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RESEARCH ARTICLE

Mixture toxicity of copper and nonylphenol on the embryo-larval development of *Rhinella arenarum*

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Abstract

Copper and nonylphenol are two commonly found chemicals in the aquatic environment, particularly in the distribution area of the amphibian *Rhinella arenarum*. The current work evaluated the lethal toxicity of equitoxic and non-equitoxic binary mixtures of copper and nonylphenol on embryos and larvae of the South America toad by means of the standardized test, AMPHITOX. Joint toxicity of mixtures was assessed in several proportions of these compounds at different exposure times and was analyzed at different level of mortality effect (LC10, LC50 and LC90). Considering the LC50, the equitoxic mixture was always antagonistic independently of the exposure time and the developmental stage. Joint toxicity showed mainly an antagonistic pattern; nonetheless, some time-dependent additive interactions were observed. Regarding the LC10, synergistic interactions were found in embryos and larvae exposed to two different mixture proportions at several exposure times. This highlights the possible synergism of these chemicals at environmentally relevant concentrations. These results point out the relevance of assessing joint toxicity of environmental pollutants for environmental risk assessment.

Keywords Amphibians · Copper · Joint toxicity · Nonylphenol · Mixtures · Synergism

Introduction

Aquatic ecosystems are frequently polluted with chemicals derived from industrial, agricultural and domestic activities. Despite the need to assess single toxicity of specific chemicals, the behavior of substances in mixtures may not correspond to that predicted from data of the individual substances. The interactions between components may cause complex and substantial changes in the apparent properties of the constituents (Altenburger et al. 2003). Evidence shows that 70–80% of chemical mixtures exhibit additive toxicity, but 10–15% are synergistic and 10–15% antagonistic (Warne 2003). This questions the ecological relevance of safety limits of single contaminants, as chemical risk management is mainly based on the toxic effects of single compounds. Determining and predicting mixture toxicity on non-target organisms, especially for contaminants that commonly coexist in the environment, have been focal points in toxicity research (Aronzon et al. 2016; Brodeur et al. 2016; Brodeur et al. 2014; Li et al. 2018; Sanches et al. 2017; Wang et al. 2018). There are several experimental and analytical models to evaluate mixture toxicity. The concentration addition (CA) and independent action (IA) are two traditional concepts that have been widely utilized (Altenburger et al. 2003). CA is based on the assumption that mixture components have the same or similar modes of action, while IA is based on the idea that each mixture component acts on a different receptor and they contribute to a common response together. However, toxicological interactions between the chemicals and their effects can occur independently of the main mode of action, and the toxic modes of action of many chemicals remain unknown. The median drug effect analysis/combination index (CI) was first described by Chou and Talalay in 1984, but more recently, it has acquired relevance by the CompuSyn's program implementation (Chou 2006) which is a widely used method in toxicology for analyzing mixture toxicity interactions (Wang et al. 2015). The method does not require previous

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knowledge of the mechanisms of action of each chemical and takes into account both the potency and the shape of the dose-effect curve of each chemical. As the type of interaction may also vary with the effect level on which the mixture is being assessed (Son et al. 2016; Yang et al. 2017), CompuSyn's also enables to analyze quantitatively the mixture interaction at different effect levels corresponding to different proportions of lethal effects (LC10, LC50 and LC90).

Copper and nonylphenol are common contaminants, particularly in Argentina because they are agro-inputs frequently applied in fields. Both substances are present in the distribution area of the native amphibian species, *Rhinella arenarum* (Babay et al. 2014; Ossana et al. 2016; Reynoso and Andriulo 2013). Copper is frequently used as fungicide, algaecide and animal feed additive (Serén 2013), while nonylphenol is commonly used as an adjuvant for pesticides formulations. They are also present in sludges employed as soil fertilizers (Hildebrandt et al. 2007). Current regulations fixed a maximal copper concentration of 2 μg/L in surface freshwater for aquatic life protection (Argentine law 24,051 decree 831/93). However, copper eventually reaches concentrations higher than 2000 mg/L in periurban surface water bodies (Ossana et al. 2016) and 31 μg/L in surface waterbodies of agriculture areas (Reynoso and Andriulo 2013), both in the distribution area of *R. arenarum*. Despite that copper is essential for living organisms, it could be toxic if water concentrations increase as a consequence of anthropogenic activities (Cappello and Fortunato 2013; USEPA 2006). Copper can catalyze the formation of highly reactive hydroxyl radicals and initiate oxidative damage, interfering with important cellular events (Gaetke and Chow 2003). Copper exposure has been shown to affect behavior, metabolism, immunity, enzyme activities, ionic regulation and epithelial cells in gills and intestine in fish (Handy 2003). A previous study on *R. arenarum* showed a lowest LC50–24 h of 17 (15.8–18.4) μg Cu^{2+}/L at organogenic stages. Moreover, copper has a high teratogenic potential, eliciting diverse adverse effects such as reduced body size, axial flexures, microcephaly, acephaly, mouth malformations, agenesis/underdeveloped gills, agenesis/underdeveloped tail and hydropsy (Aronzon et al. 2011).

Nonylphenol is one of the major degradation products of the widely used surfactant nonylphenol polyethoxylate (Soares et al. 2008). Nonylphenol has been pointed out as the most critical metabolite of alkylphenol polyethoxylates because of its enhanced resistance to biodegradation, toxicity and ability to accumulate in aquatic organisms (Arukwe et al. 2000; Tyler et al. 1998). This compound is considered an emerging pollutant and is thought to be a potential threat to ecosystems and human health. In Argentina, nonylphenol use is unrestricted and widespread, and it is not currently covered by water-quality regulations (Babay et al. 2014; Farré et al. 2008). Despite the lack of actual information, maximal concentration of 27 μg/L has been reported (Babay et al. 2008). It has been demonstrated that nonylphenol binds to amphibian estrogen receptors (Lutz and Kloas 1999), induces feminization of *Xenopus laevis* males and stimulates vitellogenin m-RNA synthesis in cultured amphibian hepatocytes (Kloas et al. 1999). Early larval stages of *R. arenarum* (S.25) were very susceptible to nonylphenol, with a 336-h LC50 of 0.11 mg nonylphenol (NP)/L. This organic compound also caused severe sublethal effects, including malformations and delayed development with a low 168-h NOEC of 25 μg NP/L (Aronzon et al. 2014).

Amphibians have high sensitivity to diverse pollutants, mainly at embryo and larval stages. So, they are widely used in ecotoxicological studies and represent a useful tool for assessing the environmental risk of different physicochemical agents (Bach et al. 2016; Ferrari et al. 2005; Ibarra et al. 2016; Pérez Coll et al. 2017; Wolkowicz et al. 2016). Moreover, amphibians breed in shallow, lentic and/or ephemeral water bodies, even at agricultural landscapes, where pollutants might be concentrated during spring/summer, time coincident with their reproductive season (Mann et al. 2009). AMPHITOX test is a battery of bioassays using embryo and larval stages of *Rhinella arenarum* (Pérez Coll et al. 2017), a representative Argentinean species. Despite that it is considered a non-threatened species (Lavilla et al. 2000) or of least concern; this status is not updated (Kwet et al. 2004) and previous studies warn about the vulnerability of this species (Bionda et al. 2013). The evaluation of joint toxicity has also been performed by means of toxicity bioassays with amphibians (Brodeur et al. 2014; Svartz et al. 2016; Wu et al. 2018; Yu et al. 2015).

The main aim of present study was to assess the joint lethal effects of equitoxic and non-equitoxic binary mixtures of copper and nonylphenol in the South American toad, *R. arenarum* by means of the standardized AMPHITOX protocol. Mixture toxicity was assessed during the embryo and larval development in order to identify the most sensitive period. The experimental design included simultaneous single bioassays with copper and nonylphenol as well as several mixture proportions of chemicals. Lethal toxicity of the mixtures was evaluated at different exposure times up to sub-chronic period.

Materials and methods

Preparation of test solutions

Solutions of individual copper and nonylphenol

Seven test solutions of copper, ranging in concentrations between 3 and 375 μg Cu^{2+}/L, were prepared by diluting a stock solution of 30 mg Cu^{2+}/L with $CuCl_2 \cdot 2H_2O$ (purity 99%, lot 11570; Riedel-de Haën) in AMPHITOX Solution (AS). AS

composition, prepared in deionized water, was NaCl 36 mg/L, KCl 0.5 mg/L, $CaCl_2$ 1 mg/L and $NaHCO_3$ 2 mg/L. Experimental copper concentrations were measured randomly four different times with an inductively coupled plasma mass spectrometer (ICPMS) with collision cell (Agilent 7500cx model). The error between nominal and measured concentrations was between 2 and 4%.

Nonylphenol (Fluka, technical grade, purity 96.9%. CAS number: 84852-15-3, marketed by Sigma-Aldrich) stock solution of 45.4 g/L was prepared in acetone. Seven test solutions of nonylphenol, ranging in concentrations between 0.025 and 4 mg NP/L, were prepared in AS. Nonylphenol in four randomly chosen test solutions was quantified according to Babay et al. (2008) by reverse-phase HPLC coupled to fluorescence detection at excitation and emission wavelengths of 230 and 300 nm, respectively. A C-8 column (250 × 4.6 mm, 5 μm, Grace, USA) and isocratic elution with MeOH/H_2O (80:20) were employed. The errors between nominal and measured concentrations were between 2 and 7%.

Solutions of equitoxic and non-equitoxic binary mixtures of copper and nonylphenol

Mixture toxicity was evaluated using a fixed ratio design according to the method described previously by Aronzon et al. (2016). Different binary mixtures were combined using different ratios. Each combination was named as the minimum entire relation of toxic units (TU) (Sprague 1970; van der Geest et al. 2000). Based on this concept, a value of 1 TU represents the concentration of the toxicant that elicits a particular response; in the case of the present study 50% mortality at 168 h (168-h LC50). First, these LC50 for embryos and larvae were estimated according to previous work (Aronzon et al. 2014; Aronzon et al. 2011), but then were recalculated according to the data of simultaneous exposure obtained in this study and specified for each assay. In the case of equitoxic mixture, copper and nonylphenol were combined in equal proportion of its toxicity, so it was named 1Cu/1NP.

Stock solutions of equitoxic and non-equitoxic mixtures were prepared dissolving single stock solutions of copper and nonylphenol in AS. Exposure solutions of the different binary mixtures were prepared by diluting the corresponding volume of mixture stock solution in AS, in order to maintain the compound proportions. Copper and nonylphenol mixture toxicity bioassays were performed according to Table 1 conditions. Mixture toxicity interactions were evaluated by means of lethal effects. Therefore, the chosen concentrations could be higher than the environmental ones, but they were selected to elicit lethal effects.

Animal acquisition and husbandry

Healthy *R. arenarum* adults, weighing approximately 200–250 g, were obtained in Lobos (Buenos Aires province, Argentina: 35° 11′ S; 59° 05′ W), where no sources of contamination are nearby. Adults were maintained in laboratory conditions for 2–3 months. Toads care, fecundation, embryo and larval husbandry and experimental protocols were conducted according to AMPHITOX protocols (Herkovits and Pérez-Coll 2003; Pérez Coll et al. 2017). Briefly, toads were kept in aquaria with AS at 20 ± 2 °C, alternating 12-h light/dark cycles and fed with cockroaches and crickets bred in laboratory. Ovulation of *R. arenarum* females was induced by means of an intraperitoneal injection of 5000 IU of human chorionic gonadotropin (Gonacor 5000 ®) per female (Mann and Bidwell 2000). Oocytes were fertilized in vitro with a 10% sperm suspension obtained by a testicular macerate homogenate in 1 mL of AS. Sperm viability was evaluated by means of spermatozoid morphology and mobility under optical microscopy (Olympus CX41, 400× magnification). Fertility success was considered acceptable with rates greater than 75%. Survival greater than 70% at neural stage was required for good embryo quality. Embryos were dejellied by means of egg ribbon immersion in 2% thioglycolic acid and 0.37 M of NaOH in AS, at pH 7.2. Then, embryos were exhaustively washed with AS and kept in shallow plastic containers with 5 L of AS at 20 ± 2 °C, alternating 12-h light/dark cycles until blastula (S.4) and larval (S.25) stages, which were defined according to Del Conte and Sirlin (1951).

Bioassay experimental design

Embryos or larvae were exposed to copper and nonylphenol independently and in mixtures from early blastula (S.4) and complete operculum (S.25) stages onwards for sub-chronic (168 h) periods.

Batches of 10 embryos or larvae were placed in covered 10-cm-diameter glass Petri dishes containing 40 mL of test solution, in triplicate. Simultaneously, triplicated control of 10 embryos or larvae was maintained in AS. A solvent control group was carried out with AS plus acetone (0.5% *v*/*v*) at the highest concentration used for nonylphenol test solution, also in triplicate (ASTM 1993). Both controls were simultaneously maintained and mortality did not differ significantly from each other.

Mortality was evaluated every 24 h by means of smooth movements of the Petri dishes, followed by stimulation with a light source. In case of no response, heartbeat was checked under a Zeiss Stemi DV4 stereoscopic microscope. 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 of balanced fish food TetraColor® every other day. Toxicity bioassays with single substances and mixtures were performed

Table 1 Conditions of equitoxic and non-equitoxic mixtures of copper (Cu) and nonylphenol (NP) in toxicity bioassays. Cu-LC50 = 0.0205 mg/L and NP-LC50 = 0.9649 mg/L for embryos and Cu-LC50 = 0.051 mg/L and NP-LC50 = 0.377 mg/L for larvae at 168 h of exposure

Developmental period	Mixture stock solution (mg/L)	Mixture stock solution in toxic units (TU)	Exposure concentrations (mg/L)
Embryo	2.58 mg/L (2.3% Cu; 97.7% NP)	1Cu/1NP	0.258; 0.516; 0.77; 1.031; 1.278; 1.547; 2.58
	1.88 mg/L (4.5% Cu; 95.5% NP)	2Cu/1NP	0.264; 0.377; 0.565; 0.753; 1.13; 1.88
	1.86 mg/L (3.4% Cu; 96.6% NP)	3Cu/2NP	0.186; 0.373; 0.559; 0.745;0.931; 1.118; 1.86
	2.18 mg/L (1.2% Cu; 98.8% NP)	1CU/2NP	0.241; 0.482; 0.724; 0.874; 1.093; 1.53; 1.857
	2.03 mg/L (1.5% Cu; 98.52% NP)	2Cu/3NP	0.203; 0.406; 0.609; 0.812; 1.015;1.421; 2,03
Larvae	0.83 mg/L (10.8% Cu; 89.2% NP)	1Cu/1NP	0.166; 0.249; 0.332; 0.415; 0.581; 0.747; 0.83
	1.3 mg/L (13.85% Cu; 85,15% NP)	4Cu/3NP	0.13; 0.26; 0.39; 0.52; 0.65;0.91; 1.3
	0.92 mg/L (19.6% Cu; 80.4% NP)	2Cu/1NP	0.092; 0.184; 0.276; 0.368; 0.46; 0.644; 0.92
	1.01 mg/L (26.7% Cu; 73.7% NP)	3Cu/1NP	0.101; 0.202; 0.303; 0.404; 0.505; 0.707; 1.01

simultaneously with embryos and larvae obtained from the same clutches, ensuring identical experimental conditions and avoiding variability in animal sensitivity (Chou 2006). All experiments were conducted according to the international standards on animal welfare (Canadian Council on Animal Care in Science 1993) and were controlled and approved by the Institutional committee for the care and use of animals in experimentation (CICUAE) of the National University of San Martín (UNSAM).

Data analysis

Lethality data were statistically analyzed by the USEPA Probit Program (USEPA 1988). LC50s were obtained for each single chemical and mixture ratio used. To establish statistical differences between the LC50 values, a comparison was made, considering the difference statistically significant when the higher LC50/lower LC50 ratio exceeded the critical value (95% confidence interval) established by the American Public Health Association (2005).

Mixture interactions were analyzed using the median-effect/combination index (CI) developed by Chou (2006). This method is based on the median-effect principle (mass-action law) (Chou 1976) that demonstrates that there is a unique and corresponding relationship between concentration and effects, independently of the number of substances and mechanism of action or inhibition. The CompuSyn's program (Chou and Martin 2005) was used for the calculation of CI values at different effect levels (Fa), with Fa = (%lethality/100). CI < 1, CI = 1 and CI > 1 indicate synergism, additivity and antagonism, respectively.

Results

The toxicity of copper and nonylphenol, determined independently and simultaneously from 72 to 168 h, is shown in Fig. 1a. As the mortality data of embryos exposed to nonylphenol at 24 h and 48 h were not appropriated for Probit analysis, the corresponding LC50s could not be obtained. Copper was between 35 and 50 times more toxic than nonylphenol to *R. arenarum* embryos at 72 h and 168 h, respectively. Toxicity of both chemicals was time-dependent and significantly ($p < 0.05$) increased with exposure time. Copper LC50 decreased from 0.14 (0.12–0.16) mg Cu^{2+}/L at 24 h to 0.0205 (0.018–0.024) mg Cu^{2+}/L at 168 h, while nonylphenol LC50 decreased from 1.5 (1.37–1.78) mg NP/L at 72 h to 0.96 (0.93–1) mg NP/L at 168 h.

In embryo assays, the toxicity of each mixture proportion was time-dependent, significantly increasing ($p < 0.05$) with exposure time (Fig. 1c). The combination index values presented in Table 2 indicate the interaction at different effect levels (Fa = 0.1, 0.5 0.9). Combination index (CI) at Fa = 0.5 was antagonistic for the equitoxic mixture toxicity independently of the exposure time. The effects of non-equitoxic mixtures of 3Cu/2NP and 2Cu/1NP on embryos were also antagonistic and independent of exposure time. However, the joint toxic effects of 1Cu/2NP were time-dependent. It was shown an antagonistic interaction at the acute period, but it became additive at the sub-chronic exposure. The joint toxic effects of 2Cu/3NP were mainly antagonistic, except for 120 h, showing an additive interaction.

The CI values at 0.9 effect level also indicated antagonism for almost all combinations and at all exposure times. The exceptions were 1Cu/2NP and 2Cu/3NP mixtures, which showed additive interactions at 144–168 h and 72–120 h, respectively.

Joint toxicity at the effect level of 0.1 showed a different pattern. Equitoxic and 2Cu/1NP mixtures were antagonistic for all exposure times. Despite that 1Cu/2NP and 3Cu/2NP were also antagonistic, an additive interaction was observed at the sub-chronic exposure in both cases. Additive effects were also observed for 2Cu/3NP mixture at acute period but synergistic effects were recorded from 120 to 168 h (Table 2).

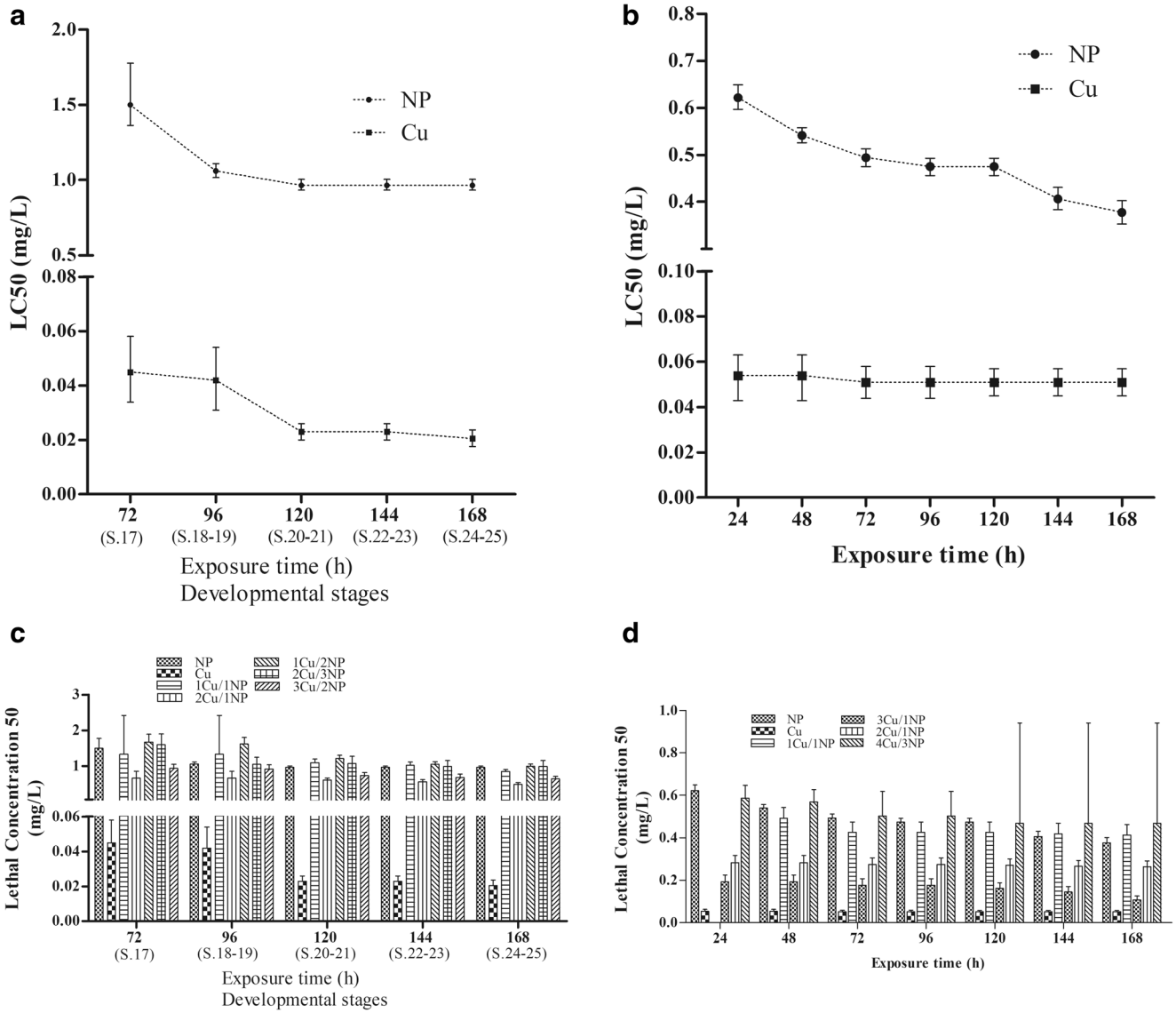

Fig. 1 Lethal concentration 50 (LC50) with 95% confidence intervals of copper (Cu) and nonylphenol (NP). **a** Embryos exposed from early blastula stage (S.4) onwards and **b** larvae exposed from complete operculum stage (S.25) onwards. LC50 with 95% confidence intervals of different mixture proportions for *R. arenarum*, **c** embryos exposed from early blastula stage (S.4) onwards and **d** larvae exposed from complete operculum stage (S.25) onwards. The corresponding stages of development are expressed with the exposure times. Larvae remain at S.25 all the time

The larval toxicity of copper and nonylphenol, which were also determined independently and simultaneously from 24 to 168 h, is shown in Fig. 1b. Copper was between 11.5 and 7.4 times more toxic than nonylphenol to *R. arenarum* larvae. The toxicity of copper remained constant along exposure time with a 24-h LC50 of 0.054 (0.043–0.063) mg Cu^{2+}/L. Nonylphenol toxicity significantly increased ($p < 0.05$) with exposure time. Thus, LC50 decreased from 0.62 (0.59–0.65) mg NP/L at 24 h to 0.38 (0.35–0.40) mg NP/L at 168 h. Toxicity of 3Cu/1NP mixture significantly increased ($p < 0.05$) along exposure time, but toxicity of 1Cu/1NP, 2Cu/1NP and 4Cu/3NP mixtures was not time-dependent (Fig. 1d).

The CI values at 0.5 effect level showed an antagonistic effect for both equitoxic and non-equitoxic mixtures of 4Cu/3NP and 2Cu/1NP at all exposure times. However, the joint toxic effect of 3Cu/1NP was time-dependent, showing antagonism at the acute period and additive interaction towards the sub-chronic exposure. The CI values at 0.9 effect level also indicated antagonism for almost all combinations and all exposure times, except for 3Cu/1NP at 120 h and 168 h, and for 1Cu/1NP at 72 h. Despite that CI values at 0.1 effect level also showed antagonist patterns for equitoxic and 4Cu/3NP at all exposure times, joint toxicity of 2Cu/1NP was time-dependent, with additive responses at the acute period and antagonism from 120 h. Moreover, joint toxicity of 3Cu/1NP was

Table 2 Combination index (CI) with 95% confidence intervals at different effect levels (Fa) for different copper (Cu) and nonylphenol (NP) mixture ratios and different exposure times on *R. arenarum* embryos exposed from early blastula stage (S.4) onwards

		Combination index (CI)					
Mixture stock solution in toxic units (TU)	Exposure time (h)	Effect level (0.1)	Interaction	Effect level (0.5)	Interaction	Effect level (0.9)	Interaction
1CU/2NP	72	1.85 ± 0.14	Antagonistic	1.53 ± 0.08	Antagonistic	1.43 ± 0.20	Antagonistic
	96	2.14 ± 0.13	Antagonistic	2.02 ± 0.18	Antagonistic	2.38 ± 0.48	Antagonistic
	120	1.90 ± 0.18	Antagonistic	1.89 ± 0.11	Antagonistic	1.99 ± 0.19	Antagonistic
	144	1.35 ± 0.28	Antagonistic	1.36 ± 0.35	Antagonistic	1.45 ± 0.78	Additive
	168	0.85 ± 0.50	Additive	1.15 ± 0.54	Additive	1.64 ± 1.53	Additive
2Cu/3NP	72	1.52 ± 0.34	Antagonistic	1.61 ± 0.35	Antagonistic	2.01 ± 1.31	Additive
	96	1.75 ± 0.41	Antagonistic	2.16 ± 0.52	Antagonistic	3.37 ± 2.48	Additive
	120	0.36 ± 0.15	Synergistic	1.22 ± 0.28	Additive	4.62 ± 4.11	Additive
	144	0.37 ± 0.15	Synergistic	1.24 ± 0.18	Antagonistic	3.79 ± 1.70	Antagonistic
	168	0.37 ± 0.15	Synergistic	1.25 ± 0.20	Antagonistic	4.41 ± 2.04	Antagonistic
1Cu/1NP	72	2.03 ± 1.07	Antagonistic	1.36 ± 0.32	Antagonistic	3.22 +/−16.87	Additive
	96	13.19 ± 6.39	Antagonistic	6.85 ± 5.11	Antagonistic	12.27 +/−63.83	Additive
	120	10.82 ± 1.967	Antagonistic	8.98 ± 0.89	Antagonistic	7.71 ± 1.35	Antagonistic
	144	10.18 ± 2.07	Antagonistic	8.72 ± 0.95	Antagonistic	7.7 ± 1.48	Antagonistic
	168	10.50 ± 1.66	Antagonistic	8.70 ± 0.80	Antagonistic	7.26 ± 0.10	Antagonistic
3Cu/2NP	72	1.34 ± 0.18	Antagonistic	1.43 ± 0.13	Antagonistic	1.81 ± 0.43	Antagonistic
	96	1.83 ± 0.16	Antagonistic	1.57 ± 0.11	Antagonistic	1.85 ± 0.41	Antagonistic
	120	1.61 ± 0.22	Antagonistic	1.81 ± 0.11	Antagonistic	2.17 ± 0.22	Antagonistic
	144	1.53 ± 0.32	Antagonistic	1.74 ± 0.14	Antagonistic	2.15 ± 0.33	Antagonistic
	168	1.09 ± 0.12	Additive	1.76 ± 0.10	Antagonistic	2.90 ± 0.40	Antagonistic
2Cu/1NP	72	2.03 ± 0.27	Antagonistic	1.60 ± 0.29	Antagonistic	1.47 ± 0.42	Antagonistic
	96	1.41 ± 0.31	Antagonistic	1.54 ± 0.21	Antagonistic	2.38 ± 0.79	Antagonistic
	120	1.33 ± 0.32	Antagonistic	1.87 ± 0.27	Antagonistic	2.87 ± 0.74	Antagonistic
	144	1.45 ± 0.24	Antagonistic	1.61 ± 0.14	Antagonistic	1.92 ± 0.26	Antagonistic
	168	1.40 ± 0.21	Antagonistic	1.63 ± 0.11	Antagonistic	1.93 ± 0.31	Antagonistic

synergistic for almost all exposure times, except at 48 h (Table 3).

Discussion

The simultaneous presence of copper and nonylphenol in the aquatic ecosystems of Argentina is coincident with the distribution area of *Rhinella arenarum*. This fact leads to the imperative need to perform joint toxicity assessment of these chemicals on amphibian species (Babay et al. 2014; Babay et al. 2008; Ossana et al. 2016), particularly in embryos and larvae because of their high sensitivity (Herkovits and Pérez-Coll 2003). We have previously shown the lethal and sublethal risk of these isolated chemicals on *R. arenarum*. Moreover, it was shown that both chemicals induce malformations and delayed development. The lethal toxicity of both chemicals at the two developmental stages was not different from that shown in previous works, highlighting the robustness and reproducibility of the AMPHITOX method (Aronzon et al. 2014; Aronzon et al. 2011). As the interaction effects might depend on the compound proportions in the mixture (Wang et al. 2009) and the environmental exposure concentrations will be dynamic and possibly change over time, the joint toxicity of copper/nonylphenol solutions was assessed in different ratios and at different exposure times. Indeed, solutions with equitoxic and non-equitoxic proportions were included; these last ones represent more environmentally realistic and relevant conditions, although the selected ratios do not cover all the spectrum of possible combinations in the environment.

Copper toxicity was 50 and almost 12 times more toxic than nonylphenol in the embryonic and larval periods, respectively. This marked differential toxicity shows the need of expressing mixture toxicity in terms of the relative toxicity of each compound, as toxic units, instead of total concentrations. Copper toxicity expressed in the sub-chronic exposure (168 h) was time-dependent only for embryo exposure, which might be due to the stage-dependent susceptibility (Aronzon

Table 3 Combination index (CI) with 95% confidence intervals at different effect levels (Fa) for different copper (Cu) and nonylphenol (NP) mixture ratios and different exposure times on *R. arenarum* larvae exposed from complete operculum stage (S.25) onwards

		Combination index (CI)					
Mixture stock solution in toxic units (TU)	Exposure time (h)	Effect level (0.1)	Interaction	Effect level (0.5)	Interaction	Effect level (0.9)	Interaction
1Cu/1NP	48	1.74 ± 0.15	Antagonistic	1.72 ± 0.09	Antagonistic	1.84 ± 0.12	Antagonistic
	72	1.63 ± 0.36	Antagonistic	1.52 ± 0.27	Antagonistic	1.48 ± 0.64	Additive
	96	1.61 ± 0.35	Antagonistic	1.62 ± 0.26	Antagonistic	1.69 ± 0.61	Antagonistic
	120	1.36 ± 0.25	Antagonistic	1.58 ± 0.27	Antagonistic	1.87 ± 0.70	Antagonistic
	144	1.59 ± 0.28	Antagonistic	2.12 ± 0.45	Antagonistic	2.9 ± 1.05	Antagonistic
	168	1.67 ± 0.28	Antagonistic	2.18 ± 0.41	Antagonistic	2.95 ± 1.05	Antagonistic
4Cu/3NP	24	8.21 ± 0.79	Antagonistic	7.11 ± 0.43	Antagonistic	6.23 ± 0.48	Antagonistic
	48	8.15 ± 0.93	Antagonistic	7.63 ± 0.49	Antagonistic	7.28 ± 0.68	Antagonistic
	72	7.05 ± 1.86	Antagonistic	6.05 ± 1.12	Antagonistic	6.07 ± 1.47	Antagonistic
	96	7.08 ± 1.93	Antagonistic	6.54 ± 1.16	Antagonistic	6.13 ± 1.54	Antagonistic
	120	5.13 ± 1.97	Antagonistic	5.13 ± 1.83	Antagonistic	5.21 ± 2.90	Antagonistic
	144	5.34 ± 1.96	Antagonistic	5.17 ± 1.78	Antagonistic	5.13 ± 2.76	Antagonistic
	168	5.38 ± 1.97	Antagonistic	5.22 ± 1.79	Antagonistic	5.17 ± 2.78	Antagonistic
2Cu/1NP	24	1.07 ± 0.31	Additive	1.36 ± 0.20	Antagonistic	1.78 ± 0.17	Antagonistic
	48	1.33 ± 0.40	Additive	1.49 ± 0.22	Antagonistic	1.81 ± 0.17	Antagonistic
	72	1.22 ± 0.34	Additive	1.57 ± 0.22	Antagonistic	2.08 ± 0.17	Antagonistic
	96	1.23 ± 0.36	Additive	1.60 ± 0.22	Antagonistic	2.15 ± 0.14	Antagonistic
	120	1.4 ± 0.28	Antagonistic	1.54 ± 0.21	Antagonistic	2.89 ± 0.29	Antagonistic
	144	1.32 ± 0.29	Antagonistic	1.60 ± 0.23	Antagonistic	2.02 ± 0.35	Antagonistic
	168	1.36 ± 0.29	Antagonistic	1.67 ± 0.19	Antagonistic	2.09 ± 0.27	Antagonistic
3Cu/1NP	24	0.75 ± 0.11	Synergistic	1.27 ± 0.21	Antagonistic	2.21 ± 0.73	Antagonistic
	48	0.94 ± 0.1	Additive	1.39 ± 0.25	Antagonistic	2.18 ± 0.77	Antagonistic
	72	0.73 ± 0.20	Synergistic	1.19 ± 0.18	Antagonistic	1.67 ± 0.36	Antagonistic
	96	0.74 ± 0.21	Synergistic	1.11 ± 0.10	Antagonistic	1.71 ± 0.38	Antagonistic
	120	0.62 ± 0.11	Synergistic	1.12 ± 0.28	Additive	2.04 ± 1.04	Additive
	144	0.66 ± 0.26	Synergistic	1.20 ± 0.26	Additive	2.47 ± 2.40	Additive
	168	0.48 ± 0.12	Synergistic	0.84 ± 0.17	Additive	1.43 ± 0.62	Additive

et al. 2011). The main biochemical toxicity of Cu is derived from its effects on the structure and function of biomolecules (DNA, proteins, membrane molecules) or through oxygen-radical mechanisms (Gaetke and Chow 2003). So, the stage-dependent toxicity might be due to the development of the antioxidant response, mainly of glutathione-related enzymes (Ferrari et al. 2008).

Nonylphenol toxicity was time-dependent for both developmental periods; in the case of larvae exposure, it might be probably due to the increase in the exposure time. Copper was the most toxic; particularly during the embryonic period while nonylphenol was more toxic during the larval period, confirming previous results (Aronzon et al. 2014). Besides, the higher toxicity of organic compounds on amphibian's larval development was previously informed (Svartz et al. 2016). This differential sensitivity might be related to the lack or insensitivity of target organs in the embryonic stages compared to the larval period (Edginton et al. 2004).The results obtained in this study become evidence of the existence of different types of interactions depending on the compound proportions in the mixture, exposure time and developmental stage. Based on the combination index at 0.5 effect level (Fa(0.5)), the joint toxicity of different copper and nonylphenol mixture proportions showed mainly an antagonistic pattern. This deviation from the additive interaction might be expected because of the different modes of action of both compounds (Kraak et al. 1999). However, mixture interactions cannot be easily explained in terms of the known primary mechanisms of action, because toxicological interactions can occur independently of the primary mode of action (Chou 2006). Indeed, lethality as an endpoint of the joint toxicity is probably a result of the malfunctioning of a wide variety of processes within the organism, caused by both primary and secondary effects (Hermens et al. 1985; van der Geest et al. 2000). Antagonistic interaction of copper in joint lethal toxicity with

other organic chemicals has been previously shown (Aronzon et al. 2016; van der Geest et al. 2000). This might be partially explained by a decreasing contribution of the essential metal to the toxicity of the mixture, so a low concentration allows the organism to regulate the metal incorporation, up to a certain concentration in the water. On the other hand, it would be in concentrations under metabolic control (Kraak et al. 1999).

At 0.5 effect level, the toxicity of equitoxic mixture on embryos and larvae was always antagonistic and independent of exposure time. Non-equitoxic mixtures of 3Cu/2NP and 2Cu/1NP on embryos, and 4Cu/3NP and 2Cu/1NP on larvae, were also antagonistic and independent of exposure time. However, the joint toxic effects of 1Cu/2NP and 2Cu/3NP in embryos and 3Cu/1NP in larvae were time-dependent, showing antagonism at acute period and additive interaction towards the sub-chronic exposure.

It is worth pointing out that most of additive interactions showed large confidence intervals (95%). This might be consequence of poor fit of lethality data to the model; this is expectable when assessing large ranges of concentration at different exposure times. However, a better adjustment of lethality might differentiate joint toxicity in another type of interaction. CompuSyn's program allowed to calculate the combination index at different effect levels. Joint toxicity at 0.1 effect level (Fa 0.1) was synergistic to 2Cu/3NP mixtures from 120 h for embryonic exposure and to 3Cu/1NP for almost all exposure times for larvae. This joint toxicity variation with effect level has been also informed for copper and other chemicals (Son et al. 2016; Yang et al. 2017). This becomes important because Fa (0.1) being equivalent to LC10, which implies lowest concentration of 0.01 mg Cu^{2+}/L and 0.2 mg NP/L. This highlights that synergism may occur at environmentally relevant concentrations of these chemicals, and more realistic scenarios where copper is in greater proportion than nonylphenol (Babay et al. 2014; Ossana et al. 2016). Moreover, LC10 is a more appropriate measure for risk assessment or environmental health purposes, as it is more conservative than LC50. Taking into account that these joint interactions were assessed only by means of lethal effects, it is interesting to point out the risk that simultaneous presence of both substances may represent for *R. arenarum* even at very low concentrations. Further studies will be required in order to explain the results of this study from a mechanistic concept. However, present findings are of immediate interest from a regulatory point of view given the environmental relevance of the assayed concentrations together with the imperative need for amphibian conservation around the world.

Conclusion

Equitoxic mixtures of copper and nonylphenol resulted antagonistic, independently of exposure time and developmental stage of the amphibian *Rhinella arenarum*. Although the most environmentally relevant non-equitoxic mixtures were also mainly antagonistic, joint toxicity was time-dependent. This highlights the relevance of assessing the joint toxic effects at different proportions of chemicals in the mixture and exposure times. The analysis of mixture toxicity at different effect levels allows detecting changes in mixture interaction. So, joint toxicity assessment becomes important as mixture exposure is a more realistic scenario.

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