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Acute and chronic ecotoxicological effects of pharmaceuticals and their mixtures in *Daphnia similis*

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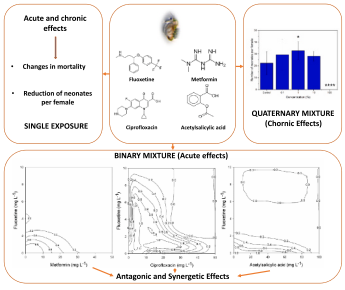
HIGHLIGHTS

- Acute and chronic toxicity of four single pharmaceuticals was evaluated to *Daphnia similis*.
- Potential adverse effects of mixtures containing the fluoxetine and other pharmaceuticals from distinct classes were also demonstrated.
- Chronic assays were also performed for the quaternary mixture at lower concentrations.

GRAPHICAL ABSTRACT



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ABSTRACT

Pharmaceuticals have increasingly received attention from the scientific community due to their growing intake, improved detection and potential ecological risks. Several pharmaceuticals, including antidepressants, anti-inflammatory and antidiabetic compounds and antibiotics, have been described as contaminants in different water matrices. In this context, the aim of the present study was to assess the acute and chronic effects of four classes of pharmaceuticals (acetylsalicylic acid, fluoxetine, metformin and ciprofloxacin) individually and in binary and quartenary mixture. Furthermore, the toxicity of binary mixtures containing the antidepressant fluoxetine was also evaluated. The results of the single acute and chronic toxicity assays indicate lower acetylsalicylic acid and higher fluoxetine toxicity towards *Daphnia similis*. Regarding the evaluated mixture toxicity, the nature of potential toxicological interactions was predicted by applying mathematical concentration addition and independent action models. The findings revealed both antagonistic and synergistic features, depending on the applied amounts and doses. Finally, the chronic assays performed with the quaternary mixture indicated the presence of a hormetic effect at low concentrations. In sum, the present study demonstrated that the effects of individual pharmaceuticals can underestimate the risk level of these contaminants in the environment.

1. Introduction

The development of pharmaceutical products has brought about important social gains, as these compounds possess a central role in contemporary therapy. Thousands of active compounds are employed at large scale for human and animal disease treatment or prevention and

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public health and life quality maintenance (Grenni et al., 2018). On the other hand, the constant increase in the production, prescription and use of many drugs have led to the contamination of many ecosystems.

Pharmaceutical compounds are one of the prevalent emerging pollutants in water samples in a few Latin America countries, such as Brazil, Mexico, Costa Rica, Colombia, Argentina (Souza et al., 2022). Occurrence rates, toxicological effects and risk assessments of multiple therapeutic drugs, such as non-steroidal anti-inflammatory (Tyumina et al., 2020) and psychiatric drugs (Castillo-Zacarías et al., 2021), as well as antidiabetics (Briones et al., 2016), and antibiotics (Singh et al., 2019), have been reported in the literature.

Depressive disorders affect about 350 million people worldwide (Rezende et al., 2021), and the consumption of antidepressant drugs has increased significantly worldwide during the recent COVID-19 pandemic (Diaz-Camal et al., 2022). According to a report from Organization for Economic Cooperation and Development (OECD) countries, the consumption of antidepressant medication has indeed doubled from 2000 to 2019 (OECD, 2021).

One pharmaceutical of particular concern is the antidepressant fluoxetine (FXT), as it has become one of the mostly widely prescribed drugs used to treat depression and anxiety. This pharmaceutical is a selective serotonin uptake inhibitor is widely employed in medicine due to its safer profile, side effects, and tolerability, and display important roles in the central nervous system, *i.e.,* neuroprotection, also exhibitingantioxidant, anti-inflammatory and antiapoptotic properties (Caiaffo et al., 2016). Incomplete FXT removal in sewage treatment plants (STPs), resulting in potential adverse biota effects and ecological risks, have been demonstrated in different studies (Castillo-Zacarías et al., 2021; de Souza et al., 2021; Ramírez-Morales et al., 2020), with adverse effects comprising both physiological and behavioral alterations in aquatic organisms at ecologically relevant concentrations (de Farias et al., 2019; Schultz et al., 2011; Sehonova et al., 2018).

Aspirin (acetylsalicylic acid, ASA) is another pharmaceutical compound widely consumed worldwide, employed as an analgesic, antipyretic and anti-inflammatory drug and also used in cardiovascular and cerebrovascular disease prevention, due to its antiplatelet properties (Montinari et al., 2019).

Metformin (MET) is widely prescribed for the prevention of chronic diseases, such as type 2 diabetes mellitus, as well as in the treatment of polycystic ovary syndrome and certain cancers (Ambrosio-Albuquerque et al., 2021). Metformin environmental impact studies are, however, still relatively recent and assessments are scarce (Ambrosio-Albuquerque et al., 2021; Elizalde-Velázquez and Gómez-Oliván, 2020). In one study, Niemuth et al. (2015) suggest estrogenic effects in male fish exposed to environmentally relevant concentrations of this drug.

The World Health Organization indicates that antibiotics, such as ciprofloxacin (CIP), represent another particularly important class of contaminants, as they may cause adverse ecosystem effects and potential risks to human health due to the development of antibiotic resistance (Kelly and Brooks, 2018).

Ecotoxicological data obtained from the assessment of pharmaceutical aquatic environment effects include adverse outcomes to several species, such as genotoxic effects, physiological and behavioral alterations (de Farias et al., 2019; Nunes et al., 2018; Schultz et al., 2011; Sehonova et al., 2018; Szabelak and Bownik, 2021). In this regard, microcrustacean *Daphnia* is one of the most studied zooplankton species, displaying a relatively well-known ecology, life history and evolutionary history (Chin and Cristescu, 2021). It is considered an important organism model in risk assessment studies and in the reproductive and developmental biology, immunology, genetics, senescence biology and chronobiology fields (Liu et al., 2021). Ecotoxicological assays employing *Daphnia similis* to assess chronic and sublethal effects, *i.e.*, reproduction and growth, have been successfully performed in recent studies regarding pollution monitoring efforts. For example, one study assessed the chronic effects of dyes and textile wastewater (Garcia et al., 2021) on these organisms, while another evaluated chronic effects following exposure to polyfluoroalkyl compounds and fire suppression agents (Cara et al., 2021).

In this context, the present study aims to evaluate acute and chronic effects following exposure to four pharmaceuticals in the cladoceran *Daphnia similis*, also evaluating acute effects of binary mixture and quaternary interactions.

2. Material and methods

2.1. Reagents

Acetylsalicylic acid (2-Acetoxybenzoic acid; CAS number 50-78-2; >99,5%) fluoxetine hydrochloride (N-methyl-3-phenyl-3-[4-(trifluoromethyl) phenoxy]propan-1-amine; CAS number 54910-89-3; 98,8%) were obtained from Labsynth and Divis Pharmaceuticals Pvt. Ltd, respectively, while metformin hydrochloride (1,1 – Dimethylbiguanide hydrochloride; CAS number 115-70-4; 97%) and ciprofloxacin (1-cyclopropyl-6-fluoro-4-oxo-7-piperazin-1-ylquinoline-3-carboxylic acid; CAS number 85721-33-1; >98%) were purchased from Sigma-Aldrich.

2.2. Toxicity assays

Acute toxicity assays with *Daphnia similis* (Claus, 1876) were performed according to the ABNT NBR 12.713/2016 standard. Twenty neonates (6–24 h) were transferred to four recipients containing 10 mL of the test concentration, comprising five organisms per replicate. All tests were performed in a dark room at 20 ± 1 °C. Organisms were not fed during the experiments. Four negative controls and replicates were applied in each test concentration/mixture. Three independent assays were carried out for the single toxicity evaluation of each pharmaceutical, while each binary mixture toxicity test was performed twice. Solution pH, conductivity and dissolved oxygen were determined at the beginning and end of each test. Daphnid immobilization after 48 h of exposure comprised the evaluated endpoint.

Acute toxicity assays of single pharmaceuticals were conducted for fluoxetine, acetylsalicylic acid, metformin and ciprofloxacin and the respective mixture of these contaminants with the evaluated antidepressant. Single nominal test concentrations for fluoxetine (0.63, 1.25, 2.50, 5.00 and 10.0 mg L^{-1}), acetylsalicylic acid (6.25, 12.5, 25.0, 50.0 and 100 mg L^{-1}), and metformin and ciprofloxacin (3.12, 6.25, 12.5, 25.0, 50.0 mg L^{-1}) were established based on a series of preliminary tests for each compound. The mixture toxicity tests with these pharmaceuticals were conducted at all 36 possible concentration combinations. No additional solvent was used.

Chronic toxicity assays of the single pharmaceuticals solutions were performed following the OECD 211 guideline (OECD, 2012). Chronic toxicity assays with the quaternary mixture were performed following the same guideline, altering the exposure time to 14 days as proposed by Vacchi et al. (2016). One neonate (<24 h old), from the third progeny was transferred to a recipient containing 25 mL of the sample solutions. Ten control and ten treatment replicates were used for each concentration. Organisms were fed daily with *Raphidocelis subcapitata* and the test medium was renewed every two/three days. Daphnids were maintained under controlled temperature (20 ± 1 °C) and photoperiod (16 h light:8 h dark) conditions. The number of living offspring produced by each parent animal was recorded. Solution pH, dissolved oxygen and conductivity were determined for each experiment. Body length measures in the single pharmaceutical toxicity assays were performed out using a Leica MZ95 magnifier (3.2 ×) coupled to Leica DFC 295 camera employing the Leica Application Suite V4.12.0 software. Measurements were taken from the base of the antenna to the beginning of the tail, and the results were expressed as millimeters.

Single nominal test concentrations for fluoxetine (0.05, 0.10, 0.20, 0.40 and 0.80 mg L^{-1}), acetylsalicylic acid (1.25, 2.50, 5.00, 10.0 and 20.0 mg L^{-1}), metformin (0.31, 0.62, 1.25, 2.50, 5.00 and 10.0 mg L^{-1})

and ciprofloxacin (0.10, 0.15, 0.30, 0.60, 1.25 and 2.50 mg L^{-1}) were established based on a series of preliminary tests for each compound. For the quaternary mixture, a stock solution containing 1000 μg L^{-1} of each pharmaceutical was prepared in natural water immediately prior to each test. The following concentrations were evaluated in: 0.1; 1.0; 10; and 100%. All toxicity assay validation criteria were applied as described in standard ABNT and OECD protocols (ABNT, 2016; OECD, 2012).

2.3. Data analysis

The EC50% (Effective Concentration 50) and the respective 95% confidence intervals were calculated from the estimated endpoint by the Trimmed Spearman-Karber method (Hamilton et al., 1977).

A one-way analysis of variance (ANOVA) was used to verify differences between the chronic assay treatments and controls. When significant ($p < 0.05$), a Dunnett's post hoc test was applied following the ANOVA assessment. The NOEC (no observed effect concentration) and LOEC (lowest observed effect concentration) were calculated for the chronic assays by this method.

Predictions concerning the acute binary mixture toxicity of the evaluated pharmaceuticals were performed by associating concentration addition and independent models and the respective deviations of each (synergism/antagonism; dose level-dependent or dose ratio-dependent) employing an automated Excel spreadsheet. Comparisons between the concentration addition and independent action models, and their eventual deviations concerning synergistic, antagonistic, dose-ratio dependent or dose-level dependent effects were calculated (Jonker et al., 2005). The best model fit was chosen through the maximum likelihood method by statistically identifying the most appropriate model for deviation description, with effects were deducted directly from the parameter values ("a" and "*b*"), as described in Table 1.

Table 1
- Interpretation of additional parameters ("*a*" and "*b*") that define the functional form of the standard deviations in the concentration addition and independent action model (IA) (Adapted from Jonker et al., 2005).

Deviation	Parameter "*a*" (CA and IA)	Parameter "*b*" (CA)	Parameter "*b*" (IA)
Synergism/ antagonism (S/A)	a >0 – antagonism a <0 – synergism		
Dose ratio-dependent (DR)	a >0 – antagonism, except for mixture ratios where a significant negative *b* value indicates synergism a <0 – synergism, except for mixture ratios where a significant positive value *b* indicates antagonism	$b_i > 0$ – antagonism where the toxicity of the mixture is mainly caused by the toxic agent *i* $b_i < 0$ – synergy where the toxicity of the mixture is mainly caused by toxic *i*	
Dose level-dependent (DL)	a >0 – low dose level antagonism and synergy at high doses a <0 – low dose level synergism and antagonism at high doses	$b_{DL} > 1$: change at lower EC_{50} level $b_{DL} = 1$: change at EC_{50} level $0 < b_{DL} < 1$: change at higher EC_{50} level $b_{DL} < 1$: No change but the magnitude of S/A is DL-dependent	$b_{DL} > 2$ – change at a lower dose than the EC_{50} $b_{DL} = 2$ – change in EC_{50} $1 < b_{DL} < 2$ – change at a higher dose than the EC50 $b_{DL} < 1$ – no change, but the magnitude of S/A is dependent on effect level

3. Results

3.1. Acute toxicity of single pharmaceuticals

A clear concentration-response relationship was demonstrated for all single chemicals in the experiments performed in the acute assay (Fig. 1). Table 2 indicates the acute toxicity results of the individual pharmaceuticals. The $EC50_{48h}$ values indicate higher acute toxicity for fluoxetine ($EC50_{48h=}$1.45 mg L^{-1}) (published in Tominaga et al., 2021), followed by metformin ($EC50_{48h=}$20.4 mg L^{-1}), ciprofloxacin ($EC50_{48h=}$23.2 mg L^{-1}), and acetylsalicylic acid ($EC50_{48h=}$86.1 mg L^{-1}) (published in Tominaga et al., 2021).

3.2. Chronic toxicity of single pharmaceuticals

Higher toxicity for the investigated antidepressant and lower effects for the anti-inflammatory compound were evidenced in the present study. No mortality was noted at the evaluated concentrations for acetylsalicylic acid (up to 20 mg L^{-1}). However, the number of neonate offspring per individual was significantly reduced from 5.0 mg L^{-1} of acetylsalicylic acid (Table 3).

Mortality increased following exposure to fluoxetine, metformin and ciprofloxacin, while the number of neonates per individual decreased with increasing concentrations (data not shown). Significantly reduced numbers of neonates per female were noted from 0.40 mg L^{-1} for fluoxetine, 0.6 mg L^{-1} for ciprofloxacin and 2.5 mg L^{-1} for metformin (Table 3).

Fluoxetine and metformin did not affect the body length of exposed organisms when compared to the controls ($p > 0.05$), while organisms exposed to ciprofloxacin at all tested concentration exhibited significant body size decreases compared to the controls ($p > 0.05$), indicating sublethal effects. With regard to acetylsalicylic acid, a significant decrease in body length was noted from 5.0 mg L^{-1} (Fig. 2).

3.3. Acute toxicity of fluoxetine mixtures towards Daphnia similis

Concerns regarding the toxicity of antidepressant mixtures in the presence of other contaminants is a growing issue, as several studies have demonstrated the effects of fluoxetine under these conditions (Flaherty and Dodson, 2005; Tominaga et al., 2018; Varano et al., 2017). However, a lack of the available data regarding several combinations is still noted.

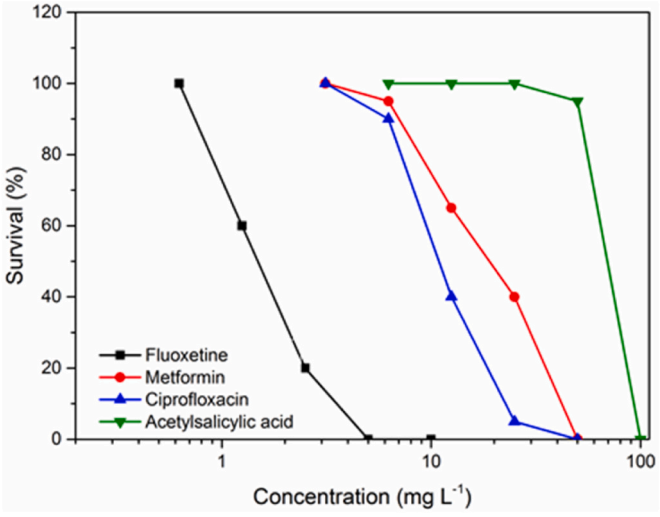

Fig. 1. Dose-response curve for acute toxicity (48 h exposure) of four pharmaceuticals (fluoxetine, metformin ciprofloxacin and acetylsalicylic acid) towards *Daphnia similis*.

Table 2
Effective concentration (EC50) values obtained from the acute toxicity tests with *Daphnia similis* exposed to the single pharmaceuticals fluoxetine, metformin, ciprofloxacin, and acetylsalicylic acid.

Pharmaceutical	EC50 (mg L^{-1})
Fluoxetine	1.45 ± 0.36[a]
Acetylsalicylic acid	86.1 ± 4.63[a]
Metformin	20.4 ± 2.83
Ciprofloxacin	23.2 ± 2.14

[a] data published in Tominaga et al. (2021).

Table 3
Chronic effect (NOEC and LOEC) values concerning pharmaceutical exposure in *D. similis* (fluoxetine, metformin, ciprofloxacin, and acetylsalicylic acid) (21 days).

Pharmaceutical	NOEC (mg L^{-1})	LOEC (mg L^{-1})
Fluoxetine	0.20	0.40
Acetylsalicylic acid	2.50	5.00
Metformin	1.25	2.50
Ciprofloxacin	0.30	0.60

The results of the mixture analysis according to the addition concentration and independent action models and their respective deviations are depicted in Table 4-6Table 4. Parameters were then estimated by analyzing the experimental data relative to the individually assessed pharmaceuticals and their mixture combinations.

3.3.1. Fluoxetine and acetylsalicylic acid mixture

The mixture toxicity results indicate that all models were statistically significant ($p < 0.05$). The derived mixture data fit revealed that the dose level-dependent deviation of the IA model was the best fit model ($SS_{res} = 1.23$; $p < 0.001$; $r^2 = 0.76$; $a = -5.82$; $b_{DL} = 1.10$; Table 4), with the lowest SS_{res} value and highest r^2 values. Furthermore, the acute toxicity results describes synergism at low mixture doses and antagonism at higher doses ($a < 0$), with the interaction change taking place at concentrations above the EC50 ($1 < bDL < 2$) (Table 4; Fig. 3).

3.3.2. Fluoxetine and metformin mixture

The results indicated that all models were statistically significant ($p < 0.05$). The derived mixture data fit revealed that dose-ratio dependent (DR) deviation of the IA model was the best fit model ($SS_{res} = 0.26$; $p < 0.001$; $r^2 = 0.95$; $a = 0.31$; $b_{DR} = -2.68$; Table 5), with the lowest SS_{res} value and highest r^2 values. Furthermore, the acute toxicity results describe the presence of antagonism ($a < 0$), except where synergy occurs mainly due to metformin interactions (Table 5; Fig. 4).

3.3.3. Fluoxetine and ciprofloxacin mixture

The results indicate that both the concentration addition and independent action models were not statistically significant ($p > 0.05$). However, after the addition of the parameters "*a*" and "*b*", the derived mixture data was demonstrated as statistically significant ($p < 0.05$) and the best fit model was the dose level-dependent deviation of the IA model ($SS_{res} = 0.52$; $p < 0.001$; $r^2 = 0.92$; $a = 7244$; $b_{DL} = 1.01$; Table 6), with the lowest SS_{res} value and highest r^2 values. Furthermore, the acute toxicity results describe antagonism at low mixture doses and synergism

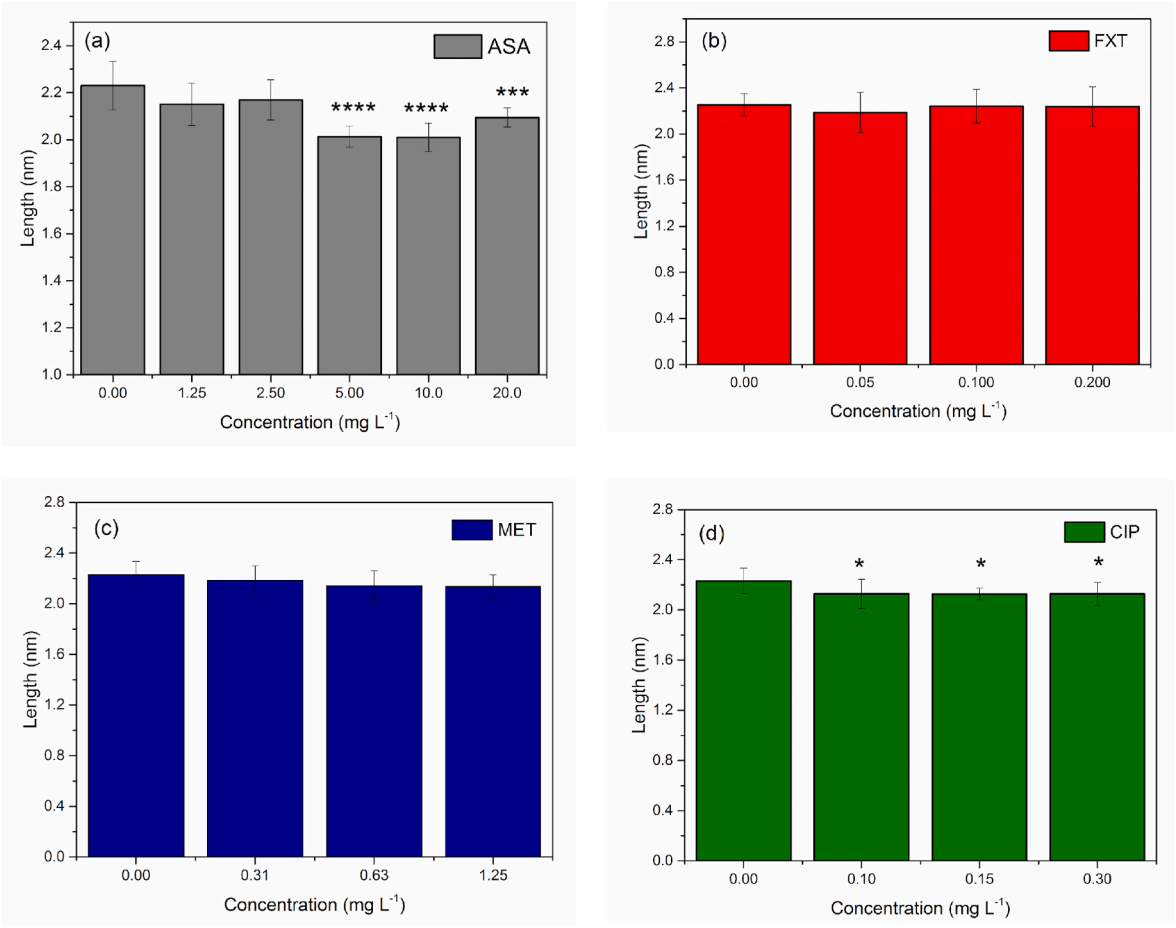

Fig. 2. Sublethal effects (body length) in *Daphnia similis* following chronic exposure (21 days) to four pharmaceuticals (fluoxetine, metformin ciprofloxacin and acetylsalicylic acid).

Table 4
Summary of the acute toxicity analysis of a fluoxetine and acetylsalicylic acid mixture towards *Daphnia similis*.

	CA	CA-S/A	CA-DR	CA-DL	IA	IA-S/A	IA-DR	IA-DL
Max	1	1	1	1	1	1	1	**1**
β_{aas}	20.02	20.02	20.02	20.02	20.02	20.02	20.02	**20.02**
β_{fxt}	3.12	3.12	3.12	3.12	3.12	3.12	3.12	**3.12**
$EC50_{aas}$	57.92	57.92	57.92	57.92	57.92	57.92	57.92	**57.92**
$EC50_{fxt}$	1.49	1.49	1.49	1.49	1.49	1.49	1.49	**1.49**
A	–	−3.85	−5.03	−9.84	–	−4.48	−3.46	**−5.82**
$b_{DR/DL}$	–	–	−15.34	0.76	–	–	−5.72	**1.10**
SS_{res}	1.64	1.58	1.48	1.43	1.93	1.30	1.23	**1.23**
r^2	0.56	0.69	0.71	0.72	0.62	0.74	0.75	**0.76**
χ^2 ou Teste F	16.04	13.17	9.67	12.21	12.54	17.31	12.41	**14.96**
Df	–	30.31	28.31	29.31	–	30.31	28.31	**29.31**
P	3.2×10^{-7}	7.9×10^{-7}	5.0×10^{-6}	7.0×10^{-7}	3.5×10^{-6}	4.7×10^{-8}	4.1×10^{-7}	**1.1×10^{-7}**

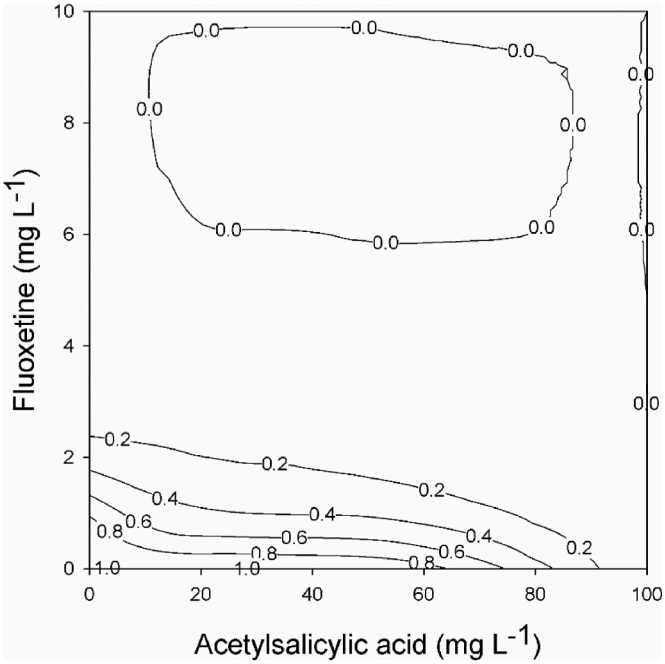

Fig. 3. Isobologram of the effects of a fluoxetine and acetylsalicylic acid mixture concerning *Daphnia similis* immobility, demonstrating a dose level-dependent (DL) deviation from independent action (IA). The linear, concave, and convex isoboles represent no interaction, synergy and antagonism, respectively.

at higher doses (a >0), and the interaction change takes place at concentrations above the EC50 (1 < bDL <2) (Table 6; Fig. 5).

3.4. Chronic toxicity of quaternary mixtures

Mortality was noted only at the highest concentration mixture (100%), while an increase in the average production of neonates compared to the controls was noted for lower concentrations. Significant differences were only demonstrated at 1% ($p = 0.0445$). The average number of neonates per female increased from 22.3 ± 9.66 (Control) to 32.9 ± 7.60 (1%) (Fig. 6). These findings seems to indicated induced reproduction due to the joint of action of fluoxetine, metformin, ciprofloxacin and acetylsalicylic acid at concentrations usually detected in surface freshwater.

4. Discussion

Concerns regarding continuous environmental contamination by pharmaceutical products is a growing issue, with many open questions still noted, mainly related to environmental pharmaceutical mixtures. The acute toxicity assays implemented herein demonstrated that the antidepressant FXT was the most toxic to *Daphnia similis*, while the anti-inflammatory ASA was the lowest, previous assessments. In this regard, $LC50_{48h}$ values of 88.31 mg L^{-1} and 88.33 mg L^{-1} have been reported for *Daphnia magna* following exposure to AAS (Cleuvers, 2004; Gómez-Oliván et al., 2014), as well as $EC50_{48h}$ values of 1.32 mg L^{-1} and 14.3 mg L^{-1} for the same species for FXT and MET, respectively (Godoy et al., 2018; Silva et al., 2016). Regarding CIP, Martins et al. (2012) reported an $EC50_{48h}$ value of 65.3 mg L^{-1} for *D. magna*, thus indicating that *D. similis* is more sensitive to this antibiotic.

High FXT toxicity to organisms belonging to different trophic levels has also been reported. Brooks et al. (2003b) reported a $LOEC_{120h}$ of 13.6 μg L^{-1} for the algae *Raphidocelis subcapitata,* a $NOEC_{7d}$ of 56 μg L^{-1} for the microcrustacean *Ceriodaphnia dubia*, an $EC50_{48h}$ of 820 μg L^{-1} for

Table 5
Summary of the acute toxicity analysis of a fluoxetine and metformin mixture towards *Daphnia similis*.

	CA	CA-S/A	CA-DR	CA-DL	IA	IA-S/A	IA-DR	IA-DL
max	1	1	1	1	1	1	**1**	1
β_{met}	2.32	2.32	2.32	2.32	2.32	2.32	**2.32**	2.32
β_{fxt}	3.12	3.12	3.12	3.12	3.12	3.12	**3.12**	3.12
$EC50_{met}$	18.24	18.24	18.24	18.24	18.24	18.24	**18.24**	18.24
$EC50_{fxt}$	1.49	1.49	1.49	1.49	1.49	1.49	**1.49**	1.49
a	–	4.60	2,88	4.57	–	0.60	**0.31**	0.003
$b_{DR/DL}$	–	–	−3.15	−0.004	–	–	**−2,68**	−367.50
SS_{res}	0.63	0.28	0.23	0.28	0.32	0.30	**0.26**	0.30
r^2	0.87	0.94	0.95	0.94	0.93	0.94	**0.95**	0.94
χ^2 ou Teste F	52.08	98.11	78.18	79.04	109	88.89	**69.34**	72.90
df	–	30.31	28.31	29.31	–	30.31	**28.31**	29.31
p	2.53×10^{-13}	1.64×10^{-8}	1.10×10^{-16}	1.22×10^{-16}	8.37×10^{-18}	4.64×10^{-17}	**5.34×10^{-16}**	3.66×10^{-16}

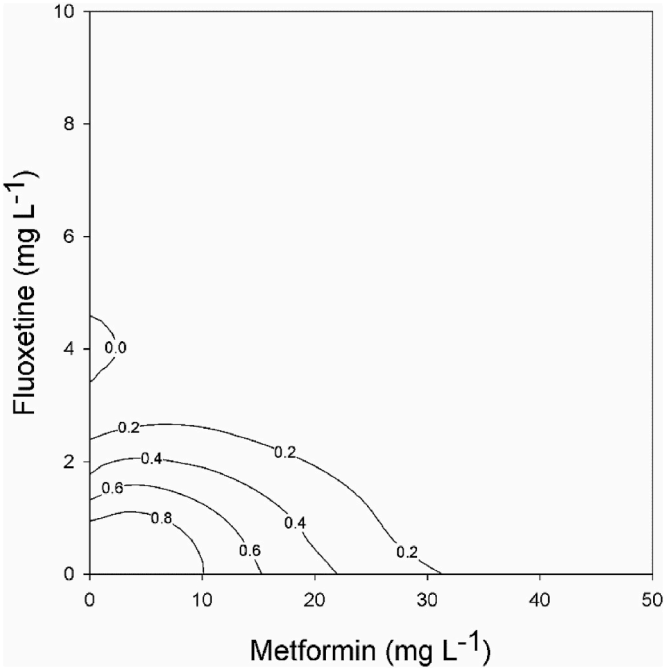

Fig. 4. Isobologram of the effects of a fluoxetine and metformin mixture towards *Daphnia similis* concerning immobility, demonstrating a dose-ratio dependent (DR) deviation from independent action (IA). The linear, concave, and convex isoboles represent no interaction, synergy and antagonism, respectively.

the microcrustacean *D. magna* and an $LC50_{48h}$ of 705 μg L^{-1} for the fish *Pimephales promelas*. In another study, Minguez et al. (2018) determined $IC50_{72h}$ values of 202.9 and 48.2 μg L^{-1} for the algae *Raphidocelis subcapitata* and *Skeletonema marinoi*, respectively. Finally, Bi et al. (2018) evaluated fluoxetine toxicity for seven algae species and demonstrated $NOEC_{96h}$ values ranging between 6.2 and 40.2 μg L^{-1}, while de Farias et al. (2019) reported a $CL50_{168h}$ of 1.18 mg L^{-1} for zebrafish embryos.

Regarding ASA, low toxicity has also been dmeonstrated for different trophic levels. For example, LC50 values of 274.6 and 567.7 mg L^{-1} have been estimated for zebrafish (*Danio rerio*) embryos and juveniles, respectively (Prášková et al. (2012), while Kusk et al. (2018) reported an $EC50_{48h}$ of 241 mg L^{-1} for the algae *Raphidocelis subcapitata* and Cleuvers (2004) estimated an EC50 of 106.7 mg L^{-1} for the algae *Scenedesmus subspicatus*.

Regarding MET and CIP, toxicities vary according to the evaluated species. For MET, Godoy et al. (2018) reported an $EC50_{7d}$ of 53.7 mg L^{-1} for the macrophyte *Lemna minor*; an $EC50_{96h}$ of 2709 mg L^{-1} for the cnidarian for *Hydra attenuatta* and a $CL50_{96h}$ 1315.5 mg L^{-1} for *Danio rerio*. In another assessment, Cleuvers (2004) reported an $EC50_{48h}$ of 64 mg L^{-1} for *Daphnia magna* and an $EC50_{72h}$ > 320 mg L^{-1} for the algae *Desmodesmus subspicatus*, while Moermond and Smit (2016) reported an $EC50_{72h}$ > 77.24 mg L^{-1} for the algae *Raphidocelis subcapitata*. Concerning CIP, Magdaleno et al. (2015) reported an $EC50_{72h}$ of 11.3 mg L^{-1} for the algae *Raphidocelis subcapitata*, while Załęska-Radziwiłł et al. (2011) reported an LC_{96h} > 100 mg L^{-1} for *Danio rerio* and *Lebistes reticulatus* fish, an $EC50_{24h}$ > 100 mg L^{-1} for the protozoan *Tetrahymena thermophila* and $EC50_{24h}$ > 100 mg L^{-1} for the marine crustacean *Artemia salina*.

These findings indicate that, according to the classification proposed by the Harmonized Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures (OECD, 2002), fluoxetine is classified as hazardous to the aquatic environment, in the acute toxicity category II (toxic to aquatic organisms), while the other drugs investigated herein are categorized in the acute toxicity category III (harmful to aquatic organisms). Thus, sublethal effect assessments through additional chronic assays become necessary.

In this regard, the chronic assay parameters obtained in the present

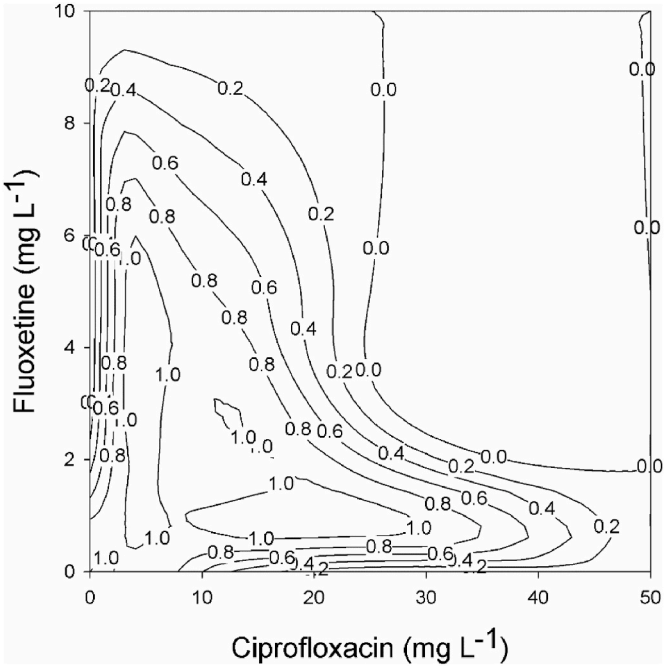

Fig. 5. Isobologram of the effects of a fluoxetine and metformin mixture towards *Daphnia similis* concerning immobility, demonstrating a dose level-dependent (DL) deviation from independent action (IA). The linear, concave, and convex isoboles represent no interaction, synergy and antagonism, respectively.

Table 6
Summary of the parameter analysis of acute toxicity of a fluoxetine and metformin mixture towards *Daphnia similis*.

	CA	CA-S/A	CA-DR	CA-DL	IA	IA-S/A	IA-DR	IA-DL
Max	1	1	1	1	1	1	1	**1**
β_{cip}	3.76	3.76	3.76	3.76	3.76	3.76	3.76	**3.76**
β_{fxt}	3.12	3.12	3.12	3.12	3.12	3.12	3.12	**3.12**
$EC50_{cip}$	11.23	11.23	11.23	11.23	11.23	11.23	11.23	**11.23**
$EC50_{fxt}$	1.49	1.49	1.49	1.49	1.49	1.49	1.49	**1.49**
A	–	21.99	14.13	43.97	–	11.02	7.12	**7244**
$b_{DR/DL}$	–	–	−5.10	0.11	–	–	−2.76	**1.01**
SS_{res}	6.95	1.79	1.58	1.55	5.34	1.51	1.28	**0.52**
r^2	–	0.71	0.75	0.75	0.14	0.76	0.79	**0.92**
χ^2 ou Teste F	–	19.23	14.24	14.24	1.29	15.55	15.55	53.46
DF	–	–	–	–	–	30.31	28.31	**29.31**
P	–	4.81×10^{-8}	1.69×10^{-7}	1.69×10^{-7}	0.29	1.92×10^{-8}	3.93×10^{-8}	**$2.27x10^{-14}$**

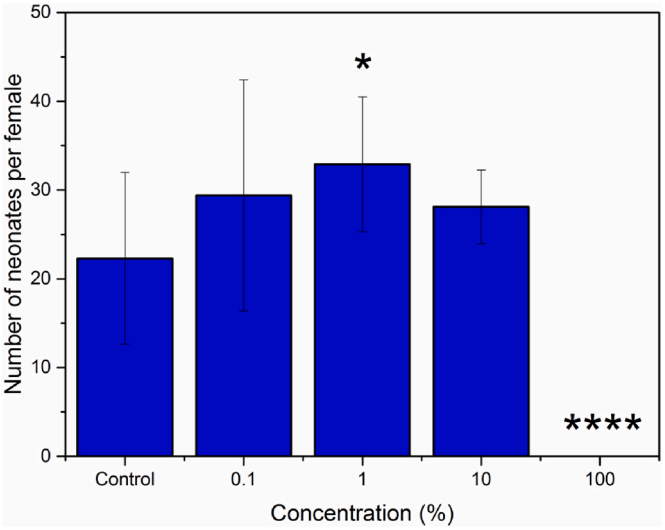

Fig. 6. Number of neonates per female Daphnia similis chronically exposed (14 days) to different dilutions of a quaternary mixture containing fluoxetine, metformin, ciprofloxacin, and acetylsalicylic acid. Asterisks (*) indicate significant differences between the replicates and the control.

study corroborate literature reports on the subject. Marques et al. (2004) estimated $LOEC_{21d}$ and $NOEC_{21d}$ ASA values of 1.8 and 1.0 mg L^{-1}, respectively, for *D. magna* and *D. longispina*, demonstrating a significant decrease in the production of neonates. Stanley et al. (2007) reported $NOEC_{21d}$ and $LOEC_{21d}$ values of 0.195 and 0.444 mg L^{-1} for a racemic mixture of FXT. With regard to MET, GODOY et al. (2018) determined a $NOEC_{14d}$ value of 4.4 mg L^{-1} for *D. similis*. Regarding CIP, Martins et al. (2012) demonstrated $NOEC_{21d}$ and $LOEC_{21d}$ values of 5.19 and 8.82 mg L^{-1} for *D. magna*, thus indicating that *D. similis* was more sensitive.

In the present study, growth effects were also noted for ASA and CIP. According to Trubetskova and Lampert (2002), a xenobiotic can be generate two different Daphnia reproduction effects, namely (1) energetic demands, delayed growth and decreased female fecundity, as females remain smaller and require longer periods of time to produce their the first brood, corroborating the pattern observed herein for acetylsalicylic and ciprofloxacin, or (2) females may grow and reproduce normally, although with lower numbers of eggs and embryos in the brood, which may affect the number of viable offspring. Moreover, physiological and/or behavioral changes in *Daphnia* spp. caused by exposure to salicylates and ciprofloxacin have described. For example, Gómez-Oliván et al. (2014) reported DNA damage and significant changes in biochemical biomarkers (increased lipid peroxidation and superoxide dismutase activity, in addition to reduced catalase activity) for *D. magna* exposed for 48 h to 8.83 mg L^{-1} of ASA. In another assessment, Szabelak and Bownik (2021) reported decreased swimming speeds, heart rates and jaw movements in *D. magna* exposed for 24 h to 5 mg L^{-1} salicylic acid solutions. Nunes et al. (2018) noted the presence of DNA damage in *D. magna* at concentrations from 13 μg L^{-1}, while Dionísio et al. (2020) reported changes in biochemical biomarkers, such as increased activity of Glutathione S-Transferase (GST) and lipid peroxidation levels and inhibition of cholinesterase activity, in *D. magna* exposed to CIP after 21 days. Furthermore, Motiei et al. (2020) verified that the antibiotic ciprofloxacin induced digestive tract microbiota alterations in *D. magna* in a concentration-dependent manner, reducing microbial biodiversity, also exhibiting a stimulatory effect on growth, fecundity and antioxidant capacity, against the authors' expectations.

Fluoxetine is known to stimulate reproduction in invertebrates at environmentally relevant concentrations. In this regard, Brooks et al. (2003a) noted a significant increase in the production of *Ceriodaphnia dubia* neonates exposed to 56 μg L^{-1} fluoxetine, while Fong (1998) reported spawning induction in male zebra mussels (*Dretssena polymorpha*) at 155 μg L^{-1}. Furthermore, different types of behavioral changes have been reported for Daphnia spp. in this regard. For example, Heyland et al. (2020) reported significant decreases in heart rates and swimming speeds of *D. magna* exposed to 5.4 ng L^{-1} FXT, while the fecundity of chronically exposed *D. magna* to 36 μg L^{-1} FXT was significantly increased according to Flaherty and Dodson (2005). Campos et al. (2012) noted that fluoxetine can alter the perception of the food environment and alter the life-history responses of *D. magna* under medium and low feeding conditions, with females reproducing earlier, producing more, albeit smaller, offspring. In addition, Campos et al. (2013) demonstrated that this antidepressant deregulates one of it targeted pathways, the serotonin metabolism, as well key genes from carbohydrate metabolism and the Krebs cycle, which results in decreased carbohydrate stores and increased respiratory metabolism. In more recent studies, Campos et al. (2016) reported that fluoxetine increases serotonin-immunoreactivity only at low-feeding conditions, increasing reproduction under conditions similar to hypothetical high-feeding conditions, in which organisms apparently exhibit saturated levels of serotonin immunoreactivity. Concerning MET, Sheng et al. (2012) noted that preconditioning with the antidiabetic metformin protected *Daphnia pulex* from hypoxic insult.

The investigation of mixture effects rather than single pharmaceuticals is paramount, as, contaminants are present at different combinations in real life-scenarios, both in conjunction with compounds displaying the same mode of action and with other contaminants (Varano et al., 2017). Because of this, the ecological risks assessment of a single pharmaceutical can underestimate the real impacts of mixtures on aquatic ecosystems (Godoy et al., 2019).

Pharmaceuticals are designed for specific activities in target organisms (Patel et al., 2019), often acting through a series of combinations of mechanisms, such as changes in intracellular levels of secondary messengers or ions and gene expression or metabolism, although, these pathways can be modified when exposed to mixtures (Varano et al., 2017).

Two classic models have been commonly employed for the prediction/assessment of mixture risks (Godoy and Kummrow, 2017). The concentration addition (CA) model assumes that pharmaceuticals in a mixture display the same mechanism of action for a specific response and act at the same site (the mixture effect is defined as the sum of the relative toxicities of individual components), while the Independent Action model (IA) assumes that pharmaceuticals affect organisms through different mechanisms of action, displaying statistically independent effects from each other without interaction (Kar and Leszczynski, 2019). Sanderson and Thomsen (2009) analyzed the mode of action of 275 drugs from distinct therapeutic classes to associate them to the mechanism of action that drives the effects of these compounds for algae, Daphnia and fish. The findings indicate non-specific narcotics seem to display an acute ecotoxicological mode of action among 70% of the analyzed pharmaceuticals. Furthermore, the authors emphasized that most these compounds are weak acids or bases and, thus, ionizable depending on the pKa. Thus, octanol-water partition coefficient (K_{ow}) changes according to pH values in the aqueous phase influence narcotic effects.

Fluoxetine is a selective serotonin reuptake inhibitor (SSRI) which can disrupt the invertebrate endocrine system and increase fecundity due to increased serotonin bioavailability (Flaherty and Dodson, 2005). Serotonin (5-HT) plays a very important role in the nervous system, performing functions such as the release of certain hormones, sleep regulation, body temperature, appetite regulation, mood, circadian rhythm, neuroendocrine functions, sexual activity, motor activity and cognitive functions (De Matos Feijó et al., 2011). In invertebrates, serotonin can stimulate ecdysteroids, and a juvenile hormone in insects responsible for controlling oogenesis and vitelogenesis (Nation, 2002).

Acetylsalicylic acid promotes the irreversible inhibition of cyclooxygenase (COX), a key enzyme that catalyzes the conversion of arachidonic acid into prostaglandins and thromboxanes (Andrade

Carvalho et al., 2004). Eicosanoids act as signaling molecules within the autocrine and paracrine systems in both vertebrates and invertebrates, comprising important mediators in reproduction mechanisms, as well as in the immune system, ion transport (Heckmann et al., 2008; Rowley, 2002) and neural transmission (Arkhipova et al., 2005). Previous studies have demonstrated behavioral and physiological changes in *D. magna* exposed to salicylic acid, such as altered swimming speedss, swimming height, travelled distances, heart rates and mandible movements (Szabelak and Bownik, 2021). It is important to note that ASA is biotransformed to salicylic acid and then conjugated to salicyluric acid, 2, 5-dihydrobenzoicacid (2,5-DHBA) (gentisic acid) and 2,3-DHBA through the intervention of CYP 2E1 and CYP 3A4 (Gómez-Oliván et al., 2014), and the presence of cytochrome P450 (CYP) and the CYP2, CYP3 and CYP4 families has been demonstrated for *D. pulex* (Baldwin et al., 2009).

Metformin is an antidiabetic drug whose mechanism of action consists in the inhibition of the complex I in the mitochondria electron transport chain, leading to depletion of adenosine triphosphate (ATP) and increased levels of adenosine monophosphate (AMP) (Foretz et al., 2014). To date, the mode of action in non-target organisms, however, remains uncertain and unexplored (Godoy et al., 2019; Moermond and Smit, 2016).

Ciprofloxacin, a quinolone antimicrobial widely employed in clinical medicine, binds to bacterial DNA gyrase, compromising its function and, consequently, preventing cell replication and bacterial proliferation (Nunes et al., 2018). Fluoroquinolones target two essential bacterial type II topoisomerase enzymes, topoisomerase IV from gram-positive bacteria, and topoisomerase II (DNA-gyrase) in gram-negative bacteria, which displays the same functions as topoisomerase IV (Hooper and Jacoby, 2016). One study has demonstrated that ciprofloxacin is genotoxic, causing DNA damage in *D. magna* (Nunes et al., 2018).

Although CA and IA models have been frequently applied to predict mixture effects of different contaminants, it is noteworthy that mixture toxicity assessments face the complex challenge of describing and modeling the joint action of multiple components from different classes that act in ecological systems with a multitude of species displaying different life cycles and diverse biochemical, physiological and genetic compositions (Backhaus, 2014). According to Berthoud (2013), mathematical synergism models are based on purely functional models with simplistic approaches, as they cannot capture the underlying biological complexity of certain systems.

Additive prediction models depend on the types and/or shapes of individual concentration-response curves, thus presenting limitations when concentration-response curves exhibit different shapes (Godoy et al., 2019; Godoy and Kummrow, 2017). This justifies the limitations of both models in accurately predicting the effects observed for the binary mixtures assessed in the present study. On the other hand, isobolograms comprise curves involving the toxicity of different ratios of contaminant mixtures and indicate whether additive, synergistic or antagonistic effects are present character (Koutsaftis and Aoyama, 2007). The acute toxicity results obtained for all mixtures containing FXT presented both antagonistic and synergistic features, depending on the ratio/dose evaluated, corroborating the literature. According to Rodea-Palomares et al. (2010) the mechanism of action of a toxicant is not useful to predict which kind of interactions the mixture will exhibit, since the nature of the interaction depends on the effect level of the mixture. Furthermore, different organisms display completely different responses. In addition, according to Cedergreen (2014), synergism occurs as a result of the interaction of one or more processes that take place as a result of chemical toxicity, namely bioavailability, uptake, internal transport, metabolization, binding and excretion, although most serious synergistic interactions probably occur due to metabolism interactions.

Chronic toxicity assays demonstrated that the interaction of the quaternary mixture at higher concentrations induced inhibitory reproduction and survival effects, while a stimulatory effect was noted at low concentrations. The determination of the hormetic effects of mixtures is a challenge, considering that the CA and IA models have been shown to be ineffective in predicting hormetic dose-response effects of mixtures (Zou et al., 2013). Hormesis is an adaptive response characterized by a biphasic dose-response curve behavior with two types of biological responses, stimulation at low doses and inhibition at high doses (Calabrese and Baldwin, 2002). These effects have been described in the literature, for example, by Fent et al. (2006), who reported the presence of stimulatory effect for a mixture of cimetidine, fenofibrate, furosemide and phenazone employing assays with recombinant yeasts, while Zou et al. (2013) demonstrated the presence of stimulatory effects for antibiotic mixtures for *A. fischeri* at low concentrations.

The findings reported herein are based on nominal concentrations, considering that, due to logistics, it would not be feasible to carry out a high number of analyses to effectively confirm the concentrations due to the experimental design and the presence of various substances. However, significant losses are not expected to occur with the studied pharmaceuticals. In this regard, Kwon and Armbrust (2006) noted that fluoxetine was hydrolytically and photolytically stable over a period of 30 days in aqueous solution. Hydrolytically losses of $<1.3\%$ at pH 5, 7 and 9 at 50 °C over 5 days and a photolysis half-life of 28.3 days have been reported for metformin (Straub et al., 2019), while ciprofloxacin is not expected undergo hydrolysis for 5 days at 50 °C (Sahlin et al., 2018). In this regard, Lin et al. (2010) determined the following half-life of ciprofloxacin at different conditions, 13.3 days for ciprofloxacin (pond water, artificial UV-A light, 10 mg L^{-1}, pH 8.4) and 47.4 days (pond water, fluorescent light, 10 mg L^{-1}, pH 8.4). Finally, aspirin is hydrolyzed in water forming salicylic acid and acetic acid (Mukherjee et al., 2016). However, aqueous acetylsalicylic solutions display a half-life of 40 days (Eberlein, 2008).

The present study demonstrated acute toxic fluoxetine effects in a binary mixture with other pharmaceutical contaminants (antibiotic, anti-inflammatory, and antidiabetic). In addition, the quaternary mixture led to stimulatory effects at ecological relevant concentrations. A lack of available information regarding the mode of action of these pharmaceuticals towards *D. similis* is, however, noted. Both CA and IA models were evaluated for acute toxicity. The results indicate that the mixtures displayed independent modes of action, which would be the required assumption to use the IA model as the reference to assess their interactive effects. Synergism/antagonism or additivity did not depend on the similarity/dissimilarity of the mode of action of the mixture compounds. The chronic assays indicated the presence of hormesis. Therefore, neither model was applied to determine potential effects, as none of the investigated models seems to fully adequate to explain the observed *D. similis* effects. However, there is still no consensus as to how strict the requirements for similarity concerning site, mechanisms, or modes of action for mixture components should be to adequately employ, while reference model detailed pharmacological information as usually absent for most environmental contaminants (Varano et al., 2017).

5. Conclusion

The present study demonstrated acute and chronic effects of four pharmaceuticals frequently detected in environmental matrices on *Daphnia similis*, determining the interaction between the evaluated antidepressant and the other pharmaceuticals. The most significant finding reported herein concern mixture interactions of pharmaceuticals from different classes, improving data on mixture effects. The experimental data indicated that the independent mode of action comprised the best model to describe binary mixture interactions. The results also revealed both antagonistic and synergistic characteristics, depending on the evaluated ratio/dose. The quaternary mixture led to a hormetic effect at low doses. Although an unequivocal conclusion cannot be achieved, the results indicate relevant implications to environmental risk assessments, as evaluations only concerning the effects of individual pharmaceuticals can underestimate the risks levels of these contaminants in the

environment, as increased or decreased effects can take place in organisms when exposed to multiple contaminants.

Authors contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Flavio Kiyoshi Tominaga, Thalita Tieko Silva, Nathalia Fonseca Boiani and Vanessa Silva Granadeiro Garcia. The first draft of the manuscript was written by Flavio Kiyoshi Tominaga and all authors commented on previous versions of the manuscript. Sueli Ivone Borrely revised it critically for important intellectual content. All authors read and approved the final manuscript.

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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