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Toxicity of binary-metal mixtures (As, Cd, Cu, Fe, Hg, Pb and Zn) in the euryhaline rotifer *Proales similis*: Antagonistic and synergistic effects

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ABSTRACT

Data regarding the effects of binary metal mixtures in marine zooplankton are scarce, particularly for rotifers. We examined the toxicity of 21 binary equitoxic mixtures of As, Cd, Cu, Fe, Hg, Pb, and Zn on the euryhaline rotifer *Proales similis*. The toxic units (TU_{50}) revealed that 20 of these binary mixtures exhibited synergistic effects (TU_{50} < 1.00). The As—Hg mixture showed a strong antagonistic effect (TU_{50} = 2.39), whereas the Hg—Cu interaction exhibited a significant synergistic effect (TU_{50} = 0.29) on *P. similis*. TU_{50} values were <0.60 in all cases that showed synergism (80 %). Regarding the MIXTOX analysis, 13 binary mixtures presented some level of synergism, while two mixtures presented only additivity. Results emphasize the need for environmental agencies to revise and readjust protection guidelines for marine biota in response to the evident synergistic effects occurring at metal mixtures concentrations below the current permissible limits.

1. Introduction

Naturally, potentially toxic elements (PTEs) are widely distributed in the environment. In addition, the anthropogenic load of these elements causes increasingly higher concentrations in aquatic environments (Ali et al., 2019). Metal(loid)s such as As, Cd, Cu, Pb, Hg, and Zn are listed as priority pollutants by the US Environmental Protection Agency (US EPA, 2011). Presumably, coastal areas are very susceptible to pollution because they receive substantial loadings of PTEs and may persist over extended periods dissolved, suspended, and accumulated in the biota and the sediments (Páez-Osuna et al., 2017).

As in other world regions, relatively high PTE concentrations have been reported in some coastal waters, lagoons, and estuaries of Mexico (Table 1). Some concentrations of PTEs detected in the Gulf of California (GC), Mexico, deserve attention; they sometimes exceed the permissible Mexican limits ((NOM-001-SEMARNAT-2021) SEMARNAT, 2022) and the international guidelines (US EPA, 2011) for discharges in marine areas, thereby posing a persistent environmental risk (García-Hernández et al., 2013; Páez-Osuna et al., 2017).

Metals such as Cu, Fe, and Zn are essential for the biological functions of aquatic organisms (e.g., in crustaceans, Cu is part of the respiratory pigment (hemocyanin)); however, when these metals, such as Zn, exceed the required levels, they can alter the osmoregulatory capacity and cause death in invertebrates (Ardiansyah et al., 2012). As, Cd, Hg, and Pb are nonessential metals and could cause disturbances in the homeostasis of marine invertebrates (Viarengo and Nott, 1993; Davis and Gatlin, 1996; Deidda et al., 2021), interfering with the expression of proteins and enzymes, compromising important pathways such as apoptosis and glucose metabolism, and inducing the expression of metallothioneins (Frías-Espericueta et al., 2022).

The high ecotoxicological relevance of PTEs is because individual chemicals cause adverse effects in aquatic organisms (de Oliveira-Filho et al., 2004; Ramírez-Pérez et al., 2004; Li et al., 2015), and these effects can be potentiated when several PTEs continuously interact in the environment (Wu et al., 2016; Jeong et al., 2023). Worldwide, many aquatic organisms are exposed to mixtures of PTEs. In a recent study, Costa et al. (2022) confirmed the presence of As, Cd, Cr, Cu, Fe, Hg, Mn, Pb, and Zn in water, sediments, phytoplankton, zooplankton, shrimp, and fish in the coastal environments of Brazil. Their investigation indicates that taxonomic groups such as zooplankton persistently interact

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Table 1
Environmental concentrations (μg/L) of As, Cd, Cu, Fe, Hg, Pb and Zn in coastal waters of Mexico.

Criterion	Metal	Habitat	Concentration	References
	As	Estuary	1869	García-Hernández et al. (2013)
Mexican limit – marine areas		Wetland	86	García-Hernández et al. (2013)
		Seawater	0.5–22.0	Jonathan et al. (2011)
Daily average	300			
Monthly average	200			
CMC	69			
CCC	36			
	Cd	Coastal lagoon	2.0–93.0	Vazquez et al. (1999)
Mexican limit – marine areas		Seawater	0.06–4.90	Jonathan et al. (2011)
		Coastal lagoon	0.05–0.13	Páez-Osuna et al. (1986)
Daily average	300	Mazatlan harbor	0.04–2.00	Osuna-López et al. (1989)
Monthly average	200		0.25–0.75	Jara-Marini et al. (2008)
CMC	40			
CCC	8.8			
	Cu			
Mexican limit – marine areas		Wetland	1992	García-Hernández et al. (2013)
		Seawater	1.61–49.0	Jonathan et al. (2011)
Daily average	5000	Mazatlan harbor	1.75–3.61	Jara-Marini et al. (2008)
Monthly average	4000		3.0–77.0	Vazquez et al. (1999)
CMC	4.8	Coastal lagoon	0.5–1.0	Páez-Osuna et al. (1986)
CCC	3.1	Mazatlan harbor	0.05–2.50	Osuna-López et al. (1989)
	Hg	Wetland	10.20	García-Hernández et al. (2013)
Mexican limit – marine areas		Seawater	0.045–0.078	Jara-Marini et al. (2012)
		Mazatlan harbor	0.64–1.05	Jara-Marini et al. (2008)
Daily average	15	Estuary	12.0	Pérez-Zapata et al. (1984)
Monthly average	10			
CMC	1.8			
CCC	0.94			
	Pb	Seawater	0.40–45.0	Jonathan et al. (2011)
Mexican limit – marine areas		Coastal lagoon	1.48–3.64	Jara-Marini et al. (2008)
		Mazatlan harbor	0.24–1.24	Osuna-López et al. (1989)
Daily average	750			
Monthly average	500			
CMC	210			
CCC	8.1			
	Zn			
Mexican limit – marine areas		Seawater	5.8–410	Jonathan et al. (2011)
		Coastal lagoon	0.31–1.79	Jara-Marini et al. (2008)
Daily average	15,000		3.0–97.0	Vazquez et al. (1999)
Monthly average	10,000	Mazatlan harbor	3.4–359	Osuna-López et al. (1989)
CMC	90			
CCC	81			
	Fe			
		Seawater	41–13,400	Jonathan et al. (2011)
		Estuary	367–1235	Vazquez et al. (1999)
		Coastal lagoon	181–1201	Vazquez et al. (1999)

Mexican limit – marine areas, is the permissible limit established by the Mexican Official Standards NOM-001-SEMARNAT-2021; CMC, US EPA criterion maximum concentration; CCC, US EPA criterion continuous concentration.

with several PTEs simultaneously. Evaluating ecosystems impacted anthropogenically is a complex issue because there are many possible stressors producing many potential effects. In the case of adverse effects (toxic effects), stressors (mainly chemical substances) are usually tested individually. However, in nature, organisms are often exposed to a cocktail of contaminants at different concentrations simultaneously (Ríos-Arana et al., 2007; Wu et al., 2016). In this context, various investigations have been carried out on the effects of binary mixtures of metals on different aquatic taxonomic groups ranging from phytoplankton and zooplankton (primary consumers) to fishes (tertiary consumers) (Cooper et al., 2009; Brix et al., 2016; Nagai and De Schamphelaere, 2016; Castaldo et al., 2021; Gebara et al., 2020; Sauliutė et al., 2020).

Data on the effects of binary metal mixtures in marine zooplankton are scarce, particularly for rotifers. Buikema Jr et al. (1977) found that in the rotifer *Philodina acuticornis,* binary metal mixtures of Cr and Cu were additive, and Zn and Cu had no interaction. In *Plationus patulus*, exposure to several mixtures of As, Cr, Cu, Ni, Pb, and Zn induced stress protein expression 60 (HSP60) (Rios-Arana et al., 2005). The reproduction of *Brachionus calyciflorus* was significantly affected by mixtures of five metals (Cu, Zn, Cd, Cr, and Mn), and the ratio of mictic to amictic females was the most sensitive endpoint and might be suitable to evaluate the effects of multimetal mixtures on rotifers (Xu et al., 2015). The marine rotifer *B. koreanus* was exposed to field seawater contaminated with mixtures of Cd, Cu, Ni, Pb and Zn, which caused decreased population growth rates and highly induced the transcription of detoxification-related genes (Jeong et al., 2019). Medina-Ramírez et al. (2022) assessed the enhanced toxicity of ZnO nanoparticles (NPs) and dissolved copper (Cu^{2+}) using the rotifer *Lecane papuana*; enhanced acute toxicity was elicited at low concentrations of Cu^{2+} and ZnO NPs; the authors concluded that the enhanced toxicity was mainly caused by the synergistic interaction of ZnO NPs and Cu^{2+}. Brown et al. (2023) investigated the toxicity of five metals (Cd, Cu, Ni, Pb, and Zn) to the rotifer *Adineta editae*, both singly and in metal mixtures; they found that the response to metal mixtures was antagonistic, with less toxicity observed than was predicted by the model developed from the single metal exposure data.

Proales similis is a euryhaline rotifer inhabiting lagoon systems (SE Gulf of California, Mexico) and other coastal marine environments worldwide; its introduction as a model organism for ecotoxicological investigation has been successful (Rebolledo et al., 2018; Snell et al., 2019; Arreguin-Rebolledo et al., 2023). Compared to other rotifer species, for example, some strains of the marine rotifer *B. plicatilis*, which is traditionally used in toxicity tests, *P. similis* is more sensitive to the individual acute toxicity of As, Cd, Cu, Fe, Hg, Pb, and Zn, and it has been found that its LC_{50} values of Cu, Hg, Pb, and Zn are below the permissible limits for Mexican marine zones ((NOM-001-SEMARNAT-2021) SEMARNAT, 2022) but above the US EPA (2011) criterion maximum concentration (CMC) for saltwater (Rebolledo et al., 2021).

This study aimed to examine the acute toxicity of 21 binary mixtures of seven PTEs (As, Cd, Cu, Fe, Hg, Pb, and Zn) commonly found in coastal environments of Mexico and other parts of the world (Páez-Osuna et al., 2017; Costa et al., 2022). We hypothesized that a combination of those PTEs with an LC_{50} value for *P. similis* above the permissible limits ((NOM-001-SEMARNAT-2021) SEMARNAT, 2022) could cause negative effects at lower concentrations of the same guideline. The second hypothesis is that the combination of the PTEs selected in this study could potentiate the toxicity of the elements at environmentally relevant concentrations.

2. Materials and methods

2.1. Rotifer culture

Proales similis de Beauchamp, 1907, used in this study, were cultured in the laboratory of Geoquímica y Contaminación Costera, ICMyL-UNAM, Mexico. This rotifer strain was initially collected from a shrimp farm whose water source is the subtropical coastal lagoon known as Estero de Urias (NW Mexican Pacific, SE Gulf of California) (Rebolledo et al., 2018). A monoclonal culture of *P. similis* was maintained in a 500-mL flask under controlled laboratory conditions at 25 ± 1 °C and 15 ppt artificial seawater. The culture medium was prepared using commercial sea salts (Instant Ocean™, Aquarium Systems) and distilled water. For experiments, synthetic sea salts were dissolved in purified Milli-Q water. Rotifers were fed with the marine microalgae *Nannochloropsis oculata* (approximately 3×10^6 cells/mL), and the medium was replaced twice per week. A salinity of 15 ppt was considered a representative scenario of estuarine and lagoon ecosystems. Furthermore, this salinity provides optimum growth conditions for *P. similis* (Rebolledo et al., 2018).

2.2. Chemical solutions

Stock solutions of As, Cd, Cu, Fe, Hg, Pb, and Zn at a concentration of 1000 μg/L (purity >99 %) supplied by Merck (Germany) were used. We utilized a high purity of the stock solution to minimize errors in the nominal concentrations. All tested nominal concentrations in this work were prepared by diluting the appropriate volume of the stock solutions in water at 15 ppt salinity.

2.3. Acute toxicity of binary metal mixtures

The 24 h LC_{50} data (at 15 ppt salinity) previously published by Rebolledo et al. (2021) were employed for testing 21 binary mixtures of As, Cd, Cu, Hg, Fe, Pb, and Zn. Table 2 displays the LC_{50} values of the selected metalloid and six heavy metals on *P. similis*. Based on these data, seven nominal concentrations of each metal were chosen by multiplying the LC_{50} values of each metal by 0.25, 0.50, 0.75, 1.00, 1.50, 2.00, and 2.50 as toxicity units (TUs) following the methodology outlined by Verslycke et al. (2003) and Frías-Espericueta et al. (2009). Therefore, binary mixture treatments were established by combining the different TUs at a 1:1 toxicity ratio. For instance, in the case of an As—Hg mixture at 0.25 TU, the LC_{50} values of As (822 μg/L) and Hg (6.0 μg/L) (Rebolledo et al., 2021) were multiplied by 0.25 TU, resulting in individual concentrations of 206 μg/L As and 1.5 μg/L Hg. At 2.50 TU, the individual concentrations were 2055 μg/L As and 15 μg/L Hg (Table 2).

Briefly, tests were conducted in sterile 24-well polystyrene plates (Corning Costar) with ten rotifer neonates (<12 h of age) per well (1.0 mL of test solution at 15 ppt salinity). The appropriate concentrations of each binary mixture (21) were added in each well, along with six replicates and a control (test medium without toxicants) per treatment. A low concentration of *N. oculata* (2.5×10^4 cells/mL) was introduced to ensure that mortality in the control groups did not exceed 10 % (Rebolledo et al., 2021). All plates were incubated at 25 °C ± 1 °C in the dark. The evaluated endpoint, mortality, was determined by visual inspection under a stereomicroscope at 24 h.

2.4. Data analysis

The study analyzes trends with a simple regression analysis: $y = a + bx$, where y = mortality caused by acute toxicity of binary mixtures; b = slope and a = y-intercept. First, the toxic unit (TU_{50} and their corresponding confidence intervals) approach was used to determine the type of joint action for each binary mixture based on the LC_{50} estimates from bioassays with mixtures and single metals. A value of 1 TU is assigned to the LC_{50} of each metal. If $TU_{50} = 1$, 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) (Spehar and Fiandt, 1986).

In a second examination, data obtained from the binary mixture toxicity tests were analyzed by using the MIXTOX modelling tool (Jonker et al., 2005), which has proven to be a useful tool to analyze mixture toxicity to a wide variety of organisms (Gebara et al., 2020, 2021; Loureiro et al., 2010; Lima et al., 2023; Mansano et al., 2020). Few mixture studies with marine organisms have used the CA and IA models to interpret toxicity data, although this approach is usually used to assess the toxicity of freshwater organisms (Damasceno et al., 2017). We use the data obtained by Rebolledo et al. (2021) to run the models and the mentioned tool. First, using the MIXTOX tool, we checked whether the data fit the concentration addition (CA) and independent action (IA) models. After that, we analyzed the deviations from the reference models, i.e., synergism/antagonism (S/A) interactions and dose-ratio (DR) and dose-level dependent (DL) deviations, which were modeled by adding the parameters “a” and “b”. More details can be found in Jonker et al. (2005). We analyzed the following binary mixtures using the MIXTOX tool: As—Cd, As—Cu, As—Fe, As—Pb, As—Zn, Cu—Cd, Cu—Fe, Cu—Pb, Cu—Zn, Fe—Cd, Fe—Pb, Fe—Zn, Pb—Cd, Zn—Cd and Zn—Pb. We did not present the analysis of the binary mixtures with Hg, i.e., As—Hg, Cd—Hg, Cu—Hg, Fe—Hg, Pb—Hg and Zn—Hg, since it was not possible to fit these ecotoxicology data to the conceptual models of CA or IA using the MIXTOX tool.

3. Results

3.1. Analysis of joint-action of binary mixtures by TU_{50}

Fig. 1 summarizes the percentage mortality of the rotifer *P. similis* exposed to the different binary mixtures of As, Cd, Cu, Fe, Hg, and Pb

Table 2
Nominal equitoxic concentrations (μg/L) using different toxic units (TU) based on the 24-h LC_{50} values (μg/L) of one metalloid and six heavy metals for *P. similis*.

Metal	24-h LC_{50}[a]	TU						
		0.25	0.5	0.75	1	1.5	2	2.5
As	822	206	411	617	822	1233	1644	2055
Cd	855	214	428	641	855	1238	1710	2138
Cu	68	17	34	51	68	102	136	170
Hg	6.0	1.5	3.0	5.0	6.0	9.0	12	15
Fe	192	48	96	144	192	288	384	480
Pb	665	166	333	499	665	998	1330	1663
Zn	662	166	331	497	662	993	1324	1655

[a] Rebolledo et al. (2021).

and seven toxic units (TUs) at 24 h of exposure. Correspondingly, the results of the regression analysis for each mixture are shown. In general, mortality in the control groups was <10 %. The observed 50 % mortality depended on the different combinations and units of toxicity.

Twenty-one combinations were analyzed. Fifty percent mortality was observed in three combinations at 0.25 TU (Cu—Pb, Fe—Hg, and Fe—Pb), three combinations at 0.5 TU (Cd—Hg, Cd—Pb, and As —Pb), twelve combinations at 0.75 TU (As—Cd, As—Cu, As—Fe, Cd—Cu, Cd—Fe, Cd—Zn, Cu—Zn, Cu-Fe; Fe—Zn, Hg—Pb, and Pb—Zn), one combination at 1.0 TU (Hg—Zn), one combination at 1.5 TU (As—Zn), and one combination at 2.0 TU (As—Hg). In most cases, 75 % of the LC_{50} value of each metal produced 50 % mortality in the mixtures. In one case, this percentage of mortality was observed in an As—Hg combination at 2.0 TU, equivalent to 1644 μg/L As and 12 μg/L Hg.

According to the TU_{50} values of binary metal mixtures, 20 of 21 induced synergism, and one showed an antagonistic effect (Table 3). The lowest TU_{50} value was observed in the Cu—Hg mixture (0.29), corresponding to 20 μg/L of Cu and 2.0 μg/L of Hg. The Pb—Fe, Pb—Cu, Pb—Cd, Pb—Hg, and Cu—As mixtures presented TU_{50} values from 0.32 to 0.40, equivalent to 32–40 % of the individual concentration of each metal (Table 4). For example, the Pb—Fe mixture had a concentration of 213 μg/L Pb and 61 μg/L Fe, and the Pb—Hg mixture had a concentration of 266 μg/L Pb and 2.0 μg/L Hg. In the Hg—Fe, Hg—Cd, Fe—Cu, Zn—Cu, Zn—Hg, and Pb—As mixtures, the TU_{50} ranged from 0.41 to 0.50. For the Cu—Cd, Fe—Cd, Zn—Fe, and Zn—Pb mixtures, the TU_{50} varied from 0.51 to 0.59. In the Cd—As, Zn—As, and Zn—Cd mixtures, the TU_{50} was observed between 0.61 and 0.76, with the Zn—As mixture in which the individual concentrations of each metal were higher than other treatments (503 μg/L Zn-625 μg/L As).

The As—Hg mixture presented an antagonistic effect at a TU_{50} of 2.39, equivalent to an individual concentration of 1965 μg/L As and 14 μg/L Hg (Table 4). In the other Hg mixtures, the TU_{50} was obtained at a concentration of 2 to 3 μg/L Hg. In the case of As, the concentrations in their mixtures varied between 321 and 625 μg/L. The Cu concentrations that reached the TU_{50} were 20 to 35 μg/L. Thus, Hg and Cu were the most toxic metals in the tested mixtures.

3.2. Prediction of toxicity by the MIXTOX model

Regarding the MIXTOX tool analyses, all the parameters, the modeled data and the significance test results are available in Tables S1 to S15 (supplementary material). The parameters were interpreted according to information available in Jonker et al. (2005). In addition to the parameters described in Tables S1 to S15 (supplementary materials), we also presented in isobolorams (Fig. 2) the model and deviation that best described the results for each binary mixture. Using the MIXTOX tool, we analyzed 15 binary mixtures: As—Cd, As—Cu, As—Fe, As—Pb, As—Zn, Cu—Cd, Cu—Fe, Cu—Pb, Cu—Zn, Fe—Cd, Fe—Pb, Fe—Zn, Pb—Cd, Zn—Cd and Zn—Pb. From Table 5, four binary mixtures (Cu—Cd, Cu—Fe, Cu—Pb, As—Cu) fit the concentration addition (CA) model (dos Reis et al., 2022), while eleven binary mixtures (As—Cd, As—Fe, As—Pb, As—Zn, Cu—Zn, Fe—Cd, Fe—Pb, Fe—Zn, Zn—Pb, Pb—Cd, Zn—Cd) fit the independent action (IA) model better (dos Reis et al., 2022).

In mixtures of As—Cd, As—Fe, As—Pb, As—Zn, Cu—Zn, Fe—Cd, Fe—Zn, and Zn—Pb, the model that best described the data was the IA model ($p < 0.0001$), followed by the dose-level dependent (DL) deviation ($p < 0.05$). In As—Cd (Fig. 2A), the IA/DL interaction (SS – the sum of squared residuals = 35.16, $p = 1.86 \times 10^{-9}$, $r^2 = 0.92$) indicates synergism at lower concentrations and antagonism at higher levels (a = −5.47), with a change at a higher dose level than the LC_{50} (b = 1.65). In As—Fe (Fig. 2B), the IA/DL (SS = 28.28, $p = 0.01$, $r^2 = 94$) indicated synergism at lower concentrations and antagonism at higher levels (a = −4.96), with a change at higher dose levels than the LC_{50} (b = 1.15). In As—Pb (Fig. 2C), the IA/DL analysis (SS = 48.38; p = 0.01, $r^2 = 0.91$) shows synergism at lower concentrations and antagonism at higher levels (a = −5.68), with a magnitude of synergism and antagonism effect level dependent (b = 0.92). In As—Zn (Fig. 2D), the IA/DL (SS = 44.72; $p = 9.34 \times 10^{-11}$, $r^2 = 0.90$) showed synergism at lower concentrations and antagonism at higher levels (a = −4.37), with changes at lower dose levels than the LC_{50} (b = 2.12). In Cu—Zn (Fig. 2E), in IA/DL (SS = 34.98 $p = 4.64 \times 10^{-5}$, $r^2 = 0.93$), there was synergism at lower concentrations and antagonism at higher levels (a = −5.00), with changes at higher dose levels than the LC_{50} (b = 1.25). In Fe—Cd (Fig. 2F), the IA/DL (SS = 31.24; $p = 4.43 \times 10^{-6}$, $r^2 = 0.94$) indicates synergism at lower concentrations and antagonism at higher levels (a = −6.56), with a change at a higher dose level than the LC_{50} (b = 1.22). In Fe—Zn (Fig. 2G), the IA/DL deviation (SS = 36.31; $p = 1.62 \times 10^{-18}$, $r^2 = 0.92$) showed synergism at lower concentrations and antagonism at higher levels (a = −7.35), with changes at higher dose levels than the LC_{50} (b = 1.68). In Zn—Pb (Fig. 2H), the IA/DL (SS = 28.20; $p = 0.000283$, $r^2 = 0.94$) showed synergism at lower concentrations and antagonism at higher levels (a = −4.31), with changes at higher dose levels than the LC_{50} (b = 1.30).

The mixtures of Pb—Cd, Zn—Cd and Fe—Pb also better fit the IA model ($p < 0.0001$). The data from Pb—Cd (Fig. 2I) and Zn—Cd (Fig. 2J) were better described by IA/SA interactions (Pb-Cd: SS = 25.06; $p = 6.95 \times 10^{-13}$, $r^2 = 0.96$; and Zn-Cd: SS = 59.39; $p = 1.11 \times 10^{-7}$, $r^2 = 0.91$), showing only synergism (a < 0). On the other hand, the Fe—Pb mixture (Fig. 2K) better fit the IA/DR deviation (SS = 23.26; $p = 0.00832$, $r^2 = 0.96$), in which antagonism occurred at high Fe and low Pb levels and synergism occurred at low Fe levels. The synergism in the mixtures was caused mainly by Pb.

The CA model better describes their data in mixtures of As—Cu, Cu—Cd, Cu—Fe, and Cu—Pb ($p < 0.0001$). Cu—Cd (Fig. 2L) and Cu—Fe (Fig. 2M) did not present statistically significant deviations from the CA model, i.e., the mixtures showed additive effects. Cu—Pb (Fig. 2N) mixture data fit the CA/SA interaction (SS: 32.40, $p = 5.63 \times 10^{-6}$, $r^2 = 0.94$), with synergistic effects (a < 0). In the As—Cu mixture (Fig. 2 O), the deviation that best described the data were CA/DL (SS: 43.84, $p = 0.01 \times 10^{-6}$, $r^2 = 0.91$), which exhibited synergism at lower concentrations and antagonism at higher levels (a = −1.98), with a change at a higher dose level than the LC_{50} (b = 0.44).

4. Discussion

Typically, the persistence and concentration of PTEs in coastal marine environments are values used to initially assess environmental risk. However, it is necessary to provide evidence of the potential risks for living organisms that inhabit polluted environments, including realistic scenarios of pollutant's toxic interactions. The findings found here indicate that binary mixtures of As, Cd, Cu, Fe, Pb, and Zn have the potential to induce synergistic effects in key organisms of coastal environments. It has been reported that when key species are affected by environmental pollution, there is a disturbance in trophic systems and biodiversity, and the fishing economy is threatened (Sharifuzzaman et al., 2016; Páez-Osuna et al., 2017).

4.1. Synergistic effect of binary mixtures

Much of the knowledge we have to understand joint effects of chemicals in mixtures for distinguishing among situations that can arise with mixtures as synergism and antagonism has been obtained from binary mixtures (Newman and Unger, 2003). In aquatic organisms, diverse studies provide helpful information between the presence of each metal and the observed effects (e.g., García-Navarro et al., 2017; Gebara et al., 2020; Bautista-Covarrubias et al., 2020; Yoo et al., 2021; dos Reis et al., 2022; Matyja, 2023), which is a more challenging task in more complex mixtures. Nonetheless, this doesn't underestimate the importance of studying multiple mixtures to simulate real-life conditions in polluted environments. Using *P. similis*, Rebolledo et al. (2021) identified synergistic effects in more complex mixtures involving four,

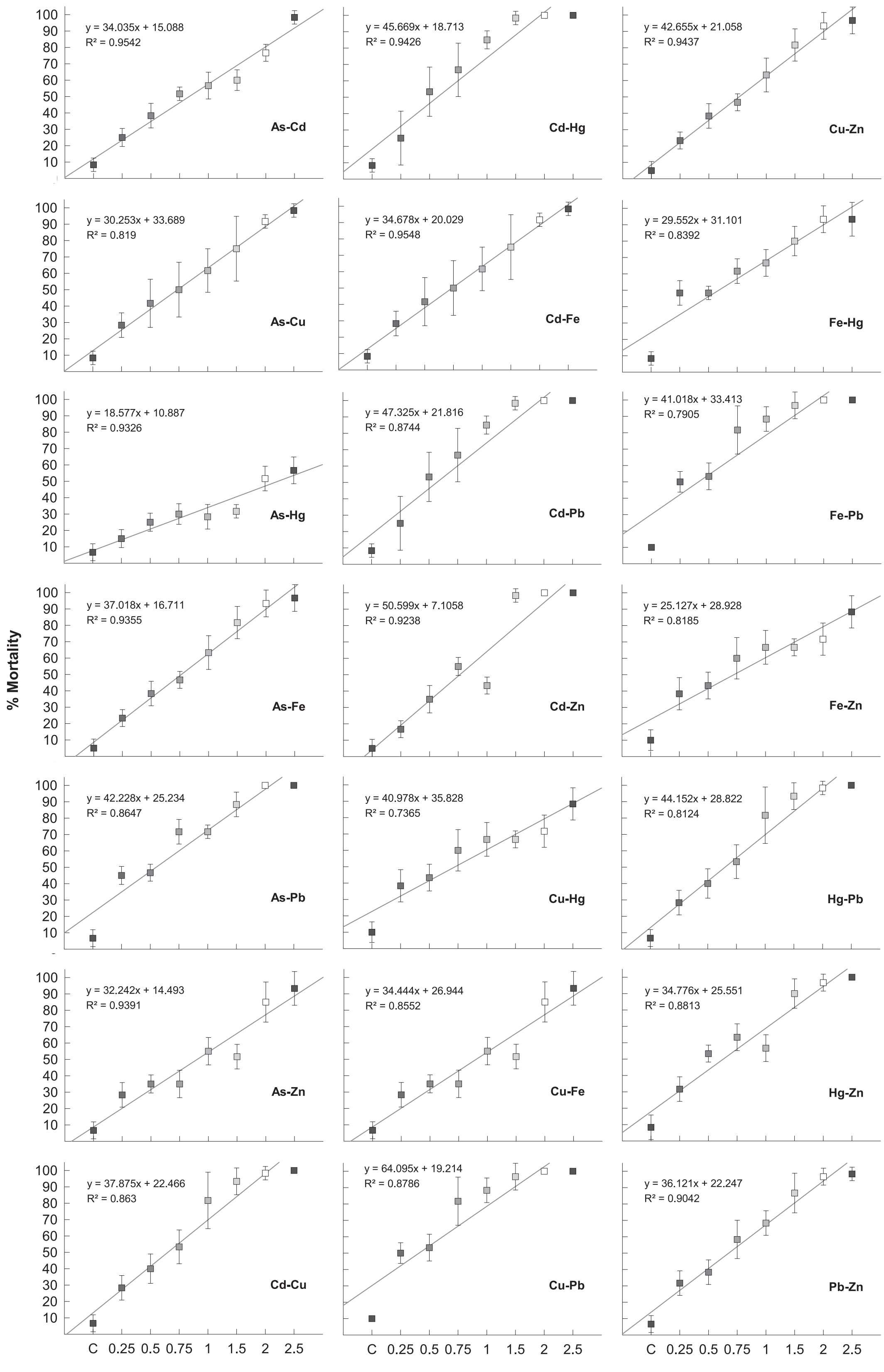

Fig. 1. Percentage of mortality observed in *P. similis* exposed to 21 binary mixtures of As, Cd, Cu, Fe, Hg, Pb, and Zn after 24 h of exposure.

Table 3
The TU_{50} values at 24 h of 21 binary metal mixtures for *P. similis*. Data are presented as the mean and their 95 % confidence intervals (in parentheses). $TU_{50} < 1$, $= 1$, and > 1 indicates synergism, additive effect and antagonism, respectively.

Metal	As	Cd	Cu	Fe	Hg	Pb
Cd	0.67 (0.54, 0.83)					
Cu	0.39 (0.31, 0.49)	0.51 (0.42, 0.61)				
Fe	0.62 (0.51, 0.74)	0.59 (0.48, 0.71)	0.45 (0.35, 0.58)			
Hg	2.39 (1.55, 3.69)	0.50 (0.41, 0.63)	0.29 (0.24, 0.36)	0.41 (0.31, 0.54)		
Pb	0.43 (0.38, 0.50)	0.38 (0.38, 0.54)	0.38 (0.24, 0.36)	0.32 (0.25, 0.40)	0.40 (0.34, 0.47)	
Zn	0.76 (0.59, 0.97)	0.61 (0.51, 0.73)	0.47 (0.34, 0.65)	0.52 (0.36, 0.75)	0.48 (0.38, 0.62)	0.52 (0.44, 0.62)

Table 4
Equivalent concentrations (μg/L) of TU_{50} of the 21 binary mixtures tested for *P. similis*.

Mixture	TU_{50}	Mixture	TU_{50}	Mixture	TU_{50}
As-Cd	551–573	Cd-Fe	504–113	Cu-Zn	32–311
As-Cu	321–27	Cd-Hg	428–3.0	Fe-Hg	79–2.0
As-Fe	510–119	Cd-Pb	325–253	Fe-Pb	61–213
As-Hg	1965–14	Cd-Zn	522–404	Fe-Zn	100–344
As-Pb	353–286	Cu-Fe	31–86	Hg-Pb	2.0–266
As-Zn	625–503	Cu-Hg	20–2.0	Hg-Zn	3.0–318
Cd-Cu	436–35	Cu-Pb	26–253	Pb-Zn	346–344

five, and nine elements. The present research demonstrates that synergistic effects can also occur in binary interactions.

Out of 21 binary mixtures tested, our results indicated that 20 induced synergistic effects in *P. similis*, according to the TU_{50} values. It is disconcerting that most mixtures tested were at concentrations below those detected in the coastal marine environments of Mexico, which is the natural home of a wide variety of aquatic organisms. The following mixtures induced synergistic effects in the rotifer *P. similis* and in other marine invertebrates: Cd—Hg, Hg—Zn, and Hg—Pb in the white shrimp *Litopenaeus vannamei* (Frías-Espericueta et al., 2009); As—Pb and Cd—Pb in the water flea *Diaphanosoma celebensis* (Yoo et al., 2021); Cu—Zn, Pb—Zn and Pb—Cd in the echinoderm *Strongylocentyotus intermedius* (Xu et al., 2011); Cd—Pb and Hg—Cu in the echinoderm *Paracentrotus lividus* (Fernández and Beiras, 2001); and Cd—Zn in the copepod *Tisbe holothuriae* (Verriopoulos and Dimas, 1988). In *P. similis*, the Cu—Hg mixture was more toxic (lowest TU value: 0.29) than the other mixtures. This could be due to the combined effect of these PTEs because Cu and Hg have deleterious effects on energy production (Hansen et al., 1992; Hatef et al., 2013). This affects a critical physiological process (respiration), which could cause *P. similis* mortality. In contrast to *P. similis*, the following mixture produces antagonistic effects: Cd—Zn in L. *vanammei* (Frías-Espericueta et al., 2009), As—Cd in *D. celebensis* (Yoo et al., 2021), Zn—Pb in *S. intermedius* (Xu et al., 2011), and Cu—Cd and Hg—Cd in the copepod *Tigriopus fulvus,* with TU values of 1.70 to 2.95 (Prato et al., 2013). It is evident that the effects of heavy metal mixture exposure in aquatic organisms vary depending on the type and number of metals, chemical speciation, exposure time, and environmental conditions (temperature, pH, salinity, redox, etc.) (Rebolledo et al., 2021; Jeong et al., 2023).

From the TU_{50} analysis, we observed that unlike in other aquatic organisms where the interaction between Zn, Cd, and Cu usually induces antagonistic effects (Pitombeira de Figuerêdo et al., 2016), in *P. similis*, all combinations with these elements resulted in synergism, as in microbial biosensors that include *Escherichia coli* HB101 pUCD607 and *Pseudomonas fluorescens* 10,586 pUCD607 (Preston et al., 2000). On the other hand, the results from the MIXTOX tool show a more complex scenario, in which Zn—Cd mixture analysis showed synergism, corroborating the TU_{50} analysis; in Zn—Cu mixtures, we observed synergism only at lower levels and antagonism at higher levels, and Cu—Cd mixtures presented additivity (without significant effects of synergism or antagonism).

Analyzing the mixture data obtained by MIXTOX was a challenge, and we ran 15 binary mixtures, resulting in large amounts of data. Although the results should be carefully analyzed, case by case, we could observe some patterns. For example, the metals As, Zn, and Pb presented synergism at lower concentrations in all 12 binary mixtures containing these metals. Furthermore, data from the literature on freshwater zooplankton show that Zn caused synergistic effects at lower concentrations on the cladocerans *Daphnia carinata* (Zn—Cu), *D. magna* (Zn—Cd), and *Ceriodaphnia silvestrii* (Zn—Al) and on the microalga *Raphidocelis subcapitata* (Zn—Al) (Cooper et al., 2009; Gebara et al., 2020; Gebara et al., 2021; Shaw et al., 2006). However, there were no interactions in the Zn—Cu mixture in the freshwater rotifer *Philodina acuticornis* (Buikema Jr et al., 1977). Additionally, studies show that mixtures containing Pb resulted in synergism on the cladocerans *C. dubia* (Cu—Pb) and *D. carinata* (Cu—Pb) and on the isopod *Asellus aquaticus* (Pb—Cd) (Cooper et al., 2009; Van Ginneken et al., 2015). However, it is important to emphasize that the type of interaction (synergism, antagonism, additivity – no interaction) may vary according to the species studied, since the response could be different even among aquatic invertebrates from the same family (Daphnidae) (Nys et al., 2017; Shaw et al., 2006). Salinity exerts a significant influence on metal speciation. In freshwater environments, there is an increased concentration of free metal ions, which reasonably anticipation that freshwater zooplankton species are more susceptible to metal toxicity than marine species (Hall and Anderson, 1995; Heugens et al., 2001). The present results emphasize that the euryhaline rotifer *P. similis* displays an elevated sensitivity to binary metal mixtures, similar to other key freshwater zooplankton species that also play an equally significant role in the ecological structure of aquatic ecosystems.

In general, synergistic interactions can involve six basic processes that determine toxicity in an organism (Cedergreen, 2014): bioavailability, absorption, internal transport, metabolism, site-specific binding, and excretion. However, the alterations of metabolic activity are the most well investigated mechanisms on synergistic behavior; in which a metal (or chemical) can either increase or decrease the metabolization rate of another metal (or another chemical) (Cedergreen, 2014). Nevertheless, the twenty synergistic interactions involved in the present study are most likely caused by interactions of one or more of these processes. Specific information on the mechanisms of heavy metals in rotifers is very rare (Li et al., 2020), particularly in binary and multiple mixtures. However, studies of binary mixtures reveal some tendencies and biochemical effects. These mechanisms involving toxic metals suggest that these PTEs may bind to proteins or enzymes with SH-containing ligands, leading to structural and functional disruptions (Landis and Yu, 1999). The combined effects of PTEs on marine invertebrates, which encompass the disruption of energy production through alterations in the oxidative phosphorylation system by inhibiting active transport mechanisms (Landis et al., 2017), the induction of oxidative stress through the inhibition of antioxidant enzyme activity (Capparelli et al., 2019), and the interference with osmoregulation by inhibiting Na-K/ATPase activity (Pequeux, 1995), offer a plausible explanation for the observed synergistic effects (Frías-Espericueta et al., 2022). For example, Yoo et al. (2021) showed that compared to a single exposure to Cd, Pb, and As, mixed exposure to Cd—Pb and As—Pb induced higher antioxidant enzyme activity in *D. celebensis*.

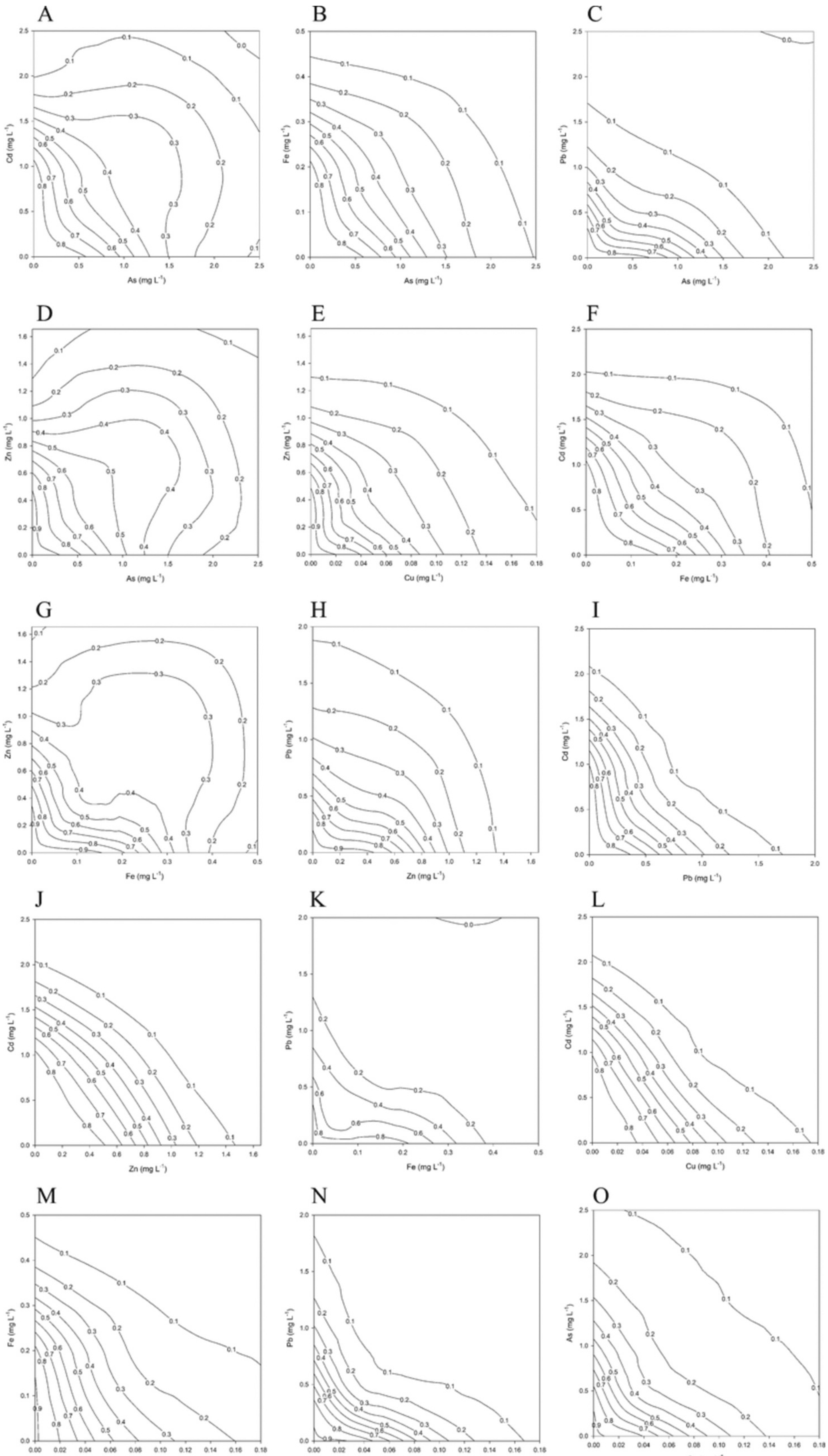

Fig. 2. Isobolograms of mortality of *P. similis* exposed to 15 binary mixtures of As, Cd, Cu, Fe, Pb, and Zn. The linear lines represent no interaction (additivity); concave and convex shapes represent synergism and antagonism, respectively. The models and deviations represented are A to H = IA/DL; I and J = IA/SA; K = IA/DR; L and M = CA; N = CA/SA; O = CA/DL, where CA = concentration addition model, IA = independent action model, SA = synergism/antagonism, DR = dose ratio-dependent deviation, and DL = dose level-dependent deviation.

Table 5
Summary of the MIXTOX tool analysis showing the best fitting models of the toxicity data of *P. similis* exposed to binary metal mixtures (As, Cd, Cu, Fe, Pb and Zn).

Mixtures	Best fitting model / deviation
As-Cd	IA / DL
As-Cu	CA / DL
As-Fe	IA / DL
As-Pb	IA / DL
As-Zn	IA / DL
Cu-Cd	CA
Cu-Fe	CA
Cu-Pb	CA, S/A
Cu-Zn	IA / DL
Fe-Cd	IA / DL
Fe-Pb	IA / DR
Fe-Zn	IA / DL
Pb-Cd	IA, S/A
Zn-Cd	IA, S/A
Zn-Pb	IA / DL

CA = concentration addition model; IA = independent action model, S/A = synergism/antagonism, DR = dose ratio-dependent deviation, and DL = dose level-dependent deviation.

4.2. Antagonistic effect of As—Hg mixture

Interestingly, the As—Hg mixture had an antagonistic effect in *P. similis* ($TU_{50} = 2.39$). It has been documented that in the amphipod *Gammarus pulex*, As induces antagonistic effects on the toxicity of Cd (Vellinger et al., 2012). Conversely, the interaction of Hg with Cd and Cu results in antagonistic effects on the copepod *T. fulvus* (Prato et al., 2013). Concerning Hg, the interaction of this metal with Se is one of the best-known examples of biological antagonism (Khan and Wang, 2009). Here, we found that As decreased the toxicity of Hg. Although As is chemically similar to phosphorus and can form phosphorus-like esters, whether this metalloid is essential for life is still under discussion (Rosen et al., 2011). According to Calabrese and Baldwin (2003), As can produce a hormesis response in animals. This was confirmed by Rebolledo et al. (2020), who noted that *P. similis* and *Brachionus ibericus* grew better at 100 and 200 μg/L As than in the control groups. The individual effects of As in *Proales* could explain the antagonistic effects in the As—Hg mixture. However, this mechanism deserves more attention.

The mechanism involved for this antagonism may be explained by one or more of the following four forms (Newman and Unger, 2003): (i) that As and Hg eliciting opposite physiological effects and, as a consequence counterbalancing each other (functional antagonism); (ii) that As and Hg react with one another to produce a less toxic product (chemical antagonism); (iii) involves the uptake, movement within the organism of the two elements, deposition at specific sites, and elimination of the toxicants (dispositional antagonism); and (iv) that As and Hg bind to the same receptor, and each toxicant blocks the other from fully expressing its toxicity (receptor antagonism). The mechanism proposed for the interaction As—Se could to be argued to explain also the interaction As—Hg. Arsenic can react with glutathione (GSH) and S-adenosylmethionine (SAM) by forming As—Hg complexes, which can be secreted extracellularly (Sun et al., 2014).

4.3. Mixtures of PTEs and the guideline permissible limits

Evidence supports that the euryhaline rotifer *P. similis* is highly sensitive to the maximum permissible levels of Cu, Hg, Pb, and Zn outlined in the Mexican guidelines for marine zones ((NOM-001-SEMARNAT-2021) SEMARNAT, 2022) (Rebolledo et al., 2021). Our findings demonstrated that even low concentrations of As, Cd, Cu, Fe, Hg, Pb, and Zn in the tested binary mixtures were sufficient to trigger synergistic effects. This study has identified that the Cu and Hg combination poses a potential ecological threat to marine invertebrates, given the frequent co-occurrence of these heavy metals in coastal marine environments. Commonly, the mixture toxicity of heavy metals in marine invertebrates, such as *P. similis*, appears to rise in conditions of lower salinity and elevated temperatures, as observed in several studies (Verslycke et al., 2003; Rebolledo et al., 2021; Frías-Espericueta et al., 2022; Jeong et al., 2023). Hence, additional research is required to determine the significance of these environmental factors in the combined toxic effects of PTEs on euryhaline invertebrates.

In certain binary mixtures, the concentrations of As and Cd that generated synergistic effects exceeded the permissible levels defined in the Mexican guidelines. Nevertheless, a synergistic effect was observed when combining As and Cd with other elements at concentrations below the allowable limit. For CMC (US EPA, 2011) guidelines, synergistic effects are observed at concentrations above these guidelines. Mercury is the only element that comes perilously close to the established limit of 1.8 μg/L set by the US EPA. At a concentration of 2.0 μg/L Hg, synergistic effects were observed in the tested mixtures. Iron is not included in saltwater quality guidelines. Nevertheless, Shuhaimi-Othman et al. (2012) suggested a CMC of 37.2 μg/L of Fe in freshwater environments according to the sensitivity of freshwater species with LC_{50} values of 0.12 to 8.49 mg/L. In the case of *P. similis*, the LC_{50} varies between 138 and 327 μg/L Fe; however, in binary mixtures, this concentration decreases significantly to just 86 μg/L, which is up to 150 times less than the actual Fe concentration detected in Mexican seawater (Jonathan et al. (2011). The toxicity of Fe for marine invertebrates such as rotifers has been relevant in recent years, given the constant persistence, abundance, and high possibility that it interacts with other elements, intensifying the vulnerability of organisms to contamination (Rebolledo et al., 2020; Han et al., 2022).

Although the results of this study indicate that the response of the model organism, *P. similis*, to various binary mixtures of potentially toxic elements cannot be extrapolated to encompass the entire spectrum of marine organisms, it is important to emphasize that the presently established permissible limits may provide protection to some species while leaving others at risk. In this sense, we make a suggestion relevant to both Mexican and international guidelines: it is crucial to consider that the individual concentrations allowed can present a significant environmental risk when they interact (combined) and disturb the ecological structure of coastal marine systems. This highlights the importance of reviewing and adjusting regulatory standards to ensure effective protection of marine biodiversity as a whole.

5. Conclusion

This study constitutes the first research study examining binary mixtures of As, Cd, Cu, Hg, Pb, and Zn at environmental concentrations in the marine invertebrate *P. similis*. MIXTOX analysis proved to be a good tool to assess the binary mixtures of the rotifer *Proales*. Synergistic effects were detected in mixtures whose elemental concentrations were below the Mexican guidelines for water discharge in coastal ecosystems ((NOM-001-SEMARNAT-2021) SEMARNAT, 2022), which confirmed our first hypothesis. When the tested elements were combined at permissible concentrations by the US EPA (2011), a synergistic effect was observed only for the combination involving Hg. For the first time, we documented the antagonistic effect of the As—Hg mixture in marine invertebrates. These results partially confirm the second hypothesis. Our findings suggest that key taxa such as *P. similis* that inhabit Mexico can live under the permissible levels of the US EPA (2011) but not the Mexican guidelines. The ecotoxicological relevance of pollutant mixtures should be considered as a more realistic scenario in current environmental risk assessments since synergistic effects on organisms at the base of the food web may lead to great disturbances in aquatic ecosystems.

CRediT authorship contribution statement

Uriel Arreguin-Rebolledo: Conceptualization, Investigation, Methodology, Data curation, Formal analysis, Writing – original draft. **Renan Castelhano Gebara:** Formal analysis, Writing – review & editing. **Valencia-Castañeda Gladys:** Conceptualization, Methodology, Writing – review & editing. **Rico-Martínez Roberto:** Conceptualization, Writing – review & editing. **Frías-Espericueta Martín Gabriel:** Conceptualization, Writing – review & editing. **Páez-Osuna Federico:** Conceptualization, Supervision, Project administration, Funding acquisition, Writing – review & editing.

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

No data was used for the research described in the article.

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Appendix A. Supplementary data

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