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Multi-and transgenerational synergistic effects of glyphosate and chlorpyrifos at environmentally relevant concentrations in the estuarine rotifer *Proales similis*☆

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ABSTRACT

We evaluated the multi-and transgenerational effects of single and combined environmentally relevant concentrations of glyphosate (GLY) and chlorpyrifos (CPF) in the estuarine rotifer *Proales similis*. The acute and chronic toxicities of GLY and CPF were determined as individual compounds and as a mixture. Rotifers were exposed to environmental concentrations of GLY (1, 10, 100, and 1000 μg/L) and CPF (0.1, 1, 5, and 10 μg/L). The main findings were as follows: (i) the LC_{50} values were 33.91 mg/L (GLY) and 280 μg/L (CPF); (ii) the toxic unit (TU_{50}) of the mixture was 0.30, corresponding to 10.17 mg/L GLY and 83 μg/L CPF; (iii) the multigenerational study indicated that the tested concentrations of GLY and CPF, both single and combined, significantly and consistently decreased the growth rates of *P. similis* from the F0 to F6 generations; (iv) in most cases, GLY and CPF mixtures induced a strong synergistic effect; and (v) transgenerational effects were detected in the F4 generation, especially GLY and CPF in higher equitoxic proportions. These effects seem to dissipate in F5. Across multigeneration, a slight recovery could indicate population resilience to pollution. Our findings suggest that a mixture of GLY and CPF at environmental concentrations is likely to occur under real field conditions, increasing the risk to marine and estuarine invertebrates such as rotifers.

1. Introduction

Pesticides are necessary to meet the food demands of the human population worldwide (Hough, 2021). They are natural or synthetic agents used and designed to kill pests that attack plant crops and organisms from aquatic farming (Matozzo et al., 2020). Glyphosate (GLY) is a nonselective contact organophosphate herbicide known globally and used in at least 130 countries (Baylis, 2000); it is extensively applied in home gardens and urban areas, as well as in the agricultural industry in major dimensions (Benbrook, 2016). In aquaculture, GLY is spread to control aquatic weeds and for algae removal in fish, crabs, and crayfish ponds (Yan et al., 2022). Many agricultural and aquaculture activities occur close to freshwater and marine environments, such as coastal lagoons and estuaries. Consequently, pesticides are present in these environments (Zhang et al., 2022). In freshwater habitats, GLY concentrations oscillated from 0.010 to 700 μg/L (half-life: 7–142 days) (Ruiz-Toledo et al., 2014; De María et al., 2021) and from 0.02 to 1377 μg/L in marine systems (half-life: 47 days at 25 °C in low light and up to 315 days at 31 °C in darkness) (Mercurio et al., 2014; Skeff et al., 2015; Wang et al., 2016).

Mexico dedicates ~32 million ha to agriculture, mainly in the northwest, and GLY is the most widely and controversial herbicide used (Alcántara-de la Cruz et al., 2021; Ávila-Díaz et al., 2021). More than 400 GLY brands are registered in commercial formulations in Mexico, whose doses for agriculture range from 700 to 2200 g/ha (Alcántara-de la Cruz et al., 2021). However, the use of GLY in Mexico is restricted as of 2024. Once the GLY ban goes into force, large industries could switch to even more harmful chemicals, resulting in a threatening environmental risk scenario.

Chlorpyrifos (CPF) is another broad-spectrum insecticide used in

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agricultural and aquaculture systems (Sun and Chen, 2008; Hites, 2021). It is estimated that approximately 200,000 tons of CPF were utilized worldwide in 2015, and these quantities are expected to increase in the following years (John and Shaike, 2015). Chlorpyrifos has contaminated rivers, lakes, and seawaters. The current levels of CPF ranged from 0.2 to 303 μg/L in freshwater (half-life: >65 days) (Racke et al., 1988; Lalah et al., 2003; Campillo et al., 2013) and 0.8–199 μg/L in seawater (half-life: <8 days and degradation can be long >28 days) (Lalah et al., 2003; Campillo et al., 2013). Detected levels of CPF in the environment have exceeded the criteria established in several parts of the world (Sumon et al., 2018; Ávila-Díaz et al., 2021). Therefore, there is growing concern regarding the effects on human health and aquatic ecosystems. Chlorpyrifos is considered highly toxic to a great variety of aquatic biota ranging from primary consumers to fishes (Huang et al., 2020). Given its toxic background, the use of CPF has been restricted in developed countries, including the USA, UK, and European countries (Noore et al., 2021). Unlike GLY, CPF is still currently used in Mexico.

Glyphosate and CPF have distinct degrees of toxicity. Nevertheless, the environmental concern is the same given their potential persistence in nature. Like many other chemicals, GLY and CPF coexist in contaminated environments (Tomé et al., 2020). Although GLY is considered a moderately toxic contaminant, the presence of this herbicide in the field is more concerning, assuming that it does not appear individually but as a cocktail of contaminants that could lead to unintended synergistic effects (Hua and Relyea, 2014; Bonifacio and Hued, 2019).

Ecotoxicological studies commonly report acute toxicity (EC_{50} or LC_{50}) as the first approximation in environmental risk assessment. Many acute test values often exceed environmental realistic concentrations of chemicals to which organisms are exposed. However, acute toxicity values can predict possible pollution scenarios (Naito et al., 2003; Shao et al., 2019). The effects of the environmental concentrations of pollutants are not visible to the naked eye, but they have prolonged exposures (chronic toxicity), as observed in multiple and transgenerational studies (Jeong et al., 2015). The first term refers to the population where each generation has been continuously exposed to contaminants, and maternal transfer of pollutants can occur. The second is for the population where only the parental generation was exposed to contaminants, and the new generations were never directly exposed (Robaire et al., 2022). Additionally, ecological and epigenetic studies are valuable tools to measure multi-and transgenerational effects (Heine-Fuster et al., 2017; Terrazas-Salgado et al., 2022).

Rotifers play a crucial role in the energy flow to higher trophic levels and are important elements in the diets of predatory fish and crustaceans. *Proales similis* is a euryhaline rotifer available for ecotoxicological studies in various parts of the world. This species inhabits estuaries and coastal marine environments (Rebolledo et al., 2021). It also assembles the requirements for testing in environmental risk assessment studies (Breitholtz et al., 2006). Currently, interest in *P. similis* has increased in traditional ecotoxicology and environmental genomics (Snell et al., 2019; Kim et al., 2021; Rebolledo et al., 2021).

There is scarce information regarding the individual and combined effects of GLY and CPF on marine organisms (Huang et al., 2020; Matozzo et al., 2020). In the present study, the estuarine rotifer *P. similis* was selected as a model species to examine the individual and combined effects of GLY and CPF at environmentally relevant concentrations through multi-and transgenerational experiments. We hypothesized that (i) the combination of environmental concentrations of GLY and CPF will affect the rate of population increase of *P. similis* to a greater extent than individual chemical exposure and (ii) continuous or parental exposure to GLY and CPF (single and combined) can induce adverse effects on the growth rates of this species.

2. Materials and methods

2.1. Rotifer maintenance

The estuarine rotifer *P. similis* was initially collected from a shrimp farm in northwestern Mexico. We maintained this species under laboratory conditions for more than four years before conducting the experiments. Rotifers were cultured in 250-mL flasks with artificial seawater (ASW) at 15 ppt and at 25 °C under a light and dark cycle of 18 h:8 h. The green marine microalgae *Nannochloropsis oculata* was used as the exclusive food ($\sim 3 \times 10^6$ cells mL^{-1}). This reconstituted saltwater works well to assess the survivorship and demography of *P. similis* at different experimental salinities (Rebolledo et al., 2020). In this manner, we ensured the health conditions of the test rotifers. To maintain the cultures, we filtered the entire contents of the flask using a 50 μm mesh, and then we transferred approximately 20% of the filtered rotifers to a flask with fresh medium. This procedure was performed twice a week.

2.2. Chemicals

Glyphosate (99% purity, CAS# 1071-83-6) was purchased from Sigma−Aldrich (St. Louis, MO, USA) and chlorpyrifos (99.5% purity, CAS# 2921-88-2) from Chem Service (West Chester, PA, USA.) were used for the analysis. The standard solution of GLY was prepared by dissolving 10 mg of salt in 10 mL of Milli-Q water (1 mg/mL). The stock solution of CPF was prepared by dissolving 10 mg of powder in 10 mL of HPLC grade acetonitrile, obtaining a final concentration of 1 mg/mL. GLY levels were measured using the enzyme-linked immunosorbent assay (ELISA) according to Mahler et al. (2017); this method is feasible since it has a negligible bias relative to liquid chromatography tandem mass spectrometry analysis. Concentrations of CPF were measured using PerkinElmer Clarus 680 Gas Chromatography-Clarus SQ8T Mass Spectrometry (Ohio, USA) according to Schäfer et al. (2018). GLY and CPF concentrations were measured at the beginning of each experiment (0 h, N = 3). It has been reported that there is no significant variation between the concentration of organophosphate pesticides in the test media at 0 and 48 h (Mottier et al., 2013).

2.3. Acute toxicity tests

To obtain neonates of known age, we separated several adult females 10 h the day before the start of the experiment and placed them in a 20 mL medium for incubation. Then, ten neonates (<12 h) were introduced into 1 mL of test medium containing the desired chemical concentrations. After a range-finding test on *P. similis*, seven concentrations of GLY (0, 1, 10, 20, 40, 50, and 60 mg/L) and CPF (0, 50, 100, 250, 500, 750, and 1000 μg/L) were selected for the acute toxicity test (24 h LC_{50}). Each concentration contained six replicates. The salinity of the tests was maintained at 15 ppt at 25 ± 1 °C. Briefly, toxicity tests were conducted in sterilized 24-well polystyrene plates (Corning Costar). Ten neonates were introduced into 1 mL of test medium containing the desired chemical concentrations. Plates were incubated at 25 °C in the dark to prevent photolysis of the chemicals for 24 h. After the incubation, the number of live and dead rotifers was quantified under a stereoscopic microscope. The LC_{50} values and their 95% confidence limits were calculated using the probit method (Finney, 1971).

The mixture toxicity experiments adhered to the same experimental design as individual chemical tests, except that in this case, mixed concentrations of GLY and CPF were used. These mixtures were established by multiplying the 24 h LC_{50} of each chemical by seven toxicity units (TU = 0.05, 0.10, 0.25, 0.50, 0.75, 1.00, and 1.25). Table 1 shows the nominal and actual concentrations of GLY and CPF according to the TU. The joint toxicities were assessed using the TU approach (Arora and Kumar, 2015). TU is the sum of the toxic contributions of each component in the mixture. The following equation provides the TU for a binary mixture:

Table 1
Nominal and actual equitoxic concentrations for each toxic unit (TU) based on the LC_{50} values of GLY and CPF.

TU	Nominal concentration (mg/L)		Actual concentration (mg/L)	
	GLY	CPF	GLY	CPF
0.05	2.00	0.014	1.92	0.015
0.10	3.00	0.028	2.89	0.029
0.25	8.00	0.070	7.70	0.073
0.50	17.00	0.140	16.38	0.146
0.75	25.00	0.210	24.08	0.218
1.00	33.00	0.280	31.79	0.291
1.25	42.00	0.350	40.46	0.364

$$TU = \frac{LC50x\ G\ (mix)}{LC50\ G\ (alone)} + \frac{LC50x\ C\ (mix)}{LC50x\ C\ (alone)}$$

where G (glyphosate) and C (chlorpyrifos) are the chemicals, LC_{50}x (mix) denotes the effect of each component in the binary mixture, and LC_{50}x (alone) and the LC_{50} of G and C are applied as single components. If TU = 1, an additive action is indicated; if TU > 1, the action is less than additive (antagonistic); and if TU < 1, the toxicity of the mixture is more than additive (synergistic), as proposed by Spehar and Fiandt (1986).

2.4. Multi-and transgenerational experiments

Concerning multigenerational testing, *P. similis* was exposed to four single and combined (1:1 toxicity ratio) environmentally relevant concentrations of GLY (1, 10, 100, and 1000 μg/L) and CPF (0.1, 1, 5, and 10 μg/L) for seven generations (F0 – F6). The same concentrations were tested for transgenerational experiments, in which the exposure lasted two generations (F0–F1); afterward, rotifers were maintained in toxic-free treatments for the next four generations (F2 – F5).

Briefly, experiments were performed in sterilized 24-well polystyrene plates (Corning Costar®). Two neonates from the initial culture were used as the parental generation (F0) and were introduced into 1 mL of test medium (15 ppt) containing the desired chemical concentrations (single or combined), as well as 1×10^6 cells/mL of *N. oculata* as food. Each generation included one control with no chemical and six replicates per treatment. The plates were incubated at 25 °C in the dark. After 48 h, the offspring of the F0 generation were transferred to the next experiment (F1) and treated identically to their parents. This method was repeated until the F6 generation was reached. The experimental procedure for transgenerational effects was similar to that used for the multigenerational bioassays, except that rotifers were exposed to pesticides during F0 and F1, while the next generations (F2 – F5) were not exposed to toxicants.

Multi-and transgenerational effects of GLY and CPF were evaluated using a chronic toxicity reproductive 2-day test based on the population growth rate of the animals exposed to environmental stress (Snell and Hicks, 2011). The population growth rate was calculated using the following formula: $r = (\ln(Nt) - \ln(N0))/T$, where $\ln(Nt)$ is the natural log of the total number of rotifers in a well after 48 h, $\ln(N0)$ is the natural log of the initial number of rotifers (2 individuals), and T is time (2 days). Interactions of GLY and CPF at environmental concentrations were assessed based on the predicted and observed effects of the mixture (Gottardi et al., 2017; da Silva Pinto et al., 2021). The following equation was used to estimate the predicted effect: Predicted effect = $Gx/C \times Cx/C$, where Gx, Cx, and C are the mean responses (population growth rate) registered for individual GLY and CPF and the control, respectively, for a specific combination. The observed effect in a mixture was calculated as the ratio of the effect observed in the mixture (M) and the untreated control (observed effect = M/C). When the predicted effect is higher than the observed effect, it is considered a synergistic effect, while an antagonistic effect occurs when the observed effect is higher than the predicted effect. According to Gottardi et al. (2017), synergy was assessed by calculating a synergy ratio using the following equation: synergy ratio = predicted effect/observed effect.

2.5. Statistical analysis

All data analysis was conducted in STATISTICA 10.0 and SigmaPlot 11.0. Statistics were carried out by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Statistical significance was set at $p < 0.05$. The results are presented as the mean ± standard error (SE).

3. Results

Proales similis was more sensitive to CPF (280 μg/L) than to GLY (33.91 mg/L) based on the 24 h LC_{50} data (Table 2). The NOEC of GLY and CPF was approximately 28% of the LC_{50} of each pesticide. The LOEC values of both chemicals were approximately 35–37% regarding the LC_{50}. The TU_{50} value of the mixture was 0.30, which corresponds to 10.17 mg/L GLY and 83 μg/L CPF (Table 2). These values are close to the NOEC data recorded separately but below the LOEC.

The population growth rate (PGR) of *P. similis* exposed to environmentally relevant concentrations of GLY and CPF and their mixture for seven generations is summarized in Table 3. In the control groups, PGRs (r, d^{-1}) oscillated from 1.47 ± 0.05 to 1.52 ± 0.03, and no significant differences (one-way ANOVA; $p > 0.05$) were found between the F0 and F6 generations. Under continuous exposure to 1 μg/L GLY, PGRs ranged from 0.80 ± 0.05 to 1.11 ± 0.05 and were reduced significantly ($p < 0.05$) from 24 to 46% compared to the controls. The most affected generation was F0 and was only significantly different (one-way ANOVA; $p < 0.05$) from F3 – F6. Growth rates increased slightly but were not significantly different (one-way ANOVA; $p < 0.05$) after F2. At 10 μg/L GLY, PGRs oscillated from 0.62 ± 0.09 to 1.08 ± 0.04 and decreased significantly ($p > 0.05$) from 29 to 58% compared to the controls. PGR was inferior in F0 and significantly different from the other generations (one-way ANOVA; $p < 0.05$). From F1 – F6, the reduction ($p < 0.05$) percentage was greater than 29% compared with the controls. At 100 μg/L GLY, PGRs were significantly ($p < 0.05$) affected in the F0 – F2 generations, as much as 42–58% of the controls. These generations were not statistically ($p > 0.05$) different from each other. From F3, the PGR increased significantly (one-way ANOVA; $p < 0.05$) and remained stable until F6. At 1000 μg/L GLY, the growth rates ranged from 0.55 ± 0.09 to 1.11 ± 0.05. The most susceptible generation was F0, which had a PGR significant decrease ($p > 0.05$) of 63% compared to the control. As in all cases, PGR increased slightly with the subsequent generations. No significant differences (one-way ANOVA; $p > 0.05$) were found between the F0 and F2 – F6 generations.

For seven successive generations, the growth rates of *P. similis* exposed to 0.1 μg/L CPF ranged from 0.85 ± 0.06 to 1.19 ± 0.05

Table 2
Toxicity thresholds of glyphosate and chlorpyrifos to the marine rotifer *P. similis*.

Chemical	NOEC	LOEC	LC_{50}	TU_{50}
Glyphosate (mg/L)				
Value	9.63	12.04	33.91	
CV%			8.5	
CL			28.02–41.02	
Chlorpyrifos (μg/L)				
Value	78	104	280	
CV%			7.6	
CL			197–405	
GLY-CPF Mixture				0.30
CL				0.21–0.44

NOEC = no observed effect concentration; LOEC = lowest observed effect concentration; LC_{50} and TU_{50} = lethal concentration and toxic units, respectively, in which 50% of individuals that are exposed die; CL, 95% confidence limits for LC_{50} values; and CV = coefficients of variation.

Table 3
Multigenerational effects of the individual and combined exposure of GLY and CPF at environmental concentrations on the estuarine rotifer *P. similis*. Data are presented as mean ± SE.

Gen	Chemicals (μg/L)		Rate of population increase (r, d^{-1})				Effect: modeled data		
	GLY	CPF	Control	GLY	CPF	Mixture	Pr.	Ob.	Effect
F0	1	0.1	1.50 ± 0.03^{a}	0.94 ± 0.06^{b}	0.85 ± 0.06^{b}	0.55 ± 0.09^{c}	1.08	0.41	S
F1			1.47 ± 0.06^{a}	0.80 ± 0.05^{b}	0.94 ± 0.06^{b}	0.80 ± 0.05^{b}	0.64	0.54	S
F2			1.47 ± 0.03^{a}	0.99 ± 0.05^{b}	0.94 ± 0.06^{bd}	0.90 ± 0.02^{d}	0.69	0.59	S
F3			1.52 ± 0.03^{a}	1.08 ± 0.04^{b}	0.99 ± 0.05^{bc}	0.85 ± 0.06^{c}	0.83	0.56	S
F4			1.52 ± 0.03^{a}	1.15 ± 0.05^{b}	1.19 ± 0.05^{b}	0.94 ± 0.06^{c}	0.95	0.62	S
F5			1.47 ± 0.05^{a}	1.11 ± 0.05^{b}	1.11 ± 0.05^{b}	0.85 ± 0.06^{c}	1.00	0.58	S
F6			1.52 ± 0.03^{a}	1.08 ± 0.04^{b}	1.08 ± 0.04^{b}	0.90 ± 0.06^{c}	0.85	0.59	S
F0	10	1.0	1.50 ± 0.03^{a}	0.90 ± 0.06^{b}	1.03 ± 0.07^{b}	0.62 ± 0.09^{c}	0.61	0.58	S
F1			1.47 ± 0.06^{a}	0.62 ± 0.09^{b}	0.94 ± 0.05^{b}	0.90 ± 0.08^{c}	0.44	0.61	A
F2			1.47 ± 0.03^{a}	1.03 ± 0.07^{b}	0.99 ± 0.06^{b}	0.69 ± 0.07^{c}	1.02	0.47	S
F3			1.52 ± 0.03^{a}	1.08 ± 0.04^{b}	1.11 ± 0.05^{b}	0.80 ± 0.05^{c}	0.99	0.53	S
F4			1.52 ± 0.03^{a}	0.90 ± 0.06^{b}	1.03 ± 0.07^{b}	0.90 ± 0.06^{b}	0.68	0.59	S
F5			1.47 ± 0.05^{a}	1.03 ± 0.07^{b}	0.94 ± 0.06^{b}	0.90 ± 0.06^{b}	0.74	0.61	S
F6			1.52 ± 0.03^{a}	1.08 ± 0.04^{b}	0.85 ± 0.06^{c}	0.85 ± 0.06^{c}	0.71	0.56	S
F0	100	5.0	1.50 ± 0.03^{a}	0.85 ± 0.05^{b}	0.80 ± 0.05^{b}	0.68 ± 0.07^{b}	0.66	0.46	S
F1			1.47 ± 0.06^{a}	0.62 ± 0.06^{c}	0.90 ± 0.07^{b}	0.62 ± 0.09^{c}	0.61	0.42	S
F2			1.47 ± 0.03^{a}	0.85 ± 0.06^{b}	0.94 ± 0.06^{b}	0.73 ± 0.09^{b}	0.72	0.48	S
F3			1.52 ± 0.03^{a}	1.19 ± 0.05^{b}	1.08 ± 0.04^{b}	1.19 ± 0.05^{b}	0.71	0.78	A
F4			1.52 ± 0.03^{a}	1.11 ± 0.05^{b}	0.94 ± 0.06^{bc}	0.84 ± 0.06^{c}	0.85	0.58	S
F5			1.47 ± 0.05^{a}	1.11 ± 0.05^{b}	0.85 ± 0.07^{c}	1.19 ± 0.05^{b}	0.54	0.81	A
F6			1.52 ± 0.03^{a}	1.11 ± 0.05^{b}	0.99 ± 0.05^{b}	0.41 ± 0.07^{c}	1.75	0.27	S
F0	1000	10	1.50 ± 0.03^{a}	0.90 ± 0.06^{b}	0.62 ± 0.09^{c}	0.48 ± 0.08^{c}	0.77	0.32	S
F1			1.47 ± 0.06^{a}	0.55 ± 0.09^{b}	0.90 ± 0.06^{c}	0.48 ± 0.07^{b}	0.69	0.33	S
F2			1.47 ± 0.03^{a}	0.99 ± 0.05^{b}	1.19 ± 0.05^{b}	0.62 ± 0.09^{c}	1.26	0.41	S
F3			1.52 ± 0.03^{a}	0.80 ± 0.05^{c}	1.11 ± 0.05^{b}	0.68 ± 0.07^{c}	0.86	0.45	S
F4			1.52 ± 0.03^{a}	0.99 ± 0.05^{b}	1.23 ± 0.04^{c}	0.85 ± 0.06^{b}	0.94	0.56	S
F5			1.47 ± 0.05^{a}	0.90 ± 0.06^{bc}	1.08 ± 0.04^{b}	0.73 ± 0.09^{c}	0.90	0.50	S
F6			1.52 ± 0.03^{a}	1.11 ± 0.05^{b}	0.80 ± 0.05^{c}	0.41 ± 0.07^{d}	1.41	0.27	S

Data are presented as mean ± SE. Values that do not share the same superscripts in the same row are significantly different at $p < 0.05$.
The predicted (Pr.) and observed (Ob.) effects are presented for the mixtures that are statistically different ($p < 0.05$) from the control or compounds alone. S = synergistic effect and A = antagonistic effect.

(Table 3), which started to increase slightly from F1. Regarding the control, as much as a 35–43% reduction ($p < 0.05$) in PGR was observed from the F0 – F3 generations. PGRs reached in the F4 to F6 generations were statistically higher (one-way ANOVA; $p < 0.05$) than those recorded in F0 – F3. The PGR reduction (22–29%) in F4 – F6 was less than that in the first generations but significantly lower ($p < 0.05$) than that in the controls. At 1.0 μg/L, growth rates (0.85 ± 0.06 to 1.11 ± 0.05) fluctuated continuously over all generations. The lowest r value was registered in the F6 generation. PGRs were statistically diminished ($p < 0.05$) compared to the control by as much as 27–44%. No significant differences (one-way ANOVA; $p > 0.05$) were found between the different generations tested. At 5.0 μg/L, the PGR was 0.80 ± 0.05 in F0 and 0.85 ± 0.06 in F6; there was a 47 and 35% reduction ($p < 0.05$) vs. the control, respectively, but it was not significantly different (one-way ANOVA; $p > 0.05$) among generations. The growth rates from F1 – F6 were 29–42% significantly lower than those of the control. Across the seven generations, a higher environmental concentration of CPF (10 μg/L) caused a significant reduction ($p < 0.05$) in the growth rates that oscillated from 19 to 59% compared to the control. The PGR was lower in the F0 generation (0.62 ± 0.09). Growth rates slightly increased (0.90 ± 0.06 to 1.23 ± 0.04) from F1 – F5. The F4 generation reached a higher r value but was not significantly different (one-way ANOVA; $p > 0.05$) from F2 – F5. The PGR recorded in F6 (0.80 ± 0.05) was not different (one-way ANOVA; $p > 0.05$) from that recorded in F0.

Continuous exposure to environmentally relevant concentrations of GLY (1 μg/L) and CPF (0.1 μg/L), which correspond to the first mixture, for seven generations caused a significant ($p < 0.05$) reduction in the PGRs of *P. similis*: 38–63% vs. controls, 0–41% vs. single GLY exposure, and 4–35% vs. single CPF exposure (Table 3). Growth rates fluctuated from 0.55 ± 0.06 to 0.94 ± 0.06. Lower r values were observed in the F0 and F1 generations; however, they were not more significant (one-way ANOVA; $p > 0.05$) than those reached in the F4 – F6 generations. The first mixture produced a synergistic effect across all generations. Growth rates ranged from 0.62 ± 0.09 to 0.90 ± 0.08 across the seven generations under a mixture of 10 μg/L GLY and 1.0 μg/L CPF (second mixture). PGRs were significantly reduced ($p < 0.05$) from 31 to 44% compared to the controls. An antagonist effect was detected in the F1 generation; PGR was 0.62 ± 0.09 at individual GLY exposure and 0.90 ± 0.08 in the second mixture. Synergistic effects were found for the remaining generations. The PGRs in the second mixture were not statistically (one-way ANOVA; $p > 0.05$) different among all generations. A combination of 100 μg/L GLY and 5.0 μg/L CPF (third mixture) substantially decreased PGRs (0.41 ± 0.41 to 1.19 ± 0.05) ($p < 0.05$), up to 73% (F7) compared to the control. Only the F3 and F5 generations were significantly different (one-way ANOVA; $p > 0.05$) from the other generations. Two antagonistic effects were detected in F3 and F5; PGRs were 9–29% higher in the third mixture than in individual CPF exposure. Regarding the fourth mixture (1000 μg/L GLY and 10.0 μg/L CPF), PGRs fluctuated from 0.41 ± 0.07 to 0.85 ± 0.06 and were considerably affected (44–73% considerable reduction ($p < 0.05$) vs. control). The highest r value was in the F6 generation, but it was only significantly different (one-way ANOVA; $p > 0.05$) from F5. Synergistic effects were detected in all generations.

The transgenerational effects of environmentally relevant concentrations of GLY and CPF and their mixture are summarized in Table 4. In the control groups (F2 – F5), growth rates ranged from 1.50 ± 0.04 to 1.52 ± 0.03, with no significant differences ($p > 0.05$). In all treatments, the F2 generation was significantly different ($p < 0.05$) from the control. In this generation, significantly reduced ($p < 0.05$) growth rates of 20–43%, 26–46%, and 37–48% were found with parental exposure to the GLY, CPF, and GLY-CPF mixture, respectively. In most cases, no significant differences ($p > 0.05$) were found between the control and parental exposure treatments of 1.0 μg/L GLY and 0.1 μg/L CPF and their mixture. Regarding individual and combined exposure at 10.0 μg/L

Table 4
Transgenerational effects of the individual and combined exposure of GLY and CPF at environmental concentrations on the estuarine rotifer *P. similis*. Data are presented as mean ± SE.

Treatments	Gen	(μg/L)		Rate of population increase (r, d^{-1})			
		GLY	CPF	Control	GLY	CPF	Mixture
Parental exposure	F0	1.0	0.1	1.50±0.03^{a}	0.94±0.06^{b}	0.85±0.06^{b}	0.55±0.09^{c}
	F1			1.47±0.06^{a}	0.80±0.05^{b}	0.94±0.06^{b}	0.80±0.05^{b}
	F2			1.50±0.04^{a}	1.11±0.05^{b}	0.94±0.06^{bc}	0.80±0.05^{c}
	F3			1.52±0.03^{a}	1.60±0.04^{a}	1.49±0.06^{a}	1.44±0.04^{a}
	F4			1.50±0.03^{a}	1.52±0.03^{ab}	1.45±0.00^{ab}	1.38±0.04^{b}
	F5			1.50±0.03^{a}	1.50±0.04^{a}	1.47±0.03^{a}	1.47±0.05^{a}
Parental exposure	F0	10	1.0	1.50±0.03^{a}	0.90±0.06^{b}	1.03±0.07^{b}	0.62±0.09^{c}
	F1			1.47±0.06^{a}	0.62±0.09^{b}	0.94±0.05^{b}	0.90±0.08^{c}
	F2			1.50±0.04^{a}	1.08±.0.04^{b}	1.11±0.05^{b}	0.94±0.06^{b}
	F3			1.52±0.03^{b}	1.54±0.05^{b}	1.69±0.03^{a}	1.67±0.03^{a}
	F4			1.50±0.03^{a}	1.50±0.03^{a}	1.50±0.03^{a}	0.55±0.24^{b}
	F5			1.50±0.03^{a}	1.52±0.03^{a}	1.52±0.03^{a}	1.44±0.06^{a}
Parental exposure	F0	100	5.0	1.50±0.03^{a}	0.85±0.05^{b}	0.80±0.05^{b}	0.68±0.07^{b}
	F1			1.47±0.06^{a}	0.62±0.06^{c}	0.90±0.07^{b}	0.62±0.09^{c}
	F2			1.50±0.04^{a}	1.19±0.05^{b}	0.80±0.05^{c}	0.94±0.06^{c}
	F3			1.52±0.03^{a}	1.67±0.03^{a}	1.54±0.06^{a}	1.67±0.03^{a}
	F4			1.50±0.03^{a}	1.52±0.03^{a}	1.23±0.04^{bc}	0.86±0.13^{c}
	F5			1.50±0.03^{a}	1.52±0.04^{a}	1.52±0.03^{a}	1.38±0.06^{a}
Parental exposure	F0	1000	10	1.50±0.03^{a}	0.90±0.06^{b}	0.62±0.09^{c}	0.48±0.08^{c}
	F1			1.47±0.06^{a}	0.55±0.09^{b}	0.90±0.06^{c}	0.48±0.07^{b}
	F2			1.50±0.04^{a}	0.85±0.06^{b}	0.90±0.06^{b}	0.78±0.10^{b}
	F3			1.52±0.03^{a}	1.44±0.06^{ab}	1.29±0.06^{b}	1.38±0.04^{ab}
	F4			1.50±0.03^{a}	1.47±0.03^{ab}	1.08±0.04^{c}	0.94±0.20^{b}
	F5			1.50±0.03^{a}	1.52±0.03^{a}	1.41±0.05^{a}	1.46±0.07^{a}

The shaded generations were continuously exposed to pesticides and their mixture. Values that do not share the same superscripts in the same row are significantly different at $p < 0.05$.

GLY and 1.0 μg/L CPF, the growth rate of the F4 generation was significantly reduced ($p < 0.05$) by approximately 63% compared to the control, single, and combined exposures of GLY and CPF (parental). This was also observed in the combined exposure of 100 μg/L GLY +5.0 μg/L CPF and 1000 μg/L GLY +10.0 μg/L CPF in the F4 generation but to a lower degree and significantly different from the control and individual exposure. In general, growth rates from the F5 generation were not significantly different ($p > 0.05$) from the control. However, the mean growth rates in the treatments of parental exposure to the mixture of GLY and CPF were slightly lower than the control.

4. Discussion

Many pesticides are present in aquatic ecosystems, including coastal marine environments. GLY is a common herbicide that inhibits the growth of weed plants, while CPF is an insecticide frequently applied to control the pest population. Directly mixed, the two agrochemicals are likely to be rarely used. However, it commonly occurs that they are used individually in such way that they can be found together in the aquatic environment. For example, in Mexico, agricultural pollution is generally associated with the continental margin of the Gulf of California, where approximately 1.7 million ha are irrigated lands. The predominant chemical classes commonly used in the region are dithiocarbamates, organophosphates, carbamates, and pyrethroids (Páez-Osuna et al., 2017). It is estimated that pesticide consumption in the region is ~4500 t annually, which includes ~900 different pesticides available for crops of maize, tomatoes, potato, chili, and beans, as well as for the control of diseases such as dengue and Zika fever (Páez-Osuna et al., 2017). However, no information is available on the specific amounts of each pesticide applied. Conversely, CPF has been detected in waters of the Gulf of California region: rivers up to 4.86 μg/L, agricultural drains up to 1.97 μg/L, coastal lagoons up to 1.71 μg/L, and open sea waters 1.50 μg/L (Arellano-Aguilar et al., 2017). CPF have also been reported at levels up to 361 ng/g in the sediments of various coastal lagoons of the Gulf of California (Páez-Osuna et al., 2017). Presumably, animals are most vulnerable to pesticide contamination in peak agricultural seasons

(spring and summer) that generate large amounts of waste that enter estuaries or coastal lagoons via freshwater runoff of the Gulf of California region. The effects of GLY and CPF are scarcely studied in marine invertebrates that are usually exposed to complex mixtures (Matozzo et al., 2020; Huang et al., 2020). Despite the widespread assumption that chemical mixtures potentiate synergistic effects in realistic scenarios, most studies are based on the toxicity of individual chemical pollutants to predict environmental risk. In the present study, the toxicity of relevant environmental concentrations of GLY and CPF and their potential mixture were investigated given their ecotoxicological relevance and global concern.

As expected, *P. similis* was more sensitive to CPF than to GLY. According to the rating scheme (GESAMP, 2002), acute aquatic toxicity was highly toxic for CPF and slightly toxic for GLY, using *P. similis* as a model organism. Considering the TU_{50} values of the GLY-CPF mixture, GLY toxicity remained slightly toxic but very highly toxic for CPF. The individual toxic unit of each chemical is very close to those found in NOEC values. The TU_{50} indicates that the GLY-CPF mixture induced a strong synergism. However, GLY concentrations are above those documented in aquatic systems (Matozzo et al., 2020; Ruiz-Toledo et al., 2014). Unlike CPF, its importance as a synergistic substance in realistic scenarios is of minor concern. Acute tests indicate that GLY can be lethal at high and unrealistic concentrations for a wide variety of aquatic invertebrates. However, chronic tests suggest that GLY can affect the biological responses of marine organisms even at low concentrations (Huang et al., 2020; Matozzo et al., 2020).

Proales similis is 43 times more sensitive to CPF than the freshwater (F) rotifer *Brachionus calyciflorus* and the marine (M) rotifer *B. plicatilis* (Ferrando and Andreu-Moliner, 1991). Compared to *Lecane quadridentata* (F), it is 4.3 times more sensitive to GLY, and it has a similar tolerance as *B. calyciflorus* (Domínguez-Cortinas et al., 2008; Ferrando and Andreu-Moliner, 1991). In the present study, *P. similis* was more tolerant to GLY than other zooplankton groups, such as the freshwater cladocerans *Daphnia magna*, *D. exilis*, and *Simocephalus mixtus* (Domínguez-Cortinas et al., 2008; Rodríguez-Miguel et al., 2021), as well as the copepods *Phyllodiaptomus annae* (F) and *Pseudodiaptomus annandalei* (M) (Deepananda et al., 2011; Lim et al., 2019). Cladocerans such as *Ceriodaphnia dubia* (F), copepods such as *Acartia tonsa* (F) and *Tigriopus fulvus* (M), amphipodans such as *Corophium insidiosum* (M), and the anostracan *Artemia franciscana* (M) are 1.4–14.5 times more resistant to GLY than *P. similis* (Tsui and Chu, 2003; Parlapiano et al., 2021). Usually, commercial formulations of GLY (e.g., Roundup and Faena) are employed to assess acute toxicity, which results in a higher toxicity than GLY as an individual chemical (Bradberry et al., 2004). Regarding CPF toxicity, *P. similis* is also more sensitive than brachionid rotifers (Ferrando and Andreu-Moliner, 1991; Kim et al., 2016). Notably, *P. similis* is much more tolerant to CFP (LC_{50} = 270 μg/L) than other zooplankton organisms. Such a list includes freshwater daphnids (0.05–0.8 μg/L) (Barron and Woodburn, 1995; Romanelli et al., 2004), marine copepods (1.3–20 μg/L) (Bejarano et al., 2005; Charry et al., 2019; Bellas and Gil, 2020), amphipods (F and M) (0.08–0.30 μg/L) (Leight and Van Dolah, 1999; Anderson et al., 2014), and the estuarine mysid *Neomysis integer* (0.08 μg/L) (Roast et al., 1999).

Compared to other rotifers, the sensitivity of *P. similis* has been attributed to the hypothesis that an illoricate body could increase the permeability of toxic substances, but this is not a generalization (Rebolledo et al., 2018; Snell et al., 2019). At least *P. similis* is more susceptible to heavy metals such as Cd, Pb, As, and Hg than species of the genus *Brachionus* (Rebolledo et al., 2021). Rotifers generally show greater tolerance to GLY and CPF than other aquatic invertebrates. An explanation is related to the mechanism of action of these pesticides and the ecophysiological response of animals. GLY has been shown to produce neurotoxicity, inhibition of the mitochondrial complex, and oxidative stress in humans, rats, and invertebrates (Costas-Ferreira et al., 2022). When GLY increases oxygen consumption, it increases ATPase activity, causing a decrease in the hepatic level of cytochrome P-450 and resulting in the uncoupling of oxidative phosphorylation.

The mode of action of CPF is similar for both target and nontarget organisms. CPF also causes acetylcholinesterase enzyme inhibition, oxidative stress, and endocrine disruption, similar to other pesticides (Huang et al., 2020). Due to their toxicity and mechanisms of action, GLY and CPF affect the behavior of animals, which comprises breathing rates, swimming, and feeding ecology, as documented in crustaceans (Banaee et al., 2019). Although *P. similis* probably shares a broad range of neurotransmitters with other metazoan species (Kim et al., 2021), it has a simplified nervous system, and it is less complex than the previously compared microcrustaceans.

The rate of population increase (*r*) was used as an endpoint to examine the multi-and transgenerational effects of environmentally relevant concentrations of GLY and CPF on *P. similis*. This ecological parameter (*r*) is a sensitive indicator that shows the response of the entire population (number of offspring, reproduction output, number of mothers, and survival) in a given time to continuous and parental exposure to toxic substances (Forbes and Calow, 1999; Heine-Fuster et al., 2017). The rotifer *P. similis* had an LC_{50} of GLY and CPF that far exceeded the environmental concentrations tested in this work. In general, multigenerational results indicate that environmental concentrations of GLY and CPF significantly inhibited the growth rates in all treatments and across generations. Subsequently, the organisms showed a slight recovery across generations; nevertheless, they were never statistically equal to the control in each generation.

Proales similis showed a similar tolerance response to GLY during the seven generations analyzed. Some animals showed a high tolerance to herbicides for more than 36 generations (Wang et al., 2020). The mechanisms of cytochrome P450 in rotifers facilitate detoxification and elimination of xenobiotics (Kim et al., 2016; Xianliang Yi et al., 2016), in which *P. similis* can probably tolerate GLY for several generations. Over time, the tolerance of organisms to anthropogenic pressures is costly, even at environmental concentrations (Heine-Fuster et al., 2017). In Mexico's water bodies near crop fields, up to 37 μg/L GLY has been detected (Ruiz-Toledo et al., 2014), an environmental concentration that significantly inhibits the growth of *P. similis* for several generations. It is likely that environmental changes such as increased temperature and water quality could exacerbate the susceptibility of *P. similis* to GLY at environmental concentrations, as exhibited in other marine invertebrates (Parlapiano et al., 2021). Thus, the multigenerational effects of GLY can change drastically as environmental stress potentiates the toxicity of pollutants (Monserrat et al., 2007).

The tested environmental concentrations of CPF for *P. similis* are considered highly toxic to aquatic organisms (Huang et al., 2020). The multigenerational effects of CPF in *P. similis* were most evident in F0, and a slight recovery was observed across generations, mainly after F2. In most cases, the growth rate constantly fluctuated but did not vary among the different generations exposed to the toxicant. However, it was never higher or equal than that achieved in the controls, as Maggio and Jenkins (2022) reported in *D. magna* at $<$ 0.1 μg/L CPF. It is important to indicate that some responses to pesticides could be related to the green microalgae added as food in the chronic tests, which tend to decrease the toxicity of the chemical (Zalizniak and Nugegoda, 2006); this was not evaluated in the present work. It has been observed that parental exposure to 0.5 μg/L CPF to cladocerans that are more sensitive than *P. similis* causes more susceptibility to the toxicant at this concentration in the second generation (Zalizniak and Nugegoda, 2006). Although *P. similis* and *D. magna* inhabit waters with different conditions (salinity, pH), their contrasting and discussion are important considering that both species are model organisms to examine the toxicity of numerous pollutants. In addition, *D. magna* can tolerate 4 g/L and can be used to monitor estuarine wetland sites (Schuytema et al., 1997). Therefore, we decided to compare these values to monitor estuarine wetland sites. In this context, multigenerational effects of CPF in *D. magna* indicate an apparent tolerance over multiple generations; that is, CPF improves reproductive success and survival (Maggio and Jenkins,

2022).

Similar to the present study, multigenerational CPF exposure has been reported not to increase the vulnerability of organisms to the two pesticides, assuming that the multigenerational effects of CPF are not significant at the population level (Zalizniak and Nugegoda, 2006). However, the potential risk that populations inhabit a contaminated environment for a prolonged time should not be discounted. According to Kim et al. (2016), the marine rotifer *B. koreanus* shows a higher tolerance to chronic CPF exposure than *P. similis*, since it can grow and reproduce even at 500 μg/L CPF. In *B. koreanus*, a concentration of 10 μg/L CPF induces depletion of antioxidant enzyme activities that enhance oxidative stress, suggesting that environmental concentrations of CPF can be stressful to rotifers and affect their biological fitness. The environmental concentrations of CPF used in this study are within those detected in northern Mexico for freshwater environments (3.43–5.49 μg/L) (Ávila-Díaz et al., 2021). According to our research, these concentrations significantly inhibit the growth rates of *P. similis*, even though it is more tolerant than several freshwater invertebrates. There is a lack of information regarding the environmental levels of insecticides in Mexico's marine environments to better understand the current conditions that aquatic organisms face in polluted systems.

We found a huge difference between the acute and chronic responses of *P. similis* exposed to GLY. This response can be analyzed through the Acute-to-Chronic Ratio (ACR). ACR is used to estimate chronic data (when this is scarce) from acute data (Ahlers et al., 2006). ACR is typically calculated by the formula ACR = 1/AF, where AF is the assessment factor, which is calculated by the formula (Raimondo et al., 2007): AF = MATC/LC50. By using the acute data in Table 2, we calculated GLY ACR = LC_{50}/MATC = 33.90/10.76 = 3.15 and CPF ACR = LC_{50}/MATC = 280/90.06 = 3.11. These ACRs are not very protective to aquatic life since an ACR of 1000 may not be sufficiently protective when aquatic organisms are exposed to active pharmaceutical principles (Vestel et al., 2016). However, when we compared LOECacute/LOECchronic (from Tables 2 and 3), we found GLY LOECacute/LOECchronic = 12.04/0.001 mg/L = 12,040 and CPF LOECacute/LOECchronic = 104/0.1 μg/L = 1040. This is an important finding since GLY has been considered moderately toxic in terms of acute toxicity in aquatic invertebrates (Gill et al., 2018). However, in terms of chronic transgenerational toxicity, *P. similis* is quite sensitive to effects at 1 μg/L. The difference in comparative toxicity ranking was clear when we used the LOECchronic as an endpoint rather than the LC_{50} for both pesticides.

The examination of the mixture of pollutants in aquatic environments has been earning more interest in recent years, although pesticides have been one of the least analyzed. In terms of ecological risk, it is necessary to design realistic ecotoxicological assessments, including the interaction of pesticides, environmental concentrations, and the exposure time of the organisms (de Souza et al., 2020). In this study, synergistic effects at low concentrations of GLY and CPF were observed in most cases. The F0 and F1 generations were the most vulnerable to the mixture, while populations recovered slightly across generations; this may be a favorable response to the detoxification mechanisms of *P. similis* (Huang et al., 2020; Kim et al., 2021). A rapid recovery response to pesticides may have resulted in the detected antagonistic effects. At higher concentrations of GLY and CPF, the growth rate for the F6 generation relapsed as in F0 and F1. The combined effects of GLY and CPF have scarcely been explored. In general, the combined individual toxicities of GLY and CPF are increased when they are present as a mixture. Individual CPF toxicity appears to exert more pressure on the combination. Significant synergism was found in populations of freshwater crayfish exposed to these environmental concentrations of pesticides (Banaee et al., 2019). At environmentally realistic concentrations (same as the present study) in the zebrafish model, Terrazas-Salgado et al. (2022) found that GLY was able to induce sublet changes in transcriptomic signaling, some of which related to important biological end reproductive parameters such as oocyte maturation, metabolic processes, histone deacetylation, and nervous system development. In this sense, the main question in our study was to evaluate whether the neurotoxic mode of action of CPF could exacerbate the toxicity of GLY in the mixture.

The interactions between GLY and CPF have been examined limitedly. Osten et al. (2005) studied the acute toxicity in the fish *Gambusia yucatana* exposed to a mixture of CPF and GLY and found synergistic effects. Banaee et al. (2019) investigated the combined effects of CPF and GLY on biochemical, immunological parameters, and oxidative stress biomarkers in the freshwater crayfish *Pontastacus leptodactylus*. They found that the co-exposure of crayfish to two pesticides increased the glutamic-oxaoacetic-transaminase (SGOT) activity and the total antioxidant (TAO) levels. CPF combined with GLY decreased γ-glutamyltransferase (GGT) activity. Finally, these last authors concluded that the mixture of GLY and CPF exhibited synergistic effects on the different toxicological biomarkers in crayfish. These results in fish are in concordance with those observed in our study, in which most cases in *P. similis*, GLY and CPF mixtures induced a strong synergistic effect. The present results support that exposure to GLY and CPF as a mixture affects the population dynamics and physiology of aquatic organisms (Bonifacio and Hued, 2019; Pinto et al., 2022). CPF is a typical organophosphate pesticide with anticholinergic effects that block many processes that produce a decrease in detoxification mechanisms. GLY is an herbicide that disrupts the shikimic acid pathway in plants through inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (Gill et al., 2018). However, there have been several reports of GLY causing death and growth inhibition in aquatic invertebrates (Gill et al., 2018). Therefore, we propose that the anticholinergic activity of CPF causes a synergistic effect with the adverse effects of GLY simply by reducing the detoxification mechanisms. In an ecotoxicological context, the sum of individual effects can exacerbate compensatory cell responses and increase the oxidative vulnerability of animals, which is of growing concern.

The transgenerational effects of pesticides in rotifers have a high cost at the population level (Heine-Fuster et al., 2017). All combined environmental concentrations of GLY and CPF induced transgenerational effects in F4, mainly above 10 μg/L GLY and 1.0 μg/L CPF, resulting in 38–63% growth rate reductions. After the third generation, a rapid population recovery was observed in parentally singly exposed to GLY and CPF, which continued up to the fifth generation. *Proales similis* has a high recovery potential from toxic stress due to its short generation time (Rebolledo et al., 2018). In F5, all populations were statistically equal to the growth rates of the control. These results suggest that the single exposure of GLY and CPF at environmental concentrations after F4 does not result in a transgenerational fitness decline. However, a clear trend was noted; in most cases, growth rates in the parental exposure treatments to CPF and the GLY-CPF mixture were lower than the controls. These findings open the field for exploring the epigenetic effects in *P. similis* exposed to pesticides, which deserves more attention. In this research, the relevance of studying the effects of environmental concentrations on realistic concentrations of pollutants and their co-interaction in the environment is emphasized. Although organisms may generate some adaptability to contaminants after successive generations, a persistent threat remains in the environment. Concentrations of GLY and CPF detected in some regions of Mexico can exert adverse effects on aquatic populations, and these effects can be potentiated when pesticides interact. To our knowledge, this is the first study to demonstrate that a mixture of GLY and CPF can have multigenerational and transgenerational effects in euryhaline invertebrates.

Specific information on the interaction of *P. similis* with GLY and CPF in the environment is not available. However, this contact is probably elevated, considering that this cosmopolitan rotifer exhibits a wide distribution in inland saline and marine waters (Rebolledo et al., 2021). *Proales similis* is a euryhaline rotifer that inhabits freshwaters to hypersaline environments in Mexico, and it has been registered in inland saline waters in the Desert and in coastal lagoons (Walsh et al., 2008;

Rebolledo et al., 2018). Additionally, it has been found in the Namib Desert Namibia (Brain and Koste, 1993), in marine waters from Bermuda (Sorenson, 2001), and in an estuary in Okinawa, Japan (Wullur et al., 2009).

Rotifers are among the most important aquatic organisms due to their ubiquitous nature in most aquatic ecosystems. *Proales similis* is a promising live food for rearing fish larvae, which by their small size (40–110 μm) results in better ingestion and digestion by fish larvae (Hagiwara et al., 2014). Numerous studies confirm that *P. similis* is a suitable initial food for a number of marine fish larvae (e.g., Wullur et al., 2011; Hagiwara et al., 2014). Considering the high evolutionary divergence between rotifers and their widespread distribution in aquatic environments and their high importance as unlimited sources of bioactive molecules (e.g., lectins), the effect on the health of rotifers has important ecological implications. For example, rotifers such as *Brachionus calcyflorus* and *P. similis* are important secretory lectins, which are proteins with remarkable carbohydrate-recognition properties involved in immunity, reproduction, self/nonself recognition and several other biological processes (Gerdol, 2022). Therefore, the impact on key species, such as rotifers, can change the food web structure and biodiversity.

5. Conclusions

This is the first study that explores the multi-and transgenerational effects of GLY and CPF, as individual compounds and as a mixture, at environmental concentrations in a marine rotifer model. Compared to other freshwater or marine rotifer species, *P. similis* is highly sensitive to GLY and CPF. Continuous exposure to GLY and CPF adversely affected the growth rates of *P. similis* through seven generations. The combined individual toxicities of GLY and CPF at environmental concentrations induce strong synergistic effects, which increase their concern in aquatic systems. Across multigeneration, a slight recovery could indicate population resilience to pollution. Strong signaling of transgenerational effects produced by the mixture of GLY and CPF and individual exposure to CPF was detected. These findings open the field for exploring epigenetic marks in *P. similis* exposed to pesticides. The importance of examining the individual and combined effects of environmental concentrations of pesticides is emphasized, thereby increasing the ecological realism usually faced by many aquatic populations.

Credit author statement

UAR: Investigation, Formal analysis, Visualization, Writing – original draft; RRM: Investigation, Validation, Writing – review & editing; FPO and MBL: Conceptualization, Methodology, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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