Environmental Toxicology and Pharmacology 105 (2024) 104326

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

Copper and cadmium, isolated and in the mixture, impact the Neotropical freshwater Calanoida copepod *Notodiaptomus iheringi*: A short-term approach with environmental concentrations

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A R T I C L E I N F O

Edited by: Ayse Basak Engin

Keywords:
Antagonism
Calanoida
Metallic mixtures
Sub-chronic toxicity
Sub-lethal effects

A B S T R A C T

Metal discharges in aquatic ecosystems are of concern since they affect different trophic levels, altering the functioning of the aquatic food chain. The metals can interact among them and with other pollutants, resulting in complex mixtures whose effects on biota are unpredictable. The impacts of copper (Cu) and cadmium (Cd), isolated and combined, on the freshwater copepod *Notodiaptomus iheringi* were assessed in acute and sub-chronic exposures. Species sensitivity distribution (SSD) curves were constructed for both metals. In the acute tests antagonism was observed in mortality, while in sub-chronic, mortality was not affected; however, the eggs produced and percentage of viable eggs were significantly altered. Our data suggest that egg production can be a detoxification route in *N. iheringi* under Cu and mixture exposure. From the SSD curves, *N. iheringi* was the most sensitive Brazilian species for Cu and the second most sensitive for Cd.

1. Introduction

Metals are ubiquitous in aquatic ecosystems, and their inputs increase with the release of agriculture, industries, and mining (Nour et al., 2019; Briffa et al., 2020). Some metals are essential due to their function in enzymes and metabolic processes (e.g., cobalt - Co, copper - Cu, zinc - Zn), while others do not present known functions in biological processes and are considered non-essential (e.g., aluminum - Al, cadmium - Cd, lead - Pb) (Nagajyoti et al., 2010). Copper and Cd are well-studied metals in the different levels of the aquatic trophic chain, and they are known to affect photosynthesis and the biochemical composition of algae (Baracho et al., 2019; Rocha et al., 2020, 2021); impairing the reproduction and secondary production of cladocerans (Souza et al., 2014; Rocha et al., 2016); and damaging the antipredator behavior, neurobehavior, and antioxidant system in fish (Pan et al., 2017; Jia et al., 2020; Volz et al., 2020). In Brazil, the concentrations of Cu can reach up to 90 μg L^{-1} in a river affected by a deactivated mine (Perlatti et al., 2021), and Cd can be detected in the range from 0.7 to 20 μg L^{-1} in water bodies close to a coal mine or intensive agriculture (Campaner et al., 2014; CETESB, 2018).

Although the most part of studies and regulations deal with the isolated contaminants, in the environment the organisms are exposed to different combinations of stressors (Kortenkamp and Faust, 2018). When there are interactions between them, the combination results in more (synergism) or less (antagonism) damage than the isolated compounds to the organisms (Backhaus et al., 2004; Martin et al., 2018). These interactions can be calculated using the concentration addition (CA) or independent action (IA) model, considering the effects of each contaminant isolated, assuming non-interaction between them (Barata et al., 2006; Gopalapillai and Hale, 2015). However, the interactions of the stressors in the mixtures and their relation to biotic and abiotic factors are not fully understood, making predictions difficult and

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https://doi.org/10.1016/j.etap.2023.104326
Received 14 September 2023; Accepted 20 November 2023
Available online 23 November 2023

generalizations very tricky.

Ecotoxicological studies use several groups of organisms (e.g., alga, zooplankton, fish) as models to assess the effects of contaminants at different trophic levels and in different environments to predict the risks of exposure to the pollutants of interest (e.g., Faria et al., 2020; Gebara et al., 2021, 2023). For freshwater environments, some well-established and standardized test organisms are the cladocerans *Daphnia magna* and *Ceriodaphnia dubia* in temperate zones (ISO, 2008; OECD, 2012), and *Ceriodaphnia silvestrii* in Brazil (ABNT, 2016, 2017). Notwithstanding, freshwater copepods play a role of high ecological significance, being sensitive, which justifies their use in ecotoxicological tests (Brown et al., 2005). In addition, they are an energy transfer link in the trophic chain; however, their use in ecotoxicology is scarcer when compared to marine copepods or other freshwater organisms, such as cladocerans (Willis, 1999; Kulkarni et al., 2013). Brown et al. (2005) and Kulkarni et al. (2013) highlighted the absence of protocols for ecotoxicological tests involving freshwater copepods, which have not yet been established. On the other hand, there have been reports recommending the use of the marine copepod *Acartia tonsa* in ecotoxicology evaluations since the 1970s, and the standards set by international regulatory agencies are related to cultivation and tests involving species of Calanoida and Harpacticoida copepods from temperate zones (UK EPA, 2007; OECD, 2011, 2014). The use of copepod species from different aquatic systems is increasing in the last years to assess their response to different chemicals; however, there are still many questions to be answered, in particular regarding (i) the toxicity routes of contaminants, i.e., via water or food intake (Bielmyer et al., 2006) and (ii) the processes involved in the internalization of metals by the copepods from water, i.e., some authors argue that the entry of metals occur via the body surface (Gutierrez et al., 2010), while others by oral route (Kadiene et al., 2019).

Recent studies indicate the high sensitivity of the freshwater Neotropical Calanoida Copepoda *Notodiaptomus iheringi* to different combinations of contaminants – pesticides (fipronil and 2,4-Dichlorophenoxyacetic acid – 2,4-D) and the mixture Cd-fipronil. While synergism was observed in the mixture of pesticides (Lopes et al., 2023), antagonism was the main response in the combination of metal-pesticide (Rocha et al., 2023). These studies reinforce the need for more studies with this species exposed to different combinations of contaminants to understand better its ecology and response to the stressors. The current study evaluated the toxicity of environmental concentrations of Cu and Cd, isolated and in the mixture, in acute and sub-chronic tests, on the survival, morphology, and reproduction of *N. iheringi*. This species is widely distributed in the Southern Hemisphere, and it is found from latitudes 5°S to 25°S, being a dominant and representative species in several Brazilian freshwater environments, especially in São Paulo state (Matsumura-Tundisi and Tundisi, 2003). In addition, we constructed species sensitivity distribution (SSD) curves to compare the sensitivity of this species with other species used in ecotoxicology tests, including Brazilian native species.

2. Material and methods

The copepods *Notodiaptomus iheringi* were collected, acclimated to laboratory conditions (25 ± 2 °C, photoperiod of 12:12 h light:dark, ASTM water - hardness 40–48 mg $CaCO_3$ L^{-1}; pH 7–7.6; conductivity: ~160 µS cm^{-1}) (ABNT, 2017) for at least six months, and cultured as described in Rocha et al. (2023). Briefly, the organisms were collected in a reservoir (Lobo Dam) located in São Paulo state, Brazil, and acclimated gradually to laboratory conditions (≈ one month of acclimation and five months of maintenance) before being used in tests. The water was totally renewed three times per week, and the organisms were fed with vitamin complex Sera Fishtamin (Sera, Germany) and the freshwater microalga *Raphidocelis subcapitata*. The age of organisms was known, and tests were performed with adult organisms ≥ 20 ≤ 30 days old, with an equal amount of males and females (i.e., 1:1 ratio). Monthly, the sensitivity of the organisms was checked with NaCl as a reference substance to ensure the health of cultures and the reliability of the tests.

Copper and Cd solutions were made by serial dilutions of $CuCl_2$ Titrisol 1000 mg L^{-1} (Merck) and $CdCl_2$ Titrisol 1000 mg L^{-1} (Merck), respectively, in ultrapure water (Barnstead Easy Pure II, Thermo Scientific, Dubuque, IA, USA). The metals were added in ASTM water 24 h before the exposure of the organisms to the contaminants, to allow the equilibrium between the metals and the medium. Before the transfer of the animals, 15 mL samples from the medium with Cu and / or Cd were taken, acidified with 1% HNO_3, kept cold (4 ºC) and measured by inductively coupled plasma – optical emission spectrometry (Thermo ICP-OES, iCAP 7000; Thermo Fischer Scientific, Madison, WI, EUA), and quantified using 324.75 nm (Cu) and 228.80 nm (Cd) emission lines. The limits of quantification (LOQ) were 0.05 and 0.01 mg L^{-1}, while the limits of detection (LOD) were 0.004 and 0.00006 mg L^{-1} for Cu and Cd, respectively.

For the acute toxicity tests, the animals were exposed for 48 h, without food supply, to the following treatments: Control (C, no Cu or Cd); 10 (Cu1); 15 (Cu2); 20 (Cu3); 25 (Cu4) and 30 µg Cu L^{-1} (Cu5); 1.25 (Cd1); 2.5 (Cd2); 5 (Cd3); 10 (Cd4) and 15 µg Cd L^{-1} (Cd5); and to the mixtures: Cu1Cd1 (M1); Cu3Cd1 (M2); Cu5Cd1 (M3); Cu1Cd2 (M4); Cu3Cd2 (M5) and Cu5Cd2 (M6). These concentrations were defined after preliminary tests with isolated metals (3 tests for each metal). Each treatment had 20 organisms, divided in five replicates with four adults (two females without eggs and two males) each. The organisms were placed in 50 mL polypropylene flasks with 40 mL of medium, and the mortality was the endpoint evaluated. In addition, we observed the production of eggs and the morphology of the organisms. In the present study, the term "aborted eggs" refers to the eggs that were expelled from the females before the eclosion, while non-viable eggs are black or white eggs, which is not the normal feature. "Impairment features" refers to protuberance/swelling that is not observed in the organisms in normal conditions. Initial pH (7.5–7.7), dissolved oxygen (7.3–7.5 mg L^{-1}), water hardness (48 mg $CaCO_3$ L^{-1}), and electric conductivity (159–164 µS cm^{-1}) were similar in the treatments.

For the sub-chronic toxicity tests, we assessed the effects of $LC_{10\text{–}48\ h}$ of Cu (16 µg L^{-1}), $LC_{10\text{–}48\ h}$ of Cd (4.0 µg L^{-1}), the mixture of these $LC_{10\text{–}48\ h}$ and the control during 8 days. As in our previous study (Rocha et al., 2023), we followed 20 couples per treatment, with one male and one female per replicate. The animals were fed every 48 h with *R. subcapitata* (10^6 cells mL^{-1}) and the test cultures were renewed every 96 h. Daily, we assessed the morphology of the organisms, the amount of eggs (produced and expelled) and nauplii, then expelled eggs and nauplii were removed. We did not continue following replicates where it was observed dead male and female without spermatophore or eggs.

The data of the acute and sub-chronic tests were tested for normality and homogeneity of variance. One-way ANOVA and Dunnett's post-hoc test were applied, with $\alpha = 0.05$ (Minitab 17). The lethal concentration of Cu and Cd to 50% of copepods ($LC_{50\text{–}48\ h}$) were calculated using a sigmoidal three parameters logistic curve (SigmaPlot 11, Systat). The obtained parameters for each metal (Cu and Cd) were used to estimate the predicted effects of concentration addition (CA) and independent action (IA) models in the MixTox tool, where the deviations synergism/antagonism (S/A), dose-ratio dependent (DR) and dose-level response (DL) were also used to define the best fit for the data (Jonker et al., 2005).

Species sensitivity distribution (SSD) curves were plotted with log-normal values of Effective/Inhibitory/Lethal concentrations to 50% of the organisms (EC50/IC50/LC50) toxicity data for Cu or Cd compiled from the USEPA ECOTOX database (http:// cfpub. epa. gov/ ecotox/). SSD curves were constructed using ETX 2.0 software (Van Vlaardigen et al., 2004) assuming a 5% log normality according to the Anderson-Darling test. In addition, we calculated the hazardous concentrations for 5 (HC_5) and 50% (HC_{50}) of the species (Aldenberg and Jaworska, 2000).

3. Results and discussion

After 48 h exposure, the physico-chemical properties of the culture medium were not affected significantly, with final pH ≈ 7.5–7.6, and the final dissolved oxygen ≈ 7.4–7.6 mg L^{-1}. The toxicity data from isolated metals indicate that $LC_{50-48\ h}$ for Cu was 27.7 μg L^{-1} (Fig. 1A), while for Cd was 8.0 μg L^{-1} (Fig. 1B). These data are in the range of these metals' toxicity to other freshwater zooplankton, where the toxicity for Cu can range from 10 to 103 μg L^{-1} and for Cd from 2 to 50 μg L^{-1} in the daphnids *Daphnia magna* and *Ceriodaphnia dubia*, which are considered model species for ecotoxicity tests (Barata et al., 2006; Cooper et al., 2009; Meyer et al., 2015a; Clément et al., 2022). At the end of the acute exposure, it was possible to observe females with eggs or spermatophores in all treatments. Aborted eggs were found in the Cu3, Cd1, Cd2, Cd4, Cd5, M1 and M2 treatments. Impairment features were found in Cu5, M3, and M6 treatments. Since the concentration of Cu was the same in these three treatments, the morphological changes were most likely caused by Cu. The same impairments were observed by Lopes et al. (2023) evaluating the impacts of the pesticides 2,4-D and fipronil, isolated and combined, to the same species, suggesting that this species is sensitive to metals and pesticides.

According to SSD curves (Fig. 2) constructed based on literature data (Tables S1 and S2, Supplementary material), it is possible to observe that *Notodiaptomus iheringi* was the Brazilian native species most sensitive to Cu and the second most sensitive to Cd, being less sensitive than the cladoceran *Simocephalus serrulatus*. The mean values and 95% confidence intervals for HC_5 and HC_{50} calculated for Cu were, respectively, 4.9 (1.3–12.6) μg L^{-1} and 180 (110−380) μg L^{-1}. For Cd, the calculated HC_5 and HC_{50} values were, respectively, 2.4 (0.53–7.31) μg L^{-1} and 220 (100−530) μg L^{-1}. The results indicate that *Notodiaptomus iheringi* would be potentially protected for both metals regarding the HC_5; however, they would not be potentially protected for any of the metals regarding HC_{50}. According to the limit levels established in Brazilian legislation for Cu (9 μg L^{-1}, Brazil, 2005) and for Cd (1 μg L^{-1}, Brazil, 2005), the recommendations would protect 50% of species according to HC_{50} for both metals, but not the 5% (HC_5) in the case of Cu. *N. iheringi* is a sensitive species to different contaminants (Lopes et al., 2023; Rocha et al., 2023), and it would be potentially protected for both metals in environments with concentrations below the recommended regulations.

The data obtained for the metal mixture using MixTox are shown in Table 1. Both models CA and IA were validated, and it was possible to calculate all of the deviations from the CA model, i.e., S/A, DR, and DL. For the IA model, the S/A deviation was not significant, and it was not possible to calculate the DR and the DL deviations. For the CA model (Table 1), the sum of squared residuals (SS) was 31.39 ($p < 0.05$; $r^2 = 0.74$). After adding the "a" parameter in the S/A deviation, the SS value decreased to 14.96 ($p < 0.05$; $r^2 = 0.87$), and it was the best deviation for CA. In DR deviation, parameters "a" and "bDR" were added, and the SS was 14.95 ($p < 0.05$; $r^2 = 0.87$), and in DL deviation, the SS was 17.43 ($p < 0.05$; $r^2 = 0.85$). Thus, based on lower p-value followed by lower residuals, the CA model was the best fit to our data, and the S/A deviation was the best fit to the model, indicating antagonism ($a > 0$) in the mixtures. In the DL deviation, it is possible to observe synergism at low doses and antagonism at high doses ($a < 0$), and this change occurred in doses lower than LC_{50} ($b_{DL} > 1$). Data obtained for S/A from the CA model are presented in the isobologram (Fig. 3).

The prediction of metallic mixtures toxicity to aquatic organisms is still challenging (Clément et al., 2022) and there is no universal pattern in the responses, even when using models to predict the interactions (Farley et al., 2015; Meyer et al., 2015b). Some complicating factors in estimating the metallic mixtures' effects in aquatic systems are related with the essentiality of some metals, their bioavailability and metals speciation (Van Genderen et al., 2015; Väänänen et al., 2018), which can change according to several characteristics, such as temperature, pH, water hardness, amount and quality of organic matter. Cooper et al. (2009) observed antagonism, synergism and additivity effects on two freshwater cladocerans exposed to Cu, Pb and Zn, and the responses were related to the metal combination, being different between species. Naddy et al. (2015) observed synergism and antagonism on a cladoceran exposed to the mixture of Cu, Cd and Zn, and the different responses were related to the water hardness. In a study with a tropical Ostracoda (*Strandesia trispinosa*), Lima et al. (2023) observed different responses of antagonism and synergism in mixtures of Cu and mercury or Cu and manganese, depending on the concentration of each metal. Reviews of metallic mixtures indicate that antagonism can range from 43% to 51%, while synergism occurs in ≈ 28%, and additivity in ≈ 22–27% (Norwood et al., 2003; Vijver et al., 2011; Cedergreen, 2014), and a severe synergism response in metallic mixtures is rare (Cedergreen, 2014).

In binary Cu-Cd mixtures, the less-than-addictive toxicity can be due to metal competition for the ligand sites (Franklin et al., 2002; Meyer et al., 2015a). Barata et al. (2006) concluded that mixtures of Cd and Cu were always less than additive in *D. magna*. Although, for Clément et al. (2022), the change on the Cu:Cd ratio impacts the response of metal interaction in daphnids. Antagonism was observed in Cd-rich mixtures and synergism in Cu-rich mixtures. Similar responses were observed by Jonker et al. (2004), where the increase of Cd decreases the toxicity, while the increase of Cu increases the toxicity on *Caenorhabditis elegans*. The results from Lima et al. (2023) in Cu-Cd mixtures to the Ostracoda

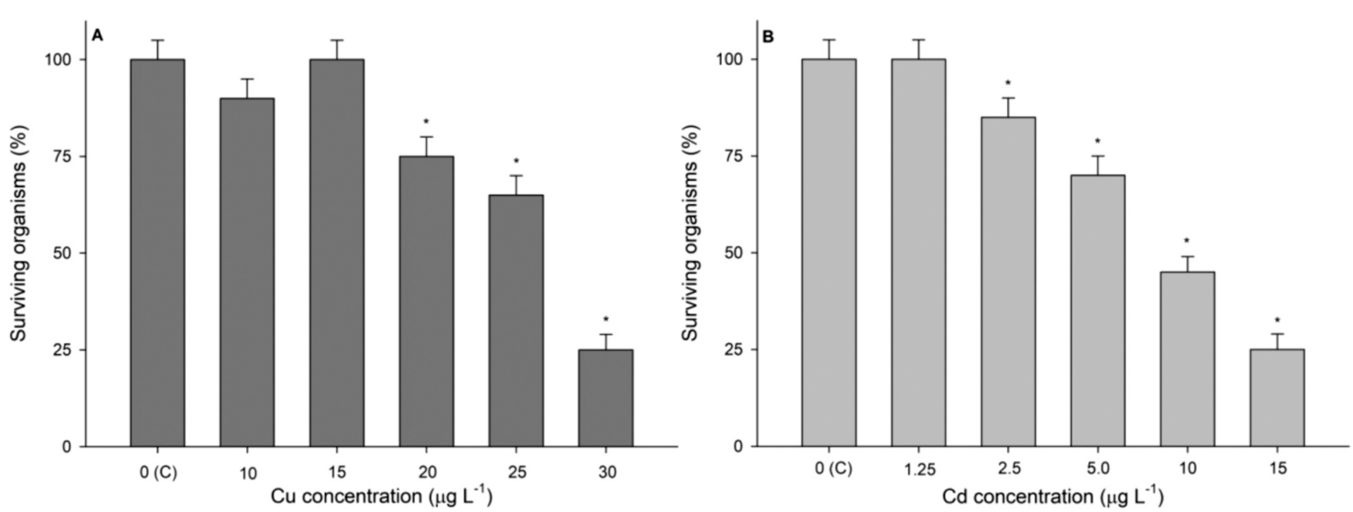

Fig. 1. Percentage of *Notodiaptomus iheringi* adults surviving after 48 h exposure to copper (A) and Cd (B). C indicates control. Error bars indicate standard deviation. * indicates values significantly different from control ($p < 0.05$; One-Way Anova, Dunnett post-test).

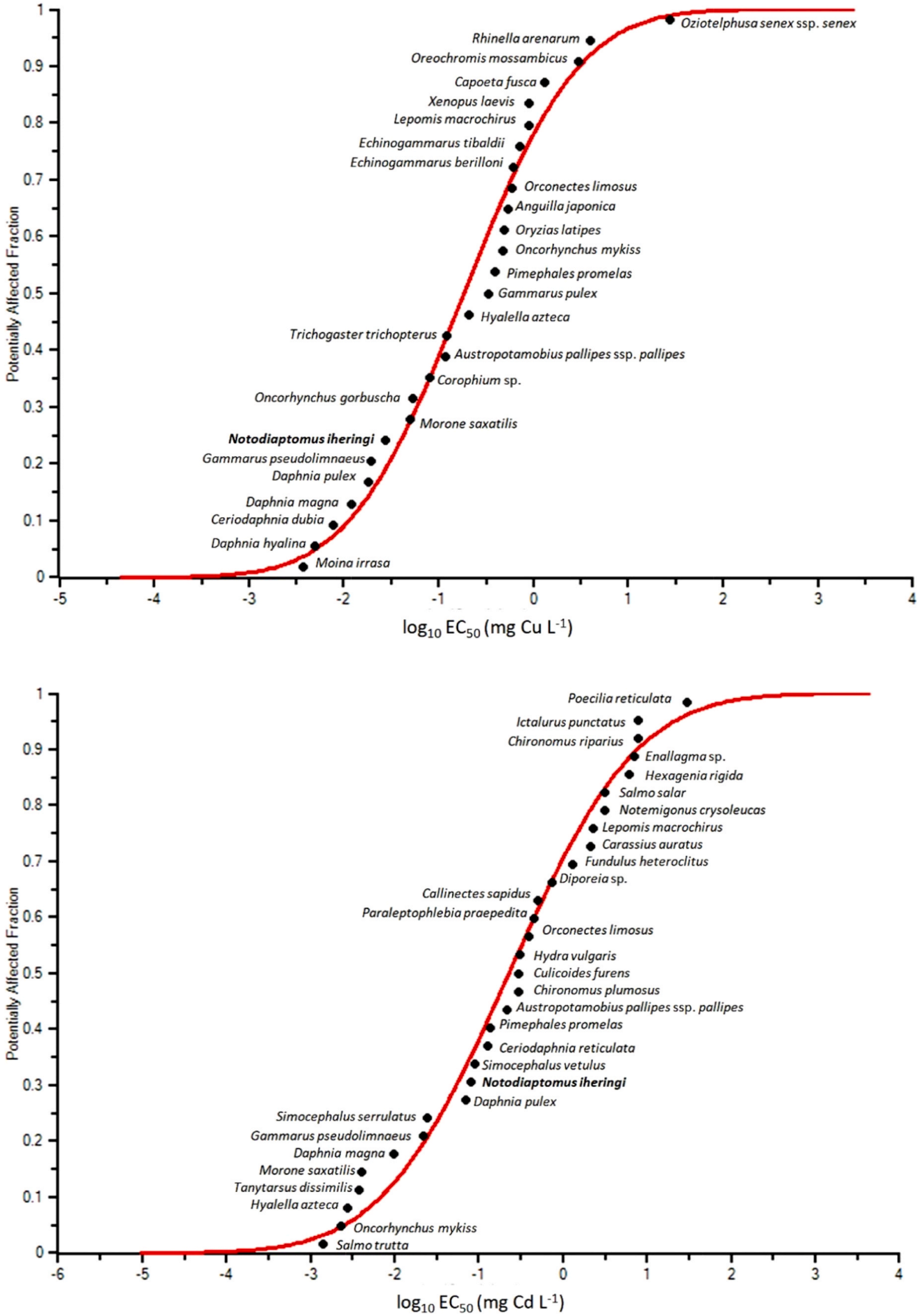

Fig. 2. Species sensitivity distribution (SSD) based on EC/LC/IC50 values (mg metal L^{-1}) of aquatic organisms – obtained from scientific literature, exposed to copper (Cu; upper panel) and cadmium (Cd; lower panel). Data from the present study (*Notodiaptomus iheringi*) are in bold.

Table 1
Data from the reference concentration addition model (CA) applied to the survival of *Notodiaptomus iheringi* (Copepoda) exposed to 48 h to the contaminants copper (Cu), cadmium (Cd), and their mixtures. These data were obtained using the MixTox tool. Max = maximum response value, β = slope response of isolated compounds, LC_{50} = median lethal concentration; *a* and $b_{DR/DL}$ = function parameters; SS = sum of the squared residuals; r^2 = regression coefficient; χ^2 or F test = statistical test; df = degrees of freedom; p (χ^2/F) = level of significance of statistical test; S/A = synergism/antagonism deviation; DR = dose ratio-dependent deviation; DL = dose level-dependent deviation.

	CA	S/A	DR	DL
Max	0.98	**0.98**	0.98	0.98
βCu	2.94	**6.12**	6.23	7.60
βCd	2.12	**2.13**	2.15	2.42
LC50 Cu	36.14	**25.97**	25.96	26.21
LC50 Cd	10.09	**8.28**	8.28	8.67
a	-	**2.00**	2.81	-0.01
$b_{DR/DL}$	-	**-**	-1.18	123.46
SS	31.39	**14.96**	14.95	17.43
r^2	0.74	**0.87**	0.87	0.85
χ^2 or F test	87.44	**16.44**	16.45	13.97
Df	-	**1**	2	2
p (χ^2/F)	4.58×10^{-18}	$\mathbf{5.02\times10^{-5}}$	2.68×10^{-4}	9.25×10^{-4}

*Interpretation of parameters of CA model based in Jonker et al. (2005)
S/A: $a > 0$ = antagonism; $a < 0$ =synergism.
DR: $a > 0$ = Antagonism. Excluding mixture ratios where a significant negative b value indicates synergism; $a < 0$ = Synergism. Excluding mixture ratios where a significant positive b value indicates antagonism; $b_{DR} > 0$ = Antagonism. The toxicity of the mixture is caused mainly by toxicant *i*; $b_{DR} < 0$ = Synergism. The toxicity of the mixture is caused mainly by toxicant *i*.
DL: $a > 0$ = Antagonism at low dose level; synergism at high dose level; $a < 0$ = Synergism at low dose level; antagonism at high dose level; $b_{DL} > 1$ = Change occurs at lower dose level than EC_{50}; $b_{DL} = 1$, The change occurs at EC_{50}; $0 < b_{DL} < 1$, The change occurs at higher dose level than EC_{50}; $b_{DL} < 0$, No change, but the magnitude of synergism/antagonism is effect level dependent.

Strandesia trispinosa were similar to ours, where the concentration addition with antagonistic deviation best explained the data. In our study, the Cu:Cd ratio ranged from 4 (M4) to 24 (M3) and antagonism was observed, which does not corroborate the previous studies where the higher Cu:Cd ratio resulted in synergism; indicating that the responses to Cu-Cd mixtures can be species-specific.

In some situations, one metal can protect the organisms from the toxicity of other contaminants or contributes to their toxicity, such as the case of Zn and Ni protecting *D. magna* from Cd toxicity at intermediate concentrations, but increasing its toxicity at higher concentrations (Pérez and Hoang, 2017, 2018). Rocha et al. (2023) suggested that Cd has a protective effect in combination with fipronil, decreasing the effects of the pesticide in *N. iheringi* in all combinations tested. In the present study, our data confirm the same behavior of Cd protection in the combination with Cu, although it is interesting to highlight that synergism occurred in the mixture of Cd with Cu3 (M2 and M5) and antagonism was observed in the other treatments. While in combination with fipronil the protection of Cd occurs in all combinations, in the present study, this protection was stronger in a higher concentration of Cd, which can indicate a concentration-dependent response in the presence of two contaminants with most likely the same mode of action. Based on our data, we suggest that Cd is the main factor driving the antagonism in mixtures.

Survival of organisms during the sub-chronic tests was not significantly different between treatments (data not shown). In control, all organisms survived, while in the treatments with the contaminants, the average survival was 85–90%. However, significant differences were observed in the reproduction of organisms (Fig. 4). Control females had an average production of 6 eggs/female with a proportion of abortion or non-viable eggs of 20%. Copper-exposed organisms produced more eggs (13.3 eggs/female) but with an abortion/non-viable eggs rate of 81%. The animals exposed to Cd had an average egg production close to that of the control (6.4 eggs/female) but with an abortion/non-viable eggs rate of 67%. The animals exposed to the mixture of metals showed a higher egg production than the control (9.9 eggs/female) but also with a high rate of abortion/non-viable eggs (75%). The non-viable eggs were usually totally white; however, we could observe black and white or smaller black eggs in smaller proportions. In some organisms, we could also observe the presence of empty egg sacs, i.e., there was the formation of egg sacs indicating that copulation occurred, but the sacs were without eggs. Our data suggest that the greater production of eggs in the treatments is a detoxification pathway for eliminating metals since the eggs produced were expelled, and the hatching of nauplii was lower than that observed in control. We did not observe nauplii with morphological changes or with altered behavior, such as erratic or slower movements.

The impairment in reproduction of freshwater invertebrates due to Cu and Cd exposure are reported in the literature (Barata and Baird, 2000; Rodgher et al., 2008; Souza et al., 2014), and the effects of Cd in the embryonic development was demonstrated (Pérez and Hoang, 2017). Our data for Cd exposure differ from those by Barata and Baird (2000), where the authors did not observe reductions in egg survival of *D. magna* exposed to Cd. According to Hook and Fisher (2001a) the vitellogenesis seems to be altered in copepods exposed to metals, resulting in lower production of eggs. Unfortunately, we were not able to measure the amount of metals in the eggs; however, the higher production of eggs can be a detoxification mechanism in *N. iheringi,* especially under Cu exposure. In the presence of metals, the animals produced a higher quantity of eggs, most of them not viable. The amount of viable eggs was significantly lower in the organisms under metal exposures compared to control; however, the amount of viable eggs produced was similar under Cu, Cd and CuCd exposures. Hook and Fisher (2001a) (2001b) observed decreased egg production and hatching rates exposing the marine copepods *Acartia tonsa* and *A. hudsonica* to dissolved Cd, silver and mercury; with no significant difference in percentage of hatched eggs under dissolved Cd or silver exposure. However, these authors observed significant decrease in hatching rate when the organisms were exposed to algae contaminated with these metals (trophic transfer).

4. Conclusion

Our data indicate that *Notodiaptomus iheringi* is a sensitive organism to metal contamination. The organisms were affected by environmental concentrations of Cu and Cd, isolated and in mixture, in acute or sub-chronic exposures. In acute tests, it was observed morphological changes in the organisms exposed to the contaminants, isolated or in mixture, and antagonism was observed in the metallic mixture. The impairments were caused by the highest Cu concentration evaluated, while Cd looks like to be the responsible for the antagonism. In the sub-chronic experiments, the reproduction of organisms was impaired under Cu, Cd or CuCd exposures, with higher amount of eggs produced in Cu and CuCd, and with higher rates of abortion/non-viable eggs in all treatments compared to control. We recommend more studies using this species, especially with regards to the production of reactive oxygen species and oxidative stress, which can provide a better understanding of the effects of the metals in the copepods.

Author contributions

GSR: Conceptualization; Formal analysis; Investigation; Funding acquisition; Writing – review and editing. LFPL: Conceptualization; Formal analysis; Investigation; Writing – review and editing. ELGE: Supervision; Funding acquisition; Writing – review and editing. GSR and LFPL contributed equally in this manuscript and should be considered as first authors.

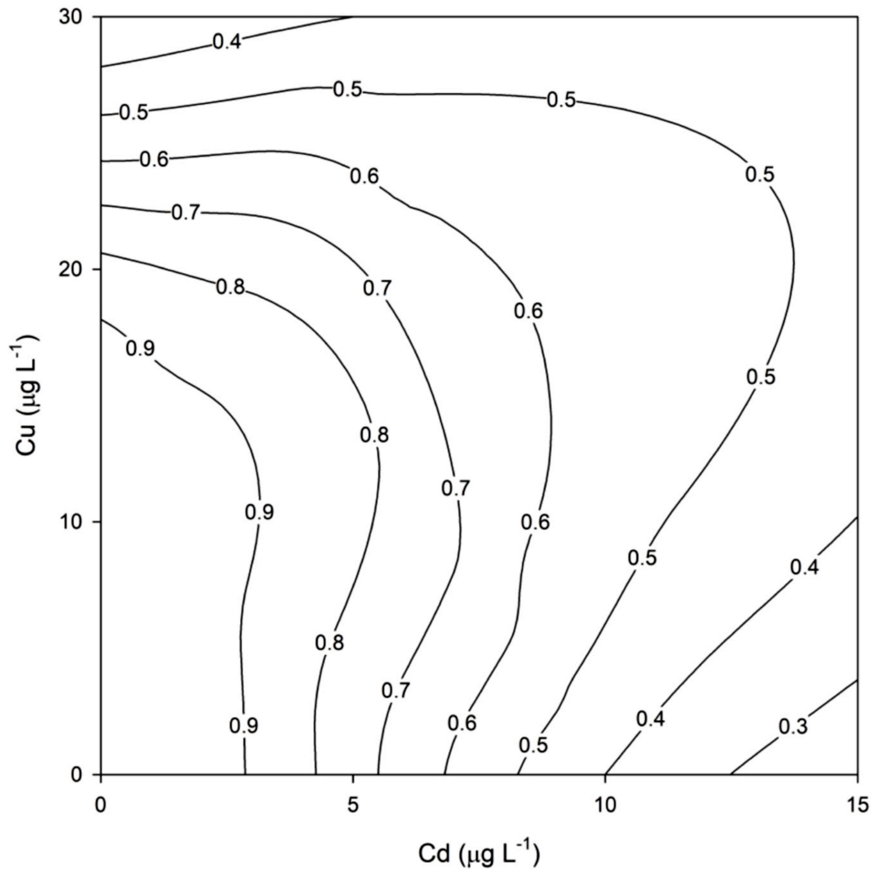

Fig. 3. Mixture data from *Notodiaptomus iheringi* exposed to copper (Cu) and cadmium (Cd) mixtures for 48 h. The isobologram represents the concentration addition model with antagonistic deviation, represented by the convex shapes. Numbers along the shapes represent the modeled mortality ratios of copepods by the deviation model.

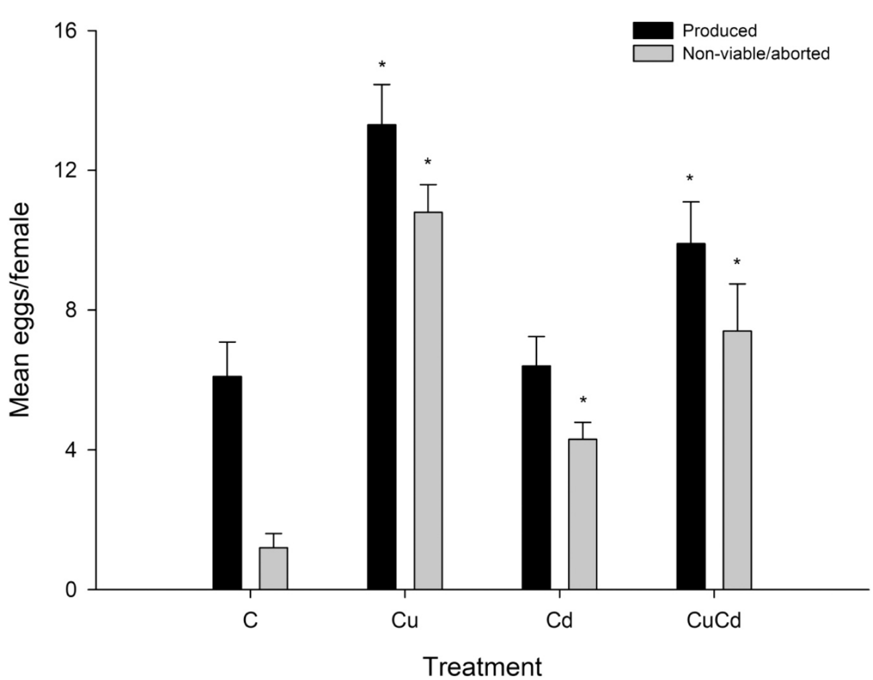

Fig. 4. Mean produced (black bars) and aborted/non-viable eggs (gray bars) by females of *Notodiaptomus iheringi* during the sub-chronic test (8 days). C (control), Cu (copper), Cd (cadmium), CuCd (copper and cadmium in mixture). Error bars indicate standard deviation. * indicates values significantly different from control ($p < 0.05$; One-Way Anova, Dunnett post-test).

Declaration of Competing Interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

Acknowledgements

The authors thank Grant #2015/25436–1 São Paulo Research Foundation (FAPESP). *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) granted a scholarship to L.F.P.L. (88882.379019/2019–01).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.etap.2023.104326.

References

ABNT - Brazilian National Standards Organization (Associação Brasileira de Normas Técnicas). 2016. Aquatic ecotoxicology - Acute toxicity - Test with *Daphnia* spp (Cladocera, Crustacea). NBR 12713. Rio de Janeiro, RJ, 27 pp.
ABNT - Brazilian National Standards Organization (Associação Brasileira de Normas Técnicas) (2017). Aquatic ecotoxicology - Chronic toxicity - Test method with *Ceriodaphnia* spp (Crustacea, Cladocera). NBR 13373. Rio de Janeiro, RJ. 20 pp.
Aldenberg, T., Jaworska, J.S., 2000. Uncertainty of the hazardous concentration and fraction affected for normal species sensitivity distributions. Ecotoxicol. Environ. Saf. 46, 1–18.
Backhaus, T., Faust, M., Scholze, M., Gramatica, P., Vighi, M., Grimme, L.H., 2004. Joint algal toxicity of phenylurea herbicides is equally predictable by concentration addition and independent action. Environ. Toxicol. Chem. 23 (2), 258–264.
Baracho, D.H., Silva, J.C., Lombardi, A.T., 2019. The effects of copper on photosynthesis and biomolecules yield in *Chlorolobion braunii*. J. Phycol. 55 (6), 1335–1347.
Barata, C., Baird, D.J., 2000. Determining the ecotoxicological mode of action of chemicals from measurements made on individuals: results from instar-based tests with *Daphnia magna* Straus. Aquat. Toxicol. 48, 195–209.
Barata, C., Baird, D.J., Nogueira, A.J.A., Soares, A.M.V.M., Riva, M.C., 2006. Toxicity of binary mixtures of metals and pyrethroid insecticides to *Daphnia magna* Straus. Implications for multi-substance risks assessment. Aquat. Toxicol. 78, 1–14.
Bielmyer, G.K., Grosell, M., Brix, K.V., 2006. Toxicity of silver, zinc, copper, and nickel to copepod *Acartiatonsa*exposed via a phytoplankton diet. Environ. Sci. Technol. 40, 2063–2068.
Brazil - Ministry of the Environment (2005). National Environmental Council - CONAMA. Resolution n. 357/2005. March 17th. Brasília, DF, 7 pp.
Briffa, J., Sinagra, E., Blundell, R., 2020. Heavy metal pollution in the environment and their toxicological effects on humans. Heliyon 6, e04691.
Brown, R.J., Rundle, S.D., Hutchinson, T.H., Williams, T.D., Jones, M.B., 2005. A microplate freshwater copepod bioassay for evaluating acute and chronic effects of chemicals. Environ. Toxicol. Chem. 24 (6), 1528–1531.
Campaner, V.P., Luiz-Silva, W., Machado, W., 2014. Geochemistry of acid mine drainage from a coal mining area and processes controlling metal attenuation in stream waters, southern Brazil. An. Acad. Bras. Ciências 86 (2), 539–544.
Cedergreen, N., 2014. Quantifying synergy: a systematic review of mixture toxicity studies within environmental toxicology. PLoS ONE 9. https://doi.org/10.1371/journal.pone.0096580.
CETESB (2018) Qualidade das águas interiores no Estado de São Paulo 2017, Relatórios. Série Relatórios/CETESB, São Paulo.
Clément, B., Felix, V., Bertrand, V., 2022. The toxicity of cadmium-copper mxtures on daphnids and microalgae analyzed using the Biotic Ligand Model. Environ. Sci. Poll. Res. Int. 29, 29285–29295.
Cooper, N.L., Bidwell, J.R., Kumar, A., 2009. Toxicity of copper, lead, and zinc mixtures to *Ceriodaphnia dubia* and *Daphnia carinata*. Ecotoxicol. Environ. Saf. 72, 1523–1528.
Faria, M., Wu, X., Luja-Mondragón, M., Prats, E., Gómez-Oliván, L.M., Piña, B., Raldúa, D., 2020. Screening anti-predator behaviour in fish larvae exposed to environmental pollutants. Sci. Total Environ. 714, 136759.
Farley, K.J., Meyer, J.S., Balistrieri, L.S., De Schamphelaere, K.A.C., Iwasaki, Y., Janssen, C.R., Kamo, M., Lofts, S., Mebane, C.A., Naito, W., Ryan, A.C., Santore, R.C., Tipping, E., 2015. Metal mixture modeling evaluation project: 2. Comp. four Model. Approaches Environ. Toxicol. Chem. 34 (4), 741–753.
Franklin, N.M., Stauber, J.L., Lim, R.P., Petocz, P., 2002. Toxicity of metal mixtures to a tropical freshwater alga (*Chlorella* sp.): the effect of interactions between copper, cadmium, and zinc on metal cell binding and uptake. Environ. Toxicol. Chem. 11, 2412–2422.
Gebara, R.C., Alho, L.O.G., Abreu, C.B., Mansano, A.S., Moreira, R.A., Rocha, G.S., Melão, M.G.G., 2021. Toxicity and risk assessment of zinc and aluminum mixtures to *Ceriodaphnia silvestrii* (Crustacea: Cladocera). Environ. Toxicol. Chem. 40 (10), 2912–2922.
Gebara, R.C., Alho, L.O.G., Mansano, A.S., Rocha, G.S., Melão, M.G.G., 2023. Single and combined effects of Zn and Al on photosystem II of the Green microalgae *Raphidocelis subcapitata* assessed by pulse-amplitude modulated (PAM) fluorometry. Aquat. Toxicol. 254, 106369.
Gopalapillai, Y., Hale, B., 2015. Evaluating the concentration addition approach for describing expected toxicity of a ternary metal mixture (Ni, Cu, Cd) using metal speciation and response surface regression. Environ. Chem. 13 (3), 447–456.
Gutierrez, M.F., Gagneten, A.M., Paggi, J.C., 2010. Copper and chromium alter life cycle variables and the equiproportional development of the freshwater copepod *Notodiaptomus conifer* (SARS). Water Air Soil Poll. 213, 275–286.
Hook, S.E., Fisher, N.S., 2001a. Reproductive toxicity of metals in calanoid copepods. Mar. Biol. 138, 1131–1140.
Hook, S.E., Fisher, N.S., 2001b. Sublethal effects of silver in zooplankton: Importance of exposure pathways and implications for toxicity testing. Environ. Toxicol. Chem. 20 (3), 568–574.
ISO (2008) ISO 20665:2008 – Water quality – Determination of chronic toxicity to Ceriodaphnia dubia.
Jia, D., Li, X., Du, S., Xu, N., Zhang, W., Ynag, R., Zhang, Y., He, Y., Zhang, Y., 2020. Single and combined effects of carbamazepine and copper on nervous and antioxidant systems of zebrafish (*Danio rerio*). Environ. Toxicol. 35, 1091–1099.
Jonker, M.J., Piskiewicz, A.M., Castella, N.I.I., Kammenga, J.E., 2004. Toxicity of binary mixtures of cadmium-copper and carbendazim-copper to the nematode *Caenorhabditis elegans*. Environ. Toxicol. Chem. 23 (6), 1529–1537.
Jonker, M.J., Svendsen, C., Bedaux, J.J.M., Bongers, M., Kammenga, J.E., 2005. Significance testing of synergistic/antagonistic, dose-level dependent, or dose ratio-dependent effects in mixture dose response analysis. Environ. Toxicol. Chem. 24, 2701–2713.
Kadiene, E.U., Ouddane, B., Hwang, J.S., Souissi, S., 2019. Bioaccumulation of metals in calanoid copepods by oral intake. Sci. Rep. 9, 9492.
Kortenkamp, A., Faust, M., 2018. Regulate to reduce chemical mixture risk. Science 361 (6399), 224–226.
Kulkarni, D., Gergs, A., Hommen, U., Ratte, H.T., Preuss, T.G., 2013. A plea for the use of copepods in freshwater ecotoxicology. Environ. Sci. Poll. Res. 20, 75–85.
Lima, J.C.S., Moreira, R.A., Gazonato-Neto, A.J., Freitas, E.C., Daam, M.A., Rocha, O., 2023. Toxicity of binary metal mixtures to the Tropical Ostracoda *Strandesia trispinosa*. Bull. Environ. Cont. Toxicol. 110 (3), 64.
Lopes, L.F.P., Rocha, G.S., Medeiros, J.F., Montagner, C.C., Espíndola, E.L.G., 2023. The acute effects of fipronil and 2,4-D, individually and in mixture: a threat to the freshwater Calanoida copepod *Notodiaptomus iheringi*. Environ. Sci. Poll. Res. Int. https://doi.org/10.1007/s11356-023-28066-y.
Martin, O.V., Bopp, S., Ermler, S., Kienzler, A., McPhie, J., Paini, A., Parrisis, N., Richarz, A.N., Scholze, M., van der Linden, S., Kortenkamp, A., 2018. Protocol for a systematic review of ten years of research on interactions in chemical mixtures of environmental pollutants. Zenodo. https://doi.org/10.5281/ZENODO.1319759.
Matsumura-Tundisi, T., Tundisi, J.G., 2003. Calanoida (Copepoda) species composition changes in the reservoirs of São Paulo State (Brazil) in the last twenty years. Hydrobiologia 504 (1), 215–222.
Meyer, J.S., Ranville, J.F., Pontasch, M., Gorsuch, J.W., Adams, W.J., 2015a. Acute toxicity of binary and ternary mixtures of Cd, Cu, and Zn to *Daphnia magna*. Environ. Toxicol. Chem. 34 (4), 799–808.
Meyer, J.S., Farley, K.J., Garman, E.R., 2015b. Metal mixtures modeling evaluation project: 1. Backgr. Environ. Toxicol. Chem. 34 (4), 726–740.
Naddy, R.B., Cohen, A.S., Stubblefield, W.A., 2015. The interactive toxicity of cadmium, copper, and zinc to *Ceriodaphnia dubia* and rainbow trout (*Oncorhynchus mykiss*). Environ. Toxicol. Chem. 34, 809–815.
Nagajyoti, P.C., Lee, K.D., Sreekanth, T.V.M., 2010. Heavy metals, occurrence and toxicity for plants: a review. Environ. Chem. Lett. 8, 199–216.
Norwood, W.P., Borgmann, U., Dixon, D.G., Wallace, A., 2003. Effects of metal mixtures on aquatic biota: a review of observations and methods. Hum. Ecol. Risk Assess. 9 (4), 795–811.
Nour, H.E., El-Sorogy, A.S., El-Wahab, M.A., Nouh, E.S., Mohamaden, M., Al-Kahtany, K., 2019. Contamination and ecological risk assessment of hravy metals pollution from the Shalateen coastal sediments, Red Sea, Egypt. Mar. Poll. Bull. 144, 167–172.
OECD, 2011. Report of progress on the interlaboratory validation of the OECD harpacticoid copepod development and reproduction test. Ser. Test. Assess. No. 158, 53.
OECD (2012) Daphnia magna reproduction test. OECD Guidelines for Testing Chemicals, Section 2, Test No. 211. 25 pp.
OECD, 2014. New guidance document on harpacticoid copepod development and reproduction test with *Amphiascus*. Ser. Test. Assess. No. 201, 36.
Pan H., Zhang X., Ren B., Yang H., Ren Z., Wang W. (2017) Toxic Assessment of Cadmium Based on Online Swimming Behavior and the Continuous AChE Activity in the Gill of Zebrafish (Danio rerio) Water Air Soil Poll., 228: 355.
Pérez, E., Hoang, T., 2017. Chronic toxicity of binary-metal mixtures of cadmium and zinc to *Daphnia magna*. Environ. Toxicol. Chem. 36 (10), 2739–2749.
Pérez, E., Hoang, T., 2018. Responses of *Daphnia magna* to chronic exposure of cadmium and nickel mixtures. Chemosphere 208, 991–1001.
Perlatti, F., Martins, E.P., Oliveira, D.P., Ruiz, F., Asensio, V., Rezende, C.F., Otero, X.J., Ferreira, T.O., 2021. Copper release from waste rocks in an abandoned mine (NE, Brazil) and its impacts on ecosystem environmental quality. Chemosphere 262, 127843.
Rocha, G.S., Tonietto, A.E., Lombardi, A.T., Melão, M.G.G., 2016. Effect of copper contaminated food on the life cycle and secondary production of *Daphnia laevis*. Ecotoxicol. Environ. Saf. 133, 235–242.

Rocha, G.S., Parrish, C.C., Espíndola, E.L.G., 2020. Shifts in photosynthetic parameters and lipid production of the freshwater microalga *Selenastrum gracile* (Chlorophyceae) under cadmium exposure. J. Appl. Phycol. 32, 4047–4055.

Rocha, G.S., Parrish, C.C., Espíndola, E.L.G., 2021. Effects of copper on photosynthetic and physiological parameters of a freshwater microalga (Chlorophyceae). Algal Res. 54 (6), 102223.

Rocha, G.S., Lopes, L.F.P., Medeiros, J.F., Montagner, C.C., Espíndola, E.L.G., 2023. Environmental concentrations of cadmium and fipronil, isolated and combined, impair the survival and reproduction of a Neotropical freshwater copepod. Environ. Poll. 336, 122415.

Rodgher, S., Lombardi, A.T., Melão, M.G.G., Tonietto, A.E., 2008. Change in life cycle parameters and feeding rate of *Ceriodaphnia silvestrii* Daday (Crustacea, Cladocera) exposure to dietary copper. Ecotoxicology 17, 826–833.

Souza, J.P., Melo, D.C., Lombardi, A.T., Melão, M.G.G., 2014. Effects of dietborne cadmium on life history and secondary production of a tropical freshwater cladoceran. Ecotoxicology 23 (9), 1764–1773.

UK EPA (2007). United Kingdom Environmental Agency. The direct toxicity assessment of aqueous environmental samples using the marine copepod Tisbe battagliari lethality test - Methods for the examination of waters and associated materials. Leicertersheine, UK, 35 p.

Väänänen, K., Leppänen, M.T., Chen, X.P., Akkanen, J., 2018. Metal bioavailability in ecological risk assessment of freshwater ecosystems: from science to environmental management. Ecotoxicol. Environ. Saf. 147, 430–446.

Van Genderen, E., Adams, W., Dwyer, R., Garman, E., Gorsuch, J., 2015. Modeling and interpreting biological effects of mixtures in the environment: introduction to the metal mixture modeling evaluation project. Environ. Toxicol. Chem. 34 (4), 721–725.

Van Vlaardigen P., Traas T.P., Wintersen A., Aldenberg T. (2004) ETX 2.0 A program to calculate hazardous concentrations and fraction affected, based on normally distributed toxicity data. RIVM report 601501028. (⟨https://rivm.openrepository.com/handle/10029/9005⟩).

Vijver, M.G., Elliott, E.G., Peijnenburg, W.J.G.M., de Snoo, G.R., 2011. Response predictions for organisms water-exposed to metal mixtures: a meta-analysis. Environ. Toxicol. Chem. 30, 1482–1487.

Volz, S.N., Hausen, J., Nachev, M., Ottermanns, R., Schiwy, S., Hollert, H., 2020. Short exposure to cadmium disrupts the olfactory system of zebrafish (*Danio rerio*) – Relating altered gene expression in the olfactory organ to behavioral deficits. Aquat. Toxicol. 226, 105555.

Willis, K.J., 1999. Acute and chronic bioassays with New Zealand freshwater copepods using pentachlorophenol. Environ. Toxicol. Chem. 18 (11), 2580–2586.