Synergistic effect of chloroquine and copper to the euryhaline rotifer *Proales similis*

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**Abstract**

Chloroquine (CQ) has been widely used for many years against malaria and various viral diseases. Its important use and high potential to being persistent make it of particular concern for ecotoxicological studies. Here, we evaluated the toxicity of CQ alone and in combination with copper (Cu) to the euryhaline rotifer *Proales similis*. All experiments were carried out using chronic toxicity reproductive five-day tests and an application factor (AF) of 0.05, 0.1, 0.3, and 0.5 by multiplying the 24-h LC50 values of CQ (4250 µg/L) and Cu (68 µg/L), which were administered in solution. The rate of population increase (*r*, d⁻¹) ranged from 0.50 to 52 (controls); 0.20 to 0.40 (CQ); 0.09 to 0.43 (Cu); and −0.03 to 0.30 (CQ-Cu) and showed significant decrease as the concentration of both chemicals in the medium increased. Almost all tested mixtures induced synergistic effects, mainly as the AF increased. We found that the presence of Cu intensifies the vulnerability of organisms to CQ and vice versa. These results stress the potential hazard that these combined chemicals may have on the aquatic systems. This research suggests that *P. similis* is sensitive to CQ as other standardized zooplankton species and may serve as a potential test species in the risk assessment of emerging pollutants in marine environments.

**Keywords** Emerging pollutants · Chloroquine · Chemical mixtures · Synergistic effect · Aquatic invertebrates · Aquatic toxicology

**Introduction**

Emerging pollutants (EPs) are new products or chemicals, though not necessarily new, without regulatory status and whose effects on the environment and human health are unknown (Deblonde et al. 2011). A similar definition is given by the EPA (2017), which defines the EPs as chemical compounds without regulations where their behaviour and environmental and public health impacts are poorly understood. Some examples of these human-made chemicals include personal care products, pesticides, nanoparticles, microplastics, and pharmaceuticals demanded by modern society (Gavrilescu et al. 2015; Egbuta et al. 2021). These chemicals in the environment are more concerning, considering that they do not appear individually but as a complex mixture, which could lead to unwanted synergistic effects (Petrie et al. 2015). There are growing concerns regarding the global production of synthetic chemicals. Four hundred million tons per year have been produced in recent years, and at least 50% are environmentally harmful chemicals (Gavrilescu et al. 2015). Additionally, human needs could increase demand in the contemporary world (Bunke et al. 2019).

Pharmaceuticals, including antibiotics, antidiabetics, antiepileptics, antimalarial, analgesics and anti-inflammatory, are frequently reported in freshwater and marine environments (Gavrilescu et al. 2015; Gu and Wang 2015). Many of these drugs are not biodegraded during wastewater treatment and are discharged in an active form to aquatic environments.
environments (Fang et al. 2012; Liu et al. 2020). Consequently, they are regularly detected in levels ranging from ng/L to mg/L. The presence of pharmaceutical drugs in aquatic ecosystems has a significant ecological effect on the aquatic biota. For instance, in primary consumers such as rotifers and cladocerans, these compounds negatively affect their biological parameters (life span, reproductive rates, generation time, and population increase) (Varano et al. 2017; González-Pérez et al. 2018). Pharmaceuticals also can alter the behaviour traits of secondary consumers (fishes) and affect their ecological interaction (Brodin et al. 2014). It is documented that some pharmaceutical drugs accumulate and biomagnify in the tissues of animals through the food chain (Zenker et al. 2014). Finally, they cause a decrease in the abundance and diversity of aquatic invertebrate communities (Ali et al. 2021).

Chloroquine is an antimalarial treatment utilized for more than seven decades, given that it is a safe drug that is easy to obtain at a relatively low cost (Plantone and Koudriavtseva 2018). It is a drug that has been successfully repurposed to treat various diseases in humans including HIV, Q fever, influenza H5N1, malaria, hepatitis C, dengue virus, zika virus, and chikungunya virus. Furthermore, it has been tested in cancer patients with promising results. From this perspective, CQ is considered a drug with current and future potential applications (Yan et al. 2013; Plantone and Koudriavtseva 2018). For diseases such as malaria and chikungunya virus, CQ is administered in 10–25 mg/kg and 250 mg daily, respectively (Taylor and White 2004; Delegue and de Lamballerie 2011). Approximately 50% of those amounts are excreted unchanged in the urine and feces; hence, substantial amounts of these wastes enter the aquatic environment due to inadequate treatment of residual waters (Kuroda et al. 2021). Chloroquine has a long half-life (1–2 months) in the human body (Olatunde et al. 2014), and in saltwater environments, the degradation can be prolonged (>19 days; Hu et al. 2022). Because of its potential to be a persistent pollutant in water, some mechanisms for CQ degradation have been proposed, but they are presently not implemented worldwide (Midassi et al. 2020).

Copper (Cu) is a ubiquitous contaminant in aquatic ecosystems but not necessarily in a bioavailable form. The US Environmental Protection Agency has listed it as one of the priority contaminants. Both naturally occurring and anthropogenic processes (e.g., mining, industry, and sewage disposal) can release Cu into aquatic systems (Páez-Osuna and Osuna-Martínez 2015). Cu is an essential trace element for many physiological functions (e.g., mitochondrial respiration, normal cell growth and development, and antioxidant defense). However, it can be harmful to human health and aquatic life at high concentrations (Ansari et al. 2003). There is growing concern about the toxicity of copper to aquatic invertebrates, including marine zooplankton, since this metal’s presence in the medium, even at low concentrations (10–50 μg/L), adversely affects the population dynamics, which translates into potential risks to the trophic structure of aquatic ecosystems (Schuler et al. 2008; Kwok et al. 2008; Bao et al. 2013; Rebolledo et al. 2021). In some cases, this risk intensifies when Cu is combined with other toxic substances such as biocides and pharmaceuticals (Bao et al. 2013; Jia et al. 2020).

Rotifers occupy a key ecological position in aquatic food webs by transferring energy content from phytoplankton to higher trophic levels. Naturally and in aquaculture, rotifers are essential in the larval nutrition of many fish and crustacean species. In particular, rotifer species such as Brachionus plicatilis and Proales similis are reliable model organisms for marine ecotoxicology. The introduction of P. similis in ecotoxicological studies is recent and promising (Rebolledo et al. 2018; Snell et al. 2019). Its high sensitivity to pollutants, easy maintenance in the laboratory for experimental work, biological characteristics (e.g., body size, reproduction, and life cycle), and wide distribution (inland saline waters/marine waters) make it relevant for ecotoxicological studies. The most used endpoints to estimate ecological risk for rotifer populations are acute (LC50) and chronic (instantaneous growth rate (r)) toxicity tests (Rico-Martínez et al. 2013; González-Pérez et al. 2018). The r is a good measure of response to toxicants since it integrates potentially complex interactions among life-history traits, including reproductive and mortality rates (Forbes and Calow 1999), which are unattainable through acute tests.

Many aquatic organisms live in environments polluted by a cocktail of toxic substances, including heavy metals and pharmaceuticals. Despite this general assumption, the mixture toxicity of heavy metals and drugs is examined separately in most cases (Watanabe et al. 2016; Lynch et al. 2016). Few studies have examined the toxicity of mixtures like the ones mentioned above (Almeida et al. 2018; Jia et al. 2020). Taking into account (1) the current relevance of CQ as an emerging pollutant, (2) widespread Cu contamination in marine and coastal environments, and (3) that in a real scenario, environmental pollutants typically occur as mixtures rather than as individual pollutants (Cedergreen 2014), this work aimed to assess the toxicity of CQ alone and in combination with Cu to the euryhaline rotifer P. similis. The hypothesis involved is that the toxicity of the mixture of CQ and Cu is higher than that of individual compounds.

Materials and methods

Test species

Proales similis de Beauchamp 1907 (Rotifera: Monogononta) was isolated from a shrimp farm of Litopenaeus
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Since then, we have kept this rotifer species under laboratory conditions. Monoclonal culture of the test rotifer was established at salinity 15‰ and 25 ± 1 °C. Rotifers were fed with single-cell marine algae Nannochloropsis oculata at ~3 × 10^6 cells mL⁻¹. Several egg-bearing individuals were separated in a 20 mL medium to obtain neonates (<8 h-old) for each experiment. Proales similis is a euryhaline rotifer suitable for ecotoxicological tests in different salinity scenarios ranging from 5 to 35‰ (Rebolledo et al. 2021). For all bioassays, a salinity of 15‰ was used to represent inland sections of estuarine and lagoon ecosystems, where significant levels of pharmaceuticals and heavy metals could be regularly discharged in municipal effluents. Saline water was made with artificial sea salts (Instant Ocean™, Aquariam Systems). NaCl is the major component of synthetic sea salts.

### Chemicals

Copper and CQ stock solutions were prepared by dissolving an appropriate quantity of CuSO₄·5H₂O (J.T. Baker, >99%) and N4-(7-Chloro-4-quinolinyl)-N1,N1-dimethyl-1,4-panlenadimine diphosphate salt (CAS 50-63-5 from Sigma-Aldrich, >98.5%), respectively, in milli-Q water to obtain a stock solution of 1 mg/mL (pH = 6.0).

### Acute toxicity test (CQ)

Initially, we performed range-finding tests of CQ concentrations for acute toxicity testing in P. similis. Subsequently, six nominal concentrations of CQ (0.5, 2.5, 5.0, 7.5, 12.5 and 20.0 µg/L) plus one negative control (0) were used to evaluate the 24-h LC₅₀. Toxicity tests were conducted in borosilicate glass vials containing 3 mL of medium (28 vials = 6 concentrations + 1 control × 4 replicates). Ten neonates were introduced into each vial. We provided a low food density (2.5 × 10^4 cells mL⁻¹ of N. oculata) during the tests in order to avoid mortality above 10%, as noted when unfed. All tests were incubated in the dark at 25 ± 1 °C for 24 h. After exposure, the LC₅₀ values of CQ and their 95% confidence limits were calculated using the Probit method (Finney 1971).

### Reproductive assay

According to Hernández-Flores and Rico-Martínez (2006), we measured the single and combined toxicity of CQ and Cu on the rotifer P. similis through chronic toxicity reproductive 5-day tests (static non-renewal testing solution). Single chronic toxicity tests were performed using an application factor (AF) of 0.05, 0.1, 0.3, and 0.5 by multiplying the 24-h LC₅₀ values of CQ (4250 µg/L) and Cu (68 µg/L (Rebolledo et al. 2021)). The combined toxicity of Cu and CQ was evaluated through five mixtures (MX-1 – MX-5) using 0.05, 0.1, 0.3, and 0.5 as AF (see Tables 1 and 2).

Nine neonates were introduced into a 3 mL medium with respective single and combined concentrations of CQ and Cu. Each treatment had one negative control group and five replicates. Compared with higher salinities (<20 g/L), a low salinity increases availability of Cu-free ions in the medium. Cu toxicity in rotifers tends to increase at lower salinity. In our study, we chose 15 g/L of salinity for two reasons: (1) it is an optimal condition for the growth of P. similis, and (2) as salinity representative of inland sections of estuarine and lagoon ecosystems. Rotifers were fed N. oculata at a density of 1.25 × 10^6 cells mL⁻¹ for five days. The highest concentrations of CQ (2.125 mg/L) and Cu (68 µg/L) used in the chronic toxicity tests are below the tolerance threshold of microalgae. According to Debelius et al. (2009), Cu concentrations below 90 µg/L do not inhibit the growth of marine microalgae compared to the control. Concerning CQ, Zurita et al. (2005) have reported that significant inhibition for microalgae is observed at 43 mg/L. The adsorption of CQ and Cu by microalgae was not measured, which in some cases reduces the concentration of toxicants in the test medium. Particularly, N. oculata has a low Cu bioabsorption (<14%) after 21-d (Martínez-Macias et al. 2019). Accordingly, a minimal reduction of chemicals in testing is expected. The pH in the tests ranged only from 7.4 to 7.6, dissolved oxygen levels were 5.8–6.2 mg/L, and salinity (15‰) was consistent. According to Rendall et al. (2011), high pH levels (>8) affect the toxicity of CQ. We did not adjust the pH level of the test medium since it is within the pH range registered for some coastal lagoons (Páez-Osuna et al. 2016). All bioassays were incubated in the dark at 25 °C for five days. After exposure, the final population densities of the rotifers were counted under a stereomicroscope. Population growth rate (r, d⁻¹) was calculated using the formula \( r = \frac{(\ln N_f - \ln N_0)}{T} \), where \( N_f \) is density at time \( T \), \( N_0 \) is density at time 0, and \( T = 5 \) days.

The toxicity of the mixtures was determined using the toxic unit (TU) approach (Broderius et al. 2005). Additive effects were obtained from the sum of dividing the growth rate (r, d⁻¹) value from the CQ-Cu mixture by the...
corresponding \( r, \text{d}^{-1} \) value from the sum of the toxicity of the individual chemicals. If the result was <0.8, the action represents potential synergism (more than additive); if it was 0.8-1.2, an additive action is indicated (concentration addition); and if it was >1.2, the action indicates potential antagonism (less than additive).

**Statistical analysis**

One-way analysis of variance (ANOVA) followed by a Tukey’s post-hoc test was used to compare the statistical differences between group means, where \( P < 0.05 \) was considered significant. All data are expressed as mean ± standard errors (SE) based on five replicate recordings.

**Results**

The 24-h LC\(_{50}\) value of CQ for *P. similis* was 4.25 mg/L, with a 95% confidence interval of 3.0 to 5.9 mg/L. The rate of population increase (\( r, \text{d}^{-1} \)) data of *P. similis* exposed to single CQ and Cu toxicity and their mixture (CQ-Cu) were summarized in Table 2. In general, growth rates varied from 0.50 ± 0.01 to 0.52 ± 0.01 in the control groups. At chronic CQ exposure, ranged from 0.20 ± 0.01 to 0.40 ± 0.01. An increase in CQ concentration from 212 (0.05 AF) to 2125 µg/L (0.5 AF) reduced the growth rates up to 60% with respect to the controls. Regarding chronic Cu toxicity, growth rates decreased from 0.43 ± 0.01 to 0.09 ± 0.03 as metal concentration increased in the medium. At 20 (0.3 AF) and 34 (0.5 AF) µg Cu/L, toxicity substantially reduced \( (P < 0.05) \) growth by 58 (0.21 ± 0.01) and 82% (0.09 ± 0.02), respectively, as compared to the controls (0.52 ± 0.01). The results of a one-way analysis showed that controls were statistically different \( (P < 0.05) \) compared to all treatments tested (Table 2).

Under the MX-1 mixture, growth rates decreased from 0.29 ± 0.01 to -0.15 ± 0.03 as AF increased in the medium (Table 2). The CQ and Cu mixture effects versus single CQ and Cu exposure showed a synergistic response (Table 3). For example, at a single exposure of CQ at low AF (0.05), the \( r \) value was 0.40 ± 0.01. Conversely, under the CQ-Cu mixture at 0.05 AF was 0.29 ± 0.01, which is a 27.5% significant reduction \( (P < 0.05) \). A significant decrease was also observed when comparing Cu’s single toxicity versus the CQ-Cu mixture at 0.05 AF (33% reduction). At single exposure of CQ and Cu at 0.3 AF, growth rates were 0.19 ± 0.01 and 0.21 ± 0.01, respectively. In contrast, under the CQ-Cu mixture at 0.3 AF, it was 0.09 ± 0.03 \( \text{d}^{-1} \), reducing significantly over 50%. The most evident synergistic effect was observed when the rotifers were exposed to a CQ-Cu mixture at 0.5 AF (Table 3). One-way ANOVA shows significant differences \( (P < 0.05) \) when comparing the individual toxicity of each chemical against the mixture at the same AF.

Regarding the MX-2 mixture, low AF (0.05) of CQ (213 µg/L) combined with Cu decreased growth rates from 0.30 ± 0.1 to -0.03 ± 0.01 as the AF for Cu increased in the medium (Table 2). Under these mixtures, growth rates decreased significantly \( (P < 0.05) \) concerning the individual

| Mixtures | Chemicals (µg/L) | Rate of population increase (\( r, \text{d}^{-1} \)) |
|----------|-----------------|---------------------------------------------|
|          | CQ   | Cu   | CQ       | Cu   | CQ-Cu                       |
|          | 213  | 3.5  | 0.50 ± 0.01\(^a\) | 0.40 ± 0.01\(^c\) | 0.43 ± 0.01\(^b\) | 0.29 ± 0.01\(^d\) |
|          | 425  | 6.8  | 0.50 ± 0.01\(^a\) | 0.33 ± 0.01\(^b\) | 0.35 ± 0.01\(^b\) | 0.22 ± 0.01\(^c\) |
|          | 1275 | 20   | 0.50 ± 0.01\(^a\) | 0.20 ± 0.01\(^b\) | 0.21 ± 0.01\(^b\) | 0.10 ± 0.02\(^c\) |
|          | 2125 | 34   | 0.50 ± 0.01\(^a\) | 0.21 ± 0.01\(^b\) | 0.09 ± 0.03\(^c\) | -0.15 ± 0.03\(^d\) |
| MX-2     | 213  | 6.8  | 0.52 ± 0.01\(^a\) | 0.40 ± 0.01\(^b\) | 0.35 ± 0.01\(^c\) | 0.30 ± 0.01\(^d\) |
|          | 213  | 20   | 0.52 ± 0.01\(^a\) | 0.40 ± 0.01\(^b\) | 0.21 ± 0.01\(^c\) | 0.22 ± 0.02\(^c\) |
|          | 213  | 34   | 0.52 ± 0.01\(^a\) | 0.40 ± 0.01\(^b\) | 0.09 ± 0.03\(^c\) | -0.03 ± 0.01\(^d\) |
| MX-3     | 425  | 3.5  | 0.52 ± 0.01\(^a\) | 0.33 ± 0.01\(^b\) | 0.43 ± 0.01\(^b\) | 0.27 ± 0.01\(^d\) |
|          | 425  | 20   | 0.52 ± 0.01\(^a\) | 0.33 ± 0.01\(^b\) | 0.21 ± 0.01\(^c\) | 0.13 ± 0.01\(^d\) |
|          | 425  | 34   | 0.52 ± 0.01\(^a\) | 0.33 ± 0.01\(^b\) | 0.09 ± 0.03\(^c\) | -0.06 ± 0.02\(^d\) |
| MX-4     | 1275 | 3.5  | 0.53 ± 0.01\(^a\) | 0.20 ± 0.01\(^b\) | 0.43 ± 0.01\(^b\) | 0.25 ± 0.02\(^c\) |
|          | 1275 | 6.8  | 0.53 ± 0.01\(^a\) | 0.20 ± 0.01\(^b\) | 0.35 ± 0.01\(^b\) | 0.17 ± 0.01\(^c\) |
|          | 1275 | 34   | 0.53 ± 0.01\(^a\) | 0.20 ± 0.01\(^b\) | 0.09 ± 0.03\(^c\) | -0.04 ± 0.02\(^c\) |
| MX-5     | 2125 | 3.5  | 0.52 ± 0.01\(^a\) | 0.21 ± 0.01\(^b\) | 0.43 ± 0.01\(^b\) | 0.22 ± 0.02\(^c\) |
|          | 2125 | 6.8  | 0.52 ± 0.01\(^a\) | 0.21 ± 0.01\(^b\) | 0.35 ± 0.01\(^b\) | 0.17 ± 0.01\(^c\) |
|          | 2125 | 34   | 0.52 ± 0.01\(^a\) | 0.21 ± 0.01\(^b\) | 0.21 ± 0.01\(^b\) | 0.04 ± 0.02\(^c\) |

Values in a row not sharing the same superscripts are significantly different at \( P < 0.05 \).
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Table 3 Effects of CQ-Cu mixtures on the growth rates of Proales similis at different application factors (AF) for each mixture

| Cu  | CQ  | AF  | 0.05 | 0.1 | 0.3 | 0.5 |
|-----|-----|-----|------|-----|-----|-----|
| CQ  | AF  | 0.05 | 0.74 | 0.75 | 0.20 | -0.71 |
|     | 0.1 | 0.84 | 0.67 | 0.39 | -0.18 |
|     | 0.3 | 1.29 | 0.87 | 0.49 | -0.23 |
|     | 0.5 | 1.05 | 0.82 | 0.20 | -0.71 |

| CQ  | Cu  | AF  | 0.05 | 0.1 | 0.3 | 0.5 |
|-----|-----|-----|------|-----|-----|-----|
| CQ  | AF  | 0.05 | 0.68 | 0.64 | 0.59 | 0.50 |
|     | 0.1 | 0.86 | 0.63 | 0.49 | 0.49 |
|     | 0.3 | 1.05 | 0.60 | 0.45 | 0.20 |
|     | 0.5 | -0.33 | -0.64 | -0.48 | -1.58 |

TU < 0.8 indicates synergy, 0.8-1.2 additive, >1.2 antagonism

toxicity of CQ at 0.05 AF and Cu at 0.1 and 0.5 AF. We did not observe significant differences (P > 0.05) between single exposure to Cu at 0.3 AF and the mixture based on 0.05 AF of CQ + 0.3 AF of Cu (5% reduction). Synergistic effects were detected when combined with 213 µg CQ/L (0.05 AF) + 0.1, 0.3, and 0.5 AF of Cu. For Cu, two synergistic and one additive effect were detected (Table 3).

The MX-3 mixture evidenced that all combinations of CQ at 0.1 AF (425 µg L) + Cu at 0.05, 0.3, and 0.5 AF produced a significant (P < 0.05) decrease in the growth rates compared to single CQ and Cu exposure (Table 2). A mixture of CQ at 0.1 AF + Cu at 0.3 AF resulted in synergistic effects; 62 and 38% significant reductions in growth rates were observed in the individual toxicity of CQ at 0.1 AF and Cu at 0.3 AF, respectively. Using a mixture of CQ at 0.1 AF + Cu at 0.5 AF, a negative r-value was observed (−0.06 ± 0.02); this was not observed in a single CQ and Cu toxicity at the same AF. A mixture based on CQ at 0.1 AF + Cu at 0.05 AF results in an additive effect for CQ and Cu at the same AF (Table 3). Synergistic effects were detected as Cu concentrations increased in the MX-3.

The MX-4 mixture indicated that growth rates were 20% significant higher (P < 0.05) when P. similis was exposed to CQ at 0.3 AF (1275 µg/L) + Cu at 0.05 AF, compared to single CQ exposure at 0.3 AF (Table 2), a case of antagonism, but a one-way ANOVA showed no significant differences (P > 0.05) among the individual CQ exposure at 0.3 AF and the mixtures of 0.3 + 0.05 AF and 0.3 + 1.1 AF (CQ-Cu). Under a mixture of CQ at 0.3 AF + Cu at 0.5 AF, a negative r-value was produced (−0.42 ± 0.02); whilst a single exposure, the r-value was 0.20 ± 0.01 at 0.3 AF of CQ and 0.09 ± 0.03 at 0.5 AF of Cu. Combinations of CQ at 0.3 AF + Cu at 0.05 AF and CQ at 0.3 + Cu at 0.1 AF caused a significant (P < 0.05) decrease of 40 to 50% in growth rates compared to single Cu exposure at the same AF. In the case of Cu, this mixture resulted in an additive effect (Table 3). Synergistic effects were observed as the concentrations of CQ and Cu increased in the medium.

Regarding the chronic effect of the MX-5 mixture, combinations of CQ at 0.5 AF (2125 µg/L) + Cu at 0.1 and 0.3 AF decreased significantly (P < 0.05) growth rates when compared to single chemical exposure (Table 2). A mixture of CQ at 0.5 AF + Cu at 0.05 (3.5 µg/L) did not differ (P > 0.05) from the individual CQ toxicity at 0.5 AF. However, it caused a 50% significant reduction in growth rates compared to single CQ exposure at the same AF. A mixture of CQ at 0.5 AF + Cu at 0.3 AF (20 µg/L) resulted in a low r-value (0.04 ± 0.02). A more noticeable synergistic response was observed under a mixture of CQ at 0.5 AF + Cu at 0.3 AF; growth rates decreased significantly more than 75% than single Cu and CQ exposure at the same AF. Two additive effects were found for CQ and one synergistic response at 0.5 AF of CQ + 0.3 AF of Cu. For Cu, all combinations induced synergistic effects (Table 3).

Discussion

In the present study, we examined the toxicity of CQ due to its current and future potential applications in treating several human diseases. Its important use and high potential to be persistent make it of particular concern for ecotoxicological studies. Typically, CQ has been used to treat malaria caused by protozoan parasites of the genus Plasmodium (Njio et al. 2022). Also, CQ is a drug widely used as a bath pharmaceutical for external protozoa infection control in aquariums (Hu et al. 2022). CQ with Cu is commonly used at public display aquariums in response to white dot diseases caused by Cryptocaryon irritans (Lee-thocharavit 2011), a ciliated protozoan ectoparasite. To remove this parasite, the concentration of CQ and Cu in the treatment baths are maintained above a therapeutic level (CQ 10 mg/L; 63 µg/L Cu) (Leethocharavit 2011; Hu et al. 2022), and after they are discharged, reaching the receiving waters, where finally are diluted, and the CQ degraded. Presumably, as has occurred with other pharmaceuticals drugs, CQ will gain more ecotoxicological relevance after current global health issues (Plantone and Koudriavtseva 2018; Essid et al. 2020; Ali et al. 2021; Kuroda et al. 2021).

Hitherto, limited information is available regarding the acute and chronic toxicity of CQ in aquatic biota. The euryhaline rotifer P. similis has a 24-h LC50 of 4.25 mg/L as a first approximation of CQ toxicity. This toxicity was observed at 7.4 pH. CQ is a weak base. Presumably, an increase in pH will intensify the toxicity of CQ (Rendal et al. 2011). Future research should examine this aspect in the context of environmental changes. Our result is very
close to that for the freshwater rotifer *Brachionus calyciflorus* (4.39 mg/L) and the anostracan *Thamnocephalus platyurus* (4.70 mg/L), organisms widely used in ecotoxicological studies (Callega et al. 1994; Nalecz-Jawecki and Persoon 2006). *Proodas similis* seems to be more sensitive to CQ than the cladoceran *Daphnia magna* (24-h EC50, 21.5 to 43 mg/L) and the crustacean *Streptocephalus proboscidus* (24-h EC50, 11.6 mg/L) (Callega et al. 1994; Zurita et al. 2005). It is documented that some vertebrates, such as *Cyprinus carpio*, have a 96-h LC50 of 31.6 CQ mg/mL (Ramesh et al. 2018) that far exceeds the tolerance of the previously mentioned invertebrates. Considering that the sensitivity of *P. similis* to CQ is within the range reported in standard test species for ecotoxicological assays, we suggest that this marine rotifer be used as an indicator for ecological risk assessment of emerging pollutants that currently deserve more attention.

The chronic toxicity of CQ has rarely been examined in aquatic organisms. CQ and its derivatives are harmful to aquatic life due to the selective accumulation of the drug in the lysosomes that may interfere with lysosomal activity and significant oxidative stress and neurotoxicity changes in animals. Furthermore, it can inhibit enzymes, causing a considerable disturbance in the structure of cell organelles or the death of cell organelles (Zurita et al. 2005; Rendal et al. 2011; Ramesh et al. 2018; Mendonça-Gomes et al. 2021). Low (<10 µg/L) concentrations of CQ have been reported in the field (Olatunde et al. 2014). It is expected that this concentration in aquatic ecosystems will increase moderately due to the increasing demands of the world population (Plantone and Koudriavtseva 2018). Recently, a predictive study by Kuroda et al. (2021) indicates that the ecotoxicological risk of CQ in receiving river waters can be medium based on low projection concentrations (<0.86 µg/L). In our study, the chronic test concentrations were based on the LC50 value of CQ for *P. similis*. However, they can be predictive for this rotifer species and other zooplankton species with a similar response to CQ. Obtained data indicate that from 213 to 2125 µg CQ/L (5 to 50% of LC50), the growth rates of *P. similis* are significantly reduced (22 to 60%). A similar amount (200 µg/L) of other pharmaceuticals, such as amoxicillin, cause significant effects on the rate of population increase (60% of reduction) on *Brachionus havanaensis* (González-Pérez et al. 2016). Unlike *P. similis*, the growth of some aquatic organisms is inhibited at pharmaceutical concentrations ranging from 5.0 to 300 mg/L (Watanabe et al. 2016; Godoy et al. 2019) or even as much as 2000 mg/L (Magdaleno et al. 2015).

Recently, Ali et al. (2021) found that chronic exposure to high hydroxychloroquine (a less toxic derivative of CQ) concentrations (31.62 and 63.24 µg/mL) impact the abundance and diversity of marine nematodes negatively. Therefore, the present data highlights the toxic potential of CQ at moderate concentrations for marine rotifer populations.

Copper toxicity is known in various aquatic organisms, including rotifers (Arnold et al. 2011). The mechanism of toxicity for Cu in marine invertebrates includes the production of reactive oxygen species and osmoregulatory stress due to the inhibition of Na+, K+-ATPase activity (Frias-Espericueta et al. 2022). In particular, Cu is more toxic to *P. similis* at low salinity, where cupric ion concentration increases (Rebolledo et al. 2021). This rotifer species grows optimally at salinity 15‰, where Cu toxicity is moderate. Our results agree with those of Rebolledo et al. (2021). *P. similis* is more sensitive to Cu than other saline or marine rotifers. For example, at 34 µg Cu/L (50% of the LC50) and salinity 15‰, growth rates of *P. similis* are reduced by as much as 83%. At the same time, populations of *B. rotundiformis* can remain unaffected at a chronic exposure of 31 to 125 µg CuSO4/L at salinity 2.5‰ (Gama-Flores et al. 2005). Conversely, it is suggested that *B. plicatilis* is even more resistant since it can grow under a chronic concentration of 500 µg CuSO4/L at salinity 36‰ that exceeds the LC50 of *P. similis* (Luna-Andrade et al. 2002; Rebolledo et al. 2021). Snell et al. (2019) found that some complex strains of *B. plicatilis* might be more sensitive to Cu than *P. similis*, suggesting that their sensitivity is not a generality compared to other marine rotifers. In this sense, the sensitivity of rotifers to toxic substances varies and may depend on the taxa, strain, biological aspects of the animals, and environmental conditions. Cu concentrations used in our chronic tests (3.5–34 µg/L) are comparable to or below some levels detected in rivers (3.6–1300 µg/L; Farag et al. 2003; Balistrieri et al. 2007) and contaminated coastal environments (1.6–49 µg/L; Vazquez et al. 1999; Jonathan et al. 2011). It is assumed that the demographic parameters of *P. similis*, as well as other zooplankton taxa with high sensitivity to Cu, are negatively affected by chronic metal exposure that exceeds the tolerance threshold of this species (Bachmann 1994; Schuler et al. 2008; Kwok et al. 2008; Arnold et al. 2011).

Usually, the toxicities of pollutants mixtures are reported more as additive than synergistic effects (Cedergreen 2014), which has led regulatory authorities to consider that the toxicity of a combination of chemicals will be approximately additive (Walker et al. 2012). To our knowledge, this is the first study to demonstrate the mixture toxicity of CQ and Cu in marine invertebrates. These findings underline the risk of the different mixtures of these two chemicals; in most cases, they induced a synergistic effect on the population growth rate of *P. similis* like that caused by heavy metal mixtures (Rebolledo et al. 2021). Contrary to Almeida et al. (2018), who found that a mixture of drugs (carbamazepine and cetirizine) with a heavy metal (Cd) had lower biological effects than the contaminants alone in the clam *Ruditapes*
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*Olatunde et al.* (2014). Thus, their importance as synergists of CQ, these are well below environmental concentrations in marine environments (*Jonathan et al.* 2011), but in the case of environmental risk assessment of chemical mixtures, toxicity to aquatic life should be considered more frequently in the environmental risk assessment of chemical mixtures.

 Tested concentrations of Cu are within those detected in marine environments (*Jonathan et al.* 2011), but in the case of CQ, these are well below environmental concentrations (*Olatunde et al.* 2014). Thus, their importance as synergists within naturally occurring realistic scenarios is a relatively minor concern (*Cedergreen* 2014). Nevertheless, this aspect should not be understated, given the current environmental threats. Moreover, in these evaluations, it must be considered that both chemicals are associated with cellular and oxidative stress in aquatic invertebrates (*Zurita et al.* 2005; *Frías-Espiricueta et al.* 2022), suggesting that the combined exposure of CQ and Cu would increase the vulnerability of this marine rotifer to chemically mediated oxidative stress. However, this explanation deserves further investigation.

**Conclusions**

According to the literature, the sensitivity of *P. similis* to CQ found in the present study is within the range reported in standard test species for ecotoxicological testing. We suggest that this rotifer species serve as a test organism to investigate the risk assessment of emerging pollutants in marine environments. In general, low tested concentrations of each chemical were enough to exert significant effects on the population growth of rotifers; these were intensified when the compounds acted in combination. CQ-Cu mixtures exhibited a strong synergistic effect on *Proales* mainly as the concentrations of each chemical increased in the medium. Cu concentrations tested are within the environmental levels documented. However, CQ levels are well above those reported in the field; in this context, its importance as a synergist substance in realistic scenarios is of minor concern. We must show the effects of global pollution from a more realistic perspective, where pollution by a single chemical is rare or does not exist.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare no competing interests.

**Consent to participate** All authors consent to participate.

**Consent for publication** All authors consent for publication.

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