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Individual and mixture toxicity of carbofuran and diuron to the protozoan *Paramecium caudatum* and the cladoceran *Ceriodaphnia silvestrii*

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ABSTRACT

The toxicity of the insecticide carbofuran and herbicide diuron (individually and in mixture) to the invertebrates *Paramecium caudatum* and *Ceriodaphnia silvestrii* was evaluated. Acute and chronic toxicity tests were carried out with the diuron and carbofuran active ingredients and their commercial products, Diuron Nortox® 500 SC and Furadan® 350 SC, respectively. Individual toxicity tests showed that *C. silvestrii* was more sensitive to both carbofuran and diuron than *P. caudatum*. In single exposures, both pesticides caused adverse effects to *C. silvestrii* in environmentally relevant concentrations (48 h EC_{50} = 0.001 mg L^{-1} and 8 d LOEC = 0.00038 mg L^{-1} for formulated carbofuran; 8 d LOEC < 0.05 mg L^{-1} for formulated diuron). For *P. caudatum*, carbofuran and diuron in single exposures were only slightly toxic (24 h IC_{50} = 5.1 mg L^{-1} and 6.9 mg L^{-1} for formulated carbofuran and diuron, respectively). Acute and chronic exposures to diuron and carbofuran mixtures caused significant deviations of the toxicity predicted by the Concentration Addition and Independent Action reference models for both test species. For the protozoan *P. caudatum*, a dose-dependent deviation was verified for mortality, with synergism caused mainly by carbofuran and antagonism caused mainly by diuron. For protozoan population growth, however, an antagonistic deviation was observed when the active ingredient mixtures were tested. In the case of *C. silvestrii*, antagonism at low concentrations and synergism at high concentrations were revealed after acute exposure to active ingredient mixtures, whereas for reproduction an antagonistic deviation was found. Commercial formulation mixtures presented significantly higher toxicity than the active ingredient mixtures. Our results showed that carbofuran and diuron interact and cause different toxic responses than those predicted by the individually tested compounds. Their mixture toxicity should therefore be considered in risk assessments as these pesticides are likely to be present simultaneously in edge-of-field waterbodies.

1. Introduction

Pesticides used in agriculture may reach edge-of-field waterbodies and cause serious effects on aquatic biota (Hasenbein et al., 2016). Most pesticide toxicity evaluations for aquatic systems are based on exposing organisms to single compounds (Kienzler et al., 2016). However, pesticides rarely occur as a single contaminant in the environment, and organisms are hence often exposed to pesticide mixtures (Choung et al., 2013; Liu et al., 2013). Interactions between components of a mixture can lead to additive effects or cause greater (synergism) or lesser toxicity (antagonism) to organisms. Risk assessments based on single pesticide exposures may, therefore not adequately evaluate the true risks to aquatic organisms (Phyu et al., 2011).

The phenylurea herbicide diuron and the carbamate insecticide carbofuran are frequently used in several agricultural crops, including sugarcane, corn, cotton and wheat, and their simultaneous presence has been reported in water bodies worldwide in concentrations ranging from 0.0002 to 3.3 μg L^{-1} of diuron and from 0.008 to 4.5 μg L^{-1} of carbofuran (Bacigalupo and Meroni, 2007; Faggiano et al., 2010; Masiá et al., 2015). In tropical regions such as Brazil, these pesticides have

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also been found together in freshwater ecosystems (Albuquerque et al., 2016; CETESB, 2018). High concentrations have been reported in surface and groundwater, with diuron varying from 0.02 to 408 μg L^{-1} (Paschoalato et al., 2008; Sposito et al., 2018) and carbofuran ranging from 0.02 to 68.8 μg L^{-1} (Carbo et al., 2008; Severo et al., 2020). The herbicide diuron (*N*-(3,4-dichlorophenyl)-*N*,*N*-dimethyl-urea) is a photosynthesis inhibitor in photosynthetic organisms (Giacomazzi and Cochet, 2004), whereas carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl-*N*-methylcarbamate) is an insecticide, nematicide and acaricide that acts by inhibiting acetylcholinesterase (AChE) (Moreira et al., 2015) and as endocrine disruptor via the thyroid pathway (Dang, 2019).

Diuron and carbofuran are among the active ingredients most frequently used in the sugarcane monoculture of São Paulo state (Brazil) (2009–2015; IEA, 2016; Pignati et al., 2017). Field recommended concentration ranges of diuron (Diuron Nortox® 500 SC) and carbofuran (Furadan® 350 SC) applied to crops are 1.6–3.2 kg active ingredient ha^{-1} and 0.7–1.75 kg active ingredient ha^{-1}, respectively (Agrofit, 2016). Maximum levels of diuron and carbofuran permitted in drinking water by Brazilian legislation are 0.09 mg L^{-1} and 0.007 mg L^{-1}, respectively (Brasil, 2011). Water body contamination by these pesticides can negatively impact organisms of different trophic levels, such as algae (Mansano et al., 2017); protozoans (Mansano et al., 2016); cladocerans (Mansano et al., 2018); benthic macroinvertebrates (Rocha et al., 2018); fish (Bretaud et al., 2000; Clasen et al., 2014; Velki et al., 2017) and amphibians (Moreira et al., 2019). These pesticides are still able to elicit effects of priority concern including endocrine disruption, neurotoxicity, carcinogenicity, among others (Schiesari and Grillitsch, 2011).

Despite the evidence that diuron and carbofuran cause toxic effects on aquatic organisms, most studies have investigated these responses when pesticides acted individually. In previous studies, we already assessed the toxicity of single exposures of carbofuran and diuron (active ingredients and commercial products) to *Paramecium caudatum* (concentration ranges: 0.47–240 mg L^{-1} for carbofuran; 0.88–224 mg L^{-1} for diuron) (Mansano et al., 2016) and *C. silvestrii* (concentration ranges: 0.00009–0.00393 mg L^{-1} for carbofuran; 0.01–32 mg L^{-1} for diuron) (Mansano et al., 2018). Subsequently, we demonstrated that the mixture of these compounds (active ingredients) can have a synergistic interaction on the toxicity to the microalgae *Raphidocelis subcapitata* (Mansano et al., 2017). Furthermore, the carbofuran and diuron combinations may cause antagonistic or synergistic interactions to the oligochaetes *Dero furcatus* and *Allonais inaequalis* depending on the dose level of the mixture (concentration ranges: 0.06–4.0 mg L^{-1} for carbofuran; 1–64 mg L^{-1} for diuron) (Rocha et al., 2018).

Protozoans are functionally very important components of aquatic ecosystems as they act as a linkage between bacterial production and secondary producers (Pomeroy et al., 2007); increase remineralization processes (Madoni, 2011); control bacterial assemblages by predation (Mansano et al., 2014); and they are prey organisms for higher organisms (Reiss and Schmid-Araya, 2011). Cladocerans occupy a key intermediate position in the food web, being important links in the phytoplankton-zooplankton-fish larvae trophic relationships (Dettmers and Stein, 1992). Thus, pesticide toxicity on protozoans and cladocerans can change trophic food-webs resulting in significantly affected aquatic system structures and functioning. In addition to their ecological attributes, protozoans and cladocerans are organisms that are easy to cultivate in laboratory, possess high reproduction rates and are sensitive to pesticides (protozoan: Mansano et al., 2016; Saib et al., 2014; cladoceran: Casali-Pereira et al., 2015; Mansano et al., 2018). These characteristics make them relevant and adequate test organisms for toxicity bioassays. Mixture toxicity studies using protozoans (Láng and Kőhidai, 2012) and tropical cladoceran *Ceriodaphnia silvestrii* (Freitas et al., 2018) as test organisms are rare. To the best of our knowledge, the toxicity of carbofuran and diuron mixtures on cladocerans and protozoans has never been evaluated.

The general aim of this study was to evaluate the individual and mixture toxicity of the insecticide carbofuran and the herbicide diuron (active ingredients and commercial products) to the invertebrates *P. caudatum* and *C. silvestrii.* For this purpose, the specific aims were i) to evaluate the effects of carbofuran and diuron on the survival and population growth of the protozoan *P. caudatum* in both single and mixture exposures; ii) to determine the effects of carbofuran and diuron on the mobility and reproduction (fecundity) of the cladoceran *C. silvestrii* in both single and mixture exposures; iii) to investigate whether single and mixture toxicity was different between the active ingredients and commercial products. We hypothesize in this study that *i*) single exposures of carbofuran and diuron cause deleterious effects in *P. caudatum* and *C. silvestrii* and these effects are more pronounced for the insecticide than for the herbicide; *ii*) mixture exposures of carbofuran and diuron cause synergistic effects to these species both in acute (mortality and immobilization) and chronic (population growth and reproduction) tests, when compared to the effects of isolated compounds; *iii*) the acute and chronic effects are more deleterious in formulated product mixtures than the active ingredient mixtures.

2. Materials and methods

2.1. Test organisms and culture conditions

Paramecium caudatum (Protozoa, Ciliophora) cultures were initiated from individuals obtained from the "Monjolinho" Reservoir (São Carlos, SP, Brazil). *Ceriodaphnia silvestrii* (Crustacea, Cladocera, Daphnidae) was collected from the "Lobo-Broa" Reservoir (Itirapina, SP, Brazil). Protozoan *P. caudatum* was cultured in tubes containing a sterile rice grain, agar (2%) and Minalba® mineral water (pH 7.5–8.0), which were supplemented with *Enterobacter aerogenes* at a concentration of 10^6 cells mL^{-1} (Mansano et al., 2016). Protozoan stock cultures were maintained in an incubator at 25 ± 1 °C temperature and 12 h: 12 h light-dark photoperiod. Following recommendations of the Brazilian Association of Technical Standards (ABNT NBR 13373, 2017), *C. silvestrii* cultures were kept at the same temperature and photoperiod in reconstituted water with a pH of 7.0–7.6 and a hardness of 40–48 mg L^{-1} (as $CaCO_3$). The cladocerans in cultures were fed daily with a suspension of the green algae *Raphidocelis subcapitata* (10^5 cells mL), plus 1 mL L^{-1} of a food supplement containing yeast and fermented fish food (ABNT, 2017; OECD, 2008). The physiological conditions of *P. caudatum* and *C. silvestrii* were checked monthly by performing acute toxicity tests with a reference substance sodium chloride (NaCl) before their use for acute and chronic tests (OECD, 2008).

2.2. Test compounds and chemical analysis

Diuron (CAS number 330-54-1) and carbofuran (CAS number 1563-66-2) analytical standards (≥98% purity) were acquired from Sigma-Aldrich. The commercial formulations evaluated were Diuron Nortox® 500 SC (Nortox S/A, Brazil; 50% mass/volume diuron) and Furadan® 350 SC (FMC, Brazil; 35% mass/volume carbofuran). Distilled water was used to prepare all stock solutions except for stock solution of the analytical standard of diuron, for which acetone (≥99.5%) was used due its low solubility in water (42 mg L^{-1}; Giacomazzi and Cochet, 2004). Test concentrations were prepared by diluting the stock solution in the test organism culture media. A solvent control (0.1% acetone v/v) was also included for the diuron analytical standard.

Tested concentration ranges for *P. caudatum* and *C. silvestrii* were chosen based on Mansano et al. (2016) and Mansano et al. (2018), respectively. The nominal concentration ranges for acute exposure to protozoan were 40–140 mg L^{-1} of diuron and 14–224 mg L^{-1} of diuron dosed as Diuron Nortox® 500 SC, 120–240 mg L^{-1} of carbofuran and 60–180 mg L^{-1} of carbofuran dosed as Furadan® 350 SC; and for chronic exposure were 0.88–28 mg L^{-1} of both diuron and diuron

dosed as Diuron Nortox® 500 SC, 1.88–60 mg L^{-1} of carbofuran and 0.47–15 mg L^{-1} of carbofuran dosed as Furadan® 350 SC. For the cladoceran, nominal concentration ranges for acute exposure were 2–32 mg L^{-1} of diuron and 0.4–2 mg L^{-1} of diuron dosed as Diuron Nortox® 500 SC, 0.00016–0.0026 mg L^{-1} of carbofuran and 0.0006–0.0039 mg L^{-1} of carbofuran dosed as Furadan® 350 SC; and for chronic exposure were 0.5–8 mg L^{-1} of diuron and 0.05–0.8 mg L^{-1} of diuron dosed as Diuron Nortox® 500 SC, 0.00015–0.00096 mg L^{-1} of both carbofuran and carbofuran dosed as Furadan® 350 SC.

To confirm nominal test concentrations, carbofuran and diuron were chemically analyzed using an Agilent Technology series 1200 HPLC-DAD (high-performance liquid chromatograph; Waldbronn, Germany). Chromatographic quantification methods and analytical conditions were described in detail by Mansano et al. (2016, 2018) and briefly in the Supplementary Material in this study. The analytical parameters used for the validation of diuron and carbofuran analysis method are shown in Table S1.

2.3. Individual toxicity tests

Acute and chronic toxicity tests with the ciliate *P. caudatum* were performed as described in Mansano et al. (2016). The test concentrations used in the single exposure tests for each compound (active ingredients or commercial products) for the protozoan are given in Table S2 of the Supplementary Material. Acute toxicity tests (mortality) with *P. caudatum* were carried out in watch-glasses that were kept individually in glass Petri dishes. Three replicates were used for each pesticide treatment and controls, with each replicate containing 1 mL test solution and ten organisms. The duration of the acute tests was 4 h. This exposure time was chosen considering the fast replication time of *P. caudatum* (~9.2 h at 25 °C; Mansano et al., 2016). The experiments were conducted at 25 ± 1 °C in complete darkness and without the addition of food. After the exposure period, the number of dead individuals was counted under a Leica MZ6 stereomicroscope (50× magnification) and used to calculate the median lethal concentration (4 h LC_{50}). Chronic toxicity tests (population growth) with *P. caudatum* had 24 h duration due to the short life-cycle duration of this species (~2.6 generations in 24 h; Mansano et al., 2016). These chronic tests were carried out in test tubes with 5 mL test solution to which 50 protozoans were transferred from a culture in exponential growth phase (stock culture < 72 h based on a preliminary growth experiment). The different pesticide treatments contained the same culture medium as the control, which was contaminated by adding the stock solutions to obtain the selected pesticide test concentrations (dilution factor of 2). Protozoans were fed during the chronic assays. To all treatment solutions, *Enterobacter aerogenes* bacteria (final concentration of 10^6 cells mL^{-1}) and a sterile rice grain were added as food. Triplicates were established for each treatment, which were maintained at the same temperature and photoperiod as described for the stock cultures. After the exposure period, protozoans were preserved (acid lugol; 0.4%) and individual numbers were counted under a Leica DMLS optical microscope (100× magnification) in a Sedgewick-Rafter chamber. Protozoan density was used to determine the median growth inhibition concentration (24 h IC_{50}). The assessed endpoints for *P. caudatum* were mortality and population growth.

Acute and chronic toxicity tests with the cladoceran *C. silvestrii* followed standardized guidelines (ABNT NBR 12713, 2016; ABNT NBR 13373, 2017; OECD, 2008). The test concentrations used in the single exposure tests for each compound (active ingredients or commercial products) for the cladoceran are given in Table S2. For *C. silvestrii*, acute toxicity tests (48 h duration) were carried out using three replicates per treatment, with each replicate containing five neonates (age: 6–24 h) in 10 mL test solution. The experiments were conducted without adding food and in darkness at a temperature of 25 ± 1 °C. After the exposure period, the number of immobile individuals were counted under a Leica MZ6 stereomicroscope and used to determine the median effective concentration (48 h EC_{50}). Individuals were considered immobilized when they were not able to swim within 15 s after gentle agitation of the test vessel. Chronic toxicity tests (8 d duration) were performed with six replicates, each containing 15 mL test solution and one neonate (age: 6–24h). The chronic tests lasted eight days and the test organisms were maintained and fed as described for the maintenance of its culture. Every two days, test solutions were renewed after recording the number of surviving adults and live newborn neonates (ABNT, 2017; OECD, 2008). The 8 d EC_{50} was calculated based on the total number of live neonates per female (fecundity). The assessed endpoints for *C. silvestrii* were immobilization and reproduction.

2.4. Mixture toxicity tests

After toxicity tests with individual pesticides revealed that the effects of active ingredients and their commercial products were different for at least one of the compounds (herbicide or insecticide), we chose to perform mixture experiments with both active ingredients and commercial formulations. The experimental design adopted for the mixture acute toxicity tests consisted of evaluating each individual pesticide and a set of the 25 possible binary combinations (full factorial design). For the chronic mixture toxicity tests, the experimental design consisted of evaluating each isolated pesticide and a set of 23 combinations (partial fixed-ratio design; Cassee et al., 1998). This design was adopted for the chronic assays in order to avoid including treatments with the highest concentrations of the two pesticides that could a priori be expected to lead to 100% mortality (for protozoan) or immobilization (for cladoceran) (Freitas et al., 2014). Concentrations of the pesticide mixture combinations were based on their expected toxic strengths: 0.375, 0.5, 0.75, 1.0, 1.5, 1.75 and 2.0 toxic units (TU) (Freitas et al., 2014; Pérez et al., 2011). One TU was equal to the 4 h LC_{50} or 24 h IC_{50} (for protozoan) or 48 h EC_{50} or 8 d EC_{50} (for cladoceran) as set in this study in the toxicity assays evaluating single exposures to each tested compound.

2.5. Data analysis

The 4 h LC_{50} (mortality) and 48 h EC_{50} (immobilization) in the acute toxicity tests and the 24 h IC_{50} (population growth) and 8 d EC_{50} (reproduction) in the chronic toxicity tests with *P. caudatum* and *C. silvestrii*, respectively, together with their respective slope and 95% confidence intervals for single and mixture exposures were determined through nonlinear regression using a three-parameter logistic curve. This curve is described by the equation: $Y_i = \max/1 + (C_i/C50_i)^{\beta i}$; where Y_i is the response of a given parameter; max is its maximum response; C_i is the concentration of chemical *i*; $C50_i$ is the effect concentration of chemical *i* (i.e. LC_{50}, IC_{50} or EC_{50}) and βi is the slope for chemical *i*. SigmaPlot version 11.0 (Systat, 2008) was used to conduct these statistical analyses.

For individual exposure data, significant differences between active ingredients and their respective commercial formulations were assessed by Student's t-test. Toxicity test data were first verified for normality (Chi squared test) and variance homogeneity (Bartlett's test). NOEC and LOEC values for tests were obtained by one-way analysis of variance (ANOVA). A post-hoc Dunnett's test was performed when significant differences between treatments and control (or between the control and solvent control for diuron) were revealed in data that met the normality and homoscedasticity criteria. For data that did not meet these requirements, the non-parametric Kruskall-Wallis test, followed by Dunn's post-hoc test, were applied. The differences were considered significant when $p \leq 0.05$. All statistical analyses were made using the SigmaPlot v. 11.0 (Systat, 2008).

Observed effects denoted in the mixture toxicity tests were compared with those expected from both the Concentration Addition (CA) and Independent Action (IA) reference models (model equations are presented in Supplementary Material) by applying the MIXTOX tool

Table 1
Acute (mortality) and chronic (population growth) toxicity values for the protozoan *P. caudatum* and acute (immobilization) and chronic (reproduction) toxicity values for the cladoceran *C. silvestrii* for each individually tested compound (in mg L^{-1}), and for the binary pesticide mixtures (in toxic units; TU). Diu = diuron; Carb = carbofuran. Values ± standard deviation.

	Paramecium caudatum		*Ceriodaphnia silvestrii*	
Compounds	**Acute Toxicity**	**Chronic Toxicity**	**Acute Toxicity**	**Chronic Toxicity**
	4 h LC_{50} [d]	**24 h IC_{50}** [e]	**48 h EC_{50}** [f]	**8 d EC_{50}** [f]
Single exposures				
Diuron (active ingredient)[a]	78.2 ± 9.0	7.2 ± 0.88	8.2 ± 0.81	6.8 ± 0.29
Diuron (Diuron Nortox® 500 SC)[a]	73.5 ± 6.2	6.9 ± 1.2	1.1 ± 0.06	0.67 ± 0.06
Carbofuran (active ingredient)[a]	174.4 ± 11.6	22.7 ± 4.1	$0.98 \times 10^{-3} \pm 0.06 \times 10^{-3}$	$0.87 \times 10^{-3} \pm 0.06 \times 10^{-3}$
Carbofuran (Furadan® 350 SC)[a]	98.0 ± 1.8	5.1 ± 0.83	$1.0 \times 10^{-3} \pm 0.08 \times 10^{-3}$	$0.85 \times 10^{-3} \pm 0.14 \times 10^{-3}$
Mixture exposures				
Diu + Carb (active ingredients)[b,c]	1.36 ± 0.05	1.16 ± 0.03	0.81 ± 0.02	1.55 ± 0.07
Diu + Carb (commercial products)[b,c]	0.89 ± 0.07	0.75 ± 0.06	1.66 ± 0.05	1.54 ± 0.19

[a] Values expressed in mg L^{-1}.
[b] Values expressed in mixture toxic units (TU_{mix}).
[c] TU_{mix} = 1 – no interaction (additive effect); TU_{mix} > 1 – antagonism; TU_{mix} < 1 – synergism (Pape-Lindstrom and Lydy, 1997).
[d] LC_{50} = median lethal concentration.
[e] IC_{50} = median growth inhibition concentration.
[f] EC_{50} = median effective concentration.

(Jonker et al., 2005). Afterward, deviations from both the CA and IA models (synergistic/antagonistic (S/A); dose-ratio dependent (DR); dose-level dependent (DL) deviations) were modelled by adding two parameters (*a* and *b*) and evaluated as detailed in Jonker et al. (2005) and deviation functions (see Table S3 of the Supplementary Material). In S/A deviation, *a* becomes negative in synergism interactions, and positive in antagonism interactions. In DR deviation, an additional parameter (b_{DR}) is included, indicating that the deviation from the reference model is controlled by the composition of the mixture. In DL deviation, parameter *a* determines the deviations in low and high doses ($a > 0$ = antagonism; $a < 0$ = synergism) and b_{DL} indicates in which dose level the deviation changed. The maximum likelihood method was used to find the best fit for the conceptual model and deviations. After identifying a statistically more descriptive deviation (i.e. the lowest "L" or "SS" and highest "r^2" values, with $p < 0.05$), the effect pattern was deduced directly from the parameter values (Table S3) and the maximum deviation was calculated in terms of effect level (Freitas et al., 2014; Jonker et al., 2005).

Dose-response curves for the pesticide mixtures on *P. caudatum* and *C. silvestrii* were plotted on a three-parameter logistic curve-fitting (SigmaPlot version 11.0). Acute and chronic toxicity values (LC_{50}, IC_{50} or EC_{50}) in the mixtures were expressed as diuron and carbofuran concentrations converted into toxic units (TU). This was necessary as several combinations of the concentrations of the two chemicals in the mixture could be derived from the same TU value. A sum of the TU contributed by each component describes the toxicity of mixture as follows (Pape-Lindstrom and Lydy, 1997):

$$TU_{mix} = \sum_{i} TU_i = \sum_{i}^{n} \frac{c_i}{EC_{50i}}$$

where *n* is the number of the mixture components, c_i is the concentration of a chemical in a mixture and EC_{50i} is the EC_{50} (replaced by LC_{50} or IC_{50}, depending on the assessed endpoint) for the respective component chemicals of the mixture. The combined pesticide effect was defined as being additive (TU_{mix} = 1), synergistic (TU_{mix} < 1) or antagonistic (TU_{mix} > 1) (Pape-Lindstrom and Lydy, 1997).

3. Results

3.1. Abiotic variables of the tests and chemical analyses

In all the protozoan tests, the pH values varied from 7.5 to 8.1; water temperature from 24.8 to 25.4 °C and water hardness of 76–80 mg $CaCO_3$ L^{-1}. In all the cladoceran toxicity tests, the pH varied from 7.2 to 7.7; water temperature from 24.6 to 25.5 °C, and the water hardness from 40 to 46 mg $CaCO_3$ L^{-1}. At the end of all toxicity tests, the mortality in the controls (negative and solvent control) never exceeded 10%. In the single and mixture experiments with the active ingredient diuron, there was no significant difference between the negative and solvent controls (Student's t-test, $p > 0.05$), excluding the possibility of solvent effects (0.1% acetone) on the toxicity test results. Therefore, negative control was used for all subsequent statistical analyses of single and mixture exposures. Subsequently, the validity criteria established by ABNT (2017) and OECD (2008) guidelines were met for all tests.

The chemical analyses of the test solutions showed that the differences between the measured and nominal carbofuran and diuron concentrations did not differ > 10% from the intended concentrations in individual (Supplementary Material Figs. S1–S2) and mixture exposures (Supplementary Material Tables S4–S11) with the protozoan *P. caudatum* (carbofuran: 97 ± 4%; diuron: 98 ± 3%) and the cladoceran *C. silvestrii* (carbofuran: 98 ± 5%; diuron: 99 ± 3%). Therefore, toxicity determinations were based on nominal concentrations, as indicated in ISO 10706 (2000).

3.2. Individual toxicity tests

The reference acute test using NaCl indicated that the protozoan sensitivity (4 h LC_{50} = 4.38 g L^{-1}) was within the required range (reference range 4.22–4.61 g L^{-1}; Mansano et al., 2016). Acute and chronic toxicity values for *P. caudatum* (4 h LC_{50} and 24 h IC_{50}, respectively) for each individually tested compound are shown in Table 1. When comparing the toxicity of active ingredients with their respective commercial products, it was found that diuron and Diuron Nortox® 500 SC did not have significantly different toxicity (Student's t-test, $p > 0.05$), while Furadan® 350 SC was significantly more toxic than the carbofuran active ingredient (Student's t-test, $p < 0.05$). The dose-response curves for each individual chemical (active ingredients and commercial formulations) are presented in the Supplementary Material Figs. S3–S4. Diuron, both the active ingredient (Figs. S4–A) and commercial product (Figs. S4–B) caused a significant decrease in population growth of *P. caudatum* at concentrations of 1.75–28 mg L^{-1} (Dunnett's test, $p < 0.05$). The NOEC for diuron (active ingredient and commercial formulation) was 0.88 mg L^{-1}, while the LOEC was 1.75 mg L^{-1}. For carbofuran, protozoan population growth was

significantly decreased at 1.88–60 mg L^{-1} of carbofuran (Figs. S4–C) and 0.94–15 mg L^{-1} of carbofuran dosed as Furadan® 350 SC (Figs. S4–D) (Dunnett's test, $p < 0.05$). The NOEC of carbofuran was < 1.88 mg L^{-1} and 0.47 mg L^{-1} for the active ingredient and commercial product, respectively. The LOEC of carbofuran was 1.88 mg L^{-1} and 0.94 mg L^{-1} for the active ingredient and commercial product, respectively.

The sensitivity of *C. silvestrii* to reference substance NaCl (48 h EC_{50} = 1.28 g L^{-1}) was also within the required range (reference range 1.00–1.32 g L^{-1}; Mansano et al., 2018). The acute and chronic toxicity values derived in the present study for *C. silvestrii* (48 h EC_{50} and 8 d EC_{50}) for each individually tested pesticide are provided in Table 1. According to the data, commercial diuron (Diuron Nortox® 500 SC) was significantly more toxic than its active ingredient (Student's t-test, $p < 0.05$), while carbofuran and Furadan® 350 SC showed no significant difference in their toxicities (Student's t-test, $p > 0.05$). Dose-response curves for each individual compound (active ingredients and commercial formulations) are presented in the Supplementary Material Figs. S5–S6. Regarding the pesticide effects on the reproduction of *C. silvestrii*, diuron (active ingredient and commercial product) caused a decrease in the number of neonates per female at the highest concentrations, but stimulating effects at the lowest concentrations tested were observed. A significant increase in reproduction was observed in cladocerans exposed to concentrations of 0.5 and 1.0 mg L^{-1} of diuron (Figs. S6–A) and 0.05 and 0.1 mg L^{-1} in its commercial form (Figs. S6–B) (Dunnett's test, $p < 0.05$). However, a significant decrease in reproduction was observed at 4–8 mg L^{-1} of diuron and 0.4–0.8 mg L^{-1} of Diuron Nortox® 500 SC. The NOEC for diuron was < 0.5 mg L^{-1} and < 0.05 mg L^{-1} for the active ingredient and commercial product, respectively, while the LOEC was 0.5 mg L^{-1} and 0.05 mg L^{-1} for the diuron active ingredient and commercial product, respectively. For carbofuran, both the active ingredient (Figs. S6–C) and commercial formulation (Figs. S6–D) significantly decreased the *C. silvestrii* reproduction at concentrations of 0.38 × 10^{-3} - 0.96 × 10^{-3} mg L^{-1} (Dunn's test, $p < 0.05$). The NOEC and LOEC values for carbofuran (both the active ingredient and commercial product) were 0.23 × 10^{-3} mg L^{-1} and 0.38 × 10^{-3} mg L^{-1}, respectively.

3.3. Mixture toxicity tests

For the acute and chronic toxicity of carbofuran and diuron combinations for *P. caudatum*, the 4h LC_{50} and 24h IC_{50} values in the mixture exposures are presented in Table 1. Dose-response curves for the experimental and modelled mixture data are shown in Fig. 1. All curve fits had determination coefficients (r^2) ranging from 0.92 to 0.99, thus allowing the valid calculation of dose-response relationships for the mixtures. Based on mixture toxicity values, a point-wise assessment was carried out of the combined action of carbofuran and diuron pesticides. The acute and chronic toxicity of the diuron and carbofuran mixtures (active ingredients) caused antagonistic effects to protozoan, since the empirically derived 4h LC_{50} (1.36) and 24h IC_{50} (1.16) values (Table 1) were greater than the expected value of 1 TU. In contrast, when analyzing the acute and chronic toxicity of the pesticide commercial product mixtures, the observed 4h LC_{50} (0.89) and 24h IC_{50} (0.75) values (Table 1) were lower than the expected values of 1 TU, indicating synergistic interactions between the pesticides.

Besides the approach of the sum of the TUs, by applying the MIXTOX tool, it was possible to verify if there were different effects on varying dose levels and proportions (DL and DR deviations) from CA and IA models, as well as the synergism/antagonism (S/A) deviation. The results obtained from the MIXTOX tool for the acute and chronic mixture toxicity tests with *P. caudatum* using the CA and IA models are shown in Table 2 (summary of the analysis) and in Tables S12–S15 (detailed analyses) of the Supplementary Material. Although both models were statistically significant ($p < 0.05$), the results obtained from the active ingredient mixtures were better explained (i.e. the lowest "L" or "SS" and highest "r^2" values) by deviations from the CA model, while the results from the commercial product mixtures were better explained by deviations from the IA model (Table 2). Fitted curves to the observed data were very close to those of the modelled data, suggesting that the chosen deviations described the experimental data well (Fig. 1).

In the acute toxicity test with the protozoan *P. caudatum* exposed to diuron and carbofuran mixtures (active ingredients) (Table 2), data fitted to the CA model yielded an *L* value of 80.73 ($p < 0.001$; $r^2 = 0.61$). After adding parameters *a* and b_{DR} to this model, the DR deviation better described the data with a decrease of *L* value to 42.72, which was statistically significant ($p < 0.001$; $r^2 = 0.79$; a = −0.99; $b_{DR} = 4.65$). Thereby, it was verified that the interaction of the pesticides in mixtures was dose-ratio dependent, and the antagonism occurred at high diuron concentrations and low carbofuran concentrations and synergism at high carbofuran concentrations and low diuron concentrations, as shown in Fig. 2A. In the chronic toxicity test with the mixtures of the pesticide active ingredients (Table 2), data fitted to the CA model yielded an *SS* value of 162.66 ($p < 0.001$; $r^2 = 0.95$). After adding parameter *a* to the CA model to describe the S/A deviation, the *SS* value decreased significantly to 143.02 ($p = 0.03$, $r^2 = 0.95$). Thus, S/A deviation from the CA model presented the best fit for the data and indicated antagonistic interactions ($a = 0.80$) between diuron and carbofuran when in mixtures (Fig. 2B).

Regarding the acute toxicity test with *P. caudatum* exposed to commercial product mixtures of diuron and carbofuran (Table 2), data fitted to the IA model yielded an *L* value of 40.27 ($p < 0.001$; $r^2 = 0.84$). After adding parameter *a* to the IA model, the S/A deviation described the data better, in which the *L* value decreased to 33.77 and was statistically significant ($p = 0.01$; $r^2 = 0.87$). The negative value of parameter *a* (a = -0.30) indicated synergism of the pesticides in mixture (Table 2; Fig. 2C). In the chronic toxicity test with the commercial product pesticide mixtures (Table 2), data fitted to the IA model yielded an *SS* value of 419.04 ($p < 0.001$; $r^2 = 0.89$). However, adding parameters *a* and b_{DR} to this model, the DR deviation better described the data since the *SS* value decreased significantly (*SS* = 241.17; p = 0.046; $r^2 = 0.94$; a = 2.20; $b_{DR} = -2.19$). In this case, the synergism was observed mostly when diuron was the dominant pesticide in the mixture, as shown in Fig. 2D.

For the acute and chronic toxicity of pesticide combinations to *C. silvestrii*, the 48 h EC_{50} and 8 d EC_{50} values in the mixture exposures are presented in Table 1. Dose-response curves for the observed and modelled data are shown in Fig. 3. The plotted logistic curves showed a good fit to the experimental and modelled mixture data ($r^2 \geq 0.89$). Based only on mixture toxicity values (Table 1), it was observed that the diuron and carbofuran combinations for both active ingredients and commercial products in chronic exposure caused antagonistic effects to cladoceran, since the experimentally derived 8 d EC_{50} values (1.55 and 1.54, respectively) (Table 1) were greater than the expected value of 1 TU. In acute exposure, the combined action of pesticide active ingredients caused synergistic effects (48 h EC_{50} = 0.81), whereas the commercial product mixtures caused antagonistic interactions between the pesticides (48 h EC_{50} = 1.66).

The results obtained from the MIXTOX tool for the acute and chronic mixture toxicity tests with *C. silvestrii* using the CA and IA models are presented in Table 3 (summary of the analysis) and in Tables S16–S19 (detailed analyses) of the Supplementary Material. The fitted curves to the observed data were close to those of the modelled data, suggesting that the chosen deviations well described the experimental data.

In the acute toxicity experiment with cladoceran involving the diuron and carbofuran mixtures (active ingredients) (Table 3), data fitted to the CA model yielded an *L* value of 27.16 ($p < 0.001$; $r^2 = 0.93$). After adding parameters *a* and b_{DL} to the CA model in order to describe the DL deviation, the *L* value decreased to 17.86 and was

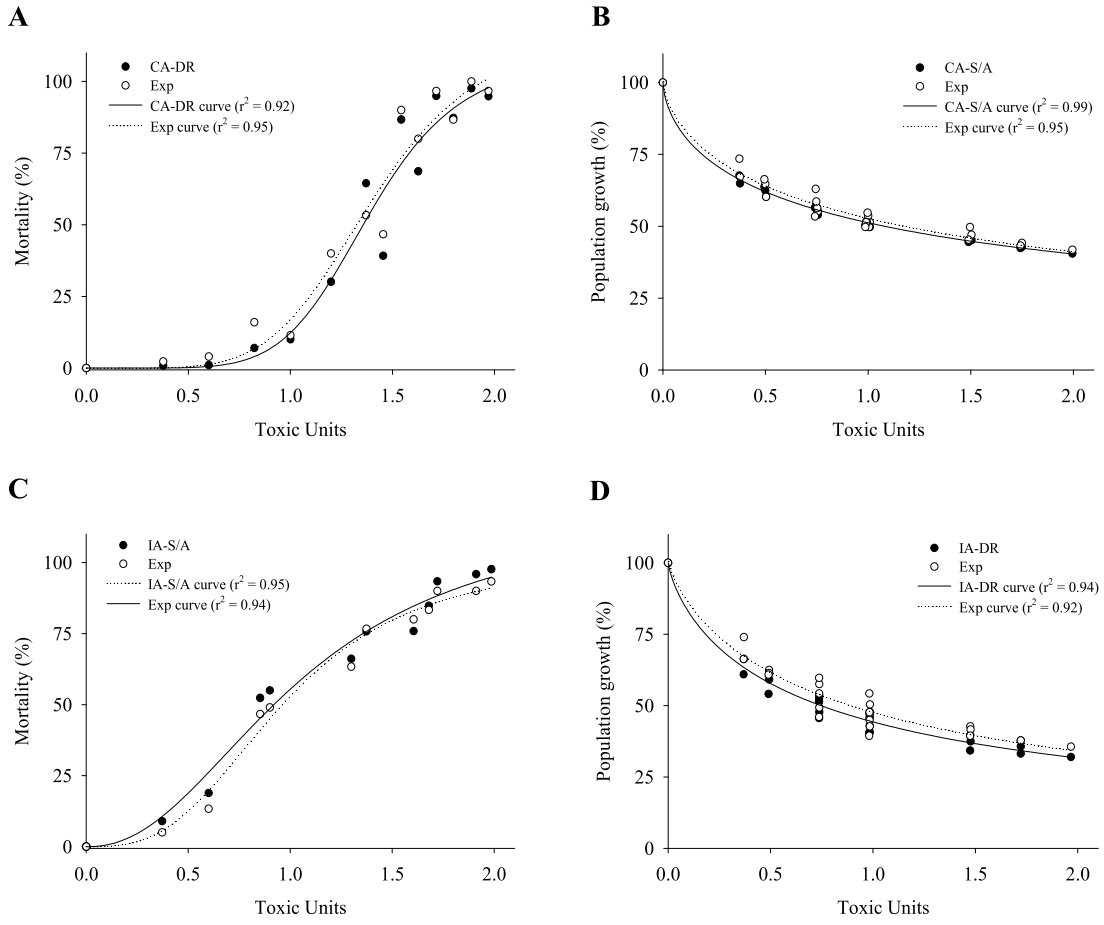

Fig. 1. Dose-response curves obtained from the observed data (Exp) and modelled data of the pesticide mixture toxicity tests on *P. caudatum* (mortality and population growth). Acute (A) and chronic (B) toxicity of the active ingredient mixtures and acute (C) and chronic (D) toxicity of the commercial product mixtures. The solid and dotted lines represent the three-parameter logistic curve-fitting from the modelled and observed data, respectively. CA-DR = dose-ratio dependent (DR) deviation from the CA model; CA-S/A = synergistic/antagonistic (S/A) deviation from the CA model; IA-S/A = S/A deviation from the IA model; IA-DR = DR deviation from the IA model.

Table 2
Summary of the analysis of the acute and chronic toxicity tests with the protozoan *P. caudatum* exposed to mixtures of carbofuran and diuron (active ingredients and commercial products) using the CA and IA models. The model and deviation that best fitted the mixture toxicity data using the MIXTOX tool (Jonker et al., 2005) are indicated in bold.

Toxicity	Mixture	Model and Deviation	*a*	$b_{DR/DL}$	*L* or *SS*	r^2	*p* (χ^2 or F)	max	LC_{50} or $IC_{50\ Diuron}$	β_{Diuron}	LC_{50} or $IC_{50\ Carbofuran}$	$\beta_{Carbofuran}$
Acute	Carbofuran and Diuron (active ingredients)	CA	–	–	80.73	0.61	7.60×10^{-26}	1.00	97.78	1.76	170.03	7.20
		CA-DR	**−0.99**	**4.65**	**42.72**	**0.79**	**$8.28x10^{-7}$**	**0.88**	**93.66**	**4.65**	**182.73**	**10.14**
		IA	–	–	73.57	0.64	2.23×10^{-27}	1.00	74.06	2.44	165.10	10.26
		–	–	–	–	–	–	–	–	–	–	–
	Carbofuran and Diuron (commercial products)	CA	–	–	90.61	0.64	2.46×10^{-34}	1.00	78.09	1.66	105.73	6.09
		CA-S/A	1.31	–	43.16	0.83	5.67×10^{-12}	0.95	80.51	3.22	98.67	8.13
		IA	–	–	40.27	0.84	3.76×10^{-45}	1.00	67.77	2.51	89.21	5.89
		IA-S/A	**−0.30**	**-**	**33.77**	**0.87**	**0.01**	**0.99**	**67.50**	**2.51**	**96.53**	**6.59**
Chronic	Carbofuran and Diuron (active ingredients)	CA	–	–	162.66	0.95	2.21×10^{-19}	61.78	7.40	0.90	25.17	0.50
		CA-S/A	**0.80**	**-**	**143.02**	**0.95**	**0.03**	**62.28**	**6.67**	**0.89**	**20.54**	**0.51**
		IA	–	–	350.27	0.89	3.03×10^{-14}	54.24	11.84	1.25	47.02	0.61
		IA-S/A	1.69	–	150.94	0.95	3.69×10^{-8}	62.37	7.31	0.83	19.07	0.48
	Carbofuran and Diuron (commercial products)	CA	–	–	331.98	0.92	3.85×10^{-16}	65.14	4.84	0.73	4.39	0.71
		CA-DR	1.95	−5.01	277.05	0.93	0.04	64.88	6.17	0.72	4.01	0.72
		IA	–	–	419.04	0.89	1.39×10^{-14}	60.09	8.35	0.82	7.69	0.72
		IA-DR	**2.20**	**−2.19**	**241.17**	**0.94**	**0.05**	**65.57**	**6.04**	**0.65**	**4.13**	**0.66**

CA is the concentration addition model and IA is the independent action model; S/A is synergism or antagonism deviation; DR is dose-ratio dependent deviation and DL is dose-level deviation; *a*, b_{DR} and b_{DL} are parameters of the functions; *L* and *SS* are the objective functions used for discrete and continuous data, respectively; r^2 is the regression coefficient; χ^2 or F test is the test statistic; and *p* (χ^2/F) is the significance level of the test statistic; max is the maximum response value; LC_{50} is the median lethal concentration; IC_{50} is the median growth inhibition concentration; β is the slope of the individual dose response curve.

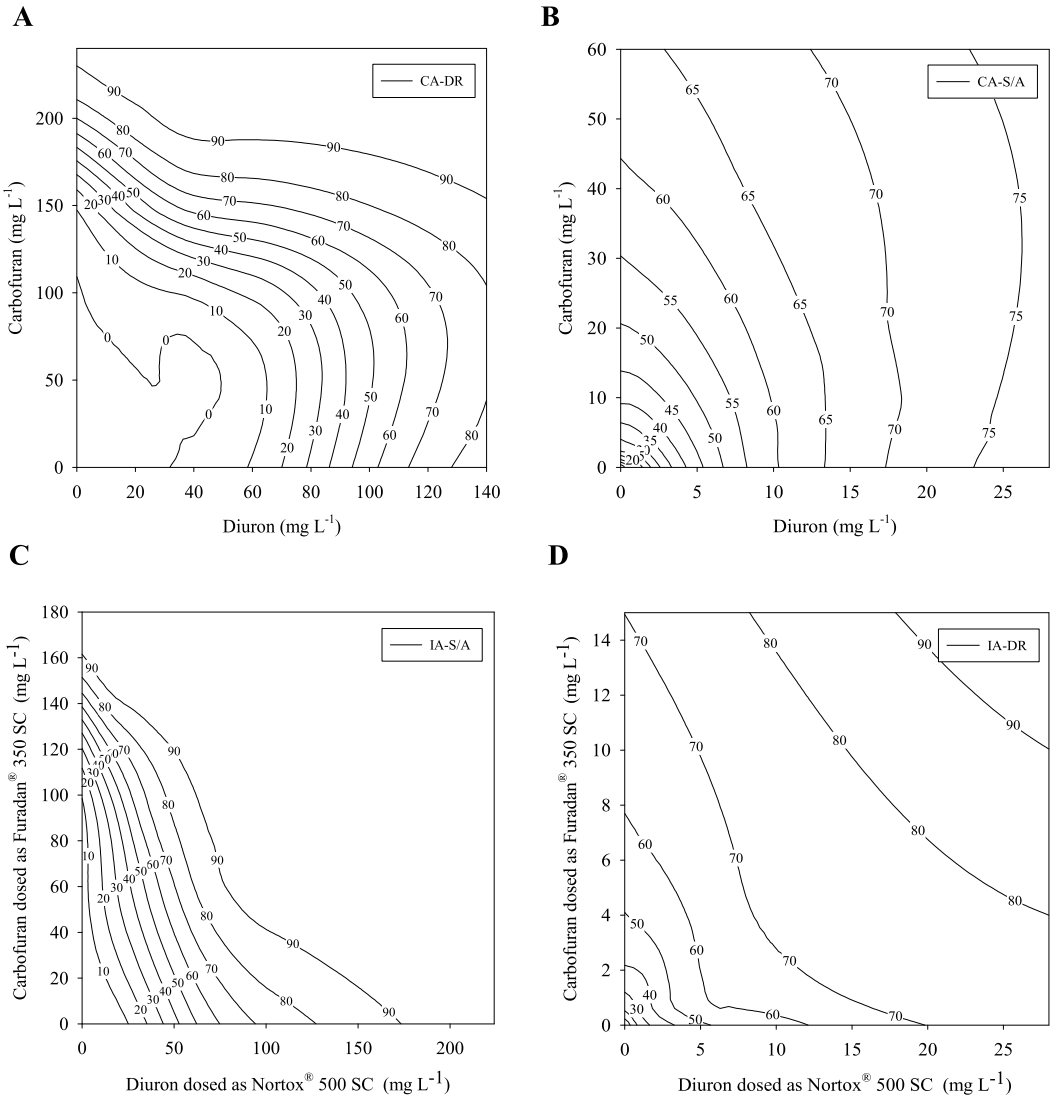

Fig. 2. Isobolograms (represented by EC_x) of the effects of the acute and chronic pesticide mixtures on *P. caudatum*. (A) dose-ratio dependent (DR) deviation from the CA model to acute active ingredient mixtures; (B) S/A deviation showing antagonism from the CA model to chronic active ingredient mixtures; (C) S/A deviation showing synergism from the IA model to acute commercial product mixtures; (D) DR deviation from the IA model to chronic commercial product mixtures. Linear, concave and convex isoboles represent no interaction, synergy and antagonism, respectively (Ryall and Tan, 2015).

statistically significant ($p = 0.04$; $r^2 = 0.95$; $a = 1.93$; $b_{DL} = 1.56$). Thus, the interaction of the pesticides in the mixture was dose-level dependent (Table 3; Fig. 4A), with antagonism occurring at low doses and synergism at high doses of the pesticides. In addition, this change in the interaction occurred at a dose level lower than EC_{50}, as shown in Fig. 4A. In the chronic toxicity test with the active ingredient pesticide mixtures (Table 3), data fitted to the CA model yielded an *SS* value of 47.68 ($p < 0.001$; $r^2 = 0.87$). After adding parameter *a* to the CA model, the S/A deviation better described the data with a decrease of *SS* value to 21.44, which was statistically significant ($p < 0.001$; $r^2 = 0.94$). Parameter *a* had a positive value ($a = 1.56$), indicating antagonism of the pesticides in mixture (Table 3, Fig. 4B).

Concerning the acute toxicity test with *C. silvestrii* exposed to commercial product mixtures of diuron e carbofuran (Table 3), data fitted the IA model ($L = 50.78$, $p < 0.001$; $r^2 = 0.85$), but a significantly better fit was obtained after adding parameter *a* to describe the S/A deviation ($L = 16.72$, $p < 0.001$; $r^2 = 0.95$). Parameter *a* was positive ($a = 4.24$), indicating antagonistic interactions of pesticide commercial formulations in the mixtures, as shown in Fig. 4C. In the chronic toxicity test with the pesticide commercial product mixtures (Table 3), the fitting of the data to the IA model yielded an *SS* of 45.12 ($p < 0.001$, $r^2 = 0.77$). After adding the parameters *a* and b_{DL} to this model, the DL deviation significantly better described the data (IA: $SS = 15.96$, $p < 0.001$; $r^2 = 0.92$; $a = -3.73$; $b_{DL} = 2.30$). Therefore, it was observed that the interaction between the pesticides in the mixtures were dose-level dependent, with synergism occurring at low doses and antagonism at high doses (Fig. 4D).

4. Discussion

4.1. Toxicity of individual pesticides

In this study, the acute and chronic toxicity values for the protozoan *P. caudatum* in single exposures of diuron and carbofuran were similar to those that we determined previously for active ingredients and commercial formulations (Mansano et al., 2016), presenting no statistical difference between the values of both studies for all tested compounds (Student's t-test, $p > 0.05$). Subsequently, the physiological health conditions of the test organisms (adequate status according to NaCl reference substance test) appeared to have been comparable between the two studies.

Our results showed that carbofuran and diuron individually tested

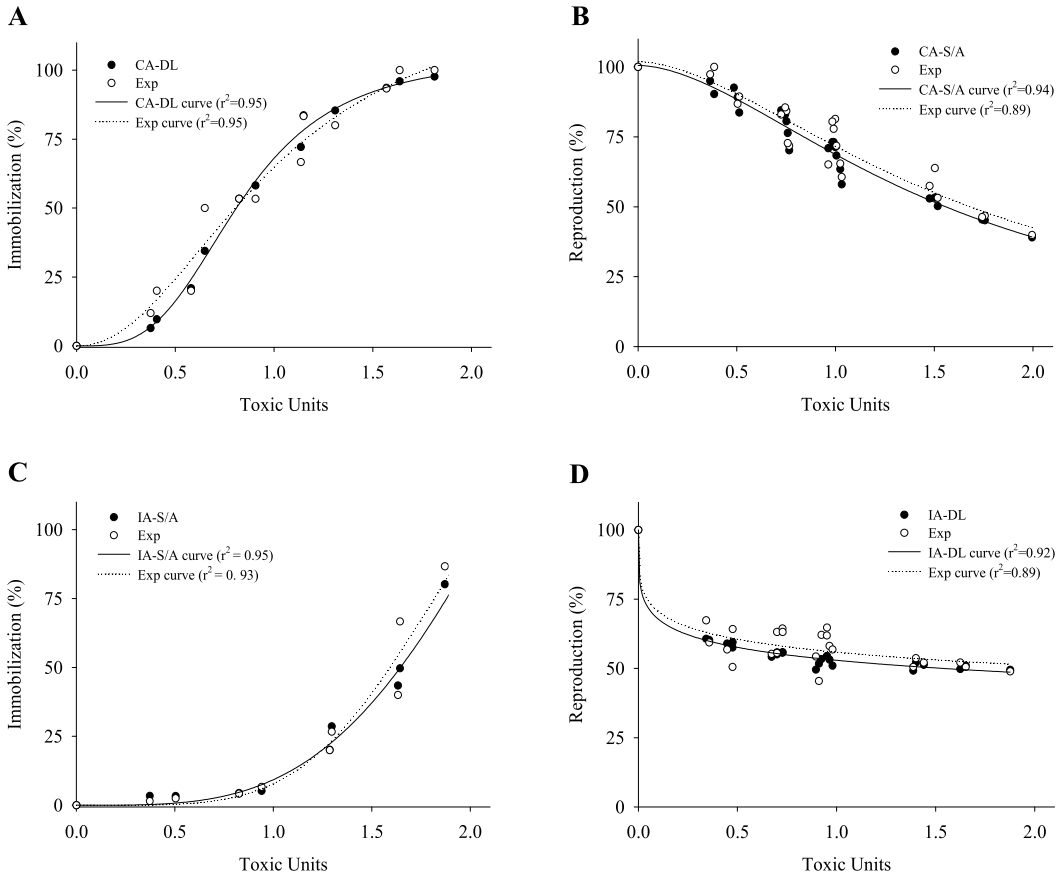

Fig. 3. Dose-response curves obtained from the observed data (Exp) and modelled data of the pesticide mixture toxicity tests on *C. silvestrii* (immobilization and reproduction). Acute (A) and chronic (B) toxicity of the active ingredient mixtures and acute (C) and chronic (D) toxicity of the commercial product mixtures. The solid and dotted lines represent the three-parameter logistic curve-fitting from the modelled and observed data, respectively. CA-DL = dose-level dependent (DL) deviation from the CA model; CA-S/A = synergistic/antagonistic (S/A) deviation from the CA model; IA-S/A = S/A deviation from the IA model; IA-DL = DL deviation from the IA model.

Table 3
Summary of the analysis of the acute and chronic toxicity tests with the cladoceran *C. silvestrii* exposed to mixtures of carbofuran and diuron (active ingredients and commercial products) using the CA and IA models. The model and deviation that best fitted the mixture toxicity data using the MIXTOX tool (Jonker et al., 2005) are indicated in bold.

Toxicity	Mixture	Model and Deviation	a	$b_{DR/DL}$	*L* or SS	r^2	*p* (χ^2 or F)	max	$EC_{50\ Diuron}$	β_{Diuron}	$EC_{50\ Carbofuran}$	$\beta_{Carbofuran}$
Acute	Carbofuran and Diuron (active ingredients)	CA	–	–	27.16	0.93	1.01×10^{-74}	0.95	6.42	2.73	0.88×10^{-3}	4.71
		CA-DL	**1.93**	**1.56**	**17.86**	**0.95**	**0.04**	**0.96**	**7.13**	**2.01**	$\mathbf{0.99 \times 10^{-3}}$	**3.59**
		IA	–	–	205.40	0.46	2.54×10^{-36}	0.99	9.03	3.50	1.05×10^{-3}	3.5
		IA-DR	−6.55	0.70	20.83	0.94	2.49×10^{-5}	0.93	9.03	3.16	0.98×10^{-3}	4.37
	Carbofuran and Diuron (commercial products)	CA	–	–	88.96	0.74	6.79×10^{-53}	1.27	1.16	1.16	0.89×10^{-3}	3.27
		CA-S/A	2.44	–	45.56	0.87	4.46×10^{-11}	0.94	1.20	4.50	1.00×10^{-3}	4.84
		IA	–	–	50.78	0.85	3.99×10^{-61}	1.06	1.16	2.37	1.14×10^{-3}	4.68
		IA-S/A	**4.24**	**-**	**16.72**	**0.95**	$\mathbf{5.36 \times 10^{-9}}$	**4.44**	**0.05**	**0.66**	$\mathbf{0.66 \times 10^{-3}}$	**4.01**
Chronic	Carbofuran and Diuron (active ingredients)	CA	–	–	47.68	0.87	5.40×10^{-13}	16.63	6.92	4.46	1.56×10^{-3}	0.98
		CA-S/A	**1.56**	**-**	**21.44**	**0.94**	$\mathbf{1.23 \times 10^{-7}}$	**16.52**	**6.43**	**3.74**	$\mathbf{0.86 \times 10^{-3}}$	**1.41**
		IA	–	–	24.52	0.93	2.69×10^{-17}	16.24	6.64	3.78	0.92×10^{-3}	1.49
		–	–	–	–	–	–	–	–	–	–	–
	Carbofuran and Diuron (commercial products)	CA	–	–	25.26	0.87	1.82×10^{-12}	16.57	0.78	1.27	1.36×10^{-3}	0.26
		CA-DL	−2.23	1.22	20.14	0.90	0.02	16.52	0.74	1.33	0.78×10^{-3}	0.56
		IA	–	–	45.12	0.77	7.28×10^{-9}	15.38	1.60	1.10	2.30×10^{-3}	0.38
		IA-DL	**−3.73**	**2.30**	**15.96**	**0.92**	$\mathbf{1.28 \times 10^{-7}}$	**16.69**	**0.74**	**1.29**	$\mathbf{0.64 \times 10^{-3}}$	**0.76**

CA is the concentration addition model and IA is the independent action model; S/A is synergism or antagonism deviation; DR is dose-ratio dependent deviation and DL is dose-level deviation; *a*, b_{DR} and b_{DL} are parameters of the functions; *L* and *SS* are the objective functions used for discrete and continuous data, respectively; r^2 is the regression coefficient; χ^2 or F test is the test statistic; and *p* (χ^2/F) is the significance level of the test statistic; max is the maximum response value; EC_{50} is the median effective concentration; β is the slope of the individual dose response curve.

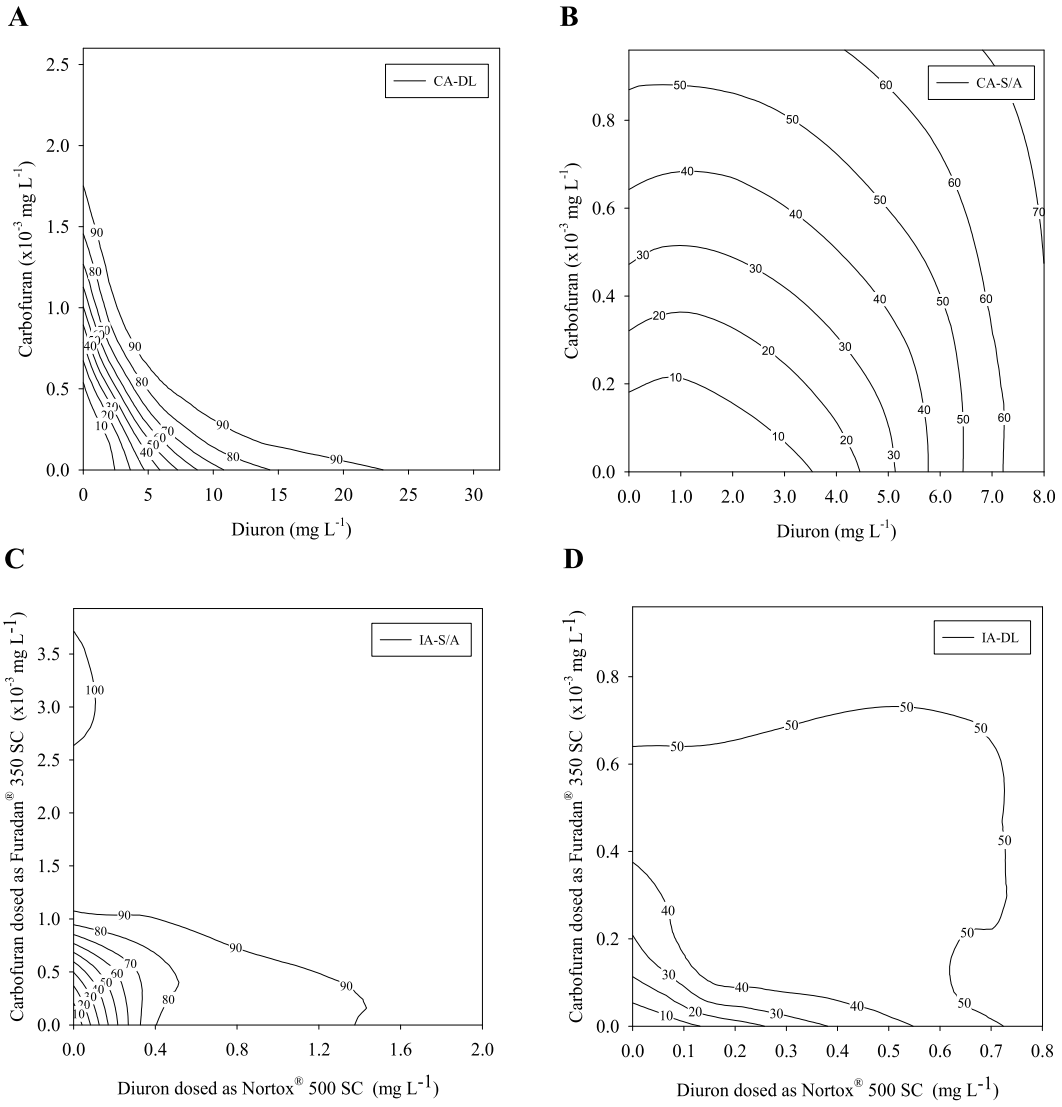

Fig. 4. Isobolograms (represented by EC_x) of the effects of the acute and chronic pesticide mixtures on *C. silvestrii*. (A) dose-level dependent (DL) deviation from the CA model to acute active ingredient mixtures; (B) S/A deviation showing antagonism from the CA model to chronic active ingredient mixtures; (C) S/A deviation showing antagonism from the IA model to acute commercial product mixtures; (D) DL deviation from the IA model to chronic commercial product mixtures. Linear, concave and convex isoboles represent no interaction, synergy and antagonism, respectively (Ryall and Tan, 2015).

caused mortality and population growth inhibition for *P. caudatum*. Despite the mode of action of herbicide diuron being well known for autotrophic (photosynthesis inhibitor) and non-target heterotrophic organisms (endocrine disruptor, AChE inhibitor), its mode of action for ciliate protozoa is still unclear. According to some studies, diuron can block the mitochondrial respiratory chain (Dragone et al., 2015; Estève et al., 2009) and generate reactive oxygen species (ROS) (Tenda et al., 2012) in heterotrophic microorganisms such as *Saccharomyces cerevisiae*. In the case of carbofuran, Hussain et al. (2008) reported that this pesticide induced inhibitory effects on phagocytosis and the pulsatile vacuole activity of *P. caudatum*. Cellular abnormalities observed in carbofuran-exposed microorganisms such as cyanobacteria, microalgae (Megharaj et al., 1993) and flagellates (Azizullah et al., 2011) indicate the interaction of carbofuran with membrane properties. Therefore, the toxicity of diuron on *P. caudatum* may have been caused by respiration inhibition and increased ROS level, whereas the toxicity of carbofuran on this ciliate may have been caused by interference of this compound in cell membrane properties.

For the cladoceran *C. silvestrii*, the acute and chronic toxicity values in single exposures of diuron and carbofuran (active ingredients or commercial formulations) found in the present study were significantly different (Student's t-test, $p < 0.05$) from those previously established in Mansano et al. (2018). Our acute values were now higher (7–14%) than those observed previously, with the exception of the carbofuran commercial product, which had a 23% lower value. For chronic toxicity, the values verified in this study for all compounds tested were lower (13–25%) than those formerly found (Mansano et al., 2018). In both studies, the physiological health conditions of *C. silvestrii* were assessed by exposure to NaCl reference substance and proved to be adequate for the toxicity tests, allowing comparisons between the results from both studies.

As expected, the insecticide carbofuran was highly toxic to cladoceran *C. silvestrii*, while the herbicide diuron was only moderately toxic to this species. Carbofuran (active ingredient or commercial formulation) caused immobility and reduced reproduction for *C. silvestrii* at very low concentrations, with toxicity increasing in a concentration-dependent manner. Carbofuran toxicity to cladoceran can be explained by the AChE inhibition. Several other studies have also shown high carbofuran toxicity to aquatic invertebrates as *Daphnia magna* (Barata et al., 2004), *D. ambigua* (Arias-Andrés et al., 2016), *Gammarus pulex* (Ashauer et al., 2011) and *Strandesia trispinosa* (Rocha et al., 2018). Differently, diuron (both forms) caused stimulatory responses

(hormesis) on the reproduction at the lowest concentrations and decreased reproduction at the highest concentrations tested. Although the hormetic effect could be interpreted as a beneficial response, this could have severe consequences for overall fitness of the species due to disproportionate energy allocation to reproduction combined with stress caused by pesticide exposure (Cedergreen et al., 2007; Mansano et al., 2018; Zalizniak and Nugegoda, 2006). Previous ecotoxicological studies have demonstrated that diuron may exert toxic effects on heterotrophic invertebrates and vertebrates through different modes of action such as immunotoxicity (Luna-Acosta et al., 2012), embryotoxicity and genotoxicity (Behrens et al., 2016) in *Crassostrea gigas*; endocrine disruption in *Xenopus laevis* (Orton et al., 2009) and *Oreochromis niloticus* (Boscolo et al., 2018) and AChE inhibition in *Bungarus sindanus* and *Electrophorus electricus* (Ahmed et al., 2012). Therefore, we suggested that the toxicity of diuron on mobility and reproduction of *C. silvestrii* in this study may have been caused by AChE inhibition and endocrine disruption.

4.2. Toxicity of pesticide mixtures

Our results showed that the carbofuran and diuron mixtures (active ingredients or commercial products) caused significant deviations in toxicity from that predicted by the CA and IA models for both the protozoan and cladoceran. In the case of *P. caudatum*, the combination of carbofuran and diuron (active ingredients) altered the acute toxicity of the compounds to the protozoan, and the type of interaction was dose-ratio dependent (Fig. 2A). When carbofuran was the prevalent pesticide in mixture, synergistic interactions between the compounds were found. The possible interaction of carbofuran with cell membranes (disruption of cellular organization; Megharaj et al., 1993) could alter the permeability and lead to a greater diuron uptake and hence be related with this synergistic interaction. However, antagonistic interactions between the pesticides occurred when diuron was the dominant compound in the mixture. The chronic test with the active ingredient mixtures also showed antagonism on the growth inhibition of *P. caudatum* (Fig. 2B). According to some studies, antagonism can be explained by an induction of P450 activity caused by herbicide (atrazine) and the consequent increase in biotransformation of insecticide (omethoate, chlorpyrifos) (Anderson and Zhu, 2004; Cedergreen, 2014; Fu et al., 2013). Furthermore, Farré et al. (2002) reported that the antagonistic effects in pesticide mixtures (endosulfan, dimethoate, fenamiphos, ametryn and chlorfenvinphos) may be caused by the association between the compound molecules, which decreased their bioavailability. A study on the inhibitory effects of binary pharmaceutical mixtures (diclofenac, ibuprofen, metoprolol and propranolol) on the proliferation of the protozoan *Tetrahymena pyriformis* revealed that the predominant interaction in mixtures was antagonism (in 59% of mixture combinations), followed by concentration addition (37%) and synergism (4%) (Láng and Kőhidai, 2012), demonstrating that antagonism could occur when the endpoint studied was protozoan population growth. As examples of other studies carried out with pesticide mixtures involving heterotrophic microorganisms, Fernández-Alba et al. (2002) found antagonistic interactions for the mixture of the herbicides Irgarol 1051 and Sea nine 211 and for the mixture of Irgarol 1051, Sea nine 211 and diuron on the bacterium *Vibrio fischeri*. Gatidou et al. (2015) observed antagonistic effects on *V. fischeri* when exposed to combination of the herbicides linuron and monolinuron, and synergistic effects when exposed to diuron and monolinuron mixture.

In evaluating the acute and chronic toxicity of carbofuran and diuron commercial product mixtures to *P. caudatum*, both the reference model and their deviations were different from those obtained in the tests with the active ingredients (Table 2, Fig. 2). In the former, the reference model that best fitted the data was IA and synergistic interactions were more apparent (Fig. 2C and D) than in the mixture tests with active ingredients that presented antagonistic interactions (Fig. 2A and B). The acute test with the commercial product mixtures showed synergism for the *P. caudatum*, while the chronic test indicated synergism caused mainly by diuron and antagonism caused mainly by carbofuran (DR deviation). Commercial pesticide formulations are a combination of the active ingredient(s) with so-called inert ingredients (e.g. emulsifiers, solvents, surfactants, etc) (Cox and Surgan, 2006), which may also contribute significantly to the overall formulation toxicity (Beggel et al., 2010; Chen and Stark, 2010; Moreira et al., 2017). When the two commercial products (Diuron Nortox® 500 SC and Furadan® 350 SC) were mixed in this study, the complexity of the mixture increased, and the protozoan was exposed to a cocktail of contaminants. Thus, the higher toxicity of the commercial product mixtures as compared with the active ingredients was probably due to interactions of diuron, carbofuran and the inert ingredients in the formulated pesticide products. Since pesticides in agriculture are applied as commercial products, this enhanced toxicity is likely to occur in natural aquatic environments. These results also support the hypothesis that complex mixture toxicity cannot be based solely on the toxicity of their single components (Fernández-Alba et al., 2002; Gomiero and Viarengo, 2014). Despite the structural simplicity of *P. caudatum* (unicellular heterotrophic organism) compared to metazoans (multicellular animals), tests with this species were able to demonstrate differential toxicity of both the individual compounds and their mixtures and may hence be considered as a suitable test organism to be used in pesticide (mixture) risk assessments.

For cladoceran *C. silvestrii*, results from the acute toxicity test with the active ingredient combinations showed that the type of interaction in the mixture was dose-level dependent, with antagonism occurring at low doses and synergism at high doses of the pesticides (Fig. 4A). Regarding chronic toxicity of the active ingredient combinations on the *C. silvestrii* reproduction, it was found that mixtures of the pesticides caused less toxicity (antagonism) than when they were tested individually (Fig. 4B). Anderson and Zhu (2004) demonstrated that the herbicide atrazine (photosynthesis inhibitor) decreased the toxicity of the insecticide omethoate (AChE inhibitor) to the midge *Chironomus tentans* (i.e., antagonism). According to these authors, atrazine decreased the omethoate toxicity through induction of P450 monooxygenases, resulting in increased omethoate biotransformation to metabolites with lower toxicity than the parent compound. Several other studies have also shown that the most common mechanism of action observed between atrazine and several organophosphorus insecticides in various invertebrates, such as *Ceriodaphnia dubia*, *Chironomus tentans* and *Hyalella azteca*, was the result of atrazine increasing the biotransformation of these compounds, converting them in less or more toxic metabolites (Anderson and Lydy, 2002; Banks et al., 2005; Belden and Lydy, 2000; Choung et al., 2011; Mehler et al., 2008). Diuron as well as atrazine induce the cytochrome P450 (CYP) enzyme activity, while many carbamate insecticides (as carbofuran) are metabolized by CYP enzymes (Abass et al., 2012, 2014). Therefore, the antagonism that we noted at all tested concentrations in the chronic tests and at low doses of the acute tests may have been due to the fact that diuron induced the cytochrome P450 and increased the carbofuran biotransformation, and consequently caused a decrease in toxicity by metabolic detoxification.

The interactions resulting from the mixtures of commercial products on *C. silvestrii* were different from those for the active ingredient mixtures (Table 3). For acute toxicity, the commercial product mixtures showed antagonistic interactions (Fig. 4C), whereas chronic toxicity showed dose-level dependent interactions (Fig. 4D) with synergism in low concentrations of the compounds, results that were not observed for the active ingredient mixtures. Rocha et al. (2018), when comparing the diuron and carbofuran mixture toxicity in their forms of active ingredients and commercial products for ostracod *Strandesia trispinosa*, also reported different toxicity deviations, as verified in this study. In active ingredient mixtures, antagonistic effects were observed on *S. trispinosa* survival, whereas in commercial product mixtures, a synergism was found in high doses of the compounds (Rocha et al., 2018). In

our study, the synergistic effects on *C. silvestrii* reproduction when exposed to commercial product mixtures could be explained by the action of the inert substances in the formulations through its intrinsic toxicity and/or by interacting with the active ingredient(s). Although many studies have demonstrated that commercial formulations exhibit more damage to non-target organisms than their corresponding active ingredients (e.g. Bach et al., 2018; Mesnage and Antoniou, 2018; Pereira et al., 2009), limited information is available regarding the mixture effects of pesticide formulation inert and active substances (e.g. Altenburger et al., 2013; Joly et al., 2013; Rocha et al., 2018).

4.3. Implications for risk assessment

Studies carried out in Brazil (e.g. Caldas et al., 2019; Carbo et al., 2008) and elsewhere, for example, in Italy (Fagnana Lake, Milan; Bacigalupo and Meroni, 2007), France (Adour-Garonne basin, Toulose; Faggiano et al., 2010); Spain (Llobregat River, northeast of Catalonia; Masiá et al., 2015) have reported the simultaneous occurrence of carbofuran and diuron in water bodies near agricultural areas. In Brazilian freshwaters, the maximum environmental concentrations of carbofuran and diuron already reported were from 0.069 mg L^{-1} (Carbo et al., 2008) and 0.408 mg L^{-1} (Paschoalato et al., 2008), respectively. At these environmental concentrations, when considering the toxicity values found in the present study, acute and chronic effects on cladoceran *C. silvestrii* could occur when exposed to insecticide carbofuran (48 h EC_{50} = 0.001 mg L^{-1} and 8 d LOEC = 0.00038 mg L^{-1} for formulated carbofuran), and also chronic effects from exposure to herbicide diuron (8 d LOEC < 0.05 mg L^{-1} for formulated diuron). For protozoan *P. caudatum*, no direct effects could be expected at environmental concentrations based on the toxicity values obtained for both pesticides in this study. Nevertheless, diuron and carbofuran may be bioaccumulated in *P. caudatum* (Mansano et al. (2016) and biomagnified along trophic chain, and consequently could affect the higher trophic levels, which enhances the importance of studying the impact of pesticide toxicity on protozoans.

In previous preliminary risk assessments (Mansano et al., 2016), we have shown that diuron and carbofuran in single exposures may pose potential ecological risks to Brazilian water bodies. In this study, our results demonstrated that carbofuran and diuron may interact and cause different toxic responses than those predicted by the individually tested compounds. In the case of pesticide formulated mixtures in chronic exposure in which synergistic interactions were observed for *C. silvestrii* at concentrations below the measured environmental concentrations (MEC) (Fig. 4D), environmental risk assessments based only on individual exposures of these pesticides may, therefore, not adequately protect aquatic ecosystems. However, to improve future environmental assessments of these pesticides in mixtures for Brazilian water bodies, more tropical species from different trophic levels should be assessed and included in the risk assessment.

5. Conclusions

The results of the present study revealed that the mixtures of carbofuran and diuron may cause greater (synergism) or lower (antagonism) toxicity to *P. caudatum* and *C. silvestrii* than when tested alone. Therefore, we reinforce that risk assessments solely based on individual pesticides may lead to an under- or over-estimation of the actual risks of the pesticide cocktail to aquatic organisms in edge-of-field waterbodies. Furthermore, in this study it was observed that the toxicity of the commercial formulation mixtures (Diuron Nortox® 500 SC and Furadan® 350 SC) was higher than that of the active ingredients due to the interactions of the active ingredients with each other and with the inert ingredients in the formulations. Since pesticides in the field are applied as commercial products, we emphasize the importance and need for studies using commercial product mixtures besides the active ingredient combinations since they can give more realistic insights into the interactions that occur under natural conditions. From the results of toxicity tests in single exposures, both pesticides caused deleterious effects to *P. caudatum* and *C. silvestrii*. Environmentally relevant concentrations of insecticide carbofuran significantly caused mortality and reduced reproduction to the tropical cladoceran *C. silvestrii*. Despite the fact that toxicity of herbicide diuron only occurs at high concentrations to the protozoan and cladoceran, this formulated pesticide at low concentrations can interact with formulated carbofuran and increase the toxicity to these species. The increased toxicity for planktonic invertebrates caused by the diuron and carbofuran formulation mixtures may pose a greater environmental risk for the aquatic biota. We believe that additional studies are needed to develop practical criteria for the selection of pesticide mixtures that demand additional efforts due to their likelihood in exerting synergistic responses in the field. As future perspectives, we suggest that molecular and biochemical studies be carried out with these pesticides, since they can contribute to the development of toxicokinetic-toxicodynamic models that are imperative for attaining a more mechanistic understanding of pesticide mixture ecotoxicology.

Credit author statement

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

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References

Abass, K., Lämsä, V., Reponen, P., Küblbeck, J., Honkakoski, P., Mattila, S., Pelkonen, O., Hakkola, J., 2012. Characterization of human cytochrome P450 induction by pesticides. Toxicology 294 (1), 17–26.

Abass, K., Reponen, P., Mattila, S., Rautio, A., Pelkonen, O., 2014. Human variation and CYP enzyme contribution in benfuracarb metabolism in human in vitro hepatic models. Toxicol. Lett. (Shannon) 224 (2), 300–309.

ABNT - Associação Brasileira de Normas Técnicas, 2016. NBR 12713. Aquatic ecotoxicology – acute toxicity – test with Daphnia spp. (Crustacea, Cladocera), rio de Janeiro, Brazil. (in Portuguese). Available in. https://www.abntcatalogo.com.br/norma.aspx?ID=355914 Accessed 04.06.2020.

ABNT - Associação Brasileira de Normas Técnicas, 2017. NBR 13373. Aquatic ecotoxicology – chronic toxicity – test with Ceriodaphnia spp. (Crustacea, Cladocera), rio de Janeiro, Brazil. (in Portuguese). Available in. https://www.abntcatalogo.com.br/norma.aspx?ID=359921 Accessed 04.06.2020.

AGROFIT - Phytosanitary Agrochemicals System, 2016. Brazil (in Portuguese). Available

in: http://agricultura.gov.br/agrofit 04.30.2016.
Ahmed, M., Latif, N., Khan, R.A., Ahmad, A., 2012. Toxicological effect of herbicides (diuron and bentazon) on snake venom and electric eel acetylcholinesterase. Bull. Environ. Contam. Toxicol. 89, 229–233.
Albuquerque, A.F., Ribeiro, J.S., Kummrow, F., Nogueira, A.J.A., Montagner, C.C., Umbuzeiro, G.A., 2016. Pesticides in Brazilian freshwaters: a critical review. Environ. Sci.: Proces. Impac. 18 (7), 779–787.
Altenburger, R., Arrhenius, A., Backhaus, T., Coors, A., Faust, M., Zitzkat, D., 2013. Ecotoxicological Combined Effects from Chemical Mixtures - Part 1: Relevance and Adequate Consideration in Environmental Risk Assessment of Plant Protection Products and Biocides. Federal Environment Agency (Umweltbundesamt) Wörlitzer Platz.
Anderson, T.D., Lydy, M.J., 2002. Increased toxicity to invertebrates associated with a mixture of atrazine and organophosphate insecticides. Environ. Toxicol. Chem. 21 (7), 1507–1514.
Anderson, T.D., Zhu, K.Y., 2004. Synergistic and antagonistic effects of atrazine on the toxicity of organophosphorodithioate and organophosphorothioate insecticides to *Chironomus tentans* (Diptera: chironomidae). Pestic. Biochem. Physiol. 80, 54–64.
Arias-Andrés, M., Rämö, R., Torres, F.M., Ugalde, R., Grandas, L., Ruepert, C., Castillo, L.E., Van den Brink, P.J., Gunnarsson, J.S., 2018. Lower tier toxicity risk assessment of agriculture pesticides detected on the Río Madre de Dios watershed, Costa Rica. Environ. Sci. Pollut. Res. 25 (14), 13312–13321.
Ashauer, R., Hintermeister, A., Potthoff, E., Escher, B.I., 2011. Acute toxicity of organic chemicals to *Gammarus pulex* correlates with sensitivity of *Daphnia magna* across most modes of action. Aquat. Toxicol. 103 (1–2), 38–45.
Azizullah, A., Richter, P., Häder, D.P., 2011. Comparative toxicity of the pesticides carbofuran and malathion to the freshwater flagellate *Euglena gracilis*. Ecotoxicology 20 (6), 1442–1454.
Bach, N.C., Marino, D.J., Natale, G.S., Somoza, G.M., 2018. Effects of glyphosate and its commercial formulation, Roundup® Ultramax, on liver histology of tadpoles of the neotropical frog, *Leptodactylus latrans* (amphibia: Anura). Chemosphere 202, 289–297.
Bacigalupo, M.A., Meroni, G., 2007. Quantitative determination of diuron in ground and surface water by time-resolved fluoroimmuno assay: seasonal variations of diuron, carbofuran and paraquat in an agricultural area. J. Agric. Food Chem. 55 (10), 3823–3828.
Banks, K.E., Turner, P.K., Wood, S.H., Matthews, C., 2005. Increased toxicity to *Ceriodaphnia dubia* in mixtures of atrazine and diazinon at environmentally realistic concentrations. Ecotoxicol. Environ. Saf. 60, 28–36.
Barata, C., Solayan, A., Porte, C., 2004. Role of B-esterases in assessing toxicity of organophosphorus (chlorpyrifos, malathion) and carbamate (carbofuran) pesticides to *Daphnia magna*. Aquat. Toxicol. 66 (2), 125–139.
Beggel, S., Werner, I., Connon, R.E., Geist, J.P., 2010. Sublethal toxicity of commercial insecticide formulations and their active ingredients to larval fathead minnow (*Pimephales promelas*). Sci. Total Environ. 408 (16), 3169–3175.
Behrens, D., Rouxel, J., Burgeot, T., Akcha, F., 2016. Comparative embryotoxicity and genotoxicity of the herbicide diuron and its metabolites in early life stages of *Crassostrea gigas*: implication of reactive oxygen species production. Aquat. Toxicol. 175, 249–259.
Belden, J.B., Lydy, M.J., 2000. Impact of atrazine on organophosphate insecticide toxicity. Environ. Toxicol. Chem. 19, 2266–2274.
Boscolo, C.N.P., Pereira, T.S.B., Batalhão, I.G., Dourado, P.L.R., Schlenk, D., Almeida, E.A., 2018. Diuron metabolites act as endocrine disruptors and alter aggressive behavior in Nile tilapia (*Oreochromis niloticus*). Chemosphere 191, 832–838.
Brasil, 2011. Brazilian Health Ministry. Portaria n° 2.914, de 12 de dezembro de 2011. Dispõe sobre os procedimentos de controle e de vigilância da qualidade da água para consumo humano e seu padrão de potabilidade. Brasil (in Portuguese).
Bretaud, S., Toutant, J.P., Saglio, P., 2000. Effects of carbofuran, diuron, and nicosulfuron on acetylcholinesterase activity in goldfish (*Carassius auratus*). Ecotoxicol. Environ. Saf. 47 (2), 117–124.
Caldas, S.S., Arias, J.L.O., Rombaldi, C., Mello, L.L., Cerqueira, M.B., Martins, A.F., Primel, E.G., 2019. Occurrence of pesticides and ppcps in surface and drinking water in southern Brazil: data on 4-year monitoring. J. Brazilian Chem. Soc. 30 (1), 71–80.
Carbo, L., Souza, V., Dores, E.F., Ribeiro, M.L., 2008. Determination of pesticides multiresidues in shallow groundwater in a cotton-growing region of Mato Grosso, Brazil. J. Braz. Chem. Soc. 19 (6), 1111–1117.
Casali-Pereira, M.P., Daam, M.A., Resende, J.C., Vasconcelos, A.M., Espíndola, E.L., Botta, C.M., 2015. Toxicity of Vertimec® 18 EC (active ingredient abamectin) to the neotropical cladoceran Ceriodaphnia silvestrii. Chemosphere 139, 558–564.
Cassee, F.R., Groten, J.P., Bladeren, P.J., Feron, V.J., 1998. Toxicological evaluation and risk assessment of chemical mixtures. Crit. Rev. Toxicol. 28, 73–101.
Cedergreen, N., 2014. Quantifying synergy: a systematic review of mixture toxicity studies within environmental toxicology. PloS One 9 (5), e96580.
Cedergreen, N., Streibig, J., Kudsk, P., Mathiassen, S.K., Duke, S.O., 2007. The occurrence of hormesis in plants and algae. Dose-Response 5, 150–162.
CETESB Companhia Ambiental do Estado de São Paulo, 2018. Qualidade das águas interiores no Estado de São Paulo. Série Relatórios. CETESB, São Paulo 284pp. Brazil (in Portuguese).
Chen, X.D., Stark, J.D., 2010. Individual-and population-level toxicity of the insecticide, spirotetramat and the agricultural adjuvant, destiny to the Cladoceran, *Ceriodaphnia dubia*. Ecotoxicology 19, 1124–1129.
Choung, C.B., Hyne, R.V., Stevens, M.M., Hose, G.C., 2011. Toxicity of the insecticide terbufos, its oxidation metabolites, and the herbicide atrazine in binary mixtures to *Ceriodaphnia cf dubia*. Arch. Environ. Contam. Toxicol. 60 (3), 417–425.
Choung, C.B., Hyne, R.V., Stevens, M.M., Hose, G.C., 2013. The ecological effects of a herbicide-insecticide mixture on an experimental freshwater ecosystem. Environ. Pollut. 172, 264–274.
Clasen, B., Leitemperger, J., Murussi, C., Pretto, A., Menezes, C., Dalabona, F., Marchezan, E., Adaime, M.B., Zanella, R., Loro, V.L., 2014. Carbofuran promotes biochemical changes in carp exposed to rice field and laboratory conditions. Ecotoxicol. Environ. Saf. 101, 77–82.
Cox, C., Surgan, M., 2006. Unidentified inert ingredients in pesticides: implications for human and environmental health. Environ. Health Perspect. 114, 1803–1806.
Dang, Z., 2019. Endpoint sensitivity in amphibian metamorphosis assay. Ecotoxicol. Environ. Saf. 167, 513–519.
Dettmers, J.M., Stein, R.A., 1992. Food consumption by larval gizzard shad: zooplankton effects and implications for reservoir communities. Trans. Am. Fish. Soc. 121, 494–507.
Dragone, R., Cheng, R., Grasso, G., Frazzoli, C., 2015. Diuron in water: functional toxicity and intracellular detoxification patterns of active concentrations assayed in tandem by a yeast-based probe. Int. J. Environ. Res. Publ. Health 12 (4), 3731–3740.
Estève, K., Poupot, C., Dabert, P., Mietton-Peuchot, M., Milisic, V., 2009. A *Saccharomyces cerevisiae*-based bioassay for assessing pesticide toxicity. J. Ind. Microbiol. Biotechnol. 36 (12), 1529–1534.
Faggiano, L., de Zwart, D., García-Berthou, E., Lek, S., Gevrey, M., 2010. Patterning ecological risk of pesticide contamination at the river basin scale. Sci. Total Environ. 408 (11), 2319–2326.
Farré, M., Gonçalves, C., Lacorte, S., Barceló, D., Alpendurada, M., 2002. Pesticide toxicity assessment using an electrochemical biosensor with *Pseudomonas putida* and a bioluminescence inhibition assay with *Vibrio fischeri*. Anal. Bioanal. Chem. 373 (8), 696–703.
Fernández-Alba, A.R., Guil, M.D.H., Lopez, G.D., Chisti, Y., 2002. Comparative evaluation of the effects of pesticides in acute toxicity luminescence bioassays. Anal. Chim. Acta 451, 195–202.
Freitas, E.C., Pinheiro, C., Rocha, O., Loureiro, S., 2014. Can mixtures of cyanotoxins represent a risk to the zooplankton? The case study *of Daphnia magna* Straus exposed to hepatotoxic and neurotoxic cyanobacterial extracts. Harmful Algae 31, 143–152.
Freitas, E.C., Rocha, O., Espíndola, E.L.G., 2018. Effects of florfenicol and oxytetracycline on the tropical cladoceran *Ceriodaphnia silvestrii*: a mixture toxicity approach to predict the potential risks of antimicrobials for zooplankton. Ecotoxicol. Environ. Saf. 162, 663–672.
Fu, Y., Li, M., Liu, C., Qu, J.P., Zhu, W.J., Xing, H.J., Xu, S.W., Li, S., 2013. Effect of atrazine and chlorpyrifos exposure on cytochrome P450 contents and enzyme activities in common carp gills. Ecotoxicol. Environ. Saf. 94, 28–36.
Gatidou, G., Stasinakis, A.S., Iatrou, E.I., 2015. Assessing single and joint toxicity of three phenylurea herbicides using *Lemna minor* and *Vibrio fischeri* bioassays. Chemosphere 119, 69–74.
Giacomazzi, S., Cochet, N., 2004. Environmental impact of diuron transformation: a review. Chemosphere 56, 1021–1032.
Gomiero, A., Viarengo, A., 2014. Effects of elevated temperature on the toxicity of copper and oxytetracycline in the marine model, *Euplotes crassus*: a climate change perspective. Environ. Pollut. 194, 262–271.
Hasenbein, S., Lawler, S.P., Geist, J., Connon, R.E., 2016. A long-term assessment of pesticide mixture effects on aquatic invertebrate communities. Environ. Toxicol. Chem. 35, 218–232.
Hussain, M.M., Amanchi, N.R., Solanki, V.R., Bhagavathi, M., 2008. Low cost microbioassay test for assessing cytopathological and physiological responses of ciliate model *Paramecium caudatum* to carbofuran pesticide. Pestic. Biochem. Physiol. 90, 66–70.
IEA - Instituto de Economia Agrícola, 2016. Banco de dados, São Paulo, Brazil (in Portuguese). Available in. http://www.iea.sp.gov.br/ 04.20.2020.
ISO 10706, 2000. Water Quality - Determination of Long-Term Toxicity of Substances to *Daphnia Magna* Straus (Cladocera, Crustacea). International Organization for Standardization, Geneve, Switzerland.
Joly, P., Bonnemoy, F., Charvy, J.C., Bohatier, J., Mallet, C., 2013. Toxicity assessment of the maize herbicides S-metolachlor, benoxacor, mesotrione and nicosulfuron, and their corresponding commercial formulations, alone and in mixtures, using the Microtox® test. Chemosphere 93 (10), 2444–2450.
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.
Kienzler, A., Bopp, S.K., van der Linden, S., Berggren, E., Worth, A., 2016. Regulatory assessment of chemical mixtures: requirements, current approaches and future perspectives. Regul. Toxicol. Pharmacol. 80, 321–334.
Láng, J., Kőhidai, L., 2012. Effects of the aquatic contaminant human pharmaceuticals and their mixtures on the proliferation and migratory responses of the bioindicator freshwater ciliate *Tetrahymena*. Chemosphere 89 (5), 592–601.
Liu, S.S., Wang, C.L., Zhang, J., Zhu, X.W., Li, W.Y., 2013. Combined toxicity of pesticide mixtures on green algae and photobacteria. Ecotoxicol. Environ. Saf. 95, 98–103.
Luna-Acosta, A., Renault, T., Thomas-Guyon, H., Faury, N., Saulnier, D., Budzinski, H., Menach, K.L., Pardon, P., Fruitier-Arnaudin, I., Bustamante, P., 2012. Detection of early effects of a single herbicide (diuron) and a mix of herbicides and pharmaceuticals (diuron, isoproturon, ibuprofen) on immunological parameters of Pacific oyster (*Crassostrea gigas*) spat. Chemosphere 87 (11), 1335–1340.
Madoni, P., 2011. Protozoa in wastewater treatment processes: a minireview. Ital. J. Zool. 78 (1), 3–11.
Mansano, A.S., Hisatugo, K.F., Hayashi, L.H., Regali-Seleghim, M.H., 2014. The importance of protozoan bacterivory in a subtropical environment (Lobo-Broa Reservoir, SP, Brazil). Braz. J. Biol. 74 (3), 569–578.
Mansano, A.S., Moreira, R.A., Pierozzi, M., Oliveira, T.M.A., Vieira, E.M., Rocha, O., Regali-Seleghim, M.H., 2016. Effects of diuron and carbofuran pesticides in their pure

and commercial forms on *Paramecium caudatum*: the use of protozoan in ecotoxicology. Environ. Pollut. 213, 160–172.
Mansano, A.S., Moreira, R.A., Dornfeld, H.C., Freitas, E.C., Vieira, E.M., Sarmento, H., Rocha, O., Seleghim, M.H., 2017. Effects of diuron and carbofuran and their mixtures on the microalgae *Raphidocelis subcapitata*. Ecotoxicol. Environ. Saf. 142, 312–321.
Mansano, A.S., Moreira, R.A., Dornfeld, H.C., Diniz, L.G., Vieira, E.M., Daam, M.A., Rocha, O., Seleghim, M.H., 2018. Acute and chronic toxicity of diuron and carbofuran to the neotropical cladoceran *Ceriodaphnia silvestrii*. Environ. Sci. Pollut. Res. 25 (14), 13335–13346.
Masiá, A., Campo, J., Navarro-Ortega, A., Barceló, D., Picó, Y., 2015. Pesticide monitoring in the basin of Llobregat River (Catalonia, Spain) and comparison with historical data. Sci. Total Environ. 503–504, 58–68.
Megharaj, M., Pearson, H.W., Venkateswarlu, K., 1993. Toxicity of carbofuran to soil isolates of *Chlorella vulgaris, Nostoc linckia* and *N. muscorum*. Appl. Microbiol. Biotechnol. 39 (4–5), 644–648.
Mehler, W.T., Schuler, L.J., Lydy, M.J., 2008. Examining the joint toxicity of chlorpyrifos and atrazine in the aquatic species: *Lepomis macrochirus, Pimephales promelas* and *Chironomus tentans*. Environ. Pollut. 152, 217–224.
Mesnage, R., Antoniou, M.N., 2018. Ignoring adjuvant toxicity falsifies the safety profile of commercial pesticides. Frontiers Public Health 5, 361.
Moreira, R.A., Daam, M.A., Vieira, B.H., Sanches, A.L.M., Reghini, M.V., Mansano, A.S., Freitas, E.C., Espindola, E.L.G., Rocha, O., 2017. Toxicity of abamectin and difenoconazole mixtures to a Neotropical cladoceran after simulated run-off and spray drift exposure. Aquat. Toxicol. 185, 58–66.
Moreira, R.A., Freitas, J.S., Silva Pinto, T.J., Schiesari, L., Daam, M.A., Montagner, C.C., Goulart, B.V., Espindola, E.L.G., 2019. Mortality, spatial avoidance and swimming behavior of bullfrog tadpoles (*Lithobates catesbeianus*) exposed to the herbicide diuron. Water, Air, Soil Pollut. 230 (125).
Moreira, R.A., Mansano, A.S., Rocha, O., 2015. The toxicity of carbofuran to the freshwater rotifer, *Philodina roseola*. Ecotoxicology 24 (3), 604–615.
OECD - Organization for Economic Cooperation and Development, 2008. Guidelines for Testing of Chemicals. *Daphnia Magna* Reproduction Test. OECD, Paris, pp. 211.
Orton, F., Lutz, I., Kloas, W., Routledge, E.J., 2009. Endocrine disrupting effects of herbicides and pentachlorophenol: in vitro and in vivo evidence. Environ. Sci. Technol. 43 (6), 2144–2150.
Pape-Lindstrom, P.A., Lydy, M.J., 1997. Synergistic toxicity of atrazine and organophosphate insecticides contravenes the response addition mixture model. Environ. Toxicol. Chem. 16 (11), 2415–2420.
Paschoalato, C., Dantas, A., Rosa, I., Faleiros, R., Bernardo, L.D., 2008. Use of activated carbon to remove the herbicides diuron and hexazinone from water. Revista DAE 179, 34–41.
Pereira, J.L., Antunes, S.C., Castro, B.B., Marques, C.R., Goncalves, A.M.M., Goncalves, F., Pereira, R., 2009. Toxicity evaluation of three pesticides on non-target aquatic and soil organisms: commercial formulation versus active ingredient. Ecotoxicology 18, 455–463.
Pérez, J., Domingues, I., Soares, A.M., Loureiro, S., 2011. Growth rate of *Pseudokirchneriella subcapitata* exposed to herbicides found in surface waters in the Alqueva Reservoir (Portugal): a bottom-up approach using binary mixtures. Ecotoxicology 20, 1167–1175.
Phyu, Y.L., Palmer, C.G., Warne, M.S.J., Hose, G.C., Chapman, J.C., Lim, R.P., 2011. A comparison of mixture toxicity assessment: examining the chronic toxicity of atrazine, permethrin and chlorothalonil in mixtures to *Ceriodaphnia* cf. *dubia*. Chemosphere 85 (10), 1568–1573.
Pignati, W.A., Lima, F.A.N.D.S., Lara, S.S.D., Correa, M.L.M., Barbosa, J.R., Leão, L.H.D.C., Pignatti, M.G., 2017. Spatial distribution of pesticide use in Brazil: a strategy for Health Surveillance. Ciência Saúde Coletiva 22, 3281–3293.
Pomeroy, L.R., Williams, P.J.I., Azam, F., Hobbie, J.E., 2007. The microbial loop. Oceanography 20 (2), 28–33.
Reiss, J., Schmid-Araya, J.M., 2011. Feeding response of a benthic copepod to ciliate prey type, prey concentration and habitat complexity. Freshw. Biol. 56 (8), 1519–1530.
Rocha, O., Neto, A.J.G., Lima, J.C.S., Freitas, E.C., Miguel, M., Mansano, A.S., Moreira, R.A., Daam, M.A., 2018. Sensitivities of three tropical indigenous freshwater invertebrates to single and mixture exposures of diuron and carbofuran and their commercial formulations. Ecotoxicology 27 (7), 834–844.
Ryall, K.A., Tan, A.C., 2015. Systems biology approaches for advancing the discovery of effective drug combinations. J. Cheminformatics 7, 1–15.
Saib, A., Berrebbah, H., Berredjem, M., Djebar, M.R., 2014. Cytotoxic study of three derivatives amidophosphonates on alternative cellular model: *Paramecium tetraurelia*. Toxicol. Res. 3 (5), 395–399.
Schiesari, L., Grillitsch, B., 2011. Pesticides meet megadiversity in the expansion of biofuel crops. Front. Ecol. Environ. 4, 215–221.
Severo, E.S., Marins, A.T., Cerezer, C., Costa, D., Nunes, M., Prestes, O.D., Zanella, R., Loro, V.L., 2020. Ecological risk of pesticide contamination in a Brazilian river located near a rural area: a study of biomarkers using zebrafish embryos. Ecotoxicol. Environ. Saf. 190, 110071.
Sposito, J.C., Montagner, C.C., Casado, M., Navarro-Martín, L., Solórzano, J.C.J., Piña, B., Grisolia, A.B., 2018. Emerging contaminants in Brazilian rivers: occurrence and effects on gene expression in zebrafish (*Danio rerio*) embryos. Chemosphere 209, 696–704.
Systat, 2008. Systat Software, Incorporation SigmaPlot for Windows Version 11.0.
Tenda, H., Alves-Pereira, I., Ferreira, R., 2012. Diuron determines *Saccharomyces cerevisiae* UE-ME3 survival at beginning of exponential phase. In: Mendez-Vilas, A. (Ed.), Microbes in Applied Research: Current Advances and Challenges. World Scientific, Singapore, pp. 80–83.
Velki, M., Di Paolo, C., Nelles, J., Seiler, T.B., Hollert, H., 2017. Diuron and diazinon alter the behavior of zebrafish embryos and larvae in the absence of acute toxicity. Chemosphere 180, 65–76.
Zalizniak, L., Nugegoda, D., 2006. Effect of sublethal concentrations of chlorpyrifos on three successive generations of *Daphnia carinata*. Ecotoxicol. Environ. Saf. 64, 207–214.