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Lethal and teratogenic effects of phenol on *Bufo arenarum* embryos

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ABSTRACT

Phenol and their derivatives are used in several industries and they have a high potential toxicity for animal and plant species. They were found in variable concentrations, as high as 1000 mg/L, in industrial wastewater and, they are often discharged into the environment.

Amphibian embryos are useful indicators of environmental pollution. However, to our knowledge, there are not studies focussed on the toxic effects of phenol on *Bufo arenarum*, which is an anuran widely distributed in South America. Therefore, the effect of phenol on the survival and morphogenesis of these amphibian embryos was evaluated by means of AMPHITOX test. Embryos at 25 stage of development (acute test) and embryos at 2–4 blastomers stage (early life stage test), were exposed to phenol solutions in concentrations ranging from 25 to 250 mg/L, which were frequently found in the environment. Mortality and malformations were registered each 24 h. LC_{50}, LC_{99}, NOEC, TC_{50} and TI_{50} values were 183.70, 250, 60, 113 mg/L and 1.62, respectively, at 96 h of treatment. Mortality and the percentage of malformations increased with increasing phenol concentrations. Teratogenic effects more frequently produced by phenol were: axial flexure, persistent yolk plug and different abnormalities which caused death of blastulaes. Moreover, other malformations were registered, such as irregular form, acephalism, edema, axial shortening and underdevelopment of gills, among others. Larvae of *B. arenarum*, at early embryonic stages (blastulae), showed higher sensitivity to phenol than tadpoles at stage 25. Results confirm high susceptibility of amphibians to phenol and that environmental concentrations of this pollutant might be harmful to these populations.

1. Introduction

Phenols are among the most common water pollutants. They arise from coking of coal, oil refineries and several industries including chemical, pesticide, wood preservation, dye manufacturing and pulp and paper production. Phenolics are also found in disinfectants and antiseptics and, consequently, they have a high potential for environmental pollution [1]. Quantitative data available for phenols in industrial wastewater are generally expressed in terms of total concentration and show a wide range of variability depending on the source. For instance, phenol concentrations for refinery effluents are approximately 50 mg/L for distillation units, in the range of 50–500 mg/L for catalytic cracking and visbreaking processes and up to 500 mg/L in the spent caustic solutions. On the other hand, waste solutions generated from coal conversion processes usually contain 200–600 mg/L of phenols which are discharged into natural water streams [2]. Thus, deliberate discharge and/or accidental release of these harmful chemical compounds into the environment have the potential to disrupt the structure and functioning of natural ecosystems [3].

With the aim of protecting human and animal health, the USEPA [4] has determined that the level of phenol in environmental waters, should not exceed 3.5 mg/L. Meanwhile, in Argentina, the law of hazardous wastes (Law 24,051, Annex II) established a guide level of 1 μg/L for protecting aquatic life in fresh surface water, and 2 μg/L for water source for human consumption [5]. However, phenol is frequently found at higher concentrations than the limit concentrations established by regulatory organizations. For example, it was found in concentrations ranging from 0.4 to 1.49 mg/L in Iguazú [6], Paraná [7], and Reconquista river [8] and in concentrations as high as 2.28 mg/L in Ctalamochita rivers [unpublished laboratory data].

Toxicity of phenolics has been studied on selected microbes (e.g. protozoa, yeast and bacteria), algae, duckweed and numerous invertebrates and vertebrates. For instance, phenols induce genotoxic effects in animals and human [9,10] and depending on the taxa, the acute toxicity of phenol can vary from 6.5 to 1840 mg/L.

Amphibians are being increasingly used for toxicity screening purposes due to their high sensitivity to physico-chemical stress [11–13]. In particular, amphibian embryos are useful indicators of environmental pollution and they are employed to assess the embryotoxicity of various chemicals including pyrethroid insecticides [14], solvents [15], metals such as copper [16], flavonoids

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such as naringenin [17] and different environmental samples, like surface waters, sediments, drinking water, groundwater, industrial effluents, urban runoff and hazardous waste sites [13,18,19]. On the other hand, various studies have focused on the effects of UV-B radiation on embryos survival [20] and photodynamic toxicity [21]. These studies have shown that resistance to environmental agents can change significantly throughout development [15,22]. Moreover, the tolerance of amphibians to different toxic substances varies depending on the concentration, exposure time, body size and environmental adaptation of the treated species [23].

Phenol can produce lethal and teratogenic effects on some amphibian species. Dumpert et al. observed that all *Xenopus* embryos exposed to 5 mg/L of phenol grew more slowly and died within 3 weeks of exposure [24]. In addition, Birge et al. and Black et al. examined the toxicity of phenol on eight species of amphibians and found LC_{50} values from 0.04 to 9.87 mg/L depending on the species studied [25,26]. Moreover, Bernardini et al. described a LC_{50} value of 178 mg/L for *Xenopus* embryos [27].

Although previous studies have been established that amphibians can be quite sensitive to phenol, the majority of studies have focused on one laboratory model (*Xenopus laevis*) and amphibians species native to Northern America. To our knowledge, no studies have investigated the effect of phenol on South America taxa, spite of the widespread use of phenolics and the high levels found in aquatic environments. Therefore, the aim of this work was to evaluate the toxicity of phenol by means of AMPHITOX bioassays using *Bufo arenarum* premetamorphic tadpoles. AMPHITOX is a set of four standardized tests employing amphibian embryos that can be used to evaluate toxicity at four different levels: acute, short-term chronic, chronic and early life stages [28,29].

2. Materials and methods

2.1. Obtaining B. arenarum embryos

In order to obtain *B. arenarum* (Hensel) embryos, adult females and males weighing approximately 200–250 g were collected in Río Cuarto (Córdoba Province, Argentina). Ovulation of the females was induced by intraperitoneal injection of a suspension containing one or two female homologous hypophysis [17,30] with 300 IU of Human Chorionic Gonadotrophin (Endocorion 5000, ELEA) [31] in 8 mL of 10% Ringer's solution (RS). This combined procedure was performed by us in order to optimize female's ovulation. Oocytes were fertilized *in vitro* with a sperm suspension in RS. After fertilization, the embryos obtained were maintained in RS at $20 \pm 2\,^{\circ}C$ until they reached the adequate development stage to perform the toxicity test (s2 and s25). Developing embryos were staged according to Del Conte and Sirlin [32].

2.2. Bioassays

Bioassays were conducted with *B. arenarum* embryos following the AMPHITOX test conditions [28]. Phenol was used as a model pollutant and was purchased from Merck Laboratories. Phenol solutions, in concentrations of 25, 50, 100, 150, 200, 230 and 250 mg/L, were prepared with distilled water.

Batches of 10 organisms (by triplicate in acute test and by quadruplicate in early life stage) were placed in 12 cm diameter glass petri dishes containing 40 mL of phenol or control solutions. Two different control solutions were used: (a) Ringer solution, which is recommended for AMPHITOX test conditions [28,29] and (b) distilled water, which was used to assess the effect of the solvent used to prepare phenol solutions, on the embryos [33].

For acute test, embryos at 25 stage of development were placed in phenol solutions for 96 h and mortality was recorded every 24 h.

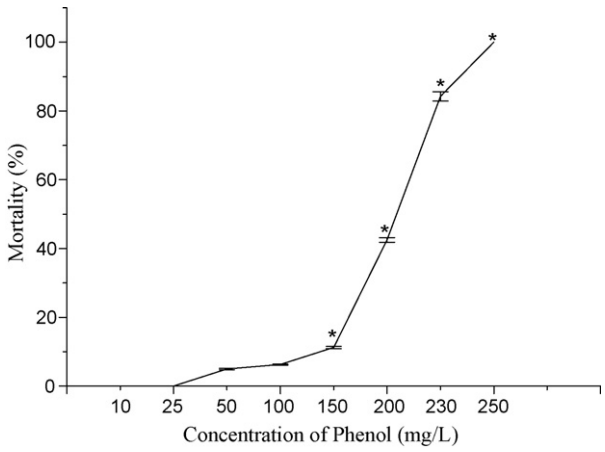

Fig. 1. Lethality exerted by phenol on *Bufo arenarum* embryos at 96 h of exposure (acute test).

For early life stage test, embryos at 2–4 blastomers stage (s2–s3) were used and jelly coats were dissolved by a treatment with 2% thioglycolic acid solution at pH 7, during 2 min followed by eggs washing with RS. Embryos were placed on phenol solutions (25–150 mg/L) for 96 h until embryos reached the 25 stage of development.

Embryos were maintained at $20 \pm 2\,^{\circ}C$ and the solutions were renewed once a day. Mortality, malformations and changes in behaviour were recorded each 24 h. Abnormalities were identified according to the "Atlas of Abnormalities" [34]. Embryos were observed using a Motic digital Microscope DM39.

2.3. Statistical analysis

Lethality and malformations data for all experiments were transformed by means of PROBIT [35] according to the American Society for Testing and Materials [33], and statically analyzed using ANOVA, in all cases $p < 0.05$ denoted significance. The results were reported as values for median lethal concentration (LC_{50}), ninety-nine lethal concentration (LC_{99}), median teratogenic concentration (TC_{50}), median teratogenic index (TI_{50}; equals LC_{50}/TC_{50}) and for no observed effects concentration (NOEC), after 96 h of exposure.

3. Results

3.1. Acute test-mortality

The development and survival of larvae (Fig. 1) were not significatively affected by phenol concentrations ranging from 10 to 100 mg/L ($p > 0.05$). Phenol solution containing 150 mg/L of the pollutant was toxic and produced a significant mortality of 11.25% ($p < 0.05$) comparing with control embryos. Probit analysis showed a LC_{50} value of 183.70 mg/L. Phenol killed all embryos at concentrations of 250 mg/L (LC_{99}) and NOEC value was 60 mg/L.

When accumulative mortality per day was analyzed (Fig. 2), phenol did not produce death at the first day, at concentrations below 200 mg/L, while a significant mortality of 5% ($p < 0.05$) was observed with phenol concentrations of 230 and 250 mg/L, after the same period of time.

The accumulative mortality of the exposed larvae at concentrations of 150 mg/L did not surpass 11.25% at 4th day, and mortality per day was 5%, approximately. Instead, solutions containing 200 mg/L of phenol produced a significant mortality of 18.75% ($p < 0.05$) on the third day of treatment, reaching a value of 42% on the fourth day of exposure.

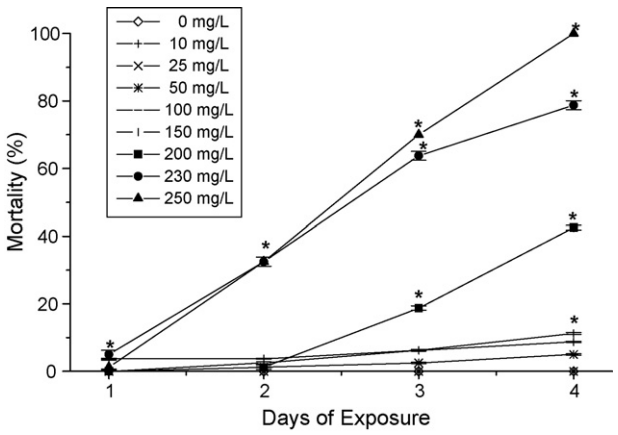

Fig. 2. Accumulative mortality exerted by phenol at 96 h of exposure (acute test).

The highest mortality was obtained at the 4th day with concentrations of 230 and 250 mg/L of phenol (78.75% and 100%, respectively, $p < 0.05$).

3.2. Early life stage test-developmental affects

TC_{50} and TI_{50} values for *B. arenarum* exposed to phenol were calculated and they were 113 mg/L and 1.62, respectively.

The most common developmental abnormalities found were: axial flexures, persistent yolk plug and different abnormalities which caused death of the blastulae (Fig. 3). Other malformations were recorded, but in low frequency such as irregular forms, acephalism, edema, axial shortening and underdevelopment of gills. In addition, other abnormalities were observed such as necrosis, abnormal pigmentation, and absence of eyes, as well as circular swimming which would be a consequence of alterations in the corporal axis or due to neurological disorders (Fig. 4).

Although profound malformations produced death of embryos in most cases, some embryos, seriously affected by the pollutant, survived until the end of the embryonic development stage. The abnormalities (Table 1) were significantly different from the control ($p < 0.05$). When embryos were exposed to phenol concentrations of 100 and 150 mg/L, high malformations were observed being the axial flexure and persistent yolk plug the more frequent. However, at concentrations ranging from 25 to 50 mg/L, some alterations were observed in embryonic development. The complete failure of brain development was observed in all phenol concentrations used and the range of embryos affected was between 1 and 6.66%, expressed as percentage relative to the controls. Different alterations in blastulae and gastrulae had an important effect (between 8.88 and 15.55%) producing, in all cases, the death of the affected embryos. The orientation of the flexure of the axis varied according to the concentration used. In solutions containing 25 and 50 mg/L

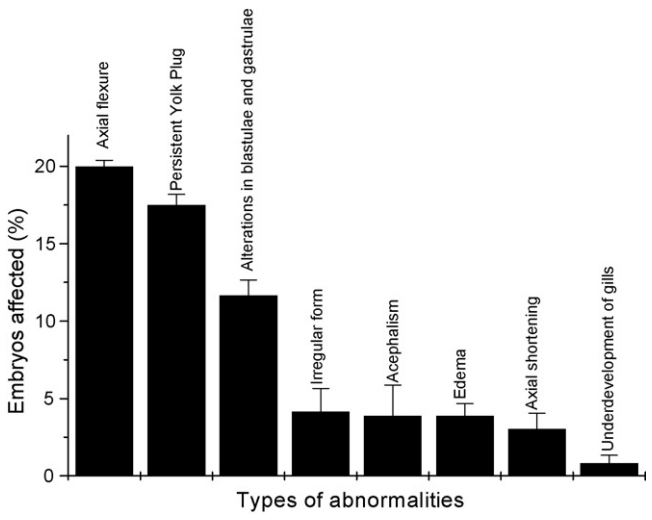

Fig. 3. Teratogenic effects produced by phenol on early life stage of *B. arenarum* embryos. Abnormalities showed correspond to total percentages of embryos which had each malformation, considering all phenol concentrations assayed.

Table 1
Percentages of abnormalities found in *Bufo arenarum* embryos treated with different phenol concentrations.

Malformation (%)	Concentration (mg/L)				
	0	25	50	100	150
Alteration in blastulae and gastrulae	0.13	15.55	8.88	6.66	15.55
Acephalism	0	3.33	1.11	6.66	4.44
Irregular form	0	1.11	2.22	2.22	11.1
Dorsal axial flexure	0	2.22	1.11	4.44	27.11
Ventral axial flexure	0	1.11	3.33	1.11	4.44
Right axial flexure	0	0	0	6.66	7.77
Left axial flexure	0	0	0	1.11	20
Axial shortening	0	0	2.22	3.33	6.66
Edema	0.2	1.11	0	1.11	13.33
Persistent yolk plug	0	4.44	11.11	27.77	26.66
Eyes absence	0	1.11	0	0	0
Necrosis	0	0	0	1.11	0
Underdevelopment of gills	0	0	1.11	2.22	0
Warts	0	0	0	0	3.33
Circulate swimming	0	0	0	1.11	0
Abnormal pigmentation	0	0	0	0	2.22
Total malformations	0.33	29.98	31.09	62.18	100

of phenol, dorsal and ventral flexures were observed, while in solutions containing 100 and 150 mg/L of the pollutant the deviations were dorsal, ventral, left and right. Dorsal flexure was the most frequent abnormality observed in all treatments. In embryos treated with 100 and 150 mg/L of phenol solutions, cutaneous alterations appeared, such as tissue necrosis, warts and abnormal pigmenta-

Fig. 4. Malformations detected in *B. arenarum* embryos in stage 24 treated with 150 mg/L of phenol solutions. (A) Control without treatment. (B) Embryo with incurvated body axis to ventral. (C) Embryo with reduced body size, acephalism, curved spine and edema.

tion. In addition, the underdevelopment of gills killed 2.22 and 1.11% of embryos exposed to 50 and 100 mg/L of phenol, respectively. These embryos did not survive beyond stage 20 of development.

Total abnormalities increased with increasing phenol concentrations and this increment was significantly different to the control ($p < 0.05$).

4. Discussion

Several organisms have been suggested as control populations in bioassays. Among them, amphibians such as *B. arenarum* which is a native South American species, showed to be suitable for toxicity evaluation of xenobiotics and environmental samples. Therefore, we evaluated lethal and teratogenic effects of phenol on embryos and young tadpoles of this native species, under laboratory conditions.

Toxic effects of phenol on *B. arenarum* embryos at stage 25 (acute tests) were observed when these embryos were exposed to solutions containing 150 mg/L of the pollutant. After 96 h of treatment, LC_{50}, LC_{99} and NOEC values (acute test) were 183.70, 250 and 60 mg/L, respectively. A concentration dependent increase in mortality was observed and these results are in agreement with those described by Bernardini et al., in *Xenopus* embryos [27], i.e. based on LC_{50} values, *B. arenarum* showed a similar phenol tolerance as this species (LC_{50} 178 mg/L). However, in other amphibian species LC_{50} values were lower and varied from 0.04 in *Rana pipiens* to 9.87 in *Rana catesbeiana* [25,26] indicating that *B. arenarum* would be less sensitive to phenol than other anuran species. These differences in the magnitude of the observed effects may also be attributed to differences in exposure protocols and duration of exposure other than an inherent characteristic of each species. For instance, in our study and that of Bernardini et al., a static renewal system, with daily test solution renewal was used, whereas Birge et al. and Black et al. used a flow-through system with a retention time of 2.5 h. So, differences observed in LC_{50} could be likely reflective of the differences in exposure regime. However, both exposure scenarios may be ecologically relevant for amphibians.

Although significant mortality occurred at the relatively high concentrations (LC_{50} 183.70), serious malformations such as axial flexure, persistent yolk plug and different abnormalities produced blastulae mortality, when they were exposed to phenol from 25 to 150 mg/L. Moreover, other malformations were recorded, like irregular forms, acephalism, edema, axial shortening and underdevelopment of gills, among others. Larvae of *B. arenarum*, in early embryonic stages, showed higher sensitivity to phenol than tadpoles at stage 25, since malformations were observed starting from concentrations of 25 mg/L of phenol, which in some cases produced the death of the treated embryos. However, concentrations of 150 mg/L of the pollutant, which produced little mortality at stage 25, caused many malformations in early stages of development. Moreover, the TC_{50} (113 mg/L) was low compared with LC_{50}, indicating a prevailing teratogenic effect.

Indeed, a TI_{50} value higher than 1.5, indicates teratogenic hazard [27]. Therefore, phenol is a teratogenic substance for *B. arenarum* embryos because its TI_{50} is above this threshold ($TI_{50} = 1.62$).

The use of acute and chronic test allows obtaining integrated information about mortality, malformations, and grown retardation of the tested organisms, which implies a vast knowledge of the toxicity of certain substances. In this sense, it is important to quantify toxicity at early embrionary stage because in most cases the concentrations of the pollutant which can affect embryos are so different than those affect adult organisms.

Teratogenic effects of phenol, including generalized edema and intestinal and ocular malformations were also described by Birge et al., Black et al. and Bernardini et al., for other amphibian species [25–27]. Moreover, the concentration which produced teratogenic effects in 50 % of exposed larvae (TC_{50}) was 113 mg/L for *B. arenarum*, which was slightly lower than TC_{50} reported for *Xenopus* species [27]. In *Xenopus* embryos, Bernardini et al., observed significant growth retardation which occurred at different exposure concentrations, beginning from 25 mg/L, the same concentration that produced malformations in *B. arenarum* embryos, at early life stages in the present study [27]. Nevertheless, Birge et al. reported that phenol produced teratogenic effects on *Rana pipiens* embryos at low concentrations (0.0047 mg/L) whereas lethal effects were observed at 0.0052 mg/L [25]. In all the examples mentioned above, teratogenic effects were detected at low phenol concentrations, while lethal effects were detected at higher phenol concentrations. By contrast, in *Rana catesbeiana* and *Bufo fowleri*, teratogenic effects were observed for higher phenol concentrations than the ones which produce lethal effects and so, their embryonic stages were less sensitive than young tadpoles [25]. In addition, Black et al. obtained similar results with *Ambystoma gracilis* and *Xenopus laevis* [26]. Therefore, determining the toxicity of phenol is complex because sensitivity varies across species and development.

It is important to note, however, that eggs and developing embryos normally have a jelly coat surrounding the eggs which removed in this study (following AMPHITOX procedures). Thus, under environmental conditions, this membrane could protect the embryo from exposure, or it could interact with the phenol in some way that we have not measured inducing stronger affects. It would be interesting to know the role the jelly coat plays in phenol toxicity.

In this study, we show that phenol induces both mortality and developmental abnormalities at ecologically relevant concentrations (e.g. 25 mg/L). As far as we know, this is the first time the effects of phenol have been studied in a South American amphibian. Considering phenol concentrations usually found in Argentinean rivers (0.4–2.28 mg/L), as well as in many other areas, and the fact that accidental or deliberated discharges can be much higher (10–1000 mg/L [36]) phenol could have important and detrimental effects on wild amphibians generally. Indeed, an enormous amount of natural and synthetic chemicals occur in the environment and could negatively affect this and other amphibian species either by inducing mortality or developmental abnormalities (teratogenesis) [4,31–34].

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References

[1] World Health Organization (WHO), Environmental Health Criteria 161: Phenol, 1994.
[2] P.S. Nayak, B.K. Singh, Removal of phenol from aqueous solutions by sorption on low cost clay, Desalination 207 (2007) 71–79.
[3] T. Vyryan, S. Coombe, M. Keith, J. Matthew, An abbreviated guide to dealing with toxicity, Water Sci. Technol. 39 (1999) 91–97.
[4] U.S. Environmental Protection Agency (USEPA), Generalized methodology for Conducting Industrial reduction evaluations TREs, EPA 600/2-88/070, 1989.
[5] Ley Nacional Argentina N° 24.051 Decreto 831/93. Reglamentación de Residuos Peligrosos, http://www.cecopal.org/derecho/DNacional/DN-831-93.htm.
[6] http://www.territoriodigital.com/nota.aspx.
[7] http://www.universia.com.ar/portada/actualidad/noticia_actualidad.
[8] P. Castañé, M. Rovedatti, M. Topalián, Al Salibián, Spatial and temporal trends of physicochemical parameters in the water of the Reconquista River (Buenos Aires, Argentina), Environ. Monit. Assess. 117 (2006) 135–144.
[9] M. Prati, E. Biganzoli, M. Boracchi, M. Tesauro, C. Monetti, G. Bernardini, Ecotoxicological soil evaluation by FETAX, Chemosphere 41 (2000) 1621–1628.

[10] M. Qiao, C. Wang, S. Huang, D. Wang, Z. Wang, Composition, sources, and potential toxicological significance of PAHs in the surface sediments of the Meiliang Bay, Taihu Lake, China, Environ. Int. 32 (2006) 28–33.
[11] C. Simms, Indications of the decline of breeding amphibians at an isolated pond in marginal land, Br. J. Herpetol. 1 (1969) 93–96.
[12] A.R. Blaustein, D.B. Wake, W. Sousa, Amphibians declines: judging stability, persistence and susceptibility of populations to local and global extinctions, Conserv. Biol. 8 (1994) 60–71.
[13] D. Huang, Y. Zhang, Y. Wang, Z. Xie, W. Ji, Assessment of the genotoxicity in toad *Bufo raddei* exposed to petrochemical contaminants in Lanzhou Region, China, Mutat. Res. 629 (2007) 81–88.
[14] A. Salibián, Effects of Deltamethrin on the South American toad, *Bufo arenarum*, tadpoles, Bull. Environ. Contam. Toxicol. 48 (1992) 616–621.
[15] O. Marquis, A. Millery, S. Guittonneau, C. Miaud, Solvent toxicity to amphibian embryos and larvae, Chemosphere 63 (2006) 889–892.
[16] J. Herkovits, C.S. Pérez-Coll, Acclimation to low level exposure of cooper in *Bufo arenarum* embryos: linkage of effects to tissue residues, Int. J. Environ. Res. Pub. Health 4 (2) (2007) 166–172.
[17] C.S. Pérez-Coll, J. Herkovits, Lethal and teratogenic effects of naringenin evaluated by means of an amphibians embryo toxicity test (AMPHITOX), Food Chem. Toxicol. 42 (2004) 299–306.
[18] J.G. Burkhart, G. Ankley, H. Bell, H. Carpenter, D. Fort, D. Gardiner, H. Gardner, R. Hale, J.C. Helgen, P. Jepson, D. Johnson, M. Lannoo, D. Lee, J. Lary, R. Levey, J. Magner, C. Meteyer, M. Shelby, G. Lucier, Strategies for assessing the implications of malformed frog for environmental health, Environ. Health Perspect. 108 (2000) 83–90.
[19] J. Tietge, G. Ankley, D. DeFoe, G. Holcombe, K. Jensen, Effects of water quality on development of *Xenopus laevis*: a frog embryo teratogenesis assays Xenopus assessment of surface water associated with malformations in native anurans, Environ. Toxicol. Chem. 19 (2000) 2114–2121.
[20] J. Herkovits, J.L. D'Eramo, O. Fridman, The effect of UV-B radiation on *Bufo arenarum* embryos survival and superoxide dismutase activity, Int. J. Environ. Res. Pub. Health 3 (1) (2006) 43–47.
[21] J. Stockert, J. Herkovits, Photodynamic toxicity and its prevention by antioxidative agents in *Bufo arenarum* embryos, Toxicology 192 (2003) 211–218.
[22] J. Herkovits, Evoecotoxicology: environmental changes and life features development during the evolutionary process—the record of the pas at developmental stages of living organisms, Environ. Health Perspect. 114 (2006) 1139–1142.
[23] J. Camargo, A. Alonso, A. Salamanca, Nitrate toxicity to aquatic animals: a review with new data for freshwater invertebrates, Chemosphere 58 (2005) 1255–1267.
[24] K. Dumpert, Embryotoxic effects of environmental chemicals: test with the South African Clawed Toad (*Xenopus laevis*), Ecotoxicol. Environ. Safe. 13 (1987) 324–338.
[25] W.J. Birge, J.A. Black, R.A. Kuehne, Effects of organic compounds on amphibians reproduction, University of Kentucky Water Resources Research Institute Research Report 121, 1980, 39 pp.
[26] J.A. Black, W.J. Birge, W.E. McDonnell, A.G. Weaterman, B.A. Ramey, D.M. Bruser, The aquatic toxicity of organic compounds to embryo-larval stages of fish and amphibians, University of Kentucky Water Resources Research Institute Research Report 133, 1982, 61 pp.
[27] G. Bernadini, O. Spinelli, C. Presutti, C. Vismara, E. Bolzacchini, M. Orlando, R. Settimi, Evaluation of the developmental toxicity of pesticide MCPA and its contaminants phenol and chlorocresol, Environ. Toxicol. Chem. 15 (1996) 754–760.
[28] J. Herkovits, C.S. Pérez-Coll, Bioensayos para test de toxicidad con embriones de anfibios ("ANFITOX"), Ingeniería Sanitaria y Ambiental 42 (1999) 24–30.
[29] J. Herkovits, C.S. Pérez-Coll, Bioensayos para test de toxicidad con embriones de anfibios ("ANFITOX"), Ingeniería Sanitaria y Ambiental 43 (1999) 50–55.
[30] J. Herkovits, C.S. Pérez-Coll, Ecotoxicological studies of environmental samples from Buenos Aires area using a standardized amphibian embryo toxicity test (AMPHITOX), Environ. Pollut. 116 (2001) 177–183.
[31] R. Mann, J. Bidwell, Application of the FETAX protocol to assesses the developmental toxicity of nonylphenol ethoxylate to *Xenopus laevis* and two Australian frogs, Aquatic Toxicol. 51 (2000) 19–29.
[32] E. Del Conte, L. Sirlin, The first stages of *Bufo arenarum* development, Acta Zoologica Lilloana 12 (1951) 495–499.
[33] American Society for Testing and Materials. Annual Book of ASTM Standard. Designation E729-80. Standard Practice for Conducting Acute Toxicity Test with Fishes, Macroinvertebrates, and Amphibians.
[34] J.A. Bantle, J.N. Dumont, R.A. Finch, G. Linder, Atlas of Abnormalities. A Guide for the Performance of FETAX, second ed., Oklahoma State University Publications, Stillwater, OK, 1998.
[35] U.S. Environmental Protection Agency (USEPA), Users Guide for a Computer Program for PROBIT Analysis of date from Acute and Short-term Chronic Toxicity test with aquatic organism. Biological Methods, Environmental monitoring and Support Lab, 1998.
[36] I. Buchanan, J. Nicell, A simplified model of peroxidase-catalyzed phenol removal from aqueous solutions, J. Chem. Technol. Biotechnol. 74 (1999) 669–674.