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Original Article

ZnO nanoparticles and Cu2+ enhanced toxicity in acute rather than chronic exposure of the freshwater rotifer *Lecane papuana*

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Abstract: Zinc oxide nanoparticles (ZnO NPs) are currently used in several fields, including removing dissolved metals and organic contaminants from wastewater by adsorption and/or photocatalytic mechanisms. Thereafter, ZnO NPs can be released into the environment and reach aquatic ecosystems, where their interaction with dissolved trace metals can alter their solubility and toxicity. For these reasons, the present study aimed to assess the enhanced toxicity of ZnO NPs and dissolved copper (Cu^{2+}), using the littoral rotifer, *Lecane papuana* as a test organism. The ZnO NPs synthesized in this research are colloidally stable at high concentrations in either distilled water or test media according to their Z-potential and hydrodynamic diameter. Thus, they remain as colloids along the duration of the exposure experiments. Acute toxicity tests showed median lethal concentrations (LC50) of 28.24×10^{-3} mg Cu^{2+}/L, 21.34 mg Zn^{2+}/L, and 78.74 mg ZnO NP/L. Enhanced acute toxicity was elicited at low concentrations of Cu^{2+} (1.42 to 5.68×10^{-3} mg/L) and ZnO NPs (0.841 mg/L). The interaction of dissolved ions, Cu^{2+} and Zn^{2+}, was discarded as the main source of toxicity towards *L. papuana* as the mixtures tested followed a concentration-addition pattern that produced ~50% mortality (1 toxicity unit [TU] = LC50). Thus, we considered that the enhanced toxicity was mainly caused by the synergistic interaction of ZnO NPs and Cu^{2+}. In contrast, chronic exposure to Cu^{2+} and ZnO NPs significantly inhibited the rotifers rate of population growth only in the groups exposed to the higher concentrations tested, representing about 10-20% of their respective LC50 values. Therefore, reports based only on acute tests might bias the ecological significance of the results as long-term and more complex matrices are required for a better understanding of the potential risks that nanoparticles and co-contaminants represent to the aquatic biota.

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Introduction

Nanostructured materials are defined based on their particle dimensions (1-100 nm), conferring physical and chemical properties completely different from the bulk or dissolved components (Khan et al., 2019). For example, nanoparticles (NPs) show high contact surface and mechanical strength, making them very attractive for several industrial applications due to their high efficiency. Nevertheless, these same properties are related to their increased toxicity to living organisms. Thus, several international legislative bodies in the European Union and the United States have focused on the risk assessment of NPs to human and environmental health (Bondarenko et al., 2013).

Zinc oxide nanoparticles (ZnO NPs) are among the most used engineered nanomaterials (ENMs), with an annual production estimated at over 550 tons (Piccinno et al., 2012). These ENMs have been used in the rubber industry (Qin et al., 2020), in antifouling paints (Kaiser et al., 2013), in textiles as antimicrobial agents (El-Nahhal et al., 2017), for UV protection (Alebeid and Zhao, 2017), and also in personal care products like sunscreen because of the UV scavenging properties of the ZnO (Subramaniam et al., 2019).

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Due to the increase in the use of ENMs, it is estimated that more than 50% of their total production enters the environment through landfills (Keller et al., 2013) and water systems (Gottschalk et al., 2013), posing a significant threat to aquatic ecosystems (Vijayakumar et al., 2017). Despite the efforts to study their (eco) toxicological characteristics, there is still a gap in aspects like the NPs fate and distribution in natural environments, their effects on key species in aquatic food webs, and their potential for biomagnification, among others.

Environmental toxicologists have selected different model organisms to assess the effects of NPs in aquatic ecosystems; among such organisms, the most commonly used are zooplankters because of their high sensitivity to environmental perturbations, their ease of handling, their short life cycles, and they represent a key component in aquatic food webs as secondary production (biomass production); besides, they serve as prey for fish and larger invertebrates. The use of rotifers as test organisms is described in the ISO guideline 19827 (ISO, 2016), and several authors have demonstrated their suitability for the standard assessment of toxic effects of chemical substances, effluents, and treated or untreated water from industrial or municipal sources (Rico-Martínez et al., 2016). The most widely used rotifers are limnetic species like *Brachionus calyciflours* (freshwater) and *B. plicatilis* (brakish and marine water), but also littoral species of the genus *Lecane* have shown high accuracy and reliability in assessing the impact of metals (Klimek et al., 2013; Hernández-Ruiz et al., 2016). They also showed higher sensitivity than the conventional test cladoceran, *Daphnia magna,* when both species were exposed to the nanocomposite Cu@ZnO (Medina-Ramírez et al., 2019).

When NPs reach aquatic environments, they can co-exist with other chemicals, dissolved inorganic and organic compounds, ionic forms of metals, and other nanomaterials. Thus, the co-occurrence of dissolved copper (Cu^{2+}) and ZnO NPs is likely because Cu^{2+} is a trace element that can be found at low concentrations in natural water systems (Rader et al., 2019), and ZnO NPs can enter aquatic environments in any part of their life cycle because of their extensive use.

Synergistic effects have been described for diverse nanomaterials and toxic compounds. Some authors have studied the interaction of nano plastics and persistent organic pollutants, which exhibited a synergistic model effect on the rotifer *Brachionus koreanus* (Jeong et al., 2018). For ZnO NPs, a similar model was described in the presence of cadmium, which promoted the lixiviation of zinc ions and enhanced toxicity toward *Phytolacca americana* (Xiao et al., 2022). The cladoceran *D. magna* exposed to ionic silver (Ag^{+}) and ZnO NPs exhibited different response patterns (antagonism or synergism) depending on the toxicity unit's ratio, with synergistic toxicity when ZnO NPs were dominant in the mixture (Baek et al., 2020).

Some studies considered that toxicity is related to ionic forms or lixiviated ions; thus, the antibacterial effect (toxicity) of ZnO NPs as the result of lixiviated Zn^{2+} rather than particles, finding Zn^{2+} concentrations of up to 4.5 mg/L after 96 hours of incubation (Wang et al., 2016). It is worth pointing out that the initial concentration (nominal) was 5.0 ZnO NPs mg/L. Nevertheless, other studies showed that the dissolution of ZnO NPs in a culture medium for *Vibrio fischeri* was about 15% (Zhang et al., 2020), which was higher than the dissolution of CuO NPs, but still lower lixiviation of Zn^{2+} than that in previous reports (Wang et al., 2016). The composite Cu@ZnO was described to exert antibacterial effects against different clinical bacterial and fungal isolates but also toxic effects on zooplanktonic species, which might be due to the lixiviation of copper ions (Medina-Ramírez et al., 2019).

For these reasons, the present work is aimed to assess the toxic effects due to the interaction of dissolved copper (Cu^{2+}) and zinc oxide nanoparticles (ZnO NPs) in the rotifer, *Lecane papuana.* Different toxicity assays were implemented: (a) acute toxicity tests (without food supplementation), (b) chronic toxicity tests, which are supplemented with food (microalgae) and might pose a factor that modifies

the toxicity of the mixture ZnO NPs-Cu^{2+} and (c) hatching of amictic eggs, to demonstrate that NPs enhance the toxicity of dissolved metals towards the aquatic biota.

Materials and methods

Chemicals: The ZnO NPs were synthesized by our research group using a microwave-activated solvothermal route. The materials have been fully characterized, exhibiting uniform size (~30 nm), mixed morphology (spheres and small rods), and high purity (Medina-Ramírez et al., 2019). $NaHCO_3$, $CaSO_4{\cdot}2H_2O$, $MgSO_4{\cdot}7H_2O$, KCl, $CuSO_4{\cdot}5H_2O$, and $ZnSO_4{\cdot}5H_2O$ were purchased from J.T. Baker and used as received. Stock solutions and further dilutions were prepared in deionized water. Work solutions were prepared in reconstituted medium-hard water, constituting the culture medium for rotifers (USEPA, 2002).

Evaluation of the Stability of ZnO nanomaterials in test media: The Dynamic Light Scattering (DLS) technique (Zetasizer ZS90, Malvern Panalytical) was used to determine the hydrodynamic diameter of ZnO NPs. The hydrodynamic diameter value was obtained by performing a second-order cumulant fit to the field correlation function measured at a scattering angle of 90° (Pecora, 2000). The zeta potential of the ZnO NPs was obtained from electrophoretic mobility measurements using O'Brien-White Theory (Delgado et al., 2005). For these analyses, ZnO NPs were suspended in deionized water, EPA medium, and EPA medium with Cu^{2+} (5 mg/L) at the highest particle concentration tested in the biological assays (100 mg ZnO NPs /L). Measurements were performed at different times (0, 24, and 48 h) to observe the size temporal evolution of the ZnO NPs and to rule out the possible destabilization of the colloidal system. The mean value is obtained by averaging six independent measurements and the error by the sample standard deviation.

Culture conditions for rotifers: The strain of *L. papuana* has been cultured for at least five years in the Laboratory of Aquatic Toxicology of the Autonomous University of Aguascalientes, Mexico. *Lecane* females were maintained in a bioclimatic chamber at 25±2ºC, photoperiod of 16 h light for 8 h dark, fed on the green algae *Nannochloropsis oculata* (UTEX strain LB2164) at 10^6 cells/mL, and reconstituted medium-hard water according to the Environmental Protection Agency o the United States (USEPA) guidelines ($NaHCO_3$ 96 mg/L, $CaSO_4{\cdot}2H_2O$ 60 mg/L, $MgSO_4{\cdot}7H_2O$ 60 mg/L, and KCl 4 mg/L; at pH 7.5) (USEPA, 2002).

Acute toxicity test of single chemicals: Twelve hours before the beginning of the test, the amictic eggs of *L. papuana* were separated and placed in a medium without food supplementation. Then, neonates (<12 h) were randomly placed in a 24-well polystyrene microplate. Five different concentrations of Cu^{2+}, Zn^{2+}, or ZnO NPs were analyzed. The negative control consisted of neonates in EPA medium without exposure to toxic substances. The conditions for the experiments were as follows: 1 mL test volume without food supplementation, at 25±2°C, for 48 h with a photoperiod of 16:8h (light/dark), and ten organisms per replica. All experiments were conducted in quadruplicate. At the end of the test, immobility and mortality were recorded, and these data were used to calculate the median lethal concentration (LC50) and lethal concentration at 10% (LC10). The lethal concentrations were estimated with the statistical package *drc* in R. All concentrations used in this study are expressed as nominal.

Chronic toxicity test of single chemical: Five different nominal concentrations were selected for Cu^{2+} (0.355, 0.710, 1.421, 2.841, and 5.683×10^{-3} mg/L) or ZnO NPs (0.984, 1.968, 3.937, 7.874 and 15.747 mg/L), which correspond to 1/80, 1/40, 1/20, 1/10 and 1/5 of their respective LC50 values. The negative control consisted of neonates reared only in an EPA medium (not exposed to any toxic substance). All controls and treatments (different concentrations of Cu^{2+} or ZnO NPs) consisted of four replicas (n = 4), and five neonates per well were placed in a 24-well polystyrene microplate with a test volume of 2 ml. The green alga *N. oculata* was

Table 1. Experimental design to assess the interaction of Cu^{2+} and ZnO NPs in acute toxicity tests with *Lecane papuana* (Rotifera: Monogononta).

Treatment	ZnO NPs + Cu^{2+}				Zn^{2+} + Cu^{2+}			
	mg/L	TUa	x10^{-3} mg/L	TUa	mg/L	TUa	x10^{-3} mg/L	TUa
A	15.747	0.20	22.731	0.80	3.617	0.20	22.731	0.80
B	31.495	0.40	17.048	0.60	7.235	0.40	17.048	0.60
C	39.369	0.50	14.207	0.50	9.043	0.50	14.207	0.50
D	47.242	0.60	11.366	0.40	10.852	0.60	11.366	0.40
E	62.990	0.80	5.683	0.20	14.470	0.80	5.683	0.20

The expected effect is based on the postulate that LC50 = 1 TUa. Thus, exposure concentrations of 1 TUa were expected to exert ~50% of immobilization/dead of exposed individuals. All treatments consisted of 1 TUa (the sum of ZnO TUa and Cu^{2+} TUa), except for the control, in which no mortality was neither expected nor observed.

added as food supplement at 10^6 cells/ml. The temperature was controlled in a bioclimatic chamber at 25±2°C, with a photoperiod of 16 h light and 8 h dark. Distilled water (100 µL) was added to the medium every other day to avoid desiccation. After five days of exposure, all living rotifers were counted. The total number of rotifers was used to estimate the intrinsic rate of natural increase (*r*) for all experimental groups (control and treatments), according to the formula of

$$r = \frac{ln(N_f/N_i)}{t}$$

Where *ln* is the natural logarithm, N_i is the initial number of rotifers, N_f is the number of organisms at the end of the test, and *t* corresponds to the exposure time (five days for these experiments).

Acute toxicity tests of binary mixtures: We assayed the following mixtures: Cu^{2+} and Zn^{2+}, and Cu^{2+} and ZnO NPs. The mixtures for the immobilization test were prepared based on their respective LC50 values, which were considered one acute toxicity unit (1 TUa). Thus, 1 TUa elicits the death of 50% of the exposed organisms. Five combinations of Cu2+ or Zn2+ with ZnO NPs were initially assayed (Table 1). This experimental setup was changed for the interaction of Cu^{2+} and ZnO NPs because of the 100% mortality in all treatments. Thus, different combinations were tested to find an interval that allowed comparisons (mortality <100%). The test conditions (final volume, no food supplementation, photoperiod, and temperature) were as aforementioned in the section on acute toxicity tests.

Chronic toxicity test of binary mixtures: Five different ratios were selected to assess the interaction of Cu^{2+} and ZnO NPs (Table 2). The control group consisted of organisms reared only in an EPA medium without exposure to toxic compounds. The test conditions were described in the aforementioned chronic toxicity assays. At the end of the exposure period, all living organisms were counted. The total number of rotifers was used to estimate the intrinsic growth rate (*r*) with the formula previously described.

Lixiviation of Zn^{2+} and toxicity towards *L. papuana*: To assess the effect of lixiviated Zn^{2+} from ZnO NPs, we experimented with the same conditions of the acute toxicity tests but without rotifers. Thus, all test concentrations of ZnO NPs were prepared in 2 ml of EPA medium, incubated at 25°C for 24 h, and 48 h, with a photoperiod of 16 h light and 8 h dark. After incubation, test solutions were transferred to microtubes and centrifuged at 12,000 g for 20 min. Supernatants were recovered and transferred to a new microplate of 24 wells, one test solution per well and four replicas per concentration. Then, 10 neonates (<24-h old) of *L. papuana* were transferred to each well and incubated for 48 h. At the end of the test, dead animals were counted, and mortality rates were estimated.

Statistical Analysis: The LC50 values were

Table 2. Assessment of the interaction of dissolved copper (Cu^{2+}) and ZnO nanomaterials in chronic toxicity tests with *Lecane papuana*.

Treatment	Cu^{2+}		ZnO NPs	
	$\times 10^{-3}$ mg/L	Acute LOEC ratio	mg/L	Acute LOEC ratio
Control	0.000	0.00	0.000	0.00
A	2.814	1.00	0.00	0.00
B	2.131	0.75	3.95	0.25
C	1.421	0.50	7.90	0.50
D	0.711	0.25	11.85	0.75
E	0.00	0.00	15.80	1.00

The highest concentration tested for Cu^{2+} (2.814×10^{-3} mg/L) and ZnO NPs (15.80 mg/L) correspond to their respective LOEC (lowest observed effect concentration) in acute toxicity tests previously performed with *L. papuana*.

Table 3. Hydrodynamic diameter and zeta potential values of ZnO NPs at time zero (at the time the sample was prepared), 24 and 48 hours in different test media.

	Hydrodynamic diameter nm			Zeta potential mV		
	0 h	24 h	48 h	0 h	24 h	48 h
Deionized water	230±22	225±13	222±8	-40.5±3.3	-38.2±1.0	-35.6±0.4
Deionized water + Cu^{2+}	235±17	221±11	218±8	-36.2±1.1	-42.0±1.6	-41.7±1.3
EPA medium	245±13	227±11	237±16	-27.5±0.9	-29.5±1.3	-29.1±0.8
EPA medium + Cu^{2+}	247±9	227±8	242±18	-27.5±0.9	-30.2±0.7	-30.7±1.3

ZnO NPs were prepared at 100 mg/L. EPA medium represents a moderate hard reconstituted water used in the culture of rotifers (USEPA, 2002). Cu^{2+} was added at 5 mg/L. All data were obtained as the average of six replicas.

Table 4. Results of the acute toxicity tests with *Lecane papuana* exposed to Cu^{2+}, Zn^{2+}, or nanoparticles of ZnO.

	$Cu^{2+} \times 10^{-3}$ mg/L	Zn^{2+} mg/L	ZnO NPs mg/L
LC1	5.574 (1.081)	0.902 (0.402)	1.873 (1.1074)
LC10	13.041 (1.355)	4.312 (1.056)	13.177 (4.192)
LC50	28.414 (1.328)	18.087 (2.142)	78.737 (8.362)

LCx, lethal median concentration that affects determined percentage of the exposed population, where *x* takes values 1, 10 and 50 for the present study. Data are presented as the mean value (standard error of the mean) (n = 4).

obtained with the help of the statistical software R and the *drc* package. The results of the chronic toxicity experiments were compared using the one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests with the help of statistical software R for Windows and the *agricolae* and the *ggplot2* packages. Significant differences were established when $P<0.05$.

Results

Stability of ZnO nanomaterials in test media: Table 3 presents the results of the stability of ZnO as suspended materials. The zeta potential of ZnO NPs in deionized water took values from -40.5 to -35.6 mV, showing slight differences in deionized water with Cu^{2+}. In both EPA media, the zeta potential showed values from -30.7 to -27.5 mV. In all cases, the zeta potential values are negative, indicating that the particles are negatively charged and higher than 25 mV in absolute values. This suggests aggregation is unlikely due to electrostatic repulsion among particles.

Acute toxicity test of single chemicals: The effective concentrations for Cu^{2+}, Zn^{2+}, and ZnO NPs are shown in Table 4, where LC50 represents the concentration that caused death to 50% of the

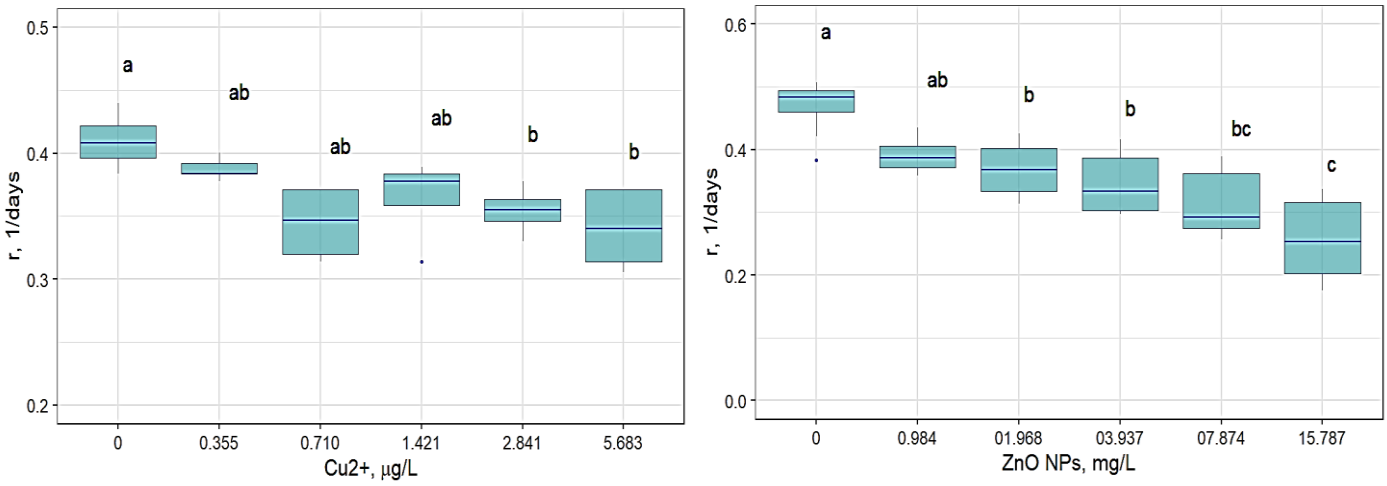

Figure 1. Results of the chronic toxicity tests with *Lecane papuana* exposed to Cu^{2+} (left) and ZnO NPs (right). Different letters represent significant differences among treatments, which were compared through one way ANOVA and multiple comparison test of Bonferroni (P<0.05).

total organisms. Copper concentrations of some micrograms can significantly affect the survival of *L. papuana* (LC50 = 28.4×10^{-3} mg/L).

Chronic toxicity test of single chemical: The results of the chronic tests are presented with their respective confidence intervals (P<0.05) in Figure 1. For these assays, we selected concentrations from 1/80 (0.0125) to 1/5 (0.20) of their respective LC50 values and found different patterns. In the case of Cu^{2+}, the population growth of *L. papuana* was significantly inhibited at concentrations above 0.1 LC50 (2.841×10^{-3} mg Cu^{2+}/L). However, ZnO NPs elicited significant changes at 0.025 LC50 (1.968 mg ZnO NPs /L) and higher concentrations.

As the initial hypothesis revolved around the interaction of Cu^{2+} and ZnO NPs and the likely synergistic effect, we first assessed the interaction of dissolved cations. For the assays with dissolved cations (Cu^{2+} and Zn^{2+}), it was not necessary to adjust the concentrations as the observed mortality was close to the expected mortality (~50%) (Fig. 2). In this experimental design, all mixtures tested (0+21.34, 7.085+16.005, 14.170+10.670, 21.255+ 5.335, 28.340+0 mg/L, Cu^{2+} at 10^{-3} mg/L and Zn^{2+} at mg/L, respectively) were expected to cause ~50% (we considered 1 TUa as equivalent to the LC50). Thus, the cations' effect was related to adding their respective toxicity units and summing ~1 TUa. The criterion of the mixture toxicity index states that if the result of dividing the observed effect of the mixture by the observed effect of the single toxic compound is comprised between 0.8 and 1.2, then it can be stated that the effect corresponds to the concentration-addition concept (Arreguin-Rebolledo et al., 2021). Therefore, the concentration-addition model can describe the effect of the mixture of dissolved Cu^{2+} and Zn^{2+}.

It was impossible to follow the same experimental design to assess the combined effect of Cu^{2+} and ZnO NPs as their mixtures elicited 100% mortality; thus, different sets of concentrations were tested to achieve those reported in Table 2 finally. These results showed that the combination of only 0.025 TUa of Cu^{2+} and 0.011 TUa of ZnO NPs produced the same effect that either 0.028 mg Cu^{2+}/L or 78.74 mg ZnO NPs/L (the respective LC50 values). The mortality of rotifers increased according to the increment of Cu^{2+} concentration, reaching the highest mortality (~100%) at 0.840 mg/L of ZnO NPs and 5.682×10^{-3} mg of Cu^{2+}/L. Thus, the concentrations in these tests correspond to approximately 1/100 of the LC50 for ZnO NPs, and from 1/40 to 1/5 of the LC50 for Cu^{2+}. Then, the combination of these metal forms expressed as TU (0.036-0.212) was expected to cause mortality between 1 and 10%, but mortality occurred at rates from 46 to 95%, which are, on average, 10-fold higher than the expected response (Table 5). Based on the aforementioned criterion (Arreguin-Rebolledo et al., 2021), the results of such a division

Table 5. Results of the acute toxicity tests with *Lecana papuana* exposed to mixtures of Cu^{2+} and ZnO NPs for 48 h.

Treatment	ZNO NP			Cu^{2+}			
	Total TU	mg/L	TU	x10^{-3} mg/L	TU	Mortality	SD
Control	0	0	0.000	0.00	0.000	n.o.	n.a.
A	0.036	0.840	0.011	0.71	0.025	0.463	0.169
B	0.061	0.840	0.011	1.42	0.050	0.700	0.200
C	0.111	0.840	0.011	2.84	0.100	0.875	0.139
D	0.162	0.840	0.011	4.26	0.151	0.713	0.136
E	0.212	0.840	0.011	5.68	0.201	0.950	0.053

LC50 = 1 TU. n.a., non-applicable; n.o., no observed. TU = Toxicity units; All combinations were expected to cause mortality ≤10% in accordance to the total TU.

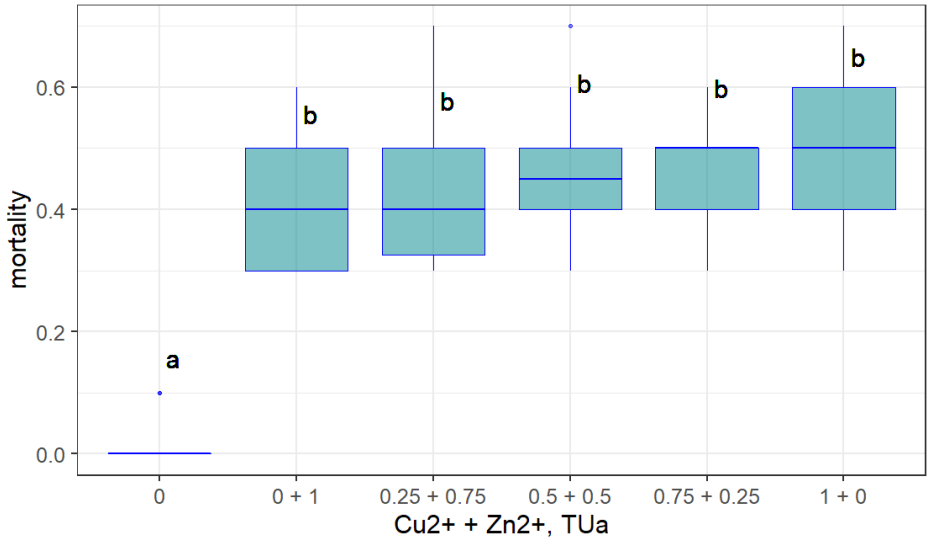

Figure 2. Results of the acute toxicity test of the mixture Cu^{2+} + Zn^{2+}. Neonates (<24 h) of *Lecane papuana* were exposed to: 0 × 10^{-3} mg Cu^{2+} /L + 21.34 mg Zn^{2+}/L (0+1 TUa), 7.085 × 10^{-3} mg Cu^{2+} /L + 16.005 mg Zn^{2+}/L (0.25 + 0.75 TUa), 14.170 × 10^{-3} mg Cu^{2+} /L + 10.670 mg Zn^{2+}/L (0.5 + 0.5 TUa), 21.255 × 10^{-3} mg Cu^{2+} /L + 5.335 mg Zn^{2+}/L (0.75+0.25 TUa), 28.340 × 10^{-3} mg Cu^{2+} /L+0 mg Zn^{2+}/L (1 + 0 TUa). 1 TUa = LC50. Control group consisted of non-exposed organisms. Different letters above indicate significant differences according to one-way ANOVA and multiple comparison test of Bonferroni (P<0.05).

are higher than 1.2, which corresponds to more than that of the additive model (synergistic effects).

Lixiviation of ions from ZnO NPs: Incubation of freshwater media with nanoparticles at the same concentrations used in the acute toxicity tests and their posterior removal through centrifugation were performed to assess the effect of ions lixiviation. Rotifers exposed to free-ZnO NPs media showed mortality rates of up to 10% at the highest ZnO NPs concentration (Fig. 3).

Chronic toxicity test of binary mixtures: Figure 4 displays the results of the chronic toxicity tests with both Cu^{2+} and ZnO NPs, showing that mixtures of Cu^{2+} and ZnO NPs elicited no significant changes in the intrinsic rate of population increase in *L. papuana*. The concentration of either Cu^{2+} and ZnO NPs were fractions of the respective LC50 values, which were expected to follow the same synergistic pattern as in the acute toxicity tests. Nonetheless, the combination of Cu^{2+} and ZnO NPs in tests with food supply (algae) showed no significant changes compared to the control group. Figure 5 presents the population growth rates of rotifers born from amictic eggs exposed to mixtures of Cu^{2+} and ZnO NPs. Here, significant changes were only observed for the two highest concentrations, corresponding to 0.162 TUa (0.840 mg ZnO NPs/L and 4.26×10^{-3} mg Cu^{2+}/L) and 0.212

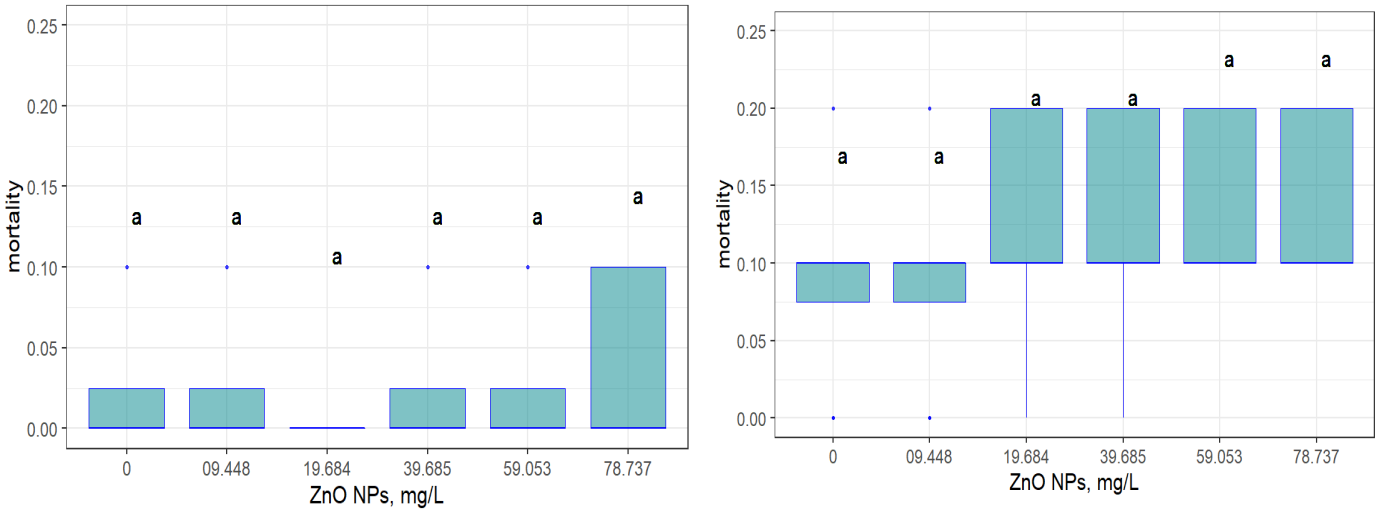

Figure 3. Results of the acute toxicity test with *Lecane papuana* exposed to media without nanoparticles. Test media consisted of reconstituted medium hard water with ZnO NPs, incubated for 24 h (left) and 48 h (right). After incubation in the same environmental conditions than rotifers culture, test media were centrifuged at 12,000×*g* and supernatants were recovered. Neonates of *L. papuana* (<24-h old) were exposed to supernatants. Mortality was recorded after 48 h. Different letters represent significant differences among treatments, which were compared through one way ANOVA and multiple comparison test of Bonferroni (P<0.05).

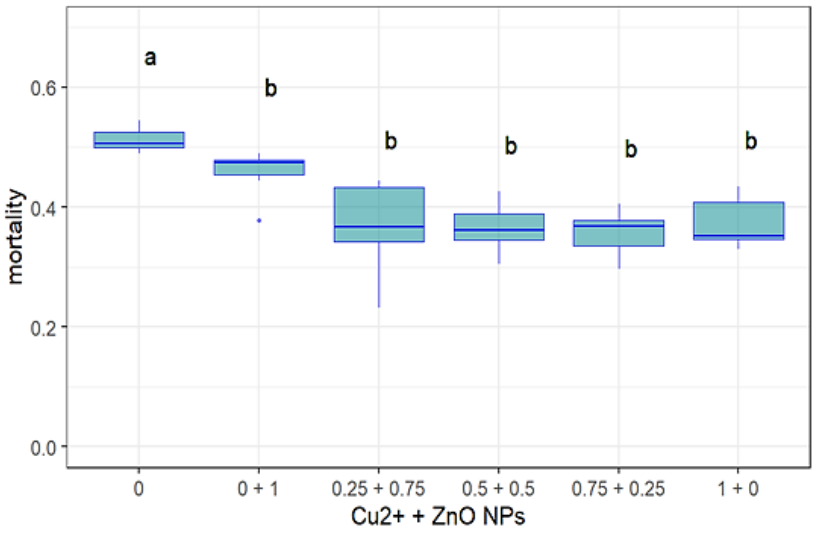

Figure 4. Results of the chronic toxicity test with the rotifer *Lecane papuana* exposed to mixtures of Cu^{2+} and ZnO NPs. Rotifers were exposed to fractions of the acute LOEC (Lowest observed effect concentration). Thus, mixtures of Cu^{2+} and ZnO correspond to: 1 + 0 (2.814×10^{-3} mg Cu^{2+}/L + 0 mg ZnO NPs /L), 0.75 + 0.25 (2.131×10^{-3} mg Cu^{2+}/L + 3.950 mg ZnO NPs /L), 0.5 + 0.5 (1.421×10^{-3} mg Cu^{2+}/L + 7.90 mg ZnO NPs /L), 0.25 + 0.75 (0.711×10^{-3} mg Cu^{2+}/L + 11.850 mg ZnO NPs /L), and 0 + 1 (0 mg Cu^{2+}/L + 15.80 mg ZnO NPs /L). Different letters represent significant differences among treatments, which were compared through one way ANOVA and multiple comparison test of Bonferroni (P<0.05).

TUa (0.840 ZnO NPs/L and 4.68×10^{-3} mg Cu^{2+}/L).

Discussion

Stability of ZnO nanomaterials in test media: The stability of ZnO NPs was further confirmed by measuring the hydrodynamic size of the nanoparticles and following its time evolution during 48 h, the duration of the acute toxicity tests. The mean hydrodynamic diameter of the ZnO NPs in deionized water was around 220-230 nm, with no significant differences when suspended in deionized water with Cu^{2+} (5 mg/L). Similar values were obtained for the dispersion of ZnO NPs in EPA medium and EPA medium + Cu^{2+}. Moreover, these values remain almost constant after 48 h of sample preparation, confirming the colloidal stability of all

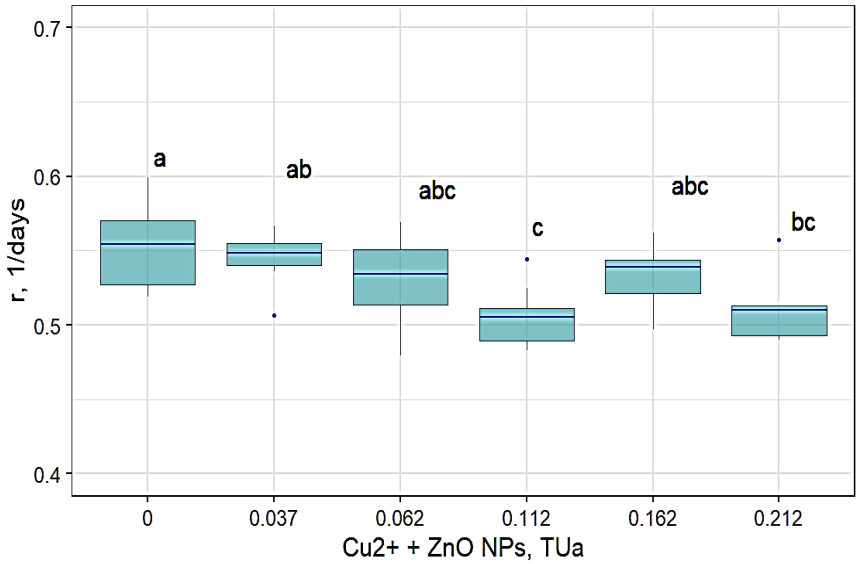

Figure 5. Reproductive performance of the rotifers hatched from amictic eggs that were exposed to Cu^{2+} (0.71, 1.42, 2.84, 4.26 or 5.68 × 10^{-3} mg/L) and ZnO NPs (0.840 mg/L), which correspond to 0.037, 0.062, 0.112, 0.162, and 0.212 TUa, respectively. Amicitic eggs were exposed until hatching and rotifers were transferred to clean EPA medium supplemented with *N. oculata* at 10^6 cells/mL. Then, the rate of population increase was estimated at the end of 120 h through the formula r = (LnNf-LnNi)/t. Different letters above the boxes refer significant differences among the treatments, which were compared through one-way ANOVA and multiple comparison test of Bonferroni (P<0.05).

the nanoparticle dispersions. The colloidal stability study was carried out using suspensions prepared with the highest concentration of both ZnO NPs and Cu^{2+}. Thus, if nanomaterials remain stable as a colloidal suspension during the exposure time under the most unfavorable conditions (salt and nanoparticle concentrations), then it can be assumed that ZnO NPs suspensions remain stable at lower concentrations of either Cu^{2+} or ZnO NPs.

Acute toxicity test of single chemicals: The LC50 values for this rotifer were very similar to data previously reported for other organisms; thus, *L. papuana* is the most sensitive to Cu^{2+} within the family Lecanidae, while their sensitivity is similar to other genera like *Brachionus* and also to some cladoceran species which are commonly used in aquatic toxicology studies (USEPA, 2017). For Zn^{2+}, *L. papuana* exhibits the highest LC50 (21.330 mg/L) compared to other zooplanktonic species. Literature reports the LC50 of ZnO NPs for freshwater invertebrates from 1.25 to 7.1 mg/L with *D. magna* (cladoceran) (Heinlaan et al., 2008, Zhu et al., 2009, Naddafi et al., 2011) and about 0.165 mg/L for *Thamnocephalus platyurus* (Heinlaan et al., 2008). Within freshwater test organisms, *L. papuana* exhibited one of the highest LC50 values, which are comparable to the results of *Artemia salina* (LC50 = 58.3 mg/L, at 96 h) (Schiavo et al., 2018). Thereafter, *L. papuana* seems e very tolerant to zinc whereas the sensitivity towards copper is similar to other freshwater species used as test organisms.

It was generally assumed that the nanomaterials of ZnO could be considered innocuous; nevertheless, nanostructured materials negatively affect the exposed organisms (Jarvis et al., 2013). The LC50 values found that *D. magna* is more sensitive (LC50 = 1.511 mg/L; (Zhu et al., 2009) than *L. papuana* (LC50 = 78.74 mg/L). In addition, all individuals of *Ceriodaphnia cornuta* died when exposed to ZnO NPs at 0.160 mg/L (Vijayakumar et al., 2016), which is nearly 500-fold lower than the LC50 of *L. papuana*. It is evident that *C. cornuta* is more sensitive than *L. papuana* to ZnO NPs, but this one is among the most susceptible organisms within Rotifera, which consists of species important for aquaculture and ecotoxicology.

Chronic toxicity test of single chemicals: Despite reports on the chronic toxicity of Cu^{2+} and ZnO NPs, only some authors reported the respective EC50 values (USEPA, 2017). In the present study, the

EC50 for either Cu^{2+} or ZnO NPs was not estimated because the population growth rate inhibition was below 50% at the concentrations tested. Higher concentrations could be required (Hernández-Flores et al., 2020) found a similar pattern with the rotifer *Euchlanis dilatata* and reported an EC50 higher than the LC50 for Cu^{2+}, stating that the alga *Nannochloropsis oculata* (the same algal species used in this study) might interfere with the bioavailability of Cu^{2+} and diminished its toxicity due to quelation or absorption of the micronutrient (because its concentration was much lower than that in the culture media for algae). In the case of ZnO NPs, it was reported that algal exudates and the interaction with their cell wall lessened the lixiviation of Zn^{2+} from the ZnO NPs and their toxicity by aggregation or adsorption (Chen et al., 2012).

Acute toxicity of binary mixtures: The increased toxicity of Cu^{2+} and ZnO NPs as mixtures could find a possible explanation for the capacity of ZnO NPs to remove metal ions, like Cu^{2+}, from the water (Le et al., 2019) and its further release within the rotifers to cause their death. Metal ions from ZnO NPs are released within the digestive tract of zooplankters and facilitate the bioconcentration of Zn, which might not generate significant effects until metals exceed threshold concentrations (Li and Wang, 2013). Baek et al. (2020) reported the enhanced toxicity of Ag^{+} and ZnO NPs, when ZnO is dominant in the mixture and suggested the formation of Ag^{+}-ZnO NPs complexes as promoters of Trojan Horse-like effects releasing cations within the gut of *D. magna*. Similarly, ZnO NPs (0.840 mg/L) enhanced the toxicity of Cu^{2+} towards *L. papuana*, diminishing the LC50 of Cu^{2+} from 0.028 mg/L to 0.71×10^{-3} mg/L, representing a 40-fold increase of toxicity. These results confirm that environmental concentrations of soluble copper and low concentrations of ZnO NPs could exert deleterious effects on rotifers.

Lixiviation of ions from ZnO NPs: Since it is of high relevance to elucidate if enhanced toxicity is due to released soluble ions (Zn^{2+}) and Cu^{2+}, the interaction of ZnO NPs and Cu^{2+}, or the participation of both mechanisms, we performed bioassays in which organisms were exposed to EPA medium in which ZnO NPs were previously dispersed and incubated for up to 48 h to allow zinc lixiviation from nanomaterials, and then, such nanoparticles were removed through centrifugation (12,000× *g*, 20 min). According to our results, mortality was low and Zn^{2+} (lixiviated from NPs) might not represent the main source of toxicity toward *L. papuana.* However, these results point out that it is certainly important to consider that Zn^{2+} can interact with the other components of the mixture and modify their toxicity. The interaction of Cu^{2+} and Zn^{2+} in assays with *L. papuana* showed that their interaction complies with the criterion of the concentration-addition model.

The stability studies, zeta potential and hydrodynamic size of ZnO NPs in pure water and the media used in this study show that nanoparticles remain dispersed during the assays; thus, rotifers are exposed and might be able to consume the ZnO NPs (Zhang et al., 2020). The estimated solubility of ZnO NPs was around 15% of the initial powder concentration, which might correspond to 0.126 mg Zn^{2+}/L in the present study. In case that ZnO NPs could be completely dissolved into the medium, Zn^{2+} concentration would correspond to 0.676 mg Zn^{2+}/L, which is 31 times lower than the respective LC50. In addition, when rotifers were exposed to mixtures of dissolved cations, Cu^{2+} (7.06 to 21.18×10^{-3} mg/L) and Zn^{2+} (5 to 16 mg/L) concentrations were higher than the result of complete solubilization of ZnO NPs. Moreover, the concentration of Cu^{2+} in the mixture ZnO NP + Cu^{2+} was set from 1 to 5×10^{-3} mg/L, representing concentrations 5 to 28 times lower than the Cu^{2+} LC50 value. Therefore, the high mortality observed due to ZnO NP+Cu^{2+} exposure cannot be related to the lixiviation of Zn^{2+} but to the interaction of Cu^{2+} and ZnO NPs.

Furthermore, lixiviation from ZnO NPs has been tested in a medium of moderately hard water (the same used in the present study) and showed that Zn^{2+} has low solubility due to carbonate precipitation and

its likely adsorption onto $CaCO_3$, thus reducing the Zn^{2+} bioavailability and its toxicity (Reed et al., 2012). Therefore, Zn^{2+} might not have an important contribution to the enhanced toxicity of the mixture ZnO NPs+Cu^{2+}. Hence, we hypothesized that the most relevant interaction could be the adsorption of Cu^{2+} on the surface of ZnO NPs, ingestion, and posterior release of Cu^{2+} within the rotifers in accordance to previous reports about the synergy of nanoparticles and dissolved ions, at least in the acute toxicity tests, similarly to those effects observed in *D. magna* (Yan et al., 2018, Park et al., 2019).

Chronic toxicity test of binary mixtures: In chronic tests, it is important to consider that microalgae can modify the solubility and bioavailability of metals and NPs. Chen et al. (2012) described that *Chlorella vulgaris* (Chlorophyceae) depleted the Zn^{2+} released from ZnO NPs (62 nm, ZnO NPs coated with 3-aminopropyl triethoxysilane) because their aggregation onto the algal cell wall, which was accomplished through algal exudates that bind the solubilized Zn^{2+}, depleting the bioavailability of Cu^{2+} and Zn^{2+}. Ionic zinc toxicity and its release from ZnO NPs can be reduced by algae due to either the aggregation of ZnO NPs or their interaction with algal exudates (McIntyre and Guéguen, 2013). Moreover, the concentration of both Cu^{2+} and Zn^{2+} and ZnO NPs used in this study were very low and did not exceed those concentrations in the Bold's basal medium (0.4 mg/L and 2 mg/L for Cu^{2+} and Zn^{2+}, respectively) (Nichols and Bold, 1965); thus, microalgae might take advantage of these metals and uptake the mineral nutrients (Cu^{2+} and Zn^{2+}) from the medium, depleting their concentration, and as a consequence, their toxicity, which resulted in the inhibition of the population growth of *L. papuana* in about 30% with respect to the controls.

Higher algal densities ameliorate the effect of toxicants and allow zooplankton species to deal with the toxicant-induced stress (Xue et al., 2021, Sun et al., 2022). The algal density was selected in the present study according to the literature. However, such a concentration can be the best for rotifers culture, but it might not be representative of natural environments unless attempting to simulate eutrophic conditions (high algal density). Therefore, although it could be convenient to assess the interaction of Cu^{2+}, Zn^{2+}, ZnO NPs, their mixtures, and the food supply, that goal is beyond the aim of this manuscript.

The intrinsic rate of population increase has been used for several years as a sensitive and environmental-relevant endpoint, which is modified by different sorts of toxic compounds. However, when population growth is diminished, it is important to consider that the exposure to toxicants can alter either hatching or fecundity. In the present study, hatching was not significantly affected by the exposure to Cu^{2+} and ZnO NPs, but the population growth suffered slight alterations. Embryonic malformations, hatching delay, and larval hyperactivity are induced in *Danio rerio* by exposition to ZnO NPs (Zhao et al., 2013, Chen et al., 2014). It is worth noting that this experimental design assessed the effects of the mixture on the rotifer eggs without food supplementation, exposed for 24 h, and then transferred to toxicant-free medium, in which altered organisms developed lower intrinsic growth rates than the control group. Therefore, the mixture of ZnO NPs and Cu^{2+} elicited pre-hatching alterations in *L. papuana* that conditioned their survival and fecundity, compromising their population growth. Biased conclusions can be achieved by only reporting effects related to acute toxicity tests as they do not consider various factors intervening in chronic toxicity tests. As aforementioned, algal cells modify the bioavailability of copper and zinc cations, probably due to mechanisms like precipitation or absorption as mineral nutrients, mainly because the concentrations tested were lower than those commonly used in algae culture medium. Higher concentrations of either Cu^{2+} or ZnO NPs could have been tested, but toxicological studies aimed to establish the potential risks of chemicals at environmentally relevant concentrations.

Some authors have described a phenomenon

called Trojan-horse effect, in which the nanoparticles act as carriers for environmental contaminants, which are then released within the exposed organisms. This route has been accepted in general terms as a possible explanation for the enhanced toxicity of nanomaterials and dissolved cations, either found in the media or released from the nanomaterials (Baek et al., 2020). Nevertheless, such estimations were based on acute toxicity tests, which do not require feeding organisms. On the contrary, chronic assays with zooplankters imply microalgae supplementation as a food source for zooplankton species. Thus, microalgae play a significant role because negative nanoparticle-associated effects depend on algal density, algal species, and phenomena of absorption and release of dissolved cations. Thereafter, the inclusion of more complex experiments will lead to a better understanding of the nanoparticles' fate and effects in aquatic systems. As observed in this study, conclusions based on acute assays overestimate the toxicity of dissolved Cu^{2+} and ZnO NPs, but chronic assays show that higher concentrations of Cu^{2+} and ZnO NPs are required to cause significant changes in the reproductive performance of *L. papuana*.

In conclusion, the interaction between Cu^{2+} and ZnO NPs in the acute toxicity tests followed a synergistic pattern, increasing their toxicity about 40 times compared to the individual chemicals. Release of Zn^{2+} from ZnO NPs was not the main toxicity mechanism on *L. papuana,* as demonstrated by the lixiviation assays that consisted in incubating suspensions of ZnO NPs (same concentration used in the acute toxicity tests and the same environmental conditions), removed them from the media through centrifugation, and found that mortality was low among the rotifers exposed to ZnO NPs-free media. In addition, the mixture of Cu^{2+} and Zn^{2+} caused mortality of *L. papuana* following a pattern that complies with the concentration-addition model, while the combination of Cu^{2+} and ZnO NPs followed a synergistic pattern. Biased conclusions are based only on the results of acute toxicity tests since chronic exposure to Cu^{2+} and ZnO NPs required higher concentrations to cause significant alterations in the population growth rate of *L. papuana*. Amictic eggs of *L. papuana* exposed for 24 h to relatively high concentrations of Cu^{2+} and ZnO NPs (those used in the acute toxicity tests) produced neonates that showed lower population growth rates than the controls; thus, those mixtures can negatively affect the population dynamics of rotifers. Although it is worth pointing out that exposure was in media without algal cells, which can modify the toxicity of metal compounds. In general, reports of the interaction of metal ions and metal oxide nanoparticles are based on acute toxicity tests rather than chronic assays, which involve factors like the interaction of food supply (algal cells) that can alter the stability of metal oxide nanomaterials and the bioavailability of metal ions, and likely reduce their toxicity as it is herein reported. Thus, the interaction of Cu^{2+} and ZnO NPs followed a different pattern than that observed in the acute toxicity tests. It might require higher concentrations or varying the ratios of both chemical species to cause significant changes in the population growth rate of *L. papuana*. Finally, we consider that the results of acute toxicity tests are relevant as a baseline for further research aimed at assessing the effects of complex systems in chronic exposure to toxic compounds.

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