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Synergy Between Diazinon and Nonylphenol in Toxicity During the Early Development of the *Rhinella arenarum* Toad

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Abstract Diazinon is an extensively applied organophosphate pesticide, and nonylphenol is one of the major degradation products of nonylphenol polyethoxylates which are commonly used as surfactant in pesticide formulations. Both pollutants are widely distributed and often coexist in agroecosystems, where they might cause toxic effects to wild biota. This study assessed single and joint toxicity of binary mixtures of these organic compounds on the early development of *Rhinella arenarum* by means of a standardized test. Joint toxicity of diazinon/nonylphenol mixtures were assessed in embryos and larvae exposed to three different proportions at different exposure times. Embryo and larval toxicity was time-dependent, and larvae were significantly more sensitive than embryos to both compounds. For both embryos and larvae, nonylphenol was between 11 and 18 times more toxic than diazinon. Joint toxicity of the chemicals showed a tendency to be significantly higher than the predicted by additivity effects highlighting the threat that diazinon/nonylphenol mixtures represent for *Rhinella arenarum* populations.

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1 Introduction

The worldwide amphibian decline and the large number of malformations found in amphibian populations are largely due to environmental degradation (Wake and Vredenburg 2008). After habitat loss, pollution is the next major threatening process to amphibians (Mann et al. 2009). Many amphibian species inhabit in shallow, lentic, ephemeral ponds, brooks, or water bodies within agricultural regions, where pollutants might be concentrated (Natale 2006). Likewise, amphibian breeding occurs in spring and summer coincident with the highest pesticide application period (Mann et al. 2009). Despite that the IUCN (2015) had classified the status of the South American toad, *Rhinella arenarum*, as "least concern" in 2004, information needs updating. Thus, *Rhinella arenarum* is the species with the highest incidence of malformations from the central region of Argentina (Peltzer et al. 2011). Moreover, projection of *Rhinella arenarum* population size exhibited a tendency to extinction in the Argentinean central region, an agriculture area dominated mainly by corn and soybean crops (Bionda et al. 2013).

Information about the toxicity of compound mixture on amphibians is still limited. Components in mixtures are likely to cause different responses when animals are exposed to each individually, but additive, synergistic, or antagonistic effects are expected when animals are

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exposed to mixtures derived of the runoff, end use formulations, or tank mixes. Current methods of risk assessment usually focus only on the analysis of a single chemical, usually the active ingredient, and toxicity which may underestimate the risk associated with toxic interaction of mixtures (Backhaus and Faust 2012). Evaluation of mixture toxicity, especially for commonly coexisting contaminants, such as pesticides and surfactants in agroecosystems, is very important to analyze the risk assessment to non-target organisms. In this context, it is relevant to reach a better understanding of the risk that mixture toxicity might exert on native amphibian species.

Diazinon is a moderately persistent organophosphate pesticide extensively applied to control insects (Banaee et al. 2011). The main mechanism of toxicity of this pesticide is based on its ability to inhibit acetylcholinesterase (AChE) (Fulton and Key 2001), the enzyme responsible for inactivating the neurotransmitter acetylcholine. Diazinon has been classified by the World Health Organization (WHO) as moderately hazardous, Class II. Moreover, the International Agency for Research on Cancer (IARC) has recently classified diazinon as probably carcinogenic to humans (Group 2A) (International Agency for Research on Cancer 2015). Diazinon toxicity on other amphibians was evaluated; acute parameters as the LC50-96 h of 0.44, 7.49, and 9.84 mg/L for *Bufo regularis* adults, *Rana booylii* larvae, and *Xenopus laevis* embryos were informed (Ezemonye and Tongo 2010; Modra et al. 2011; Sparling and Fellers 2007). Moreover, chronic exposure of *Bufo melanostictus* with LC50-30 days of 6 and 7.5 mg/L for embryos and larvae, respectively, was also reported (Sumanadasa et al. 2008). *Rhinella arenarum* was identified as one of the most sensitive amphibians to the pesticide (Aronzon et al. 2014b). It has been previously shown that diazinon caused diverse sublethal effects in amphibian embryos and larvae. The characteristic diazinon sublethal effect on larvae was an abnormal behavior related to neurotoxicity (Aronzon et al. 2014b).

Nonylphenol polyethoxylates (NPEOs) are an important group of non-ionic surfactants widely used as detergents, emulsifiers, wetting and dispersing agents, antistatic agents, demulsifiers, and solubilizers (Soares et al. 2008). Nonylphenol, as one of the major degradation products of NPEOs, enters into the aquatic environment through wastewater discharges but also by drift and runoff of applied field products (Naylor 1995). Nonylphenol has been regarded as the most critical metabolite of alkylphenol polyethoxylates because of its enhanced resistance to biodegradation, toxicity, and bioaccumulation ability in aquatic organisms (Arukwe et al. 2000). Nonylphenol is an emerging pollutant not currently covered by water quality regulations; nevertheless, it might be a potential threat to ecosystems and human health (Farré et al. 2008). Concerning amphibians, several toxicity studies have just reported the acute effects of nonylphenol on specific periods of the early life cycle of different species as *X. laevis* (LC50-96 h of 3.9 mg/L), *Criniain signifera* (LC50-140 h of 6.4 mg/L), and *Litoria adelaidensis* (LC50-140 h of 9.2 mg/L), respectively (Mann and Bidwell 2000). Among them, *Rhinella arenarum* was one of the most sensitive amphibian species (Aronzon et al. 2014a; Mann and Bidwell 2000). Nonylphenol and diazinon might co-occur in aquatic environment, as consequence of the independent runoff, and because NPEOs are routinely included as wetting agents and dispersants in pesticide formulations (Mann and Bidwell 2000) or tank mixes.

The main aim of the present study was to assess the potential interaction effects of diazinon and nonylphenol mixtures based on lethality data in *Rhinella arenarum* embryos and larvae by means of a laboratory standardized protocol. Simultaneous to single exposure, bioassays at different mixture proportions were performed.

2 Materials and Methods

2.1 *Rhinella arenarum* Embryos and Larvae

Three independent clutches were obtained from three different couples of healthy, males and females, *Rhinella arenarum* adults, weighing approximately 200–250 g obtained in Buenos Aires (Argentina, 35° 11′ S; 59° 05′ W). Ovulation of females was induced by means of an intraperitoneal injection of 5000 IU of human chorionic gonadotropin (Mann and Bidwell 2000). Oocytes were fertilized in vitro with a 10 % sperm suspension in AMPHITOX solution (AS), which composition (mg/L) is as follows: Na^+, 14.75; Cl^-, 22.71; K^+, 0.26; Ca^{2+}, 0.36; and HCO_3^-, 1.45. After fertilization, embryos were kept in AS at 20±2 °C until reaching blastula

S.4 (embryo) or S.25 (larval) stages (Del Conte and Sirlin 1951). For toxicity experimental protocols from blastula stage (S.4), 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 saturated NaOH solution every 100 mL of AS, and then thoroughly washed. 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).

2.2 Test Solutions

Diazinon (purity 99 %, CAS number: 333-41-5, Supelco Analytical) final stock solution of 3 g/L was prepared in acetone. For single diazinon exposure, nine test solutions, ranging between 1.5 and 45 mg diazinon/L, were prepared by diluting the stock solution in AS. Diazinon concentrations in test solutions were analyzed by HPLC-ESI-MS in SIM mode, positive detection. The error between nominal and measured concentrations did not exceed 5 %.

Nonylphenol (technical grade, purity 96.9 %; CAS number: 84852-15-3, Fluka marketed by Sigma-Aldrich) stock solution of 45.4 g/L was prepared in acetone. A final stock solution of 800 mg nonylphenol/L was also prepared in acetone. For single nonylphenol exposure, nine test solutions ranging in concentrations between 0.025 and 4 mg nonylphenol/L were prepared by diluting the second stock solution in AS. Nonylphenol in test solutions was quantified by reverse-phase HPLC coupled to fluorescence detection (Babay et al. 2008, 2014). The errors between nominal and measured concentrations did not exceed 10 %.

For joint exposure, stock solutions of diazinon/nonylphenol mixtures were prepared by dissolving diazinon and nonylphenol stock solution in AS. Binary mixtures were prepared using different diazinon/nonylphenol proportions based on the corresponding LC50-168 h for each chemical (Table 1), which were obtained independently and simultaneously for the corresponding clutches. Thus, the proportions were expressed as the minimum entire relationship of toxic units (TU) (Sprague 1970; van der Geest et al. 2000). This concept, which was first described by Sprague (1970), assigns a value of 1 TU to a toxicant concentration that elicits a particular response—in the case of the present study, 50 % lethality at 168 h (LC50-168 h). For equitoxic mixtures, diazinon and nonylphenol were combined in equal proportions of their respective toxicity. For example, in the case of embryos, stock solution contained 33.4 mg diazinon/L and 2.175 mg nonylphenol/L, 1.5 times their respective LC50-168 h, (1 TU diazinon/1 TU nonylphenol). For non-equitoxic mixtures, the proportions 1:2 (1 TU diazinon/2 TU nonylphenol) and 2:1 (2 TU diazinon/1 TU nonylphenol) were assayed for embryos and 2:3 (2 TU diazinon/3 TU nonylphenol) and 3:2 (3 TU diazinon/2 TU nonylphenol) for larvae (Table 1).

Table 1 Concentration of diazinon (Dz) and nonylphenol (NP) in the stock solution of each mixture proportion

Developmental stage	Mixture ratio (TU)	Diazinon (mg/L)	Nonylphenol (mg/L)
Embryos	1 Dz/1 NP	33.4	2.17
	1 Dz/2 NP	22.3	3
	2 Dz/1 NP	45	1.5
Larvae	1 Dz/1NP	12.5	0.89
	2 Dz/3 NP	12.5	1.35
	3Dz/2 NP	25	1.2

The toxicity of the mixtures was determined using a fixed ratio design. Nine test solutions for each mixture proportion were prepared by diluting the corresponding volume of the final stock solutions in AS (dilution factor = 0.9), maintaining the chemical proportions. Embryos and larvae were exposed to nine dilutions of each stock mixture solution up to 168 h.

2.3 Toxicity Experimental Protocols

Rhinella arenarum embryos and larvae obtained were exposed to diazinon and nonylphenol independently and in mixtures from S.4 (embryos) and S.25 (larvae) stages onward for acute (96 h) and short-term chronic (168 h) periods.

For each experimental condition, triplicate batches of ten embryos or larvae were placed in covered 10-cm-diameter glass petri dishes containing 40 mL of test solution. A solution of AS plus acetone at the highest concentration used for diazinon or nonylphenol solutions was added as a carrier control. Acetone concentration in test solutions was always lower than 1.1 % (ASTM 2004). Lethality was evaluated and dead individuals were removed every 24 h. Test solutions were renewed every other day, and temperature was maintained at 20 ± 2 °C. Larvae were fed with 6 ± 0.5 mg

balanced fish food TetraColor® for 24 h every other day. The survival of AS and acetone controls did not significantly differ from each other ($p = 0.05$); therefore, the results were pooled for the analysis. The survival of embryos and larvae in control groups was between 95 and 100 %. The experimental protocol was conducted for each of the three clutches.

2.4 Statistical Analysis

Lethality data were statistically analyzed by the USEPA Probit Program (US EPA 1988). LC50s were obtained for each single chemical and mixture proportion. Isotoxicity curves were plotted based on the LC50 at different exposure times. To compare LC50 values of embryos and larvae, and at different exposure times, differences were considered to be statistically significant when the higher/lower ratio exceeded the corresponding critical value established by APHA et al. (2005). Differential sensitivity inter-clutches were expressed as a coefficient of variation.

Marking's additive index of coeffects for aquatic toxicology was used to evaluate joint toxicity (1977). The additive index (S) and its statistical significance were calculated for each mixture proportion at different exposure times (Marking 1977):

$$S = (A_m/A_i) + (B_m/B_i) \quad (1)$$

where S is the joint toxicity of the paired compounds, A and B are the experimental compounds, A_i is the LC50 of compound A when used alone, A_m is the LC50 of compound A when used jointly, B_i is the LC50 of compound B when used alone, and B_m is the LC50 of compound B when used jointly.

In general terms, when $S > 1$, the coeffects were taken as antagonistic; when $S < 1$, the coeffects were taken as synergistic; and when $S = 1$, the coeffects were taken as adding effects (Li et al. 2014).

The two-sided effect isobole model was used to schematize the effective toxicity of the two components in the mixture (Fig. 1). A straight line joining the LC50s of single chemical A and single chemical B represents the expected LC50s of different A/B proportions, assuming that the interactions are due to simple additive effects. The empirical LC50 of the mixture is the concentration of chemical A and chemical B in the mixture that cause 50 % lethality (for example, point M in Fig. 1).

3 Results

3.1 Mixture Toxicity to *Rhinella arenarum* Embryos

Concentrations of nonylphenol and diazinon causing the mortality of 50 % of embryos (LC50) from 72 to 168 h are shown in Fig. 2. Comparatively, nonylphenol was between 16 and 11 times more toxic than diazinon to *Rhinella arenarum* embryos. Toxicity of both chemicals significantly increased with exposure time. Thus, diazinon LC50 significantly decreased from 32.50 to 24.89 mg diazinon/L and 22.27 mg diazinon/L at 72, 96, and 168 h, respectively. Likewise, nonylphenol LC50 significantly decreased from 2.25 mg nonylphenol/L at 72 h, to 1.45 mg nonylphenol/L at 168 h (Fig. 2). There were no significant differences in susceptibility between clutches, and the coefficients of variation were always lower than 10 %.

Additive index (S) of the joint toxicity of diazinon/nonylphenol assessed at three proportions at different exposure times is shown in Table 2. Joint toxicity of diazinon/nonylphenol was mainly additive but showed a tendency to be synergistic after the acute period.

The isobolograms shown in Fig. 3a, b illustrate the results obtained for the equitoxic and two non-equitoxic diazinon/nonylphenol mixtures at 96 and 168 h, respectively. The fact that equitoxic mixture causing 50 % of lethality lies below and to the left of the additivity line is suggestive of synergistic interactions (Fig. 3a). The mixture of 1 Dz/2 NP showed a synergistic toxicity from the acute to short-term chronic period (Table 2 and Fig. 3b).

3.2 Mixture Toxicity to *Rhinella arenarum* Larvae

Nonylphenol and diazinon concentrations causing 50 % of larval lethality from 72 to 168 h of exposure are shown in Fig. 4. In the same way that embryos exposure, nonylphenol was between 14 and 18 times more toxic than diazinon to larvae. Toxicity of both chemicals was time-dependent, as it also increased with exposure time. Thus, diazinon LC50 decreased from 13.32 to 12.25 mg diazinon/L and 8.34 mg diazinon/L at 72, 96, and 168 h, respectively. However, nonylphenol LC50 diminished from 0.87, to 0.72 and 0.59 mg nonylphenol/L at 72, 96, and 168 h, respectively (Fig. 4). There were no significant differences in larvae susceptibility between clutches, and the

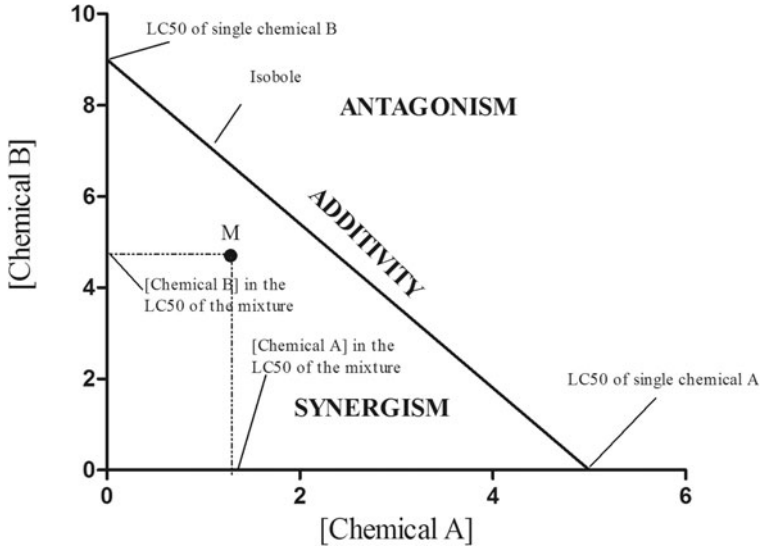

Fig. 1 Example of an isobologram of binary mixtures. A straight line joining the LC50s of single chemical A and single chemical B represents the expected LC50s of different A/B ratios, assuming the interactions are due to simple concentration-additivity of the two chemicals. The empirical LC50 of the mixture is the concentration of chemical A and chemical B in the mixture that results in 50 % lethality (for example, point M)

coefficients of variation for both compounds were always lower than 11 %.

Additive index (*S*) of the joint toxicity of diazinon/nonylphenol on *Rhinella arenarum* larvae assessed at three proportions at different exposure times are shown in Table 3. The joint toxicity of diazinon/nonylphenol at the beginning of larval development (S.25) showed a general tendency to be synergistic mainly at the acute period.

The isobolograms shown in Fig. 5a, b illustrate results obtained for equitoxic and non-equitoxic mixtures at 96 and 168 h, respectively. The fact that all nonylphenol/diazinon mixture proportions causing 50 % lethality lie below and to the left of the additivity line suggests synergistic interactions (Table 3 and Fig. 5a).

4 Discussion

Organisms in natural ecosystems are commonly exposed to chemical mixtures rather than single compounds. This is particularly true for amphibians living in agricultural areas where different agrochemicals are simultaneously applied. The study of non-equitoxic mixtures provides better information on mixture toxicity than just equitoxic mixture assays. Indeed, this approach is more environmentally realistic because it is highly unlikely that chemicals in a mixture will occur in equitoxic ratios (Warne 2003).

Some procedures predict the effects of mixtures using toxicological data of individual compounds obtained from standardized bioassays (Nowell et al. 2014). Furthermore, from experiment to experiment,

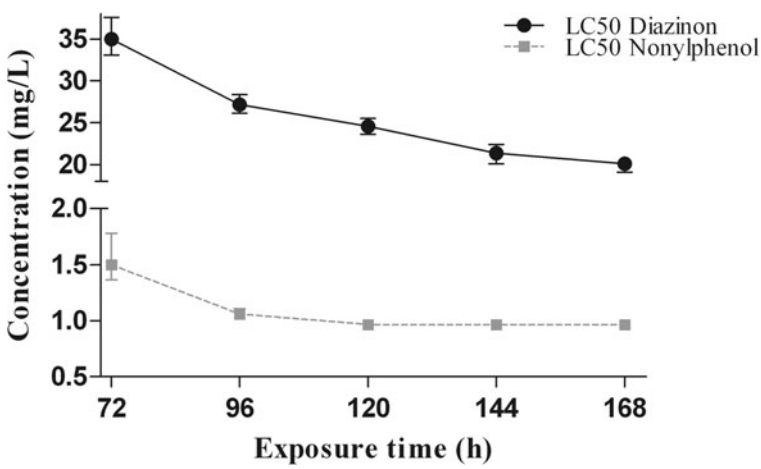

Fig. 2 Lethal concentration 50 (LC50) of single diazinon and nonylphenol for *Rhinella arenarum* embryos. *Bars* show 95 % confidence intervals

Table 2 Additive indexes (*S*) for different diazinon (Dz) and nonylphenol (NP) mixture proportions at different exposure times for *Rhinella arenarum* embryos

Mixture ratio (TU)	Exposure time (h)	Additive index (*S*)	Interaction
1 Dz/1 NP	72	0.86	Additivity
1 Dz/2 NP	72	0.99	Additivity
2 Dz/1 NP	72	0.91	Additivity
1 Dz/1 NP	96	0.57*	Synergism
1 Dz/2 NP	96	0.74*	Synergism
2 Dz/1 NP	96	0.87	Additivity
1 Dz/1 NP	168	0.84	Additivity
1 Dz/2 NP	168	0.67*	Synergism

Joint toxicity of 2 Dz/1 NP mixture at 168 h could not be calculated

*Significant differences ($p = 0.05$) from expected additivity

complexity, variability, and reproducibility might change when toxicity results are obtained in high biological levels, such as lethality (Barata et al. 2006). Accounting for these issues, in this study, toxicity of mixtures and single components was simultaneously assessed by employing embryos and larvae obtained from the same clutches by means of LC50 values. This methodology reduces the chances of false positive or negative results and gives strength to the prediction, because inter-test variability of test population may lead to misinterpret the joint toxicity (De Laender et al. 2009).

Most toxicity studies focus just on acute effects during a certain period of the life cycle. However, the present results showed that larvae are significantly more sensitive than embryos specially at short-term chronic exposure; this should be taken into account in regulatory decisions for nature conservation purposes (Greulich and Pflugmacher 2003; Hutler Wolkowicz et al. 2014). Although embryonic development has been shown as the most sensitive life cycle stage to different pollutants (Aronzon et al. 2011a, b; Sztrum et al. 2011), in present study, larvae were till 2.6 times more sensitive than embryos to both organic compounds. This finding is consistent with literature data for a freshwater fish Japanese medaka exposed to diazinon (Hamm and Hinton 2000), and also for *Rana clamitans*, *Rana pipiens*, *Rana sylvatica*, *Bufo americanus*, and *X. laevis* exposed to glyphosate formulations with different surfactants (Edginton et al. 2004; Howe et al.

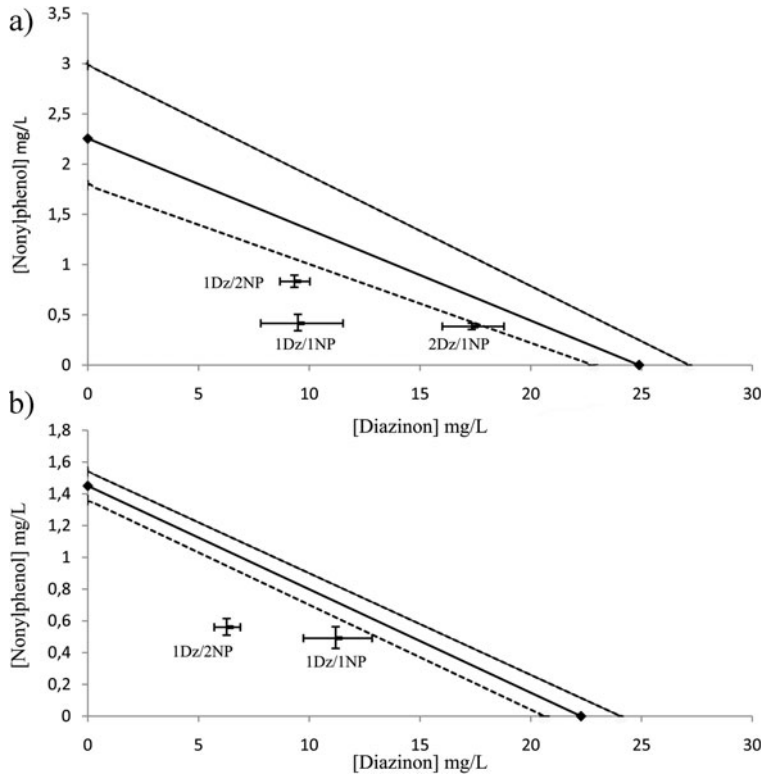

Fig. 3 Isobolograms for lethal concentration 50 (LC50) of different diazinon (Dz) and nonylphenol (NP) mixture proportions at **a** 96 h and **b** 168 h, for *Rhinella arenarum* embryos. LC50 points with 95 % confidence limits are plotted

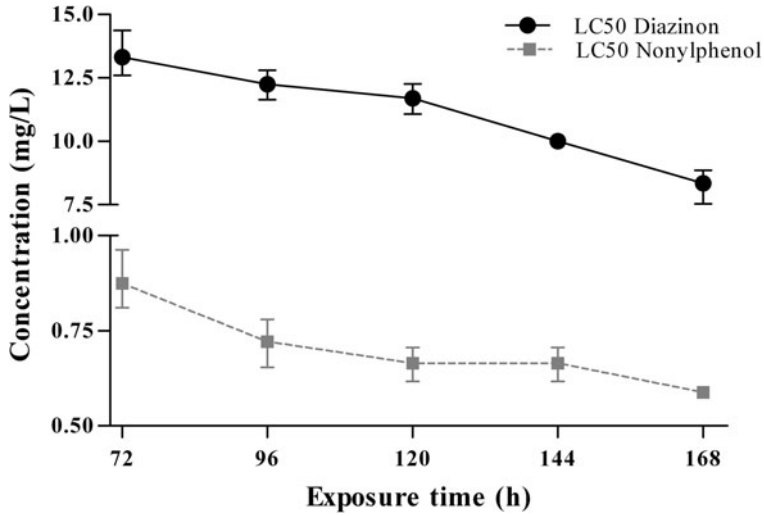

Fig. 4 Lethal concentration 50 (LC50) of single diazinon and nonylphenol for *Rhinella arenarum* larvae. *Bars* show 95 % confidence intervals

2004). This differential stage sensitivity might be due to the slightest contact between embryo and the exposure medium and the possible exclusion of chemicals by embryonic membranes (Edginton et al. 2004; Howe et al. 2004). Also, it might be due to a lack or insensitivity of target organs in the embryonic stages compared to the larval period, leading to differential exposure times of sensitive target organs (Edginton et al. 2004). Particularly, in the case of diazinon, this might be related to the developmental beginning of AChE activity, which is well correlated to amphibian muscular and nerve development (Gindi and Knowland 1979).

Table 3 Additive indexes (*S*) for different diazinon (Dz) and nonylphenol (NP) mixture proportions for *Rhinella arenarum* larvae

Mixture ratio (TU)	Exposure time (h)	Additive index (*S*)	Interaction
1 Dz/1 NP	72	0.60*	Synergism
2 Dz/3 NP	72	0.79*	Synergism
2 Dz/1 NP	72	0.81*	Synergism
3 Dz/2 NP	72	0.77*	Synergism
1 Dz/1 NP	96	0.67*	Synergism
2 Dz/3 NP	96	0.90	Additivity
3 Dz/2 NP	96	0.85*	Synergism
2 Dz/1 NP	96	0.86*	Synergism
1 Dz/1 NP	120	0.76*	Synergism
2 Dz/3 NP	120	1.01	Additivity
3 Dz/2 NP	120	0.93	Additivity
2 Dz/1 NP	120	0.92	Additivity
1 Dz/1 NP	144	0.76*	Synergism
2 Dz/3 NP	144	1.01	Additivity
3 Dz/2 NP	144	0.90	Additivity
1 Dz/1 NP	168	0.71*	Synergism
3 Dz/2 NP	168	0.94*	Additivity

Joint toxicity of 2 Dz/1 NP mixture proportion at 144 and 168 h and 2 Dz/3 NP ratio at 168 h could not be calculated

*Significant differences ($p = 0.05$) from expected additivity

Moreover, most toxicity bioassays are set at acute exposure time; however, nonylphenol and diazinon toxicity was clearly time-dependent, and the most severe effects were observed toward the end of short-term chronic exposure. This fact brings attention on the value of extending the exposure time, and it also highlights the relevance of looking for the most sensitive life cycle stage for species conservation purposes. In the case of nonylphenol and diazinon, the most sensitive period was the larval one, reaching LC50s values of 0.59 mg nonylphenol/L and 8.34 mg diazinon/L at 168 h. Moreover, previous studies on *Rhinella arenarum* show an increased toxicity of both substances toward the chronic exposure, with LC50 of 0.11 mg nonylphenol/L at 336 h (Aronzon et al. 2014a) and LC50 of 1.9 mg diazinon/L at 504 h (Aronzon et al. 2014b).

Despite that *Rhinella arenarum* embryos were more resistant to diazinon during the acute period than *X. laevis* and *Rana booylii* (Modra et al. 2011; Sparling and Fellers 2007), larvae resulted significantly more sensitive than other anurans such as *B. melanostictus* (Sumanadasa et al. 2008). *Rhinella*

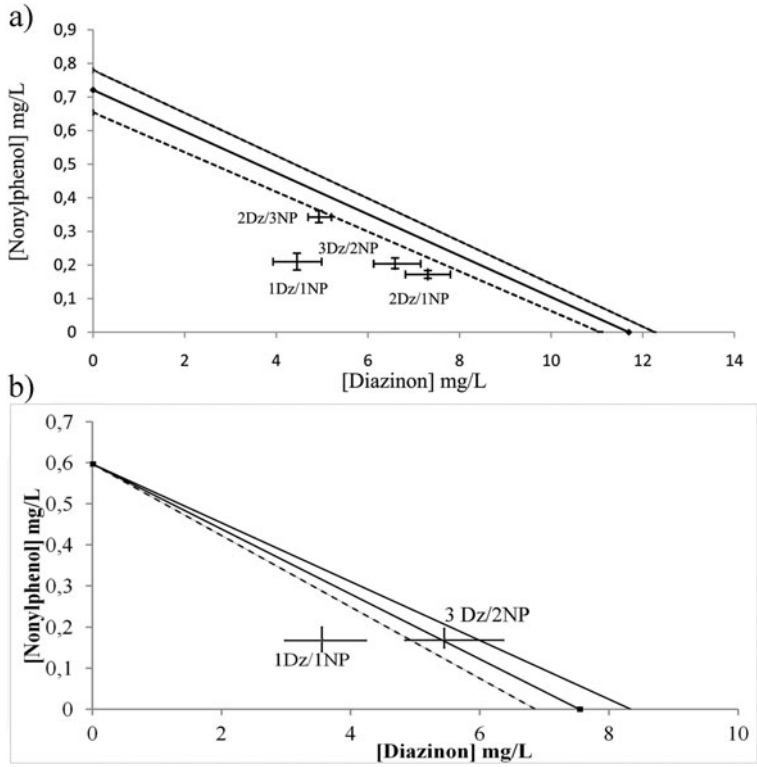

Fig. 5 Isobolograms for lethal concentration 50 (LC50) of different diazinon (Dz) and nonylphenol (NP) mixture proportions at **a** 96 h and **b** 168 h, for *Rhinella arenarum* larvae. LC50 points with 95 % confidence limits are plotted

arenarum was also more sensitive to nonylphenol than *X. laevis*, *C. signifera*, and *L. adelaidensis* (Mann and Bidwell 2000). It is noteworthy that single nonylphenol was between 18 and 14 times more toxic than diazinon to *Rhinella arenarum* larvae. Nonylphenol is an emerging pollutant, not currently covered by water quality regulations, and is thought to be a potential threat to ecosystems and human health (Farré et al. 2008). Thus, Europe has followed the recommendation of phasing out the use of alkylphenol ethoxylate surfactants in domestic and industrial cleaning agents. Moreover, Canada has recently adopted nonylphenol guidelines for aquatic life protection (Berryman et al. 2004). Nevertheless, the use of alkylphenol ethoxylates is still completely unrestricted in Latin American countries. These results are particularly relevant for Argentina and other developing countries where large agricultural areas are treated with pesticides containing non-ionic surfactants. Despite that some active ingredients of pesticide are reported of low toxicity, the surfactants added may be a health risk to aquatic biota as is shown in this study. In the case of amphibians, this is more relevant because pesticides are applied around standing or ephemeral waters with low dilution capacity (Mann and Bidwell 1999).

Present results on both equitoxic and non-equitoxic diazinon/nonylphenol mixtures suggest synergism (Tables 2 and 3, and Figs. 3 and 5). These synergistic toxic effects give evidence of the possible higher toxicity of commercial formulations. Moreover, such synergistic interactions take relevance as generally represent a minority of the cases, as evidence shows that 70–80 % of chemical mixtures have additive toxicity, 10–15 % have antagonistic toxicity, and 10–15 % have synergistic toxicity (Warne 2003).

The synergistic interactions might be the result of three different processes: (i) those involved in determining external exposure in environmental media; (ii) uptake rates, assimilation distribution, and excretion (toxicokinetics), and (iii) the association of the chemical with relevant receptors (toxicodynamics) (Spurgeon et al. 2010). These facts highlight the relevance of assessing mixture toxicity of surfactants and active ingredient pesticides commonly present in the environment.

5 Conclusion

Rhinella arenarum larvae were more sensitive than embryos as consequence of the exposure to both

compounds. Single nonylphenol was more toxic than diazinon, which also highlights the importance of assessing surfactant toxicity on non-target organisms.

The toxicity of diazinon/nonylphenol mixtures provides some perspective on the implications of these contaminants in aquatic environmental quality, highlighting the threat that both chemicals represent for *Rhinella arenarum* populations and the relevance of evaluating mixture toxicity for risk assessments and wildlife preservation purposes.

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References

American Public Health Association, American Water Works Association & Water Pollution Control Federation (2005). Standard methods for the examination of water and wastewaters, In A. P. H. Association (Ed.), (p. 1200 pp). Washington DC.

Aronzon, C., Sandoval, M., Herkovits, J., & Pérez-Coll, C. (2011a). Stage-dependent toxicity of 2,4-dichlorophenoxyacetic on the embryonic development of a South American toad. *Rhinella arenarum, Environmental Toxicology, 26*, 373–381.

Aronzon, C. M., Babay, P. A., & Pérez Coll, C. S. (2014a). Developmental toxicity and risk assessment of nonylphenol to the South American toad. *Rhinella arenarum, Environmental Toxicology and Pharmacology, 38*, 634–642.

Aronzon, C. M., Marino, D. J., Ronco, A. E., & Pérez Coll, C. S. (2014b). Differential toxicity and uptake of diazinon on embryo-larval development of *Rhinella arenarum*. *Chemosphere, 100*, 50–56.

Aronzon, C. M., Sandoval, M. T., Herkovits, J., & Pérez-Coll, C. S. (2011b). Stage-dependent susceptibility to copper in *Rhinella arenarum* embryos and larvae. *Environmental Toxicology and Chemistry, 30*, 2771–2777.

Arukwe, A., Thibaut, R., Ingebrigtsen, K., Celius, T., Goksøyr, A., & Cravedi, J. P. (2000). In vivo and in vitro metabolism and organ distribution of nonylphenol in Atlantic salmon (Salmo salar). *Aquatic Toxicology, 49*, 289–304.

ASTM (2004). Standard guide for conducting the frog embryo teratogenesis assay-*Xenopus* (FETAX).American Society for Testing and Materials. Standards on aquatic toxicology and hazard evaluation. E 1439 – 98 (2004), Philadelphia, PA, pp. 1-16.

Babay, P. A., Itria, R. F., Ale, R., Emiliano, E., Becquart, E. T., & Gautier, E. A. (2014). Ubiquity of endocrine disruptors nonylphenol and its mono- and di-ethoxylates in freshwater, sediments, and biosolids associated with high and low density populations of Buenos Aires, Argentina. *CLEAN-Soil, Air, Water, 42*, 731–737.

Babay, P. A., Romero-Ale, E. E., Itria, R. F., Becquart, E. T., Thiele, B., & Batistoni, D. A. (2008). Simplified determination of lipophilic metabolites of nonylphenol ethoxylates: method development and application in aqueous samples from Buenos Aires, Argentina. *Journal of Environmental Monitoring, 16*, 443–452.

Backhaus, T., & Faust, M. (2012). Predictive environmental risk assessment of chemical mixtures: a conceptual framework. *Environmental Science and Technology, 46*, 2564–2573.

Banaee, M., Sureda, A., Mirvaghefi, A. R., & Ahmadi, K. (2011). Effects of diazinon on biochemical parameters of blood in rainbow trout (*Oncorhynchus mykiss*). *Pesticide Biochemistry and Physiology, 99*, 1–6.

Barata, C., Baird, D. J., Nogueira, A. J. A., Soares, A. M. V. M., & Riva, M. C. (2006). Toxicity of binary mixtures of metals and pyrethroid insecticides to *Daphnia magna* Straus. Implications for multi-substance risks assessment. *Aquatic Toxicology, 78*, 1–14.

Berryman, D., Houde, F., DeBlois, C., & O'Shea, M. (2004). Nonylphenolic compounds in drinking and surface waters downstream of treated textile and pulp and paper effluent: a survey and preliminary assessment of their potential effects on public health and aquatic life. *Chemosphere, 56*, 247–255.

Bionda, C., Lajmanovich, R., Salas, N., Martino, A., & di Tada, I. (2013). Population demography in Rhinella arenarum (Anura: Bufonidae) and Physalaemus biligonigerus (Anura: Leiuperidae) in agroecosystems in the province of Córdoba, Argentina. *International Journal of Tropical Biology and Conservation, 61*, 1389–1400.

Canadian Council on Animal Care in Science (1993). Guide to the care and use of experimental animals, p. 298. http://www.ccac.ca/Documents/Standards/Guidelines/Experimental_Animals_Vol291.pdf.

De Laender, F., Janssen, R., & De Schamphelaer, K. A. C. (2009). Non-simultaneous ecotoxicity testing of single chemicals and their mixture results in erroneous conclusions about the joint action of the mixture. *Chemosphere, 76*, 428–432.

Del Conte, E., & Sirlin, L. (1951). The firts stages of *Bufo arenarum* development. *Acta Zool Lilloana, 12*, 495–499.

Edginton, A. N., Sheridan, P. M., Stephenson, G. R., Thompson, D. G., & Boermans, H. J. (2004). Comparative effects of pH and Vision® herbicide on two life stages of four anuran amphibian species. *Environmental Toxicology and Chemistry, 23*, 815–822.

Ezemonye, L., & Tongo, I. (2010). Acute toxic effects of endosulfan and diazinon pesticides on adult amphibians (*Bufo regularis*). *Journal of Environmental Chemistry and Ecotoxicology, 2*, 73–78.

Farré, M. l., Pérez, S., Kantiani, L., & Barceló, D. (2008). Fate and toxicity of emerging pollutants, their metabolites and transformation products in the aquatic environment. *TrAC, Trends in Analytical Chemistry, 27*, 991–1007.

Fulton, M. H., & Key, P. B. (2001). Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophophorus insecticide exposure and effects. *Environmental Toxicology and Chemistry, 20*, 37–45.

Gindi, T., & Knowland, J. (1979). The activity of cholinesterases during the development of *Xenopus laevis*. *Journal of Embryology and Experimental Morphology, 51*, 209–215.

Greulich, K., & Pflugmacher, S. (2003). Differences in susceptibility of various life stages of amphibians to pesticide exposure. *Aquatic Toxicology, 65*, 329–336.

Hamm, J. T., & Hinton, D. E. (2000). The role of development and duration of exposure to the embryotoxicity of diazinon. *Aquatic Toxicology, 48*, 403–418.

Howe, C. M., Berrill, M., Pauli, B. D., Helbing, C. C., Werry, K., & Veldhoen, N. (2004). Toxicity of glyphosate-based pesticides to four North American frog species. *Environmental Toxicology and Chemistry, 23*, 1928–1938.

Hutler Wolkowicz, I. R., Herkovits, J., & Pérez Coll, C. S. (2014). Stage-dependent toxicity of bisphenol-a on *Rhinella arenarum* (Anura, Bufonidae) embryos and larvae. *Environmental Toxicology, 29*, 146–154.

International Agency for Research on Cancer (2015). Evaluation of five organophosphate insecticides and herbicides. In World Health Organization (Ed.), Lyon, France, IARC Monographs.

IUCN (2015). http://www.iucnredlist.org, in The IUCN Red List of Threatened Species (Ed.), Version 2015-4. Downloaded on 19 November 2015.

Li, W. E. I., Wei-Wei, S. H. A. O., Guo-Hua, D. I. N. G., Xiao-Li, F. A. N., Miao-Ling, Y. U., & Zhi-Hua, L. I. N. (2014). Acute and joint toxicity of three agrochemicals to Chinese tiger frog (*Hoplobatrachus chinensis*) tadpoles. *Zoological Research, 35*, 272–279.

Mann, R. M., & Bidwell, J. R. (1999). The toxicity of glyphosate and several glyphosate formulations to four species of southwestern Australian frogs. *Archives of Environmental Contamination and Toxicology, 36*, 193–199.

Mann, R. M., & Bidwell, J. R. (2000). Application of the FETAX protocol to assess the developmental toxicity of nonylphenol ethoxylate to *Xenopus laevis* and two Australian frogs. *Aquatic Toxicology, 51*, 19–29.

Mann, R. M., Hyne, R. V., Choung, C. B., & Wilson, S. P. (2009). Amphibians and agricultural chemicals: review of the risks in a complex environment. *Environmental Pollution, 157*, 2903–2927.

Marking, L. L. (1977). Method for assessing additive toxicity of chemical mixtures. Aquatic toxicology and hazard evaluation (ASTM) American Society for Testing and Materials.

Modra, H., Vrskova, D., Macova, S., Kohoutkova, J., Hajslova, J., Haluzova, I., & Svobodova, Z. (2011). Comparison of diazinon toxicity to embryos of *Xenopus laevis* and *Danio rerio*; degradation of diazinon in water. *Bulletin of Environmental Contamination and Toxicology, 86*, 601–604.

Natale, G. S. (2006). Análisis ecotoxicológico de una comunidad de anuros de la Región Pampeana - Efecto del Cr(VI) sobre embriones y larvas de distintas especies de una taxocomunidad., *Tesis de Doctorado. Facultad de Ciencias Naturales y Museo. Universidad Nacional de La Plata, La Plata, p. 312*.

Naylor, G. C. (1995). Environmental fate and safety of nonylphenol ethoxylates. *Textile Chemist and Colorist, 27*, 29–33.

Nowell, L. H., Norman, J. E., Moran, P. W., Martin, J. D., & Stone, W. (2014). Pesticide toxicity index—a tool for assessing potential toxicity of pesticide mixtures to freshwater aquatic organisms. *Science of the Total Environment, 476–477*, 144–157.

Peltzer, P. M., Lajmanovich, R. C., Sanchez, L. C., Attademo, A. M., Junges, C. M., Bionda, C. L., Martino, A. L., & Bassó, A. (2011). Morphological abnormalities in amphibian populations from the mid-eastern region of Argentina. *Herpetological Conservation and Biology, 6*, 432–442.

Soares, A., Guieysse, B., Jefferson, B., Cartmell, E., & Lester, J. N. (2008). Nonylphenol in the environment: a critical review on occurrence, fate, toxicity and treatment in wastewaters. *Environment International, 34*, 1033–1049.

Sparling, D. W., & Fellers, G. (2007). Comparative toxicity of chlorpyrifos, diazinon, malathion and their oxon derivatives to larval *Rana boylii*. *Environmental Pollution, 147*, 535–539.

Sprague, J. B. (1970). Measurement of pollutant toxicity to fish. II Utilizing and applying bioassays results. *Water Research, 4*, 3–32.

Spurgeon, D. J., Jones, O. A. H., Dorne, J.-L. C. M., Svendsen, C., Swain, S., & Stürzenbaum, S. R. (2010). Systems toxicology approaches for understanding the joint effects of environmental chemical mixtures. *Science of the Total Environment, 408*, 3725–3734.

Sumanadasa, D. M., Wijesinghe, M. R., & Ratnasooriya, W. D. (2008). Effects of diazinon on larvae of the Asian common toad (*Bufo melanostictus*, Schneider 1799). *Environmental Toxicology and Chemistry, 27*, 2320–2325.

Sztrum, A. A., D'Eramo, J. L., & Herkovits, J. (2011). Nickel toxicity in embryos and larvae of the South American toad: effects on cell differentiation, morphogenesis, and oxygen consumption. *Environmental Toxicology and Chemistry, 30*, 1146–1152.

US EPA (1988). Users guide for a computer program for PROBIT analysis of data from acute and short-term chronic toxicity test with aquatic organisms', Biological Methods, Environmental Monitoring and Support Lab., United States Environmental Protection Agency.

van der Geest, H. G., Greve, G. D., Boivin, M.-E., Kraak, M. H. S., & van Gestel, C. A. M. (2000). Mixture toxicity of copper and diazinon to larvae of the mayfly (*Ephoron virgo*) judging additivity at different effect levels. *Environmental Toxicology and Chemistry, 19*, 2900–2905.

Wake, D. B., & Vredenburg, V. T. (2008). Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proceedings of the National Academy of Sciences, 105*, 11466–11473.

Warne, M. S. J. (Ed.). (2003). *A review of the ecotoxicity of mixtures, approaches to, and recommendations for their management*. Australia: Adelaide.