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Developmental Toxicity and Risk Assessment of

Nonylphenol to the South American Toad, *Rhinella*

arenarum

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Abstract:

The toxicity of Nonylphenol, an emerging pollutant, on the common South American toad *Rhinella arenarum* was stage and time dependent, thus Median Lethal Concentrations (LC50) for acute (96h), short-term chronic (168h) and chronic exposure (336h) were 1.06; 0.96 and 0.17 mgNP/L from embryonic period (S.4), whereas for exposure from larvae (S.25), LC50 remained constant at 0.37 mgNP/L from 96h to 168h, decreasing to 0.11 mgNP/L at 336h. NOEC-168h for exposure from embryos was

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0.025 mgNP/L. The Teratogenic Potential (NOEC-lethality/NOEC-sublethal effects) was 23 times higher than the threshold value, indicating a high risk for embryos to be malformed in absence of significant lethality and representing a threat for the species conservation. By comparing with other amphibians, the early development of *R. arenarum* was very sensitive to NP. The results highlight the relevance of extending the exposure time and look for the most sensitive stage in order to perform the bioassays for conservation purposes.

Keywords: Amphibians, embryo-larval development, stage-dependent susceptibility, teratogenesis, nonylphenol

1. Introduction

Nonylphenol polyethoxylate (NPEO), synthesized in 1940, is a surfactant with exceptional performance and widely used in industrial, commercial and household applications such as detergents, emulsifiers, wetting and dispersing agents, antistatic agents, demulsifiers and solubilisers (Soares et al., 2008). Some of the industrial applications, include pulp and paper, textiles, coatings, lube oils and fuels, metals and plastics (Ying et al., 2002). Nonionic surfactants, such as NPEO, are routinely included as wetting agents and dispersants in pesticide formulations. As one of the major degradation products of NPEO, Nonylphenol (NP) enters the aquatic environment through wastewater discharges but also by drift and runoff of applied products in the field (Naylor, 1995). Once NP reaches the atmosphere, it can be transported to aquatic and terrestrial ecosystems by wet deposition (Fries and Puttmann, 2004). Significant amounts of these alkylphenols were reported in river waters, groundwater, surface waters, and drinking water sources, and even in aquatic sediments (Fernández et al.,

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2007). NP has been regarded as the most critical metabolite of alkylphenol polyethoxylates because of its enhanced resistance to biodegradation, and its toxicity and ability to bioaccumulate in aquatic organisms (Arukwe et al., 2000; Tyler et al., 1998). NP is considered an endocrine disruptor, as it was found to mimic the natural hormone 17β-oestradiol (Soares et al., 2008). NP is an emerging pollutant, not currently covered by water-quality regulations, and is thought to be potential threat to ecosystems and human health (Farré et al., 2008).

In Argentina, the presence of NP was reported in some creeks and rivers of Buenos Aires province, reaching a maximal value of 27 μg/L in Morón creek (Babay et al., 2013; Babay et al., 2008). Although Europe has followed the recommendation of phasing out the use of alkylphenol ethoxylates surfactants in domestic and industrial cleaning agents and Canada has recently adopted NP guidelines for the protection of aquatic life, in the case of Latin American countries, the use of alkylphenol ethoxylates is still completely unrestricted.

Most toxicity studies are performed in specific periods of the life cycle, but treating amphibian organisms at two different early life periods, embryos and larvae, allows the evaluation of an eventual differential susceptibility, providing possible explanations based on developmental features and toxicity mechanisms (Aronzon et al., 2011b; Greulich and Pflugmacher, 2003). Furthermore, it is very relevant to identify the most sensitive period to the noxious agent and select it as the most appropriate to perform toxicity bioassays and provide recommendations on safe environmental concentrations.

An alarming amphibian population decline has been reported worldwide since the 60´s (Simms, 1969). There are several hypotheses to explain this phenomenon, including toxicity produced by chemical contaminants (Wake and Vredenburg, 2008). Several lines of evidences indicate that this fact could be related to their high susceptibility to

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contaminants, in particular during early life stages (van der Schalie et al., 1999). The risk of amphibians to get adverse effects might be increased by their preference to breed in shallow, lentic, or ephemeral water bodies, in which pollutants might be concentrated (Natale, 2006). Moreover, breeding and larval development of amphibians occurs in spring and summer, coincident with the application of pesticides and fertilizers on agricultural lands (Mann et al., 2009). Particularly, the South American toad *Rhinella arenarum*, is one of the species with the highest incidence of malformations as it was reported in a morphological study of amphibians from the middle region of Argentina (Peltzer et al., 2010). Projection of *R. arenarum* population size showed a tendency to extinction in sites dominated by crops in the central region of the Córdoba Province, Argentina (Bionda et al., 2013).

Several toxicity studies have mainly reported acute effects of NP on exotic amphibian's species in specific periods of their early life cycle as the cases of *Rana spenocephala*, *Bufo boreas, Crinia insignifera, Litoria adelaidensis* and *Xenopus laevis* (Bridges et al., 2002; Mann and Bidwell, 2000). Nevertheless, there is no data on the toxic effects of NP in the embryo-larval development of native amphibian species of South America as *Rhinella arenarum.*

The main aim of present study was to evaluate the toxic effects of NP on the South American toad, *Rhinella arenarum,* reporting lethal and sublethal effects on two different developmental periods, embryos and larvae in search of an eventual stage-dependent susceptibility. Moreover, the ecological risk for the species was calculated. Results were discussed in relation to environmental concern and the toxicity mechanisms of the substance.

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2. Materials and methods.

2.1 *Rhinella arenarum* embryos and larvae.

Healthy *Rhinella arenarum* adults, weighing approximately 200 - 250 g were obtained in Lobos (Buenos Aires province, Argentina: 35º 11´ S; 59º 05´ W). Adults were maintained in aquariums with AMPHITOX (AS) solution at a 20 ± 2ºC, alternating 12 h light/dark cycles for short periods. AS composition is (in mg/L): Na^+ 14.75; Cl^- 22.71; K^+ 0.26; Ca^{2+} 0.36; HCO_3^- 1.45. Ovulation of *R. arenarum* females was induced by means of an intraperitoneal injection of a suspension of one homologous hypophysis in 1mL per female (Pisanó, 1957), plus 500 IU of human chorionic gonadotropin (GONACOR 5000 Massone®) (Mann and Bidwell, 2000). Oocytes were fertilized *in vitro* with sperm´s suspensions prepared by mincing one testicle in 1 ml of AS. Each clutch was obtained by a unique and the same pair of adults. After fertilization, embryos were kept in AS at 20 ± 2°C until blastula (S.4) and larval (S.25) stages. Stage of embryos and larvae were defined according to Del Conte and Sirlin (1951). Embryos were dejellied by means of a 2-min treatment with 2% thioglycolic acid solution, neutralized at pH 7.2-7.4 with 1.35 mL of concentrated NaOH solution every 100 mL in AS, and then thoroughly washed (Herkovits and Pérez-Coll, 1999). Embryos and larvae were kept in shallow plastic containers with 5 L of AS until their use in the bioassays. All experiments were conducted in accordance to international standards on animal welfare (Canadian Council on Animal Care in Science, 1993)

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2.2 Test solutions.

A NP (Fluka, tecnical grade, purity 96.9 %. CAS number: 84852-15-3, marketed by Sigma-Aldrich) stock solution of 45.4 g/L was prepared by dissolving the corresponding mass, in acetone. A second stock solution of 800 mg NP/L was prepared by dissolving the corresponding volume of the first one in acetone. Seven to nine test solutions, ranging in concentrations between 0.0025 and 4 mg NP/L, were prepared by diluting the corresponding volume of the second stock solution in AS.

NP in test solutions was quantified by reverse-phase HPLC coupled to fluorescence detection at excitation and emission wavelengths of 230 and 300 nm, respectively. A C-8 column (250 x 4.6 mm, 5 μm, Grace, USA) and isocratic elution with MeOH/H$_2$O (80:20) were employed (Babay et al., 2013; Babay et al., 2008). The errors between nominal and measured concentrations did not exceed 10%.

2.3 Toxicity Experimental Protocols.

Rhinella arenarum embryos and larvae obtained from six different clutches were continuously exposed to NP from early blastula (S.4) and complete operculum (S.25) stages onwards for acute (96 h), short-term chronic (168 h) and chronic (336 h) periods (US EPA, 2002).

For each experimental condition, triplicate batches of 10 embryos or larvae were placed in covered 10-cm-diameter glass Petri dishes containing 40 mL of test solution. Each experimental design accordingly included, together with a control group exposed to AS

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only, a solvent control group that was exposed to AS containing acetone 0.5% v/v, the highest concentration used for NP test solutions (ASTM, 1993). Lethal and sublethal effects were evaluated and dead individuals were removed every 24 h. Test solutions were renewed every other day and temperature was maintained at 20 ± 2ºC. Larvae were fed with 6 ±0.5 mg of balanced fish food TetraColor® *ad libitum* for 24 h every other day.

Sublethal effects were studied with Stereoscopic Microscopy. Images of embryos and larvae were digitally recorded with a Sony DSC-S90 camera mounted on a Zeiss Stemi DV4 stereoscopic microscope. Sublethal effects were classified into different categories as abnormal development, retarded or delayed stage development, reduced body size, reduced tail size; and other morphological abnormalities as axial flexures, extrusion of the fin axis, agenesia/underdeveloped gills, malformed mouth/adhesive structures, hydropsy and others, which were identified according to the ''Atlas of abnormalities''(Bantle et al., 1992). After 4% formalin fixation of individuals exposed for 168 h to different NP concentrations, total lengths, as straight line distances, were measured from the tip of the snout to the tip of the tail under the stereoscopic microscope. As a measure of developmental hazard, the teratogenic potential (TP) (Aronzon et al., 2011a) was estimated as the ratio between the NOEC value for lethality and sublethal effects and it was compared with the maximal value of 1.5 (ASTM, 1993) which implies large separation of the mortality and malformations concentration ranges and, therefore, a great potential for all embryos to be malformed in absence of significant embryo mortality. Behavioral alterations such as abnormal fast rotations which are a sign of neurotoxic stress; lying on the lateral or dorsal side, abnormal breathing, feeding and swimming patterns were evaluated (Denoël et al., 2012). Smooth movements of the Petri-dishes, followed by stimulation with a light source were done.

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In case of no response, soft mechanic stimulation with a glass rod was made and finally heartbeat was checked under Zeiss Stemi DV4 stereoscopic microscope.

Ecological risk can be numerically estimated using the Hazard Quotient (HQ) approach (US EPA, 1998) taking the worst case scenario based on the "precautionary principle" to provide a more meaningful, yet conservative, estimation of the effect. In that way, the ratio between an Expected Environmental Concentration (EEC) and a standard toxicity endpoint (e.g. LC50) was obtained. We estimated a HQ for NP exposure to *Rhinella arenarum* embryos and larvae based on the maximal NP concentrations reported in Buenos Aires (Babay et al., 2013). The standard toxicity endpoint was the LC50s calculated for each developmental period at different exposure times. HQ was also calculated, for some cases, using a sublethal effect value (LOEC; NOEC, EC) as standard toxicity endpoint.

After HQ was calculated, it was compared with the USEPA Level of Concern (LOC). The LOC is a policy tool that the Agency uses to interpret the risk quotient and analyze the potential risk to non-target organisms and the need to consider regulatory action. The LOC value for risk is 1. If HQ>1, harmful effects are likely to result from the contaminant in question.

2.4 Statistical analysis.

Lethality data were statistically analyzed by the USEPA PROBIT Program (US EPA, 1988).Toxicity Profiles (TOPs), as isotoxicity curves (Herkovits and Helguero, 1998) were plotted based on LC50 at different times. To compare LC50 values, differences were considered to be statistically significant when the ratio of the higher and the

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lower LC50 exceeded the corresponding critical value established by the American Public Health Association *et al*. (APHA, 2005). To obtain NOEC and LOEC values the percentages of affected individuals were compared using Pearson's chi-square (χ^2) test to establish significant differences between exposed and control embryos. Kruskal-Wallis analysis was used to assess significant differences of the lengths of embryos exposed for 168 h to different conditions. Multiple comparisons were performed using Dunn test. Differential sensitivity inter-clutches was expressed as a coefficient of variation.

3. Results.

3.1. Embryo toxicity.

Lethal toxicity of NP on *Rhinella arenarum* embryos exposed from blastula stage (S.4) onwards was time-dependent. NOEC-24 h and 48 h remained at 1.5 mg/L, while NOEC -168 h and 336 h diminished to 0.75 and 0.025 mg NP/L, respectively. Acute toxicity increased from a LC50-72 h of 1.5 mg NP/L to 1.06 mg NP/L at 96 h. Toxicity was greatest at the end of short-term chronic period with a LC50 of 0.96 mg NP/L (Figure 1). When exposure was extended for a chronic period, toxicity increased even more to reach a LC50-336 h=0.17 mg NP/L. There were no differences in susceptibility among clutches, and the coefficients of variation were always lower than 9%.

3.2. Larval toxicity.

NP toxicity on *Rhinella arenarum* larvae, exposed from the beginning of larval development (S.25) was not time-dependent from 72 h to 168 h (Figure 1). In contrast to individuals exposed from the embryonic period, there were no significant differences in LC50 for larvae along the exposure time, and LC50 remained constant at 0.37 mg

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NP/L up to 168 h. Nevertheless, when exposure was extended for a chronic period, toxicity increased to a LC50-336 h= 0.11 mg NP/L. NOEC-24 h and 48 h were 0.45 and 0.4 mg/L respectively, while NOEC from 72 h to 168 h remained constant at 0.25 mg NP/L, and increased to a NOEC-336 h of 0.005 mg NP/L. There were no differences in susceptibility between clutches of larvae, and the coefficients of variation were always lower than 17%.

3.3. Sublethal effects.

Rhinella arenarum embryos exposed to NP from blastula stage (S.4) onwards showed different sublethal effects which were concentration-dependent. The main abnormalities of exposed embryos from blastula stage (S.4) were persistent yolk plugs that led to failed gastrulation process, within the first hours of exposure. Then, the main sublethal effects are summarized in table 1 and were: reduced body/tail sizes, microcephaly, underdeveloped gills, axial flexures, different edemas, like cardiac/pericardial edema, malformed mouth/adhesive structures, gut miscoiling, and atypical extrusion of the caudal fin axis (Figure 2). Moreover, a conspicuous retarded or delayed stage development was observed. Thus, when control embryos were at gill circulation stage (S.20, 96-120 h post-fecundation) embryos exposed to 1 and 1.5 mg NP/L were delayed in muscular response (S.18, 48-72 h post-fecundation) and heart beat (S.19, 72-96 h post- fecundation) stages, respectively. The statistical analysis of the total lengths of embryos exposed to NP for 168 h, when control embryos reach larval stage (S.25), showed that individuals exposed to 0.5 and 1 mg NP/L were significantly (p<0,05 and p<0,001, respectively) shorter than controls (Figure 3). LOEC-24 h was 1.5 mg NP /L, decreasing to 0.5 mg NP/L at 96 h and 0.05 mg NP/L at 168 h. NOEC-24 h for sublethal effects was 0.75 mg NP/L, decreasing to 0.25 mg NP/L at 96 h and to 0.025 mg NP/L at

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168 h. EC50-96 h for sublethal effects was 0.97 (0.90-1.19) mg NP/L. TP-96 h and 168 h were 3.6 and 34.4, respectively.

Rhinella arenarum larvae exposed to NP from the complete operculum stage (S.25) onwards showed a high incidence of axial flexures, fins with irregular borders, blisters and edemas (Figure 2h) at short exposure times. Since the first five hours of exposure, axial flexure incidence increased from 30% to 100% of larvae exposed from 0.24 to 0.4 mg NP/L, respectively.

No differences were observed between AS controls and acetone controls for all treatments, neither in mortality nor in the percentage of abnormalities.

3.4. Hazard Quotients.

The Hazard Quotients (HQ) for *Rhinella arenarum* exposed from embryos (S.4) and larvae (S.25) to NP were calculated at different exposure times (Figure 4) based on the maximal NP concentration reported in Buenos Aires province (27 µg/L) (Babay et al., 2013). HQ obtained from the LC50s for both embryos and larvae treatment remained under the LOC value. HQ values for embryos, exposed from blastula stage (S.4) onwards, increased along the exposure time. HQ for larvae, exposed from de complete operculum stage (S.25) onwards, remained constant along the short-term chronic exposure time but showed a high increase from the 192 h onwards.

HQ obtained from the LOEC value of sublethal effects for embryos remained under the LOC value at 0.018; 0.054 and 0.54 at 24 h, 96 h and 168 h, respectively. HQ calculated from NOEC value of sublethal effects were 0.036 and 0.108 at 24 h and 96 h, while at 168 h this value increased to 1.08, reaching the LOC value.

4. Discussion

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The development of the toad *Rhinella arenarum* was highly sensitive to NP. Particularly; larvae were six times more sensitive than embryos. NP also caused sublethal effects on the normal growth and development of exposed individuals. The toxicity of NP on embryos exposed from blastula stage onwards was time-dependent; in that sense, the LC50 significantly decreased 1.5 times up to 120 h of exposure. In line with toxicity of NP to *Xenopus laevis* (Mann and Bidwell, 2000), present study shows evidences of a stage-dependent susceptibility to NP. Although the early organogenic period has been shown as the most sensitive to different noxious agents (Aronzon et al., 2011a; Aronzon et al., 2011b; Sztrum et al., 2011), in this case, larvae resulted almost six times more sensitive than embryos as consequence of NP exposure. This increased sensitivity at larval period was also observed in *Rana clamitans, R. pipiens, R. sylvatica, Bufo americanus and Xenopus laevis* exposed to different glyphosate formulation with different surfactants (Edginton et al., 2004; Howe et al., 2004). One of the possible explanations given for the higher tolerance is that during the embryonic development, embryos receive nourishment from a yolk sac and they do not consume potentially contaminated food sources (Howe et al., 2004). Moreover, embryos start to increase their contact with the exposure medium when reach the gill circulation (S.20) and open mouth (S.21) stages. Indeed, maximal contact with the exposure medium reaches when individuals are able to be feed, in this study individuals were fed from complete operculum stage (S.25) onwards, coincident with the second significant increase in toxicity at 192 h. Another possible reason is the exclusion of the chemical by embryonic membranes (Edginton et al., 2004). Another reason for this differential sensitivity is a lack or insensitivity of target organs in the embryonic stages compared to the larval period, leading to differential exposure times

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of sensitive target organs (Edginton et al., 2004). It is noteworthy to point out that when NP exposure was extended from the early developmental stage until 336 h, toxicity was increased more than 10 times. This higher susceptibility might be related to the inclusion of the larval period in the exposure.

Even though larvae were the most sensitive, toxicity from the beginning of larval development did not show significant changes from 72 h up to 168 h, indicating that NP toxicity reaches its maximum value within the first 72 h. This pattern of fast maximal toxic effect of NP on *Rhinella arenarum* larvae is similar to the results reported for metals such as copper and cadmium (Aronzon et al., 2011b; Pérez-Coll and Herkovits, 1996) and for organic compounds as the herbicide 2,4-Dichlorophenoxiacetic acid (Aronzon et al., 2011a). Nevertheless, the toxicity significantly increased when exposure was extended to a chronic period of 336 h. Despite that most toxicity studies focus just on acute effects in a certain period of the life cycle, this study points out the importance of performing toxicity bioassays at different stages of the life cycle of a species and for an extended term. These allow identifying the most sensitive period to recommend tolerance thresholds, conservative and realistic for the species preservation.

NP proved to be teratogenic, as the Teratogenic Potential (TP) reached values 23 times higher than the value based on ASTM (ASTM, 1993), which implies a high risk for embryos to be malformed in the absence of significant embryonic lethality. The large difference between the lethal and sublethal concentrations contributes to consider sublethal effects as a relevant endpoint for population viability as they might reduce the fitness of individuals, at least to *R. arenarum*. Park et al (2010) reported no teratogenicity in *Bombina orientalis* embryos exposed just to one sublethal concentration ten times lower than the LC50-96 h. On the other hand, Mann and Bidwell (2000) indicated either no or low teratogenicity in *X. laevis, Litoria*

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adelaidensis and *Crinia insignifera*. In that sense, *R. arenarum* was five, six and nine

times more sensitive than *Xenopus laevis* (LC50-96 h= 3.9 mg/L), *Crinia insignifera*

(LC50-140 h=6.4 mg/L) and *Litoria adelaidensis* (LC50-140 h= 9.2 mg/L), respectively

(Mann and Bidwell, 2000). Although the maintaining media of FETAX and

AMPHITOX tests have different salinity and temperature conditions (Herkovits and

Pérez-Coll, 2003), which might modify toxicity results, these significant differences

might be rather related to the species' specific sensitivity.

Some of the main sublethal effects observed in the study were retarded or delayed stage

development and reduced body length, this last one was significant and important on

embryos exposed to NP from blastula stage (S.4) to concentrations as low as 0.5 and 1

mg NP/L at 168 h. These effects are in agreement with other studies showing that

inhibition of body growth is one of the most sensitive indicators of developmental

toxicity (Richards and Kendall, 2003; Sayed et al., 2012). Moreover, Park et al. (2010)

and Bevan et al (2003) have also shown growth inhibition in *Bombina orientalis* and

Xenopus laevis. Microcephaly, underdeveloped gills, axial flexures, different kinds of

edemas, malformed mouth and adhesive structures, and gut miscoiling, were also

observed. These anomalies may be considered as nonspecific effects of this toxic agent,

given that they were reported for metals (Aronzon et al., 2011b; Pérez-Coll and

Herkovits, 1990) and other organic compounds on *Rhinella arenarum* development

(Aronzon et al., 2011a; Hutler Wolkowicz et al., 2014; Pérez-Coll and Herkovits, 2004;

Svartz et al., 2012). These sublethal effects were also reported for *Xenopus laevis*

embryos and *Bufo regularis* larvae exposed to NP (Sayed et al., 2012; Sone et al.,

2004). In contrast, exposure to NP also caused an atypical extrusion of the fin axis

(Figure 2.), which has been also observed in *Rana pipiens* exposed to a commercial

glyphosate pesticide (Howe et al., 2004), but never reported for any of many

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physicochemical agents evaluated on *R. arenarum.* Some authors suggest that this extrusion might be cause by an inhibition in the synthesis of collagen, which would cause a defect in the development of early vertebral primordial (Birge et al., 1983), nevertheless, this abnormality was also observed in the body axis of larvae exposed from the end of embryo development (S.25) at short time exposures. Other authors have pointed out that this abnormality in the tail might be due to myotome disruption caused by an inhibition of acetylcholinesterase activity (Bonfanti et al., 2004; Li, 2008). However, this sublethal effect might be related to any mechanism capable to cause an excessive flow of calcium, which can generate an overstimulation of myocyte contractile apparatus and even muscle necrosis (Leonard and Salpeter, 1979). It has been reported that exposure of resting skeletal muscle cells to NP might cause a loss of calcium balance, possibly due to the alteration of the cell membrane and an adverse effect on the active calcium transport (Gong et al., 2008; Kirk et al., 2003; Michelangeli et al., 1990).

The teratogenic effects and the short time needed to be observed in NP exposure, highlight the relevance to consider these parameters in risk assessment.

Despite the toxicity of NP on native amphibian species has not been reported, *Rhinella arenarum* were much more sensitive than most of amphibians. These results strongly support the importance of assessing the toxicity of different noxious agents on native species, such as the common South American toad, *R. arenarum.* This provides a representative overview of the implications of xenobiotic contamination on ecosystems and species of Argentina, in order to make decisions, set thresholds, and develop management plans in line with the region situation.

The HQs obtained for the local scenario and their comparisons with the LOC value showed that, at least for the NP concentration reported in Buenos Aires province (Babay

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et al., 2013) there is little threat for *R. arenarum* populations. Coinciding with the breeding season, NP might present seasonal variations with higher concentrations in the summer, due to an increase in microbial activity at warmer temperatures (Li, 2008). Besides, environmental levels might be higher in some areas as the efficiency of water treatment plants at removing NP was found to be highly variable ranging from 11% to 99% depending on the type of unit treatment process employed (Berryman et al., 2004). In particular, NP is produced during the NPEO degradation process and is often find it in higher concentrations in the effluents than in the influents (Soares et al., 2008). Moreover, HQ showed an important increase at chronic period. It is noteworthy that despite LC50 at 336 h for embryos and larvae did not show significant differences, HQs for larvae at 336 h were 1.5 times higher than the respective values for embryos. This fact highlights the importance of reporting the most sensitive period of a life cycle to a determined chemical.

These results are very important for Argentina and other developing countries with large agricultural areas because nonionic surfactants are commonly included as wetting agents and dispersants in pesticide formulations. Despite that some active constituents of pesticide are reported of low toxicity, the additive surfactant components may be a health risk to aquatic fauna, as is shown in this study for NP, especially in the case of amphibians because pesticides are applied around or over standing or ephemeral waters with a low capacity for dilution (Aronzon et al., 2011a; Mann and Bidwell, 1999).

This study highlights the toxicity of NP on embryos and larvae, not only in a direct way for the survival of the common toad, but it also can indirectly affect the normal development and behaviour of this native amphibian species of South America, affecting the ability of organisms to avoid predation and subsequently impairing their

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ability for future reproduction (Carey and Bryant, 1995; Little et al., 1990) contributing to the decline of *Rhinella arenarum* populations.

5. **Conclusion**

The early development of the toad *Rhinella arenarum* was very sensitive to NP and showed an increased susceptibility from embryonic to larval development. The xenobiotic also caused a diversity of sublethal effects, including an atypical extrusion of the fin axis. By comparing with other amphibians, it is one of the most sensitive species to NP.

These results are very important for countries with large agricultural areas due to the use of nonionic surfactants as wetting agents and dispersants in pesticide formulations, and because the increasing environmental concentrations of this emerging pollutant, as a consequence of their still completely unrestricted use. Considering the lethal and sublethal effects caused by NP, this study shows the health risk that surfactants might represent to the native toad *Rhinella arenarum* populations, despite that some active constituents of pesticides are reported of low toxicity.

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Figure 1. Lethal Concentration 50 (LC50) of Nonylphenol in *Rhinella arenarum* embryos and larvae exposed from early blastula stage (S.4) and complete operculum stage (S.25) onwards, respectively. Bars show 95% confidence intervals.

Table 1. Summary of the main sublethal effects of embryos exposed for 96 h to different Nonylphenol concentrations. Notice that an individual might present more than one abnormality.

Figure 2. Panoramic views of malformed larvae of *Rhinella arenarum* as a result of Nonylphenol exposure (Stereoscopic Microscopy): a) Control. Embryos become larvae by continuous exposure from the blastula stage for 168 h to: b) 0.25 mg NP/L, c), d) 0.75 mg NP/L, d), e) 0.75 mg NP/L; f) 0.75 mg NP/L, detail of the extrusion of the caudal fin, g) 1 mg NP/L. Larvae exposed for 5 h to: h) 0.45 mg NP/L. Observe the reduced body size, axial flexures (af), microcephaly (m), gut miscoiling (gm), underdeveloped gills (ug), abdominal edema (ae), generalized edema (e) and the extrusion of the fin axis (efa). Scale: 1 mm.

Figure 3. Effects of Nonylphenol on the length of *Rhinella arenarum* embryos exposed to different Nonylphenol concentrations for 168 h from early blastula stage (S.4) onwards. *Significant differences from control (p <0.05).

Figure 4. Hazard Quotients (HQ) of Nonylphenol in *Rhinella arenarum* embryos and larvae exposed from early blastula stage (S.4) and complete operculum stage (S.25) for 336 h. HQ-based on the maximal NP concentration reported in Buenos Aires province.

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Highlights:

- Significant lethal and sublethal effects of Nonylphenol on Rhinella arenarum embryos and larvae were reported.

- Stage-dependent susceptibility to Nonylphenol was informed.

- A high teratogenic potential of Nonylphenol was reported.

- The study showed the high risk that Nonylphenol might represent to amphibian's populations.
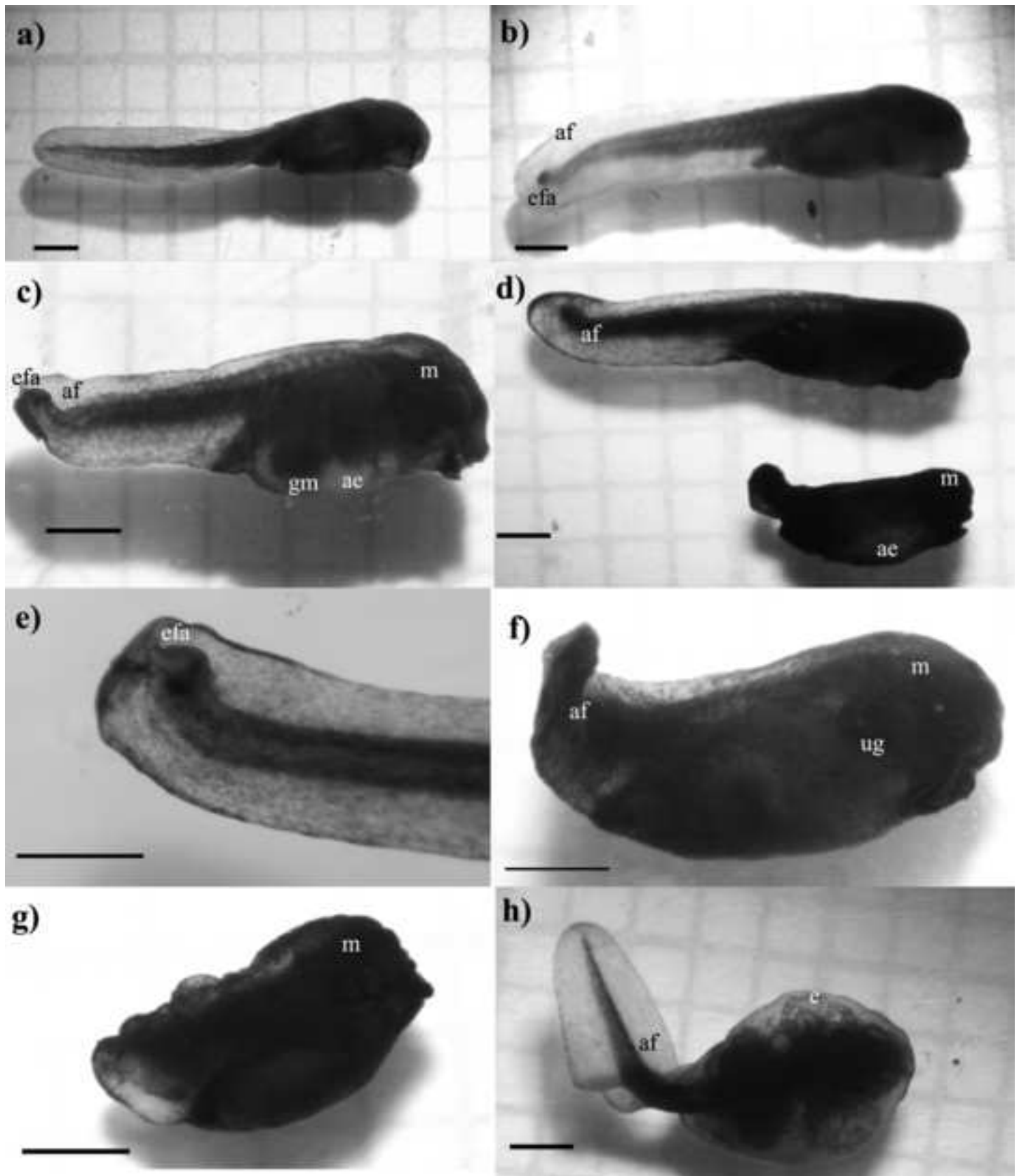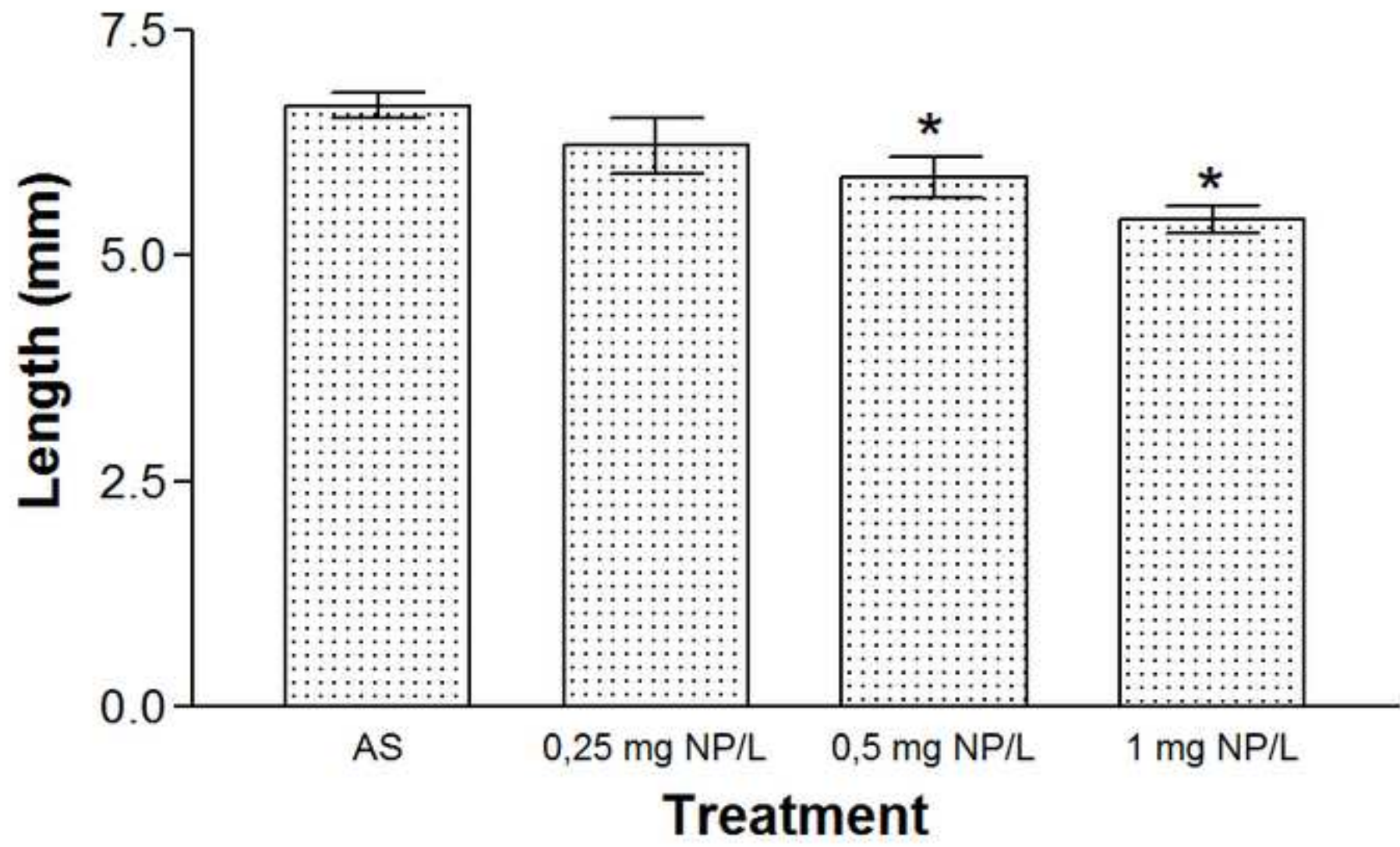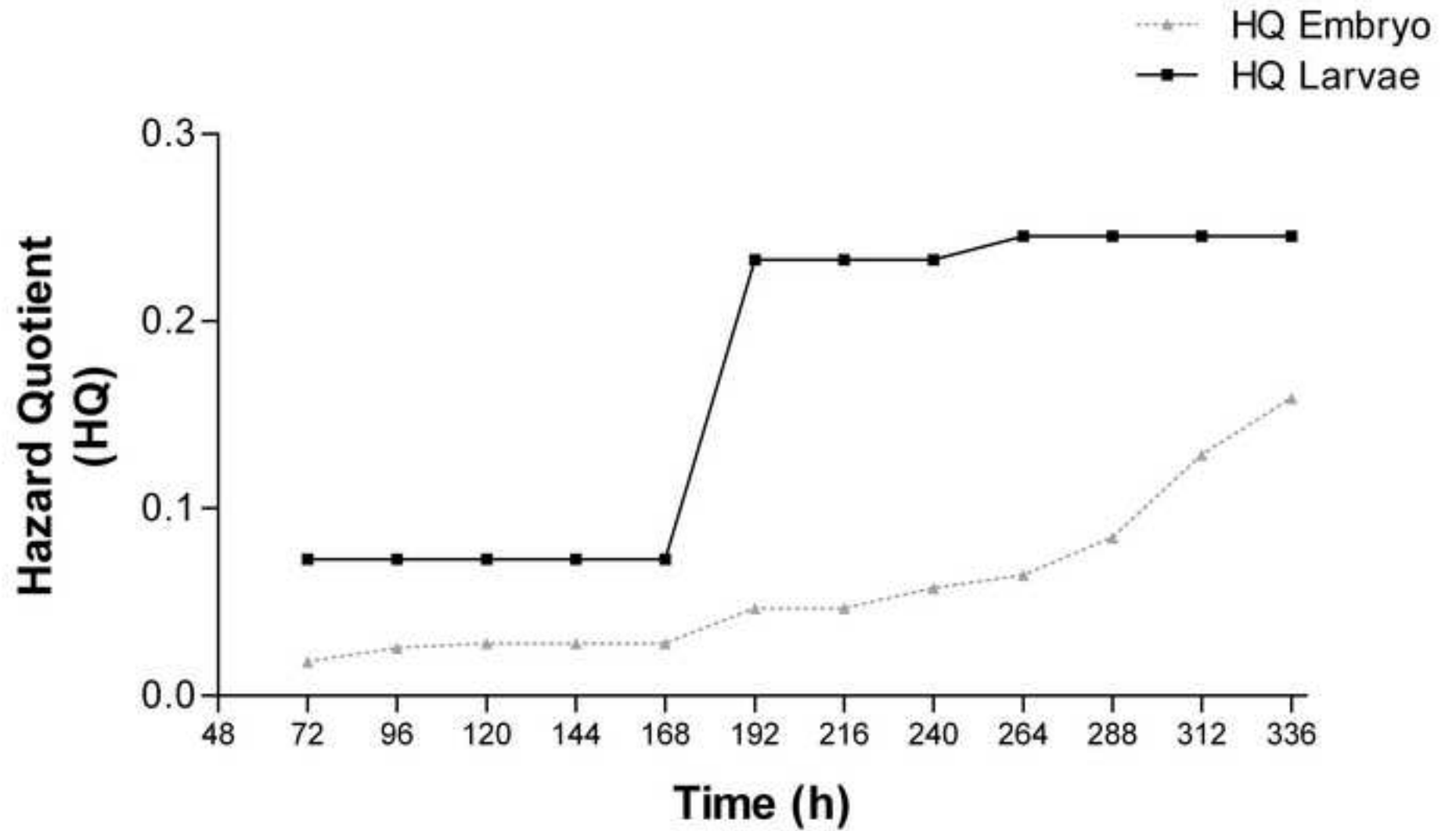
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Figure1

Figure 2

Figure 3

Figure 4

Table 1
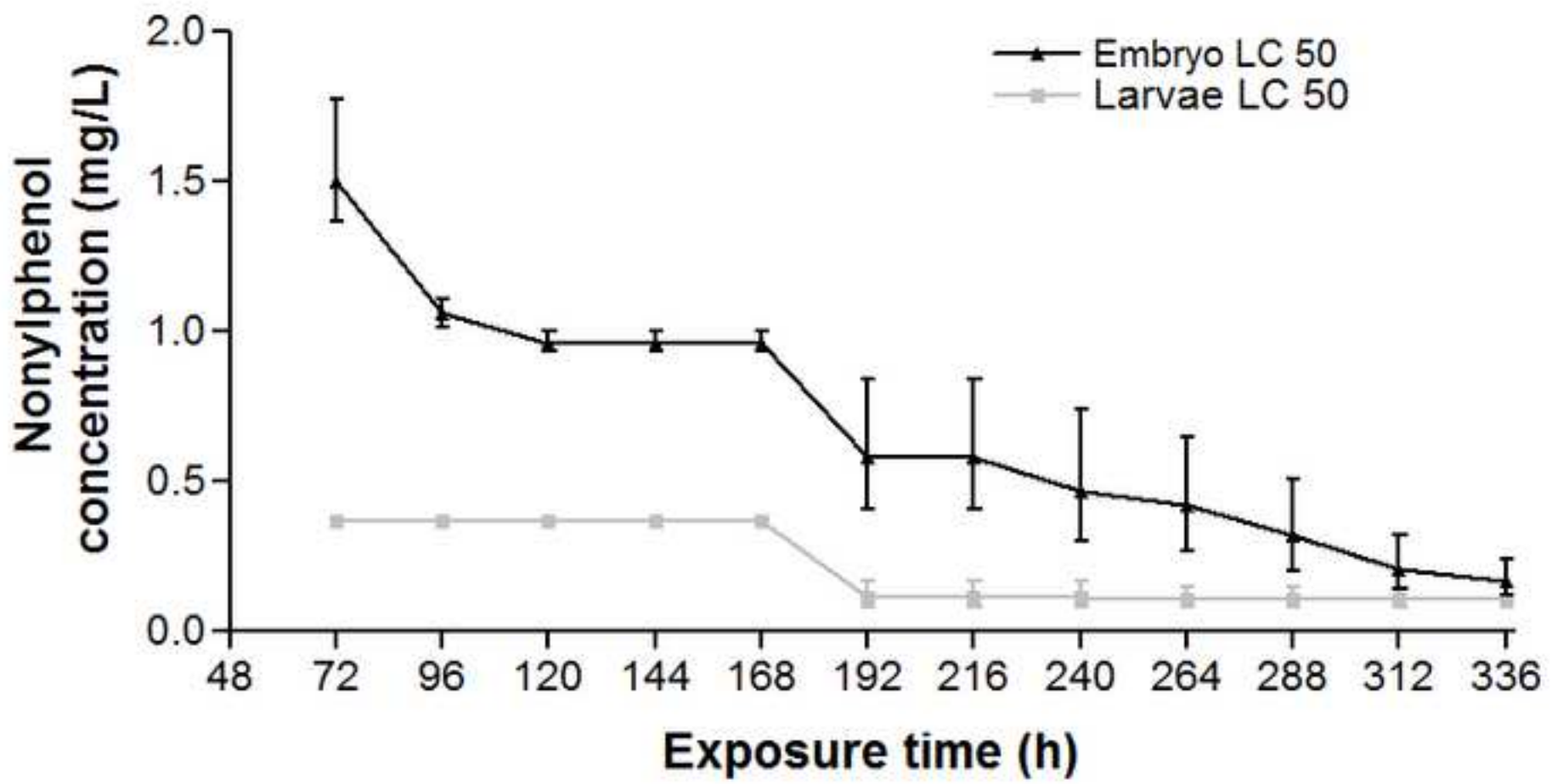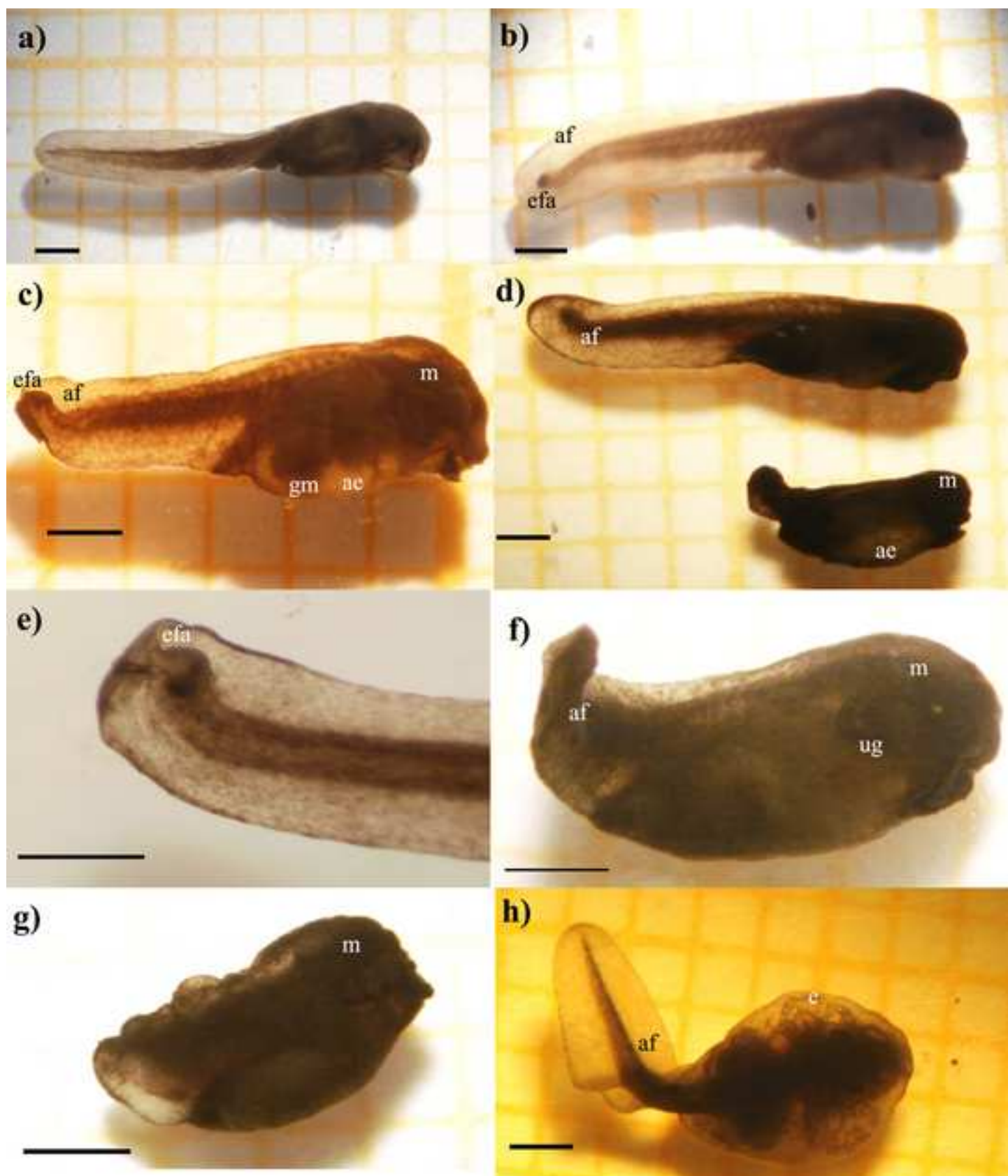
Main abnormalities (%)	(mg NP/L)			
	Control	0.25	0.75	1
Abmormal development	10	11	37	100
Delayed development	0	0	0	100
Reduced body size	7	7	38	100
Reduced tail size	0	0	38	100
Axial flexures	7	11	38	100
Extrusion of the fin axis	0	0	0	100
Agenesia/underdeveloped gills	0	0	38	86
Malformed mouth/adhesive structures	0	0	38	100
Hydropsy	0	0	38	86
Others	3	7	20	40