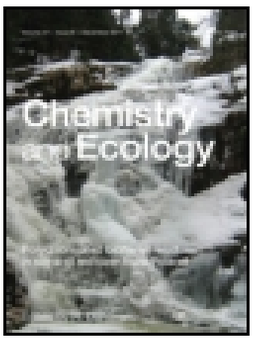

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Joint toxicity of copper and diazinon during embryonic and larval development of the common South American toad, *Rhinella arenarum*

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ABSTRACT

Copper (Cu) and diazinon are two widely distributed pollutants; they coexist in agro-ecosystems and cause toxicity to wild biota. This study proposes to analyse the joint toxicity of binary mixtures of Cu and diazinon on the early development of the South American toad, *Rhinella arenarum* by means of a standardised test. Cu was always more toxic than diazinon. Cu was more embryotoxic while the pesticide was more toxic during the larval exposure than during the embryonic period. The different Cu/diazinon mixtures proportions assayed were significantly less toxic than expected by additive effects. Thus, an antagonistic interaction pattern was observed. This pattern was independent of the assayed proportion, the exposure times and the exposure developmental periods. In the risk assessment analysis to establish water quality criteria, the joint toxicity should be considered at different ratios, exposure time and life period for a certain species, in order to preserve wild species.

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KEYWORDS
Copper; diazinon; joint toxicity; antagonistic interaction; amphibians

1. Introduction

Aquatic ecosystems are often polluted with a wide variety of contaminants from diverse activities, as agriculture, industry and urban activities. The presence of these contaminants can have a direct impact on the health of aquatic organisms and may present a threat to humans through contamination of drinking water supplies. Beyond the huge number of pollutants present in aquatic ecosystems, metals and pesticides are two types of contaminants that often coexist mainly in agro-ecosystems.[1] Most ecotoxicological studies and chemical risk assessment only focus on the exposure and effects of single chemicals. Unfortunately, the behaviour of chemicals in mixtures may not correspond to that predicted from data on single compounds. Indeed, interactions of components in a mixture can cause complex and substantial changes in the apparent properties of its chemical constituents.[2] Therefore, the evaluation of mixture toxicity, especially for contaminants that commonly coexist in the environment, is very important for assessing risk, as it provides a slightly more realistic approach of the toxicity of chemicals in the environment.

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Copper (Cu) is an essential trace element for all living systems and crucial for many cellular processes and metabolism. The levels of this metal in water are increased by anthropogenic activities, including agricultural applications.[3] Studies in our laboratory have shown that Cu is highly toxic to *Rhinella arenarum* embryos and larvae, even at very low and environmentally relevant concentrations.[4] Cu exposure caused sub-lethal effects, such as teratogenesis, delayed development, reduced body size and behaviour alterations.[4] Diazinon is an organophosphate pesticide that has been extensively applied to control insects in a wide diversity of crops and to treat ectoparasites in livestock and domestic pets.[5] After application diazinon is easily washed into surface waters and may reach ground waters; moreover, it is one of the most stable water organophosphates. Its main mechanism of toxicity is based on the ability to inhibit acetylcholinesterase (AChE), an enzyme which inactivates the neurotransmitter acetylcholine.[5] *R. arenarum* was reported as one of the most sensitive amphibian species to this pesticide.[6] A diversity of sub-lethal effects of diazinon on embryos and larvae and a significantly high Teratogenic Index were reported. The severe abnormal behaviour related to the neurotoxicity was the characteristic sub-lethal effect of diazinon exposure on larvae.[6] Cu and diazinon may enter to ephemeral pools or aquatic systems through drift, accidental overspray or run-off. Considering the different modes of action of both compounds, a joint action deviating from concentration addition would be expected.[7]

There is an increasing global concern about declines in amphibian populations, and the large number of malformed individuals found since the early 1970s [8,9]; the environmental degradation is one of the major causes of worldwide amphibian decline. In this context, the impact of diverse human activity such as industry, mining and agriculture in the natural environment is also highly noticeable by chemical pollution and the effects on amphibians.[10] This is of special concern for Argentinean wild life conservation due to its intensive agriculture.

A large proportion of a toad's life cycle occurs in shallow, lentic or ephemeral water bodies, within agricultural regions, where different pollutants may concentrate, making toads more vulnerable to toxic effects.[11] In addition, their breeding period coincides with the period of highest dosage and frequency of agrochemicals application.[10] Taking into account the high sensitivity of amphibians to a wide diversity of environmental pollutants, mainly at embryonic and larval stages, they are considered good bio-indicators of pollution; and are widely used in ecotoxicological assessment studies.[12,13] In this paper the common South American toad, *R. arenarum* was chosen as a test organism, because it is native and is one of the species with the highest incidence of malformations of the middle region of Argentina.[14] Moreover, projection of R. arenarum's population size showed a tendency to extinction in sites dominated by crops in the central region of Córdoba Province, Argentina.[15]

Most toxicity studies explore adverse effects only in acute exposure condition, focusing on a single chemical, during a certain life cycle period. Nevertheless, the possibility to assess the toxicity of pesticides and metals, both single and in mixtures, on different stages of amphibian's life cycle would be relevant in order to find the most sensitive life period for management and conservation decisions.

As little information exists regarding the toxicological interactions prevailing in mixtures of Cu and diazinon on amphibians, the aim of the current study was to evaluate the toxicity of equitoxic and non-equitoxic binary mixtures of these chemicals on

embryos and larvae of the common South American toad, *R. arenarum*. The experimental design included simultaneous single bioassays with Cu and diazinon, as well as several mixtures of both toxicants in different proportions.

2. Experimental design

2.1. Acquisition of R. arenarum embryos and larvae

Four pairs of healthy *R. arenarum* adults, weighing approximately 200–250 g were obtained in Lobos (Buenos Aires province, Argentina: 35° 11′ S; 59° 05′ W) from a local provider. Ovulation of females was induced by means of an intraperitoneal injection of one homologous hypophysis suspension in 1 mL of AMPHITOX solution (AS) per female preserved according to Pisanó,[16] plus 2500 IU of human chorionic gonadotropin.[17] AS composition is (in mg/L): Na^+ 14.75; Cl^- 22.71; K^+ 0.26; Ca^{2+} 0.36; HCO_3^- 1.45. Oocytes were fertilised *in vitro* with a fresh sperm suspension, obtained from a testicular macerate in 1 mL of AS. After fertilisation, embryos were kept in AS at 20 ± 2°C until blastula (S.4, embryos) or complete operculum (S.25, larvae) stages. The stages of embryos and larvae were defined according to Del Conte and Sirlin.[18] For toxicity experimental protocols initiating from S.4, embryos were dejellied by means of a 2-min treatment with 2% thioglycolic acid solution, neutralised at pH 7.2–7.4 with 1.35 mL of saturated NaOH solution every 100 mL in AS, and then thoroughly washed. The role of the jelly coat is still controversial; some authors suggested that it could provide protection against some chemicals,[19] while other authors showed that it is not relevant for protection.[20] In this study, jelly coat was removed to randomly select healthy embryos in order to use them as control and exposed organisms, providing homogeneous high-quality biological material. For toxicity experimental protocols initiating from S.25, the individuals hatched spontaneously. All experiments were conducted in accordance with international standards on animal welfare.[21]

2.2. Test solutions

Cu ($CuCl_2{\cdot}2H_2O$: purity 99%; Riedel-de Haën) stock solution of 1.5 g/L was prepared in distilled water. Hydrochloric acid was added until pH 1.9. Test solutions, ranging in concentrations between 3 and 375 µg Cu^{2+}/L, were prepared by diluting a secondary stock solution of 30 mg Cu^{2+}/L in AS. Experimental Cu solutions were measured four times with a Perkin Elmer atomic absorption spectrophotometer; error between nominal and measured concentrations did not exceed 5%.

Diazinon (purity 99%, CAS number: 333-41-5, Lot LB75417, Supelco Analytical) stock solution of 3 g/L was prepared in acetone. Test solutions, ranging in concentrations between 1.5 and 45 mg diazinon/L, were prepared by diluting the stock solution in AS. Experimental diazinon solutions were measured three times by High Performance Liquid Chromatography -Electrospray Ionization- Mass Spectrometry, in Selected-ion monitoring mode, positive detection[6]; error between nominal and measured concentrations did not exceed 5%.

Stock solutions of mixtures of Cu and diazinon were prepared by dissolving each chemical in AS.

Binary mixtures were combined using different ratios based on the corresponding 168-h LC50 for each chemical, obtained independently and simultaneously for the

corresponding clutch. Thus, ratios were expressed as the minimum entire relation of Toxic Units (TU).[7,22] This concept, which was first described by Sprague,[22] assigns a value of 1 TU to a concentration of toxicant that elicits a particular response, in the case of the present study 50% mortality at 168 h (168-h LC50). In the case of equitoxic mixtures, the pesticide and the metal were combined in equal proportion of their respective toxicity. More explicitly, for embryos exposure, stock solution contained 0.03 mg Cu^{2+}/L and 27.15 mg diazinon/L, 1.5 times their respective 168-h LC50, the proportion employed were 1:1 (1 TU Cu/1 TU diazinon). For non-equitoxic mixtures 3 TU Cu/ 2 TU diazinon and 2 TU Cu/1 TU diazinon were tested for embryos, while 2 TU Cu/ 3 TU diazinon and 1 TU Cu/ 2 TU diazinon were assessed for larvae.

Mixture toxicity was evaluated using a fixed ratio design.[23] Test solutions of each mixture were prepared by diluting stock solutions in AS with a dilution factor of 0.9. Embryos and larvae were exposed to nine dilutions of each stock mixture solution for 168 h.

2.3. Toxicity experimental protocols

R. arenarum embryos and larvae, obtained from four different clutches, were exposed to Cu and diazinon independently and in mixtures from early blastula (S.4) and complete operculum (S.25) stages onwards for 168 h.

For each experimental condition and control, triplicate batches of 10 embryos or larvae were placed in covered 10-cm-diameter glass Petri dishes containing 40 mL of test solution or AS, respectively. A solution of AS plus acetone at the highest concentration used for diazinon was added as a carrier control. Acetone concentration in test solutions was always lower than 1.1%.[24] Densities of embryos and larvae were always lower than 3.2 mg/mL; both of them successfully developed under those conditions. Moreover in nature, they develop in very shallow water bodies.[25] To ensure oxygen consumption and fresh media, test solutions were renewed every 48 h and temperature was maintained at 20 ± 2°C. Responses in both AS and acetone controls did not significantly differ ($p > .05$), therefore, the results were pooled for the analysis. Survival of embryos and larvae in the control groups was between 95% and 100%. Lethality was evaluated and dead individuals were removed daily. Larvae were fed with 6 ± 0.5 mg of balanced fish food TetraColor® for 24 h every other day.

2.4. Statistical analysis

Lethality data were statistically analysed by the USEPA Probit Program [26] and LC50s were obtained for each single chemical and mixture ratio used. Toxicity profiles, as isotoxicity curves were plotted based on LC50 at different exposure times. To compare LC50 values, differences were considered to be statistically significant when the higher/lower ratio exceeded the corresponding critical value established by the American Public Health Association et al.[27] Differential sensitivity inter-clutches were expressed as a coefficient of variation.

Marking's additive index of the effects for aquatic toxicology was used to evaluate joint toxicities.[23] Additive index (*S*) was calculated for each mixture ratio [23] at different exposure times. This value is the sum of the toxic effects of a mixture. It was calculated

as the sum of the ratio between the concentration of each chemical in the LC50 of the mixture and the LC50 of each single chemical.

$$S = (Am/Ai) + (Bm/Bi) \quad (1)$$

where S is the joint toxicity of the paired compounds; A and B are the experimental compounds; Ai is the LC50 of compound A when used alone; Am is the LC50 of compound A when used jointly; Bi is the LC50 of compound B when used alone and Bm is the LC50 of compound B when used jointly.

In general terms, when $S > 1$, the effects were taken as antagonistic, when $S < 1$, the effects were taken as synergistic and when $S = 1$, the effects were taken as adding effects.[28] The two-sided effect isobole model was used to schematise the effective toxicity of the two components in the mixture (Figure 1). A straight line joining the LC50s of single chemical A and single chemical B represents the expected LC50s of various A/B ratios, assuming that the interactions are due to simple concentration addition. The empirical LC50s of the mixtures are the concentrations of chemical A and chemical B in the mixture that cause 50% lethality (e.g. point M in Figure 1).

3. Results

3.1. Mixture toxicity on embryos

Concentrations causing the mortality of 50% of embryos at different times for Cu and diazinon exposure are shown in Figure 2. Cu was between 629 and 928 times more toxic than diazinon to embryos at 72 and 168 h, respectively. The toxicity of both chemicals increased along time. Thus, the LC50 values obtained for Cu decreased from 0.14 mg Cu^{2+}/L to 0.037 Cu^{2+} mg/L and 0.02 mg Cu^{2+}/L at 24, 96 and 168 h, respectively. In the case of diazinon,

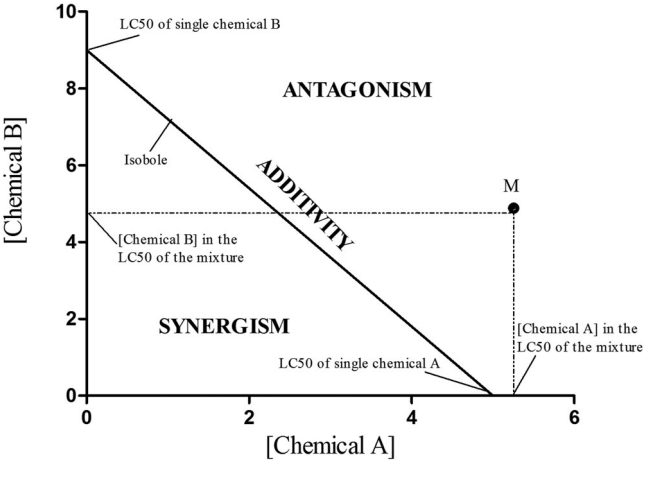

Figure 1. Example of an isobologram of binary mixtures. A straight line joining the LC50s of single chemical A and single chemical B represents the expected LC50s of various A/B ratios, assuming the interaction are due to simple concentration-additivity of the two chemicals. The empirical LC50 of the mixtures are the concentrations of chemical A and chemical B in the mixture that cause 50% mortality (e.g. point M).

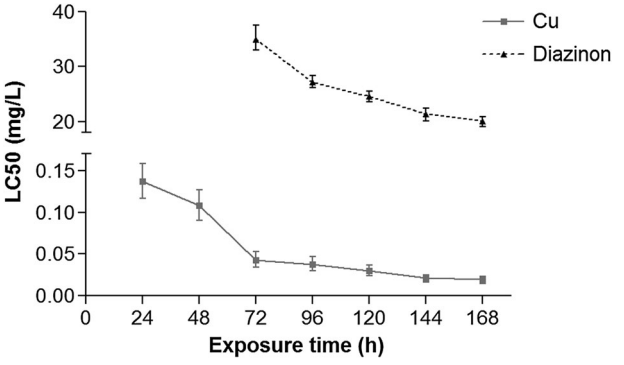

Figure 2. Lethal concentration 50 (LC50) of copper (Cu) and diazinon (Dz) at different exposure times on *R. arenarum* embryos. Bars show 95% confidence intervals. Diazinon mortality data do not allow to perform the USEPA probit analysis at 24 and 48 h. No effect Concentration-48 h was 30 mg diazinon/L.

LC50 values decreased from 31 mg diazinon/L to 23.6 mg diazinon/L and 18.1 mg diazinon/L at 72, 96 and 168 h, respectively. There were no significant differences in susceptibility between clutches, and the coefficients of variation for both compounds were always lower than 12%.

Additive index (*S*) of the joint toxicity of Cu and diazinon assessed at three ratios at different exposure times is given in Table 1. For equitoxic mixtures, toxic effects were significantly less than that predicted by the concentration addition effect. Thus, mixture toxicity at the equitoxic ratio was antagonistic for all exposure times. In the case of non-equitoxic mixtures, the same antagonistic pattern was observed. Toxicity of 2 Cu/1 diazinon ratio for the first 120 h was not significantly different from the sum of the individual toxicities, indicating that this mixture ratio was additive just for this exposure time. Nevertheless, when exposure was extended to 144 and 168 h the same mixture ratio showed antagonistic interactions. Moreover, for 3 Cu/2 diazinon mixtures, antagonistic interactions were observed for all exposure times.

The isobolograms shown in Figure 3 illustrate the results obtained for equitoxic and two series of non-equitoxic mixtures at 96 (a) and 168 h (b), respectively. The fact that almost

Table 1. Additive indexes (*S*) for different mixture ratios of copper (Cu) and diazinon (Dz) at different times on *R. arenarum* embryos.

Exposure time	Mixture ratio (TU)	Additive index (*S*)	Interaction
96 h	1Cu/1Dz	1.71*	Antagonism
	3Cu/2Dz	1.75*	Antagonism
	2Cu/1Dz	1.14	Additivity
120 h	1Cu/1Dz	2.01*	Antagonism
	3Cu/2Dz	1.82*	Antagonism
	2Cu/1Dz	1.16	Additivity
144 h	1Cu/1Dz	1.89*	Antagonism
	3Cu/2Dz	1.66*	Antagonism
	2Cu/1Dz	1.40*	Antagonism
168 h	1Cu/1Dz	2.02*	Antagonism
	3Cu/2Dz	1.64*	Antagonism
	2Cu/1Dz	1.46*	Antagonism

*Significant differences ($p = .05$) from expected additivity ($S = 1$).

all combinations of Cu and diazinon causing 50% of mortality lie above and to the right of the additivity line is suggestive of antagonistic interactions.

Similar to the toxicity pattern of single substances, mixtures caused significantly increased mortality of *R. arenarum* embryos over exposure time.

3.2. Mixture toxicity on larvae

Figure 4 shows the Cu and diazinon concentrations causing 50% of larvae mortality from 24 to 168 h of exposure. Cu was 161 and 208 times much toxic than diazinon at 24 and 168 h, respectively. Cu toxicity remained constant during the exposure period with an LC50 of 0.054 mg Cu^{2+}/L at 24 h and 0.051 mg Cu^{2+}/L at 96 h, without changes at 168 h. In contrast, diazinon toxicity significantly increased with exposure time with LC50s of 11.2; 9.6 and 8.2 mg diazinon/L at 24, 96, and 168 h, respectively. Despite that Cu was always more toxic than diazinon, Cu was significantly more toxic during the embryonic exposure, while diazinon toxicity was significantly higher for larvae. 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 Cu and diazinon assessed at three ratios at different exposure times is given in Table 2. For all mixture combinations, mortality was significantly less than that predicted by the concentration addition model for all exposure times, thus resulted in an antagonistic interaction.

The isobologram shown in Figure 5 illustrates the results obtained for equitoxic and two series of non-equitoxic mixtures at 96 (a) and 168 h (b), respectively. All combinations of equitoxic and non-equitoxic mixtures causing 50% mortality lie above and to the right of the additivity line; this suggests antagonistic interactions for all exposure times.

Table 2 Additive indexes (S) for different mixture ratios of copper (Cu) and diazinon (Dz) at different times on *R. arenarum* larvae.

Exposure time	Mixture ratio (TU)	Additive index (*S*)	Interaction
24 h	1Cu/1Dz	1.63*	Antagonism
	2Cu/3Dz	1.65*	Antagonism
	1Cu/2Dz	1.53*	Antagonism
48 h	1Cu/1Dz	1.56*	Antagonism
	2Cu/3Dz	1.47*	Antagonism
	1Cu/2Dz	1.73*	Antagonism
72 h	1Cu/1Dz	1.58*	Antagonism
	2Cu/3Dz	1.57*	Antagonism
	1Cu/2Dz	1.45*	Antagonism
96 h	1Cu/1Dz	1.39*	Antagonism
	2Cu/3Dz	1.67*	Antagonism
	1Cu/2Dz	1.45*	Antagonism
120 h	1Cu/1Dz	1.32*	Antagonism
	2Cu/3Dz	1.67*	Antagonism
	1Cu/2Dz	1.39*	Antagonism
144 h	1Cu/1Dz	1.16*	Antagonism
	2Cu/3Dz	1.51*	Antagonism
	1Cu/2Dz	1.42*	Antagonism
168 h	1Cu/1Dz	1.16*	Antagonism
	2Cu/3Dz	1.45*	Antagonism
	1Cu/2Dz	1.43*	Antagonism

*Significant differences ($p = .05$) from expected additivity ($S = 1$).

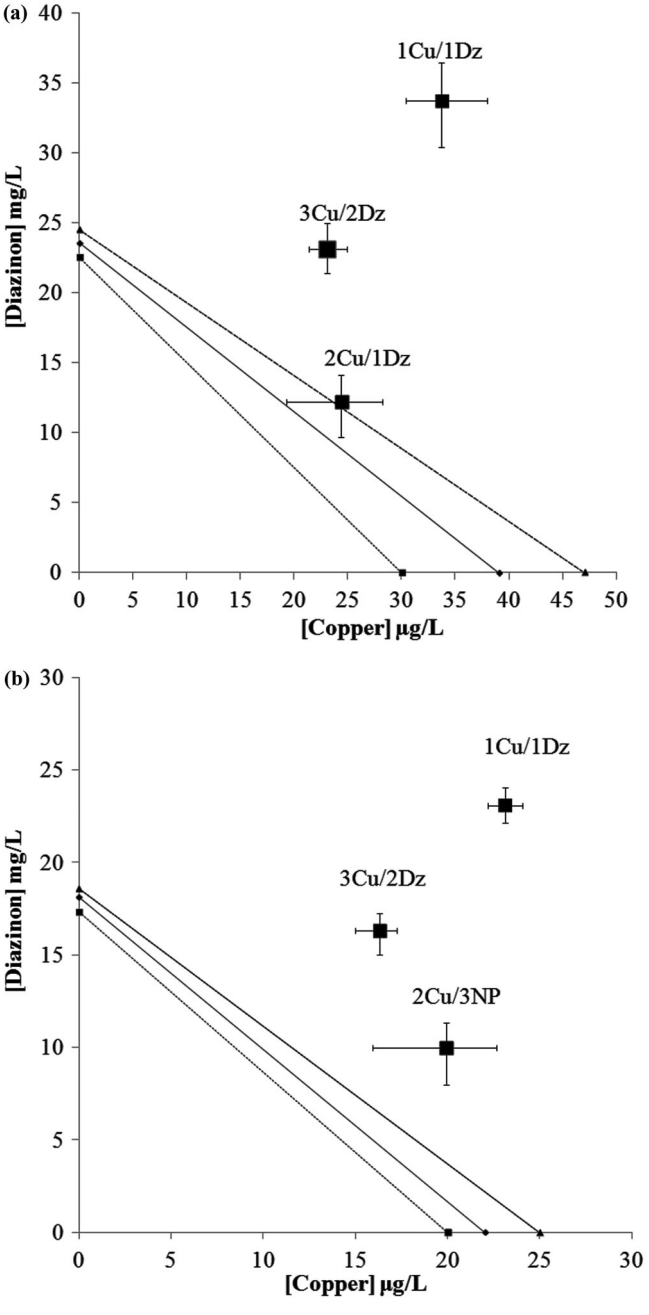

Figure 3. Isobologram plotted for LC50 of single and different mixture ratios of copper (Cu) and diazinon (Dz) at different exposure times (a = 96 h; b = 168 h) on *R. arenarum* embryos. LC50 points with corresponding 95% confidence intervals are plotted.

4. Discussion

In the environment, organisms are commonly exposed to mixtures rather than single chemicals. The current study examined the interactions in equitoxic and non-equitoxic

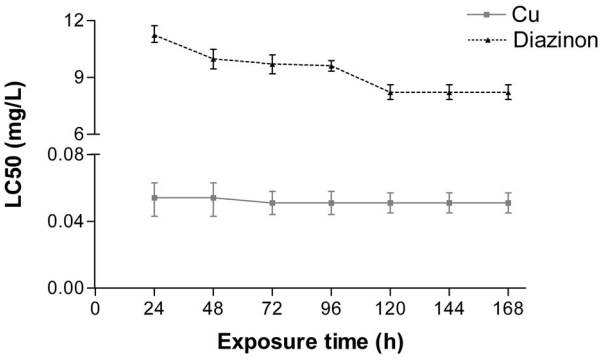

Figure 4. Lethal concentration 50 (LC50) of copper (Cu) and diazinon (Dz) on *R. arenarum* larvae. Bars show 95% confidence intervals.

mixtures of Cu and diazinon on *R. arenarum* embryos and larvae. This approach is more realistic because it is highly unlikely that chemicals occur in a unique proportion in the environment.

In order to study the mixture interaction, it was essential to precisely quantify the individual toxicity of Cu and diazinon. Results obtained in this study indicated that Cu was more toxic than diazinon in all cases, reaching a maximal difference on toxicity of 1031 times in the embryonic period. For both single chemicals, toxicity significantly increased with exposure time from blastula to larval development. Previous studies have shown that Cu exerts a higher toxicity during the organogenic period; this fact might justify the increased toxicity observed along exposure time towards the larval development.[4]

The toxicity of diazinon significantly increased with exposure time during the larval period. Because larvae do not suffer major developmental changes during this stage, this increase only shows a time-dependent toxicity. Conversely, Cu toxicity did not significantly change along exposure time during larval stage, showing that Cu achieves its maximum effect within the initial 24 h of exposure at this stage.

Despite that Cu was always more toxic than diazinon on *R. arenarum* embryos and larvae, Cu was more toxic during the embryonic period while diazinon was more toxic for larvae. This differential sensitivity to diazinon might be related to the beginning of AChE activity which is well correlated with the muscular and neuro-development, as has been shown for other amphibians.[29]

Several studies have shown that concentration addition or less than concentration addition is commonly observed when combinations of chemicals are tested.[1,7] The mixture effects of Cu and diazinon observed in this study corroborate these findings, as the toxicity of the two products was less than additive. The joint action, deviated from concentration addition, observed in the present work is in agreement with the expected result based on the different primary action modes of both compounds.[7] Nevertheless, it is worth pointing out the importance of performing the experimental testing of mixture toxicity interactions because there are many chemicals with similar, dissimilar and unknown mode of action. In particular, mortality, as an end point of evaluating mixing effects, is probably the consequence of diverse organic failures, caused by specific and non-specific effects of single compounds.[30] In that sense, secondary mechanisms of action must not be excluded, since acetylcholinesterase inhibition can be also caused by metal exposure,

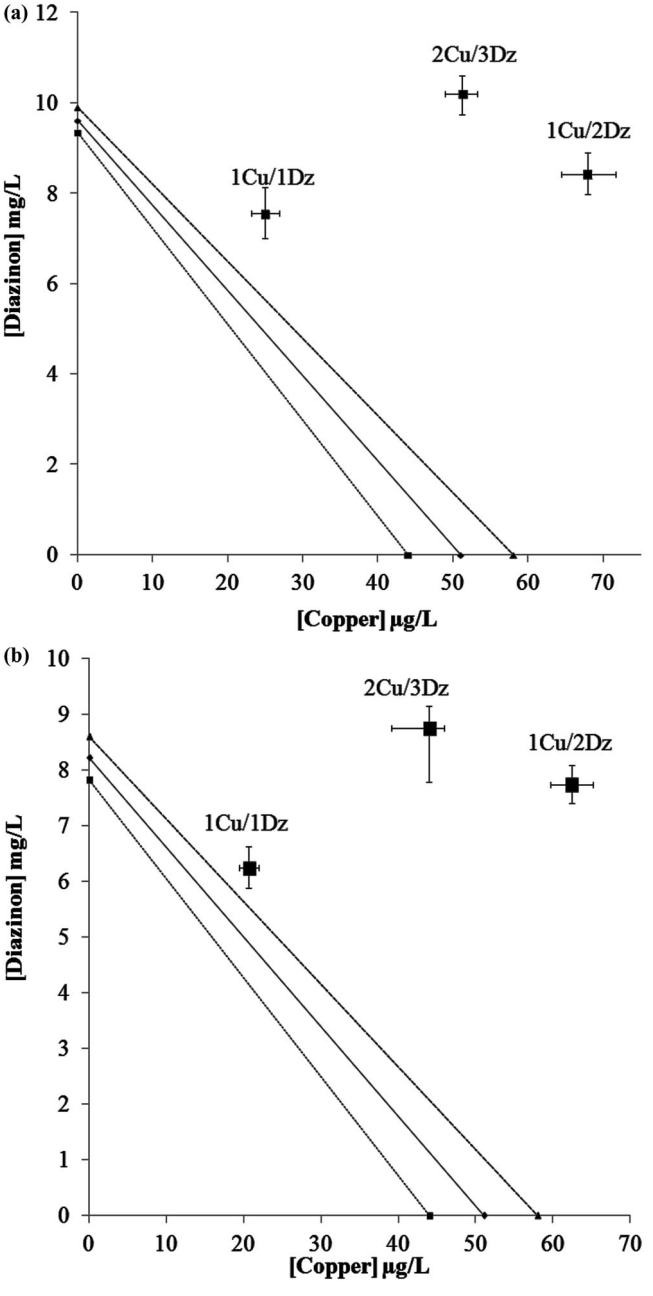

Figure 5. Isobologram plotted for LC50 of single and different mixture ratios of copper (Cu) and diazinon (Dz) at different exposure times (a = 96 h; b = 168 h) on *R. arenarum* larvae exposed from complete operculum stage (S.25) onwards. LC50 points with corresponding 95% confidence intervals are plotted.

besides organophosphates.[7,31] Strong synergistic mixture effects of metals and organophosphorous insecticides were also observed.[32] Despite that there were no data about the joint toxicity of Cu and diazinon on amphibians, the joint toxicity on *R. arenarum* was

mainly antagonistic in agreement with other studies performed on mayfly and water flea.[1,7] This antagonistic interaction might be the result of three types of processes: those involved in determining external exposure in the environmental media; uptake rate, assimilation, distribution and excretion (toxicokinetics), and the chemical association with relevant receptors (toxicodynamics).[33] Further studies will be required before this result can be explained from a mechanistic point of view.

Because that interaction effects might depend on the compounds ratio in the mixture,[34] we analysed the joint toxicity of different Cu and diazinon proportions. Nevertheless, joint toxicity of Cu and diazinon on *R. arenarum* was independent of the mixture ratio assayed. Even though the different proportions tested do not cover the large number of mixtures which can be found in the environment, these results are evidences of the kind of interaction that can be expected of them. Moreover, despite that toxicity of single compounds was stage dependent the type of joint toxicity was independent of the developmental stage.

For most mixture proportions, the interaction was independent of the exposure time, but it is noteworthy pointing out that mixture toxicity of 2 Cu/1 diazinon ratio changed with time, ranging from an additive response till 120 h of exposure, to an antagonistic interaction since 144 h of exposure. This fact highlights the relevance to extend the exposure period. As we previously described, Cu was more toxic than diazinon on *R. arenarum* embryos and larvae. As Cu was more embryotoxic, it is interesting to point out that the LC50s of Cu were nearly constant for all mixture proportions for embryo exposure while the LC50s of diazinon varied almost threefold among mixtures. Moreover, diazinon was more toxic to larval period. Thus, the LC50s of diazinon were nearly constant across the mixture proportions, while there was a threefold drop for the LC50s of Cu.

Barata et al. [35] have pointed out that if toxicity results are obtained from high biological levels as lethality, it is expected that the complexity, variability and reproducibility may vary from experiment to experiment.[35] Indeed, inter-test variability on the sensitivity of the test population may be misinterpreted.[36] Furthermore, some procedures predict the effects of pesticide mixtures using toxicological information of single compounds obtained from standardised toxicity bioassays.[37] The relevance of this work is that taking into account these issues toxicity was simultaneously evaluated in the same clutches for single compounds and binary mixtures in different proportions. The interaction effects were analysed by means of the LC50 values obtained simultaneously. This methodology gives strength to the prediction, since it decreases the chances of false positive or negative results.

The Hazard Quotient (HQ),[38] a numerical expression of ecological risk, was obtained for *R. arenarum* exposed at early developmental stages to Cu. This quotient was calculated as the ratio between an expected environmental concentration, in this case 64 µg/L found in an urban river of Argentina,[39] and a standard toxicity end point, Cu 168-h LC50 = 0.0195 mg/L, resulting in an HQ of 3.28 times higher than the fixed USEPA's Level of Concern. In a similar way, the calculated diazinon HQ based on local applications was 2.73 times higher than the USEPA's Level of Concern on *R. arenarum* larval development.[6] These facts highlight the adverse effects that Cu and diazinon might represent to *R. arenarum,* one of the species with the highest incidence of malformations in Argentina.[14] The present study demonstrated that exposure of single Cu and diazinon and combinations of them may result in adverse effects. Mixture toxicity of Cu and diazinon

observed in this study provides some perspective on the implications of these contaminants in aquatic environment, highlighting the threat that both chemicals represent for *R. arenarum* populations and the relevance of evaluating mixture toxicity for risk assessments and wildlife preservation purposes.

5. Conclusion

This study demonstrates the relevance of assessing the potential risk of binary mixtures at different ratios, exposure times and periods of the life cycle of an amphibian key species. Moreover, this work highlights the importance of simultaneous evaluation of the toxicity of single chemicals. Thus, Cu was always more toxic than diazinon on *R. arenarum* embryos and larvae. Cu was more toxic during the embryonic period than the larval one, while diazinon was more toxic during the larval period than the embryonic one. Cu and diazinon mixture interactions were antagonistic for almost all conditions.

The implications of mixture effects at different proportions, exposure times and life cycle periods of exposure for certain species should be considered for the risk assessment analysis in relation to establishing water quality criteria, particularly for wildlife preservation purposes.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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