arenarum*. Thus, it was evaluated the differential susceptibility to ECH throughout the early embryo-larval development, by means of continuous treatments and 24 h pulse-exposures during several developmental stages.

Regarding continuous exposure, lethal and sublethal effects estimated from LC50 and LOEC values, revealed that early blastula, characterized by the segmentation of embryo, was the most sensitive stage. This is most likely related to the ECH interference with DNA whose synthesis plays a crucial role in the earlier embryonic development [19].

Early larvae in continuous treatment were more resistant than blastula, but only during the acute period. The pattern of increased toxicity over time suggests that the bioaccumulation of ECH or more likely, its metabolites occurs until a threshold level, from which the toxicity becomes incompatible with life. To our knowledge, there is no data about the metabolism of ECH in aquatic biota. However, information of mammalian models indicates that ECH has a short half-life and the major metabolic route is GSH conjugation and hydrolysis reaction such as epoxide hydrolase, leading to a much more persistent metabolite, alpha-chlorohydrin [8].

LC50 is the usual acute toxicological parameter to estimate the lethal toxicity of a xenobiotic. However, the curve obtained from the LC50 values for different exposure times between 24 h and the end of chronic exposure yielded very valuable additional data, by informing the concentrations exerting the same degree of adverse effects during different exposure periods; otherwise, the overall toxic potential of a xenobiotic could not be apparent if it is consider an unique acute value. In this case, the LC50-48 h indicates that the ECH concentration necessary to cause lethal effect is twice for amphibian larvae than embryos. Also, the curve describes that after the first 24 h, lethality gradually occurred in embryos until 7 d, coinciding with the onset of larval development. Nevertheless, the LC50 profile curve for larvae at S.25 shows a constant survival rate during the first 96 h, but then susceptibility significantly increases and, as ECH exposure progresses, LC curves reach a similar profile both for embryos and larvae but even significantly different between them. This fact indicates the concentrations that produced

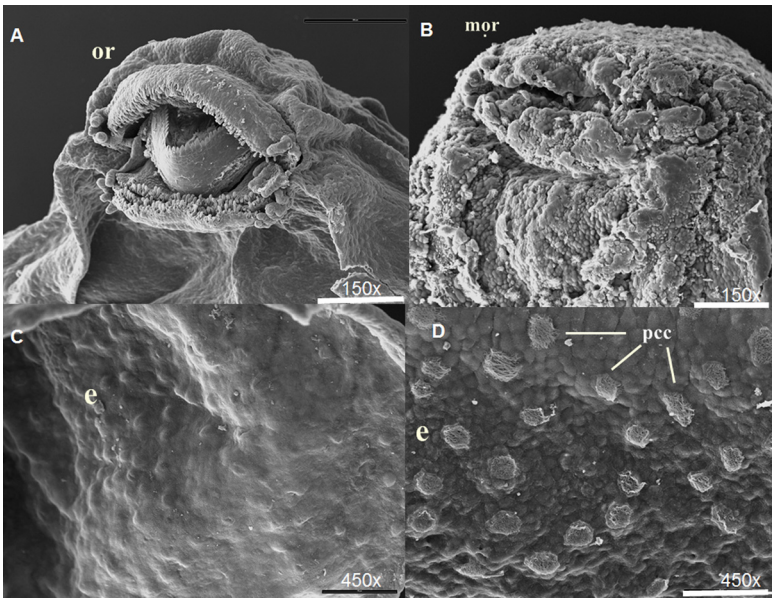

Fig. 6. Scanning electron microscopy of *R. arenarum* continuously exposed to ECH from S.3–S.4. (A) Oral region (or) of a control larva (S.25). (B) Malformed oral region (mor) of a larva exposed to 30 mg L^{-1}. (C) Epithelium (e) of a control larva tail. (D) Epithelium of a larva tail exposed to 10 mg L^{-1}. Note the persistent ciliated cells (pcc) which indicate delayed development.

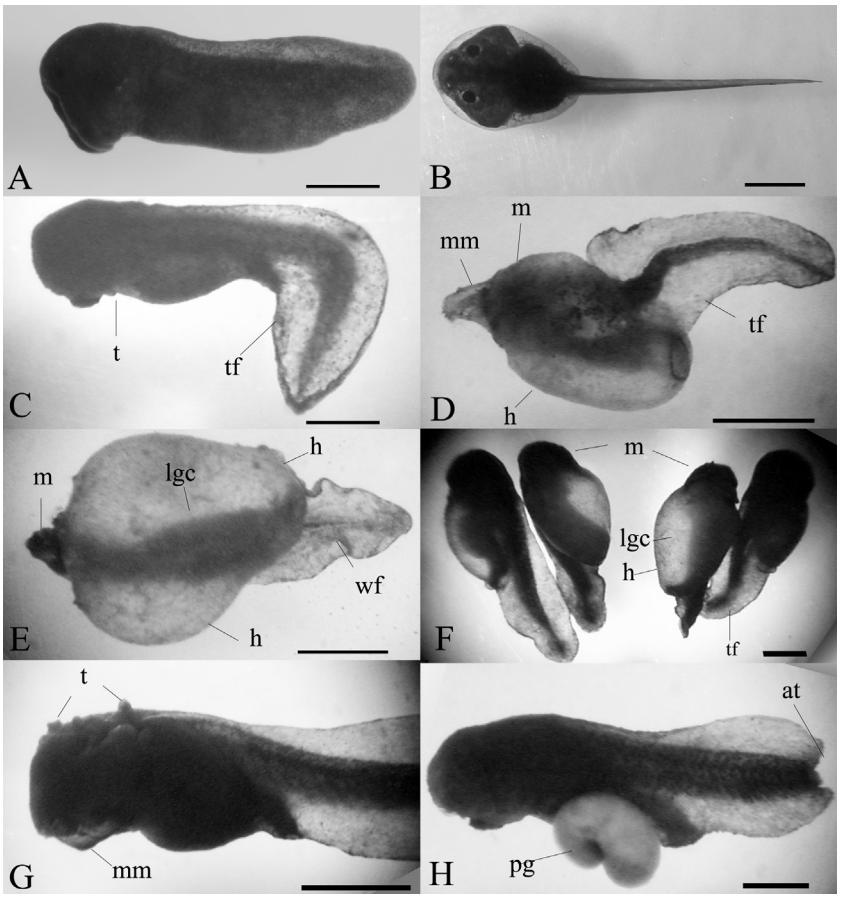

Fig. 7. Stereoscopic microscopy of 24 h pulse-exposed *R. arenarum* embryos to ECH at different developmental stages: (A) Control embryo in S.18. (B) Control embryo in S.25. (C) S.3–S.4 (30 mg L^{-1}) Observe tail flexures (tf) and tumor (t). (D) S.10–S.12 (45 mg L^{-1}). Note tail flexures (tf), microcephaly (m), malformed mouth (mm), hydropsy (h) and reduced body size. (E) S.17 (60 mg L^{-1}). Notice microcephaly (m), hydropsy (h), lack of gut coiling (lgc) observed by transparency, waving fin (wf) and the reduced body size and tail. (F) S.18 (75 mg L^{-1}). Point out the hydropsy (h), microcephaly (m), lack of gut coiling (lgc) observed by transparency and reduced body size and tail. (G) S.20 (75 mg L^{-1}). Notice several tumors (t) and malformed mouth (mm). (H) S.23 (60 mg L^{-1}). Note the protrusion of gut (pg) and abnormal end tail (at). Scale bar 1 mm.

Table 1
LOEC values and most common sublethal effects produced by ECH 24 h pulse-exposures at different developmental stages of *Rhinella arenarum* embryos and larvae.

	LOEC-24 h (mg L^{-1}) ECH	Main sublethal effects at 24 h	Main sublethal effects at 168 h
S.3–S.4 (early blastula)	30	Cell dissociation Persistent yolk plug	Delayed development Oral desquamation Reduced body size Waving fin Axial and tail flexures Tumors Microcephaly Scarce response to stimuli
S.10–S.12 (gastrula)	45	Cell dissociation Reduced body size Axial flexures	Delayed development Reduced body size Oral desquamation Hydropsy Anencephaly Microcephaly Malformed mouth Lack of gut coiling Prominent abdomen Waving fin Axial and tail flexures Erratic and shortening swimming
S.17 (tail bud)	50	Delayed development Reduced body size Prominent abdomen Scarce response to stimuli	Reduced body size and tail Waving fin Hydropsy Microcephaly Malformed mouth Lack of gut coiling Tumors Axial flexures Scarce response to stimuli
S.18 (muscular response)	50	Reduced body size	Reduced body size and tail Tail flexures Reduced tail Hydropsy Abnormal skin pigmentation Microcephaly Malformed mouth Lack of gut coiling Scarce response to stimuli
S.20 (gill circulation)	70	Skin surface irregularities Axial flexures Scarce response to stimuli	Reduced body size and tail Microcephaly Malformed mouth Irregular borders of tail Abnormal end tail Lack of feeding Scarce response to stimuli
S.21 (open mouth)	60	Tumors Hydropsy Waving fin	Reduced body size and tail Tumors Neurotoxicity
S.23 (opercular fold)	50	Tumors Oral desquamation Edema Scarce response to stimuli	Reduced body size Abnormal end tail Skin surface irregularities Protrusion of gut Neurotoxicity
S.25 (complete operculum)	50	Abnormal skin pigmentation Tail flexures Spasmodic contractions Scarce response to stimuli	Reduced body size Neurotoxicity

50% lethality in embryos are similar to those causing lethality on larvae by extending the exposure time.

Pulse-exposures to high concentrations of a xenobiotic during certain embryonic and early larval stages extend toxicological data on differential susceptibility for early life cycle of a species. This is relevant due to amphibians, key components of ecosystems, have several characteristics of their inherent nature such as external development, biphasic life cycle, *etc.*, which make them extremely sensitive to eventual spills with high concentrations of a specific xenobiotic. This information could be of important value in risk assessment analysis of emergent contaminants as in this case, ECH or its metabolites. Moreover, this experimental design allows associating determined effects with characteristic facts of their morphogenesis. Moreover, the stage-dependent exposure allows finding toxic effects not only during the exposure but even later, on subsequent developmental stages. Indeed, this delayed effect was most pronounced in blastula throughout time than the remainder stages.

Embryogenesis is a critical period in normal morphogenesis including the development of the incipient central nervous system. Abnormal embryos affected by ECH had, in most cases, a combination of different malformations and neurological effects. In this

context, the first manifestations of teratogenicity caused by ECH in embryos were the persistent yolk plugs, cell dissociation and tumors. Although the mode of action of ECH is not very clear, the tumors observed in experimental animals may be a result from its direct interaction with genetic material [18,19]. Thus, most epoxide compounds including ECH, showed direct mutagenic effects on mammals, as rat and mice, invertebrates as *Drosophila* and different prokaryotic models as *Salmonella* strains and *Escherichia coli*. [19]. ECH also belongs to the so-called "radiomimetic" genotoxic chemicals which induce similar biological end points as ionizing radiation including gene mutation, DNA strand breaks and neoplastic cell transformation [43]. Moreover, several studies were focused on ECH's ability to induce DNA damage, like in ECH-workers in which were highlighted chromosomal aberrations even at exposures below 0.26 mg L^{-1} [44] and the development of central nervous system neoplasmas [45]. On the other hand, Laskin et al. [10] reported that a high dose (100 mg L^{-1}) for 30 d produced a much greater tumor response than low dose (10–30 mg L^{-1}) for longer exposures (250–290 d) in rats. Then, Ginsberg et al. suggested that ECH dose rate (peak dose achieved) is more important than the cumulative dose in determining the degree of carcinogenic effect at contact sites. In present study, tumors also appeared in embryos exposed for short time periods, even at 24 h confirming that is not necessary to extend exposure to obtain carcinogenic effects of ECH [8].

As development advances, skin surface irregularities, waving fin, hydropsy, microcephaly, anencephaly, oral region desquamations and tail flexures were reported. As previously discussed, most information about ECH's toxicity is related to its carcinogenicity and mutagenicity and there is scarce information related to morphological effects. Previous reports on ECH toxicity in mammals during a short exposure time showed that 100 mg L^{-1} ECH increased kidney weight, produced dilatation of the renal tubules and swellings of tubular epithelial cells [11]. Also, rats exposed to lower ECH doses such as 2 mg L^{-1} and 10 mg L^{-1} during 2 years showed clinical symptoms included dyspnea, weight loss, decrease in leukocytes and hyperplasia in the forestomach [12].

The reduced body size was the most conspicuous effect of embryos continuously exposed from early blastula, reaching 100% of the organisms at 7 d. It was also remarkable the delayed development observed at cellular level, as the persistence of ciliated cells especially in early exposed embryos. Both delayed development and reduced body size are usual adverse effects produced during early life stages of this species by environmental stressors, for example agrochemicals as atrazine [46] and triphenilthin [25], other organic compounds as bisphenol A [23], phenol [29] nanomaterials [28] and metals as copper [47,48]. In the case of ECH, it was frequently observed alterations in the eating behavior with a clear cut suspension of food intake in the larval stage undoubtedly associated with the reduced body size. There are few reports related to body weight reduction after ECH exposure but only in adult's rats [11,49,50].

The Teratogenic Index (TI) is a measure of hazard of a determined chemical on the early development of a species; if it is higher than 1.5 signifies a greater potential to obtain all embryos malformed in absence of significant mortality [38]. The wide range between lethal and effective concentrations of ECH indicates teratogenesis is a relevant end point to assess the population viability that might be affected by reduced fitness of individuals.

With respect to neurological effects, scarce response to stimuli, reduction in the food intake, general weakness, shortening, erratic or circular swimming and spasmodic contractions were expressed in advanced stages. It is noteworthy that embryos exposed only during blastula developed in larvae with central nervous system defects. This fact might be due to the neurotoxic effect of ECH on cells destined to become neurons. As it was mentioned above, reductions in food and water consumption were also reported in ECH exposed adult rats [51,52]. Moreover, depression and piloerection were reported in females after ECH exposure during reproduction [53]. In conclusion, the teratogenic and neurotoxic effects of ECH on *R. arenarum* embryos and early larvae demonstrate the importance of evaluating not only lethal but also sublethal effects of this emergent contaminant.

Nowadays, the discharge of chemicals into the environment is a serious problem affecting both water and soil quality. The use of ECH as clarifier in drinking water and the possibility to find some uncoagulated ECH available [5] highlights the importance of reporting ECH's effects on human but also on aquatic organisms. During the last decades amphibians have been threatened by multiple environmental stressors, including industrial contaminants. These xenobiotics contribute strongly to the decline of their populations not only by direct lethality of eggs, embryos or larvae but also indirectly by means of sublethal exposure which produce malformations, delayed growth and/or metamorphosis, affecting the normal behavior, the ability of organisms to avoid predation and subsequently impairing their ability for future reproduction [54]. Finally, to our knowledge, these are the first results of the developmental toxicity of ECH on an amphibian species, the South American toad *R. arenarum*.

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