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Acute and chronic toxicity of 2,4-D and fipronil formulations (individually and in mixture) to the Neotropical cladoceran *Ceriodaphnia silvestrii*

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Abstract

Brazil is the largest producer of sugarcane and the world's top pesticide market. Therefore, environmental consequences are of concern. The aim of the present study was to evaluate the acute and chronic toxicity of pesticide formulations largely used in sugarcane crops: the herbicide DMA® 806 BR (a.i. 2,4-D) and the insecticide Regent® 800 WG (a.i. fipronil), isolated and in mixture, to the Neotropical cladoceran *Ceriodaphnia silvestrii*. Toxicity tests with the individual formulated products indicated 48h-EC_{50} values of 169 ± 18 mg a.i./L for 2,4-D and 3.9 ± 0.50 µg a.i./L for fipronil. In the chronic tests, the 8d-EC_{50} values for reproduction were 55 mg a.i./L (NOEC/LOEC: 50/60 mg a.i./L) and 1.6 µg a.i./L (NOEC/LOEC: 0.40/0.80 µg a.i./L) for 2,4-D and fipronil, respectively. A significant decrease in reproduction of *C. silvestrii* in all concentrations tested of fipronil, except at the lowest, was observed. Regarding 2,4-D, the organisms had total inhibition of reproduction in the two highest concentrations. Probably your energy reallocation was focused (trade-off) only on its survival. The acute pesticide mixture toxicity (immobility) revealed a dose level dependent deviation with antagonism at low and synergism at high concentrations. For chronic mixture (reproduction) toxicity, antagonism occurred as a result of the interaction of the pesticides. Based on our results and concentrations measured in Brazilian water bodies, fipronil represents ecological risks for causing direct toxic effects on *C. silvestrii*. These results are worrisome given that agricultural production is likely to increase in the coming years.

Keywords DMA® 806 BR · Regent® 800 WG · Herbicide · Insecticide · Reproduction · Aquatic ecotoxicology

Introduction

Nowadays, Brazil is the world's largest producer of sugarcane and ethanol (renewable biofuel produced by the fermentation of sugarcane extract) (OECD/FAO 2019). Thus, this monoculture farming is quite extensive, being classified as the third largest crop after corn and soybean (UNICA 2015). The annual production is approximately 640 thousand tons and the area of occupation is 900 thousand hectares. This production has a market that tends to increase considerably due to the incentive of replacing fossil fuels (Conab 2019).

Agricultural expansion has been considered one of the main threats to the conservation of biodiversity in continental waters around the world (Lacher and Goldstein 1997; FAO 2019). In addition to the numerous problems related to the destruction of natural vegetation, the intensification of agriculture has led to an exponential growth in the use of pesticides, compounds with high environmental toxicity (Carvalho 2006; Daam and Van den Brink 2010). In line with this, for sugarcane there are currently 333 active ingredients registered in Brazil, composing 2316 formulated products (AGROFIT 2020). Among the active ingredients most used in crops in the state of São Paulo, which

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represents 52% cultivated area in the country from 2009 to 2015, are the herbicide 2,4-D and the insecticide fipronil (Institute of Agricultural Economics-IEA 2016).

The herbicide 2,4-D of the chemical group of phenoxyacetic acids is an active ingredient that targets dicotyledonous plants. It acts by deregulating their growth and subsequent death through uncontrolled cell division in vascular tissue (Jervais et al. 2008). Currently 2,4-D is the second most used herbicide for sugarcane in Brazil (Li et al. 2017) and can be applied in pre- or post-emergency conditions for crops (MAPA 2019). Its exacerbated use is related to low cost, selectivity, efficiency, wide spectrum and high solubility in water (Islam et al. 2018). On the other hand, its high application in crops leads to its presence and persistence in the environment (Messing et al. 2014; Ismail et al. 2015). Fipronil is an insecticide of the phenylpyrazole class that acts on the central nervous system by blocking the chlorine channels present in the GABA (gamma-aminobutyric acid) receptor, causing the death of the target organisms through paralysis and hyperexcitation (Tingle et al. 2003). However, several studies have already demonstrated the risk of exposure of non-target organisms to this active ingredient (Tingle et al. 2003; Gunasekara et al. 2007; Gibbons et al. 2015). In the cultivation of sugarcane, 2,4-D and fipronil may be concomitantly present mainly at planting, since at this stage the monoculture is more susceptible to be attacked by pests, diseases and invasive plants (Townsend 2000).

In natural aquatic systems located near areas with predominance of sugarcane crops in the state of São Paulo, 2,4-D is commonly detected, where for example, the high concentration of 366.6 µg/L has already been recorded (CETESB 2018). Furthermore, 2,4-D is found in combination with several other pesticides, such as fipronil, which has already been detected at an environmental concentration of up to 465 µg/L (CETESB 2018). Several studies point to the risk of pesticide mixtures occurring in the environment, which can lead especially to biodiversity loss (Relyea 2009; Pavlaki et al. 2011). In the aquatic environment these substances negatively affect different levels of the trophic chain as primary producers, e.g. *Raphidocelis subcapitata* (Mansano et al. 2017), primary consumers, e.g. *Macrothrix flabelligera* (Moreira et al. 2017) and secondary consumers, e.g. *Danio rerio* (Sanches et al. 2017). In this sense, this is of especial concern when the expansion and intensification of agriculture takes place in biodiversity-rich tropical countries (Vörösmarty et al. 2010).

For pesticides, the geoclimatic conditions directly influence their toxic effects on non-target organisms (Silva et al. 2020). Factors such as soil quality that interferes with the mobility of compounds, the higher temperatures that increase the solubility of the products in water and the absorption by organisms, intensify the environmental risks of pesticides in the tropical region (Sanchez-Bayo and Hyne 2011). Still, there is a higher frequency of application and in a large part of the year, compared with those of temperate regions (Ecobichon 2001; Satapornvanit et al. 2004). Thus, the use of temperate toxicity data in tropical risk assessments has often been disputed (Pham and Bui 2018; Wang et al. 2019).

Considering this scenario, there is a growing need to obtain ecotoxicological data under tropical conditions using indigenous test organisms to increase our understanding of the sensitivity of these species and use them for the environmental risk assessment of pesticides. Most studies into pesticides toxicity on primary consumers have focused on species distributed in temperate regions, such as *Daphnia magna* and *Ceriodaphnia dubia* (US EPA 2002a, b). *Ceriodaphnia silvestrii* presents a wide geographic distribution in South America, has a short life cycle, is easy to maintain and culture in the laboratory, and belongs to one of the most sensitive group of organisms for a variety of toxic substances, and is therefore an excellent test organism for tropical regions (Casali-Pereira et al. 2015; Mansano et al. 2018). In addition, guidelines for toxicity testing have previously been developed for this species (ABNT 2017). For these reasons, this study aimed to evaluate the acute and chronic toxicity of two commercial pesticide formulations commonly used in sugarcane crops in Brazil: the herbicide DMA® 806 BR (a.i. 2,4-D) and the insecticide Regent® 800 WG (a.i. fipronil), isolated and in mixture, to the Neotropical cladoceran *Ceriodaphnia silvestrii*.

Materials and methods

Test organism and culture conditions

The experiments were carried out at the Center for Water Resources and Environmental Studies (NEEA/CRHEA) of the São Carlos School of Engineering, University of São Paulo (EESC/USP), located in the municipality of Itirapina, Brazil (22°01'22"S, 43°57'38"W). The organisms used in the tests were obtained through continuous cultures established in the laboratory, and cultivation procedures and toxicity tests followed the recommendations of standard 13373: 2016 (ABNT 2017).

C. silvestrii cultures were kept under controlled temperature (25 ± 2 °C) and photoperiod (12:12-h light/dark) in reconstituted water with pH 7.0–7.6, conductivity of 160 µS/cm, and hardness of 40–48 mg/L (as $CaCO_3$). The organisms were fed daily with the chlorophycean algae *Raphidocelis subcapitata* (10^5 cells/mL), which was grown in LC Oligo medium (AFNOR 1980), and a suspension

containing yeast (0.5%) and fermented fish food (0.5%) was added as a food supplement (1 mL/L) (ABNT 2017).

Chemical analysis of the pesticides

The commercial formulation DMA® 806 BR (Dow AgroSciences Industrial Ltda., Brazil) contains 67% m/v of active ingredient (a.i.) −2,4-D acid equivalent (80.6% m/v of −2,4-D, dimethylamine salt) (41.9% m/v of inert ingredients). Regent® 800 WG (BASF, Brazil) contains 80% m/v of active ingredient (fipronil) and 20% m/v of inert ingredients.

The quantification of 2,4-D and fipronil was performed with Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) using chromatography Agilent 1200 coupled to a Triple Quadrupole mass spectrometer with electrospray ionization (ESI) (Agilent 6410) in negative mode. The Limit of Quantification (LOQ) of the method to 2,4-D and fipronil was 1 and 0.1 µg/L, respectively.

Acute toxicity tests

Preliminary acute toxicity tests were performed to define the sensitivity range of the species for the isolated exposure to the pesticides. For definitive bioassays, one control and six concentrations (100, 125, 150, 175, 200 and 225 mg a.i./L dosed as DMA® 806 BR) of the herbicide were prepared from a stock solution of 6.7 g a.i./L. For the insecticide, one control and five concentrations (0.8, 1.6, 3.2, 6.5 and 13 µg a.i./L dosed as Regent® 800 WG) were prepared from a stock solution of 1.3 mg a.i./L. All acute toxicity tests were made in quadruplicate, with five newborns/replica (6–24 h old) in nontoxic polypropylene plastic cups containing 10 mL of test solution for 48 h, without feeding, 12:12 light/dark and temperature of 25 ± 2 °C. The acute toxicity tests were performed three times for each pesticide and the endpoint observed was the immobility of the organisms to obtain the mean 48 h-EC_{50} (effect concentration that causes acute effects to 50% of the test population). The acute mixture test was realized using the full factorial method (Cassee et al. 1998), resulting in 30 combinations of the concentrations and one control, with three replicates each. The endpoint was immobility (48 h-EC_{50}). Tests with the individual compounds and full factorial mixtures were conducted simultaneously to avoid any influence of eventual differences in sensitivity of *C. silvestrii* and experimental conditions (Pavlaki et al. 2011). Water quality parameters (pH, conductivity and dissolved oxygen) were verified at the beginning and end of the experiments. Acute toxicity tests with the reference substance sodium chloride (NaCl) were carried out monthly to evaluate the physiological condition of the organisms (ABNT 2017).

Chronic toxicity tests

For the chronic toxicity tests with the isolated formulations, five concentrations with 10 replicates containing one newborn (6–24 h old) each were selected based on the EC_{10} values of the acute tests. Concentrations of 40, 50, 60, 70 and 80 mg a.i./L from a stock solution of 6.7 g a.i./L and one control were tested for the herbicide. For the insecticide, one control and concentrations of 0.4, 0.8, 1.2, 1.6 and 2 µg a.i./L were used from a 1.3 mg a.i./L stock solution. The duration of the tests was 8 days and the organisms were fed and maintained under the same conditions (temperature, photoperiod, medium) described previously for culture maintenance. The test solutions were renewed every 48 h after the number of surviving adults and newborns had been recorded. The endpoints evaluated were EC_{50} (reproduction), EC_{20}, EC_{10}, LOEC (Lowest Observed Effect Concentration) and NOEC (No Observed Effect Concentration). The age of first reproduction, survival percentage and the number of neonates per female were daily observed under a stereomicroscope, and the 8-d EC_{10} and EC_{50} were calculated based on the fecundity. The intrinsic rate of population increase (r) were calculated using Euler's equation 1 (Marinho et al. 2019):

$$1 = \sum_{x=0}^{n} e^{-rx} l_x m_x, \quad (1)$$

where "r" is the intrinsic rate of population increase (day^{-1}), "x" is the age of the organisms (days), "l_x" is the probability of survival at the age "x", and "m_x" is the number of neonates at the age x.

The chronic mixture test was performed using the partial fixed-ratio method (Cassee et al. 1998), which included the isolated evaluation of 2,4-D and fipronil and 23 binary combinations of the pesticides with five replicates/concentration. Water quality parameters (pH, conductivity and dissolved oxygen) were checked at the beginning, end and at each renewal to ensure that these were at optimum as recommended for the species.

Statistical analysis

The EC_{50}, EC_{20} and EC_{10} values of the acute tests with the isolated formulations were analyzed by nonlinear regression using the three-parameter logistic curve through the software Statistica version 7.0 (StatSoft 2004). For values of the chronic tests, we analyzed significant differences between controls and treatments for age of first reproduction, fecundity and intrinsic rate of population increasing. The normality (Shapiro–Wilk) and homogeneity of the data (Levene) were verified and differences between treatments were assessed by analysis of variance (ANOVA). This was

followed by the post-hoc Dunnett's test in case of data that met the normality and homoscedasticity criteria. For data that did not meet these requirements, the nonparametric Kruskall–Wallis test, followed by Dunn's Method post hoc test, were used. The mixtures of both acute and chronic formulations were analyzed by means of conceptual concentration addition (CA) and independent action (IA) models using the MIXTOX tool (Jonker et al. 2005). The analysis was then extended, as described in Jonker et al. (2005) and the three deviations from the reference models, i.e. synergetic/antagonistic interactions (S/A), deviation dose ratio-dependent (DR) and dose level-dependent (DL), were modeled by adding two parameters ("a" and "b"). Further details on the deviation functions can be obtained from Jonker et al. (2005).

Results and discussion

Abiotic variables of the toxicity tests and chemical analyses

The water quality parameters evaluated were in accordance with the criteria established by ABNT (2017): pH (7.3–8), dissolved oxygen (6.8–7.9 mg/L), conductivity (153–238 µS/cm) and hardness (42–48 mg$CaCO_3$/L). For all experiments, the nominal concentrations of fipronil and 2,4-D were measured from stock solutions (1.3 mg a.i./L of fipronil and 6.7 g a.i./L of 2,4-D), shortly after their preparation, and 1.3 ± 10.4 mg a.i./L and 5.3 ± 0.16 g a.i./L, respectively, were quantified. Thus, analysis of the stock solutions of the pesticides confirmed the prepared concentrations. The toxicity values in the acute and chronic toxicity tests with fipronil and 2,4-D were calculated based on measured concentrations of the stock solutions.

Acute tests with isolated formulations

Acute toxicity tests of the pesticides DMA® 806 BR (a.i. 2,4-D) and Regent® 800 WG (a.i. fipronil) showed 48 h-EC_{50} (mean ± SD) for *Ceriodaphnia silvestrii* of 169 ± 18 mg a.i./L (95% CI 138–192 mg ai/L) and 3.9 ± 0.50 µg a.i./L (95% CI 2.5–4.9 µg ai/L), respectively. The results indicate that the insecticide fipronil was more toxic (43 times) than the herbicide 2,4-D. The acute toxicity values of 2,4-D for cladocerans found in the literature range from 20 to 422 mg a.i./L whereas for fipronil and their formulations, the sensitivity range for cladocerans species is between 1 and 190 µg a.i./L (see US EPA 2019).

For 2,4-D, EFSA (2014) presents an 48 h-EC_{50} value of 134.2 mg/L for *D. magna* at 20 °C in a 48-h test, a value matching the LC_{50} of 135 mg/L obtained by Benijts-Claus and Persoone (1975). In addition, some studies evaluated the acute effect of 2,4-D only up to a specific value, for example Crosby and Tucker (1966) and Sanders (1970) with values of 26 h-EC_{50} and 48 h-LC_{50} > 100 mg/L for *D. magna*. Nelson and Roline (1998) also represented their 48 h-EC_{50} result > 422 mg/L for *C. dubia*. EC and LC values below 100 mg/L were obtained for *D. magna* with 48 h-LC_{50} of 25 and 36.4 mg/L (Alexander et al. 1985) and 48 h-LC_{50} of 20 mg/L for *Daphnia lumholtzi* (George et al. 1982). Differences in the toxicity of a compound on species of the same taxonomic group can be explained due to laboratory test conditions, such as exposure time, culture water constitution, hardness, photoperiod and temperature (Moreira et al. 2014). Présing (1981), for example, observed differences in toxicity with changes in temperature and time of exposure of *D. magna* to 2,4-D. This author denoted an increase of up to 16% in toxicity with a change in temperature from 15 to 20 °C and difference of 47% in LC_{50} values between 24 and 96 h. Milam et al. (2005) also evaluated the effects of 2,4-D on *D. magna* and obtained an average 24 h-LC_{50} of 415.7 mg/L at 22 °C while Présing (1981) obtained an 48 h-LC_{50} value of 417.8 mg/L at 20 °C. For *Ceriodaphnia dubia*, this comparison can also be made with the studies by Milam et al. (2005) and Oris et al. (1991) who obtained 24 h-LC_{50} values of 272.5 and 48 h-LC_{50} of 236 mg/L, respectively, in which the average temperature between the two studies differed by 3 °C. In the present study, for 2,4-D, the sensitivity values obtained for *C. silvestrii* is within the values established for the other species of the cladocerans, although further studies are needed to rule out influences of physical and chemical characteristics, such as temperature.

In this study, *C. silvestrii* was more sensitive to fipronil when compared to other cladocerans. A greater sensitivity of the tropical species *C. silvestrii* than some standard temperate test species such as *D. magna* was also noted in previous studies. For example, *C. silvestrii* was demonstrated to be more sensitive than *D. magna* to acetaminophen, propranolol and diclofenac (Oliveira et al. 2018), to bisphenol and nonylphenol (Spadoto et al. 2017) and to carbofuran and diuron (Mansano et al. 2018).

Regarding temperature, for insecticides such as fenvalerate, cypermethrin, deltamethrin and malathion the relationship between temperature and toxicity to *D. magna* appears to be positive (Ratushnyak et al. 2005; Willming et al. 2013). In this sense, evaluations of temperature-dependent toxicities with the standard species are also necessary to reduce the gaps regarding the toxicity of fipronil to cladocerans. In the case of the native cladoceran *C. silvestrii*, in a previous study by Silva et al. (2020), the greater sensitivity of the species was verified with respect to toxic effects and temperature influence. The same was

Table 1 EC values of toxicity to *Ceriodaphnia silvestrii* exposed for acute and chronic tests to pesticides DMA® 806 BR (a.i. 2,4-D) and Regent® 800 WG (a.i. fipronil)

	Fipronil (µg a.i./L)			2,4-D (mg a.i./L)		
	Mean	SD[a]	CI 95%[b]	Mean	SD[a]	CI 95%[b]
Immobility						
EC10	2.1	0.68	0.76–3.4	133	15	97–163
EC20	2.6	0.65	1.3–3.6	145	18	111–175
EC50	3.9	0.50	2.5–4.9	169	18	138–192
Reproduction						
EC10	0.22	0.10	0.01–0.42	43	3.2	36–49
EC20	0.45	0.14	0.16–0.74	47	2.7	41–52
EC50	1.6	0.23	1.1–2.1	55	1.8	51–59

[a]Standard deviation

[b]Confidence interval

evaluated for *C. dubia* (Tsui and Chu 2003), which showed no variation in sensitivity in exposure to an herbicide with the same active ingredient as in the previous experiment, demonstrating once again that the native species seems to be more sensitive than the temperate species.

In the case of studies with fipronil, we observed that *D. magna* has average 48 h-EC_{50} and 48 h-LC_{50} values that are higher than those of other cladocerans. Based on the values denoted in studies for this species, we calculated an average of 132 µg/L (US EPA 1992; EFSA 2006; Nakagome and Noldin 2006; Hayasaka et al. 2012), whereas for *D. pulex* and *C. dubia* the average is 28 µg/L (Stark and Vargas 2005; Hayasaka et al. 2012) and 17 µg/L (Konwick et al. 2005; Wilson et al. 2008; Hayasaka et al. 2012), respectively. For two other cladocerans, *Ceriodaphnia reticulata* and *Moina macrocopa*, values of 48 h-EC_{50} of 9 and 29 µg/L were determined, respectively (Hayasaka et al. 2012). Based on these values, it can be noted that *D. magna* is five to over ten times more tolerant to fipronil than the other species. Hayasaka et al. (2012), when assessing fipronil toxicity to five cladocerans, observed that the toxicity 48 h-EC_{50} was positively correlated with the body size of the organisms, with the smaller species belonging to the genus *Ceriodaphnia* as the most sensitive. The 48 h-LC_{50} values these authors reported for this genus (0.99 and 8.8 µg/L for *C. dubia* and *C. reticulata*, respectively) are indeed in the same order of magnitude as that denoted in the present study for *C. silvestrii*. Thus, the more since the genus *Ceriodaphnia* is considered a bioindicator equivalent to *D. magna* in terms of environmental risk assessment (Versteeg et al. 1997; Pakrashi et al. 2013), the use of ecotoxicological assays with native species (belonging to this genus) is crucial for a better ecological risk assessment in tropical contaminated areas.

Chronic tests with isolated formulations and its implications

With respect to chronic pesticide toxicity tests, EC_{50}-Reproduction values for DMA® 806 BR (a.i. 2,4-D) and Regent® 800 WG (a.i. fipronil) were 55 ± 1.8 mg a.i./L (95% CI 51–59 mg a.i./L) and 1.6 ± 0.23 µg a.i./L (95% CI 1.1–2.1 µg a.i./L), respectively (Table 1). The calculated values of EC_{10} and EC_{20} were 43 and 47 mg a.i./L for the herbicide and 0.22 and 0.45 µg a.i./L for the insecticide (Table 1). NOEC e LOEC were 50 and 60 mg a.i./L for the herbicide and 0.40 and 0.80 µg a.i./L for the insecticide. Figure 1 shows the average of newborns per concentration in the chronic toxicity test with the DMA® and Regent®. For the insecticide, the number of newborns decreased gradually with increasing test concentrations, with at least one brood less in the lowest three concentrations, and two in the highest two concentrations, when compared with controls (Kruskall–Wallis; $H_5 = 43.66$ $p < 0.05$; Dunn's method, $p < 0.05$; Fig. 1e). For the herbicide, reproduction decreased significantly from 60 mg a.i./L (7 ± 6 newborns) in relation to the control (20 ± 3 newborns). At this concentration and higher concentrations (70 and 80 mg a.i./L), the females practically did not reproduce (Kruskall–Wallis; $H_5 = 41.27$ $p < 0.05$; Dunn's method, $p < 0.05$; Fig. 1b), keeping unviable eggs in their body cavity during the exposure (Fig. 2).

The comparison of data from the first reproduction showed significant differences only for the highest concentration of DMA® (Kruskall–Wallis; $H_5 = 19.772$; Dunn's method, $p < 0.05$; Fig. 1a) while no differences were denoted at any Regent® concentration (Kruskall–Wallis; $H_5 = 8.530$; $p = 0.129$; Fig. 1d). Regarding the intrinsic rate of population increase (r), the statistical analysis showed a decrease of 41% at 60 mg/L and 100% in the highest two concentrations of the herbicide (Kruskall–Wallis; $H_5 = 55.790$; Dunn's method, $p < 0.05$; Fig. 1c). In the case of the insecticide, significant differences were observed in all treatments with a decrease of 3 to 36% in r values throughout the concentrations (One way ANOVA; $F_5 = 944.682$; $p < 0.05$; Dunnet test, $p < 0.05$; Fig. 1f). Other insecticides such as chlorpyrifos, cyfluthrin, cypermethrin, endosulfan, etofenprox and lindane have been shown to change the age to the first brood and the intrinsic rate of population increase of *Daphnia* when compared to uncontaminated treatments (Fernández-Casalderrey et al. 1993; Ferrando et al. 1995; Brausch and Salice 2011; Rajini et al. 2016; Sancho et al. 2018). In the case of herbicides, diuron, molinate and propanil also changed the two rates evaluated for *Daphnia* (Pereira et al. 2007; Sánchez et al. 2004; Brausch and Salice 2011). For diuron, the study carried out with *D. magna* further demonstrated that the toxic effects can be mitigated over generations (Brausch and Salice 2011). The delay of the first brood can affect the r values by

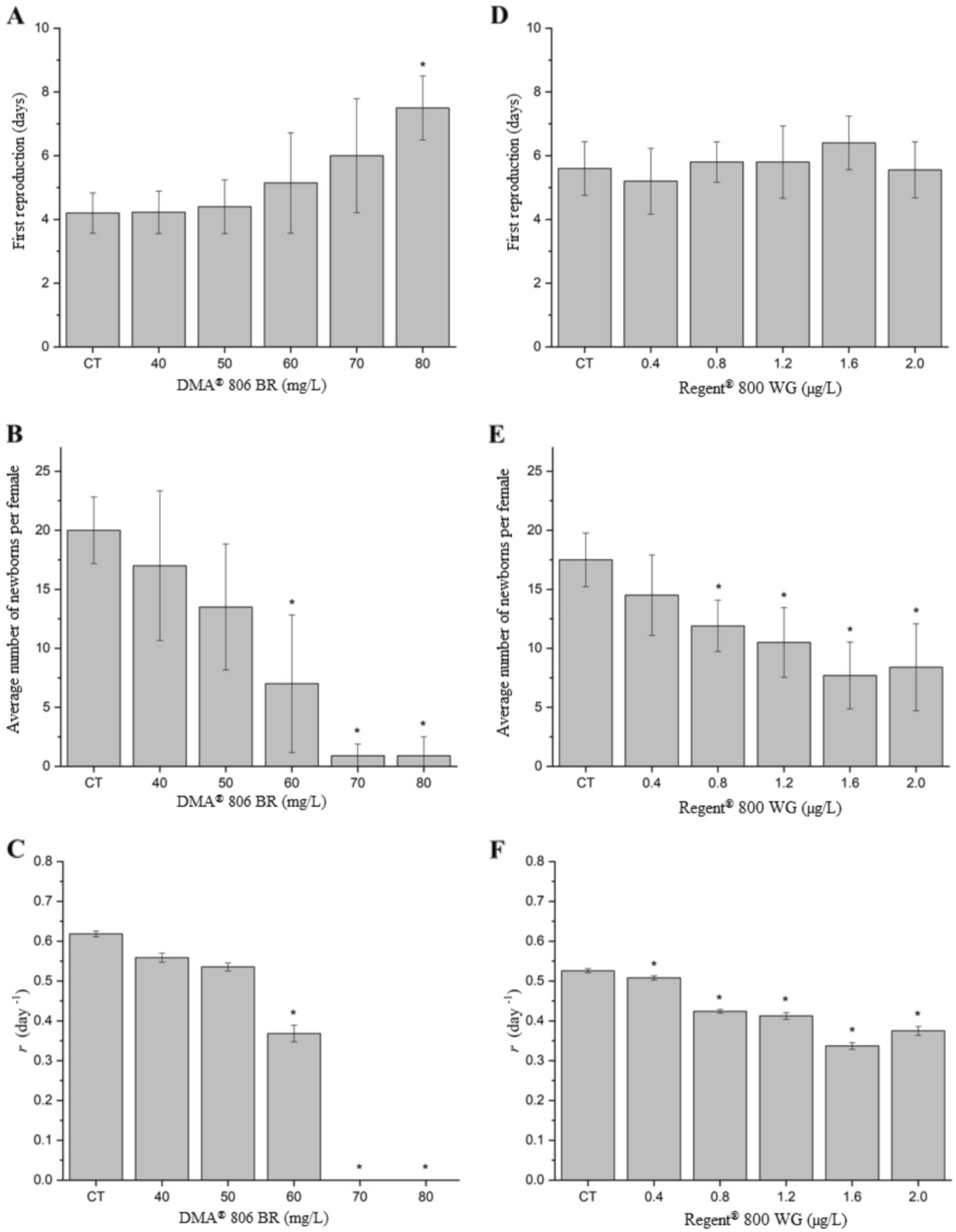

Fig. 1 Fertility (mean ± SD number of newborns per female), first reproduction and intrinsic rate of population increase (r) of *Ceriodaphnia silvestrii* after exposure to different concentrations of **a** DMA® 806 BR (a.i. 2,4D) and **b** Regent® 800 WG (a.i. fipronil). The asterisk indicates the value significantly different from control ($p \leq 0.05$, Dunnett's test)

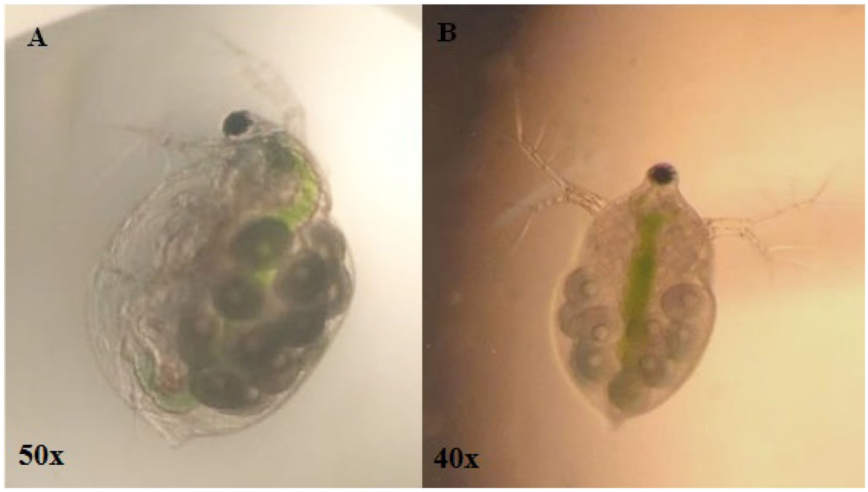

Fig. 2 Females at the end of the chronic test. **a** Control female after 3rd brood. **b** Female with unviable eggs (abortions) on exposure to herbicide DMA® 806 BR (a.i. 2,4D)

directly interfering with the speed at which the population expands in the ecosystem. In the present study, *C. silvestrii*, despite surviving the highest concentrations of DMA®, had its reproduction compromised. In real-world aquatic ecosystems, this time without reproduction may be sufficient to contribute to the disappearance of the species. In the case of Regent®, the decrease in fertility and intrinsic rate of population increase at low insecticide concentrations can similarly be an important factor in the rapid disappearance of *C. silvestrii* in contaminated areas.

With respect to reproduction data available in the literature for 2,4-D, not many studies were found evaluating its chronic toxicity despite that this compound is a relatively old molecule and cladocerans are widely used in toxicity tests. The range of reproductive effects reported for standard cladoceran test species is 10 to 151 mg/L (US EPA 1992). For *D. magna*, two references point to LOEC values for species with a difference of 15 times. US EPA (1992) showed NOEC and LOEC values of 79 and 151 mg/L, while Matsumoto et al. (2009) obtained a LOEC of 10 mg/L. For *C. dubia*, IC_{50} (median inhibitory concentration) values for reproduction obtained by Oris et al. (1991) were 86.8 mg/L (4 days) and 69.8 mg/L (7 days), values at which *C. silvestrii* drastically reduced reproduction in the present study.

For this finding of severe inhibition of reproduction at non-lethal 2,4-D concentrations, we hypothesize that these concentrations are stressful in such a way that the energy allocation of *C. silvestrii* is focused (trade-off) only on its survival and as such compromises its reproduction. Gottardi et al. (2017) observed that *D. magna* exposed to the fungicide epoxiconazole initially allocated its energy for growth and reproduction suggesting a trade-off with subsequent impairments to immune responses and mobility. In the case of *C. silvestrii*, we did not evaluate other sublethal endpoints such as length of organisms, enzymatic activity or swimming behavior that collaborate to affirm the adequacy of the results obtained with the energy allocation (Forbes 2000). However, the inhibition of reproduction without any acute (lethal) effect on females exposed to 2,4-D demonstrates a high metabolic and physiological effort of the organism to survive at these herbicide concentrations. In addition, comparing the results of the present study with those presented by Oris et al. (1991) for *C. dubia*, it is noted that *C. silvestrii* is apparently more sensitive to 2,4-D than its relatives of the same genus.

For fipronil, the sublethal concentrations tested in the chronic test were close to those tested in the acute toxicity test, thus demonstrating the high toxicity of the compound to *C. silvestrii*. Other cladocerans species showed effects on their life parameters at concentrations of fipronil up to 110 times those denoted in the present study to exert effects. For example, for *D. pulex* exposed to the formulated product Regent® 4 SC in a 10-day experiment, a NOEC and LOEC of 30 and 50 µg/L have been reported, respectively, with extinction of the species at a concentration of 80 µg/L (Stark and Vargas 2005). In the case of *D. magna*, developmental and survival data indicated LOEC values of 19 and 27 µg/L, respectively, with a developmental NOEC of 9.6 µg/L (US EPA 1992). For its temperate relative *C. dubia*, Wilson et al. (2008) evaluated the sublethal effects of fipronil and its enantiomers on mobility, development and reproduction. The LOEC value for racemate reproduction was 15 µg/L, which is 33 times higher than the LOEC for *C. silvestrii*. The enantiomers S−(+) and the R−(−) showed LOEC values of 2 and 30 µg/L.

Mixture toxicity tests

All parameters and significance test results obtained by fitting the MIXTOX tool are shown in Table 2. In the acute test with the mixture of pesticides the derived data fitted the

Table 2 Parameters and fit tests of the reference models concentration addition and independent action applied to acute test of *Ceriodaphnia silvestrii* exposed for 48 h to mixtures of DMA® 806 BR (a.i. 2,4-D) and Regent® 800 WG (a.i. fipronil)

	Concentration addition				Independent action			
	CA	S/A	DR	DL	IA	S/A	DR	DL
Max	0.85	0.85	0.85	0.98	0.95	0.92	0.92	0.92
βDMA	10.49	8.90	8.72	5.84	7.02	8.03	8.02	5.43
βRegent	3.59	7.78	8.99	1.09	2.75	3.17	3.12	2.11
EC_{50} for DMA (mg/L)	187.50	176.86	178.73	163.13	162.51	174.14	173.55	172.02
EC_{50} for Regent (µg/L)	41.36	31.53	31.32	32.96	17.38	22.35	22.66	22.78
a	–	0.88	0.65	8.19	–	−2.19	−1.91	3.51
*b*DR/DL	–	–	0.63	0.72	–	–	−0.67	2.78
SS	75.40	65.05	64.75	50.91	64.20	59.65	59.63	50.85
r^2	0.80	0.83	0.83	0.87	0.83	0.84	0.84	0.87
χ^2 or *F* test	308.01	10.34	10.65	14.14	319.20	4.55	0.015	8.79
df	–	1.00	1.00	1.00	–	1.00	1.00	1.00
p (χ^2/*F*)	2.03×10^{-65}	0.0012	0.5805	0.0001	7.78×10^{-68}	0.0328	0.9008	0.0030

Max is the maximum response value; β is the slope of the individual dose response curve; EC_{50} is the median effect concentration; *a*, *b*DR and *b*DL are parameters of the function; SS is the sum of squared residuals; r^2 is the regression coefficient; χ^2 or *F* test is the test statistic; df is the degrees of freedom; and p (χ^2/*F*) is the significance level of the test statistic. CA is concentration addition model and IA is independent action model, S/A is synergism or antagonism deviation, DR is dose-ratio dependent deviation and DL is dose-level deviation

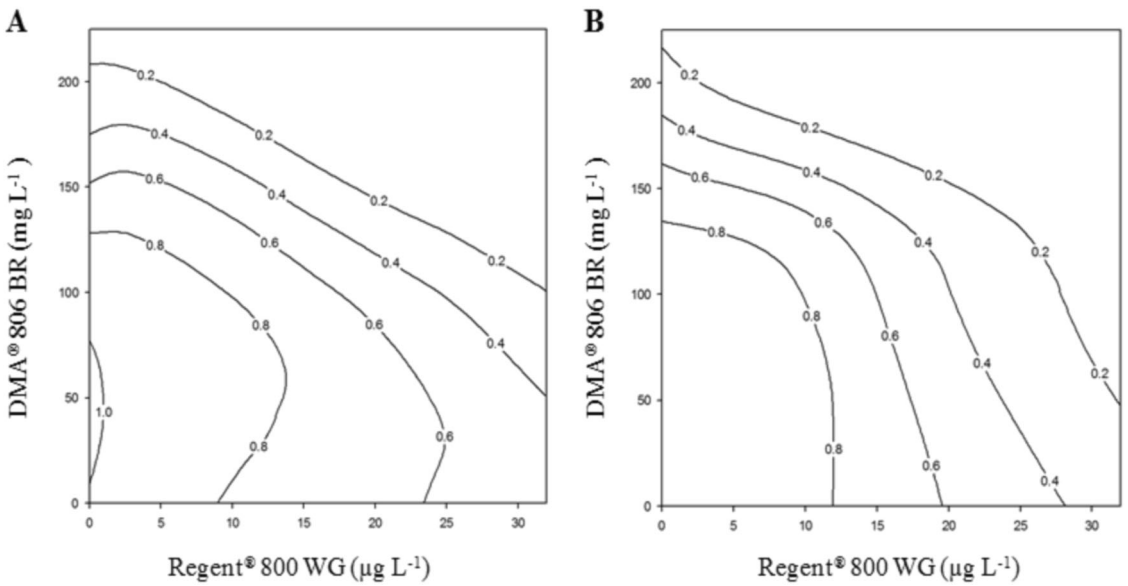

Fig. 3 Isobolograms of the pesticide mixture effects on immobility of *Ceriodaphnia silvestrii*. **a** Dose-level dependent (DL) from the concentration addition model (CA) and **b** dose-level dependent (DL) from the independent action model (IA)

IA and CA models. The model that best described the observed effects for the tested concentrations was the concentration addition (CA) model (Fig. 3a), which yielded a sum of squared residuals (SS) of 75.40 ($p < 0.05$; $r^2 = 0.80$). The dose level dependent deviation (DL) decreased the SS value to 50.91, being statistically significant ($p < 0.05$; $r^2 = 0.87$). The independent action model (IA) (Fig. 3b) yielded a sum of square residuals (SS) of 64.20 ($p < 0.05$; $r^2 = 0.83$). Dose level dependent deviation (DL) decreased the SS value to 50.85 ($p < 0.05$; $r^2 = 0.87$). The interaction of concentrations demonstrated antagonism at low doses and synergism at high doses, and the change from antagonism to synergism is at doses higher than the EC_{50} values (Fig. 3). As both models described the toxicity of the mixture, we chose CA as the best descriptive model because of the higher statistical significance in DL (Table 2).

Table 3 Parameters and fit tests of the reference models concentration addition and independent action applied to chronic test of *Ceriodaphnia silvestrii* exposed for 8 days to mixtures of DMA® 806 BR (a.i. 2,4-D) and Regent® 800 WG (a.i. fipronil)

	Concentration addition				Independent action			
	CA	S/A	DR	DL	IA	S/A	DR	DL
Max	86.24	84.73	84.15	84.47	90.62	86.28	84.96	87.81
βDMA	9.37	8.89	9.37	9.03	5.71	6.42	7.49	6.34
βRegent	1.14	1.96	2.16	2.21	1.17	2.05	2.47	1.12
EC_{50} for DMA (mg/L)	72.88	72.66	73.01	72.68	69.58	71.01	71.84	70.85
EC_{50} for Regent (mg/L)	0.015	0.007	0.006	0.007	0.008	0.007	0.006	0.010
a	–	1.16	2.22	0.65	–	−1.21	1.46	1.37
*b*DR/DL	–	–	−1.70	−0.71	–	–	−5.60	5.70
SS	2771.85	2438.41	2404.16	2434.30	2558.02	2469.03	2331.3	2395.42
r^2	0.69	0.73	0.73	0.87	0.72	0.73	0.74	0.74
χ^2 or *F* test	16.53	4.35	4.83	4.41	18.52	1.20	3.15	2.23
df	–	1.00	1.00	1.00	–	1.00	1.00	1.00
p (χ^2/*F*)	3.65×10^{-7}	0.03	0.09	0.10	1.17×10^{-7}	0.27	0.33	0.31

Max is the maximum response value; β is the slope of the individual dose response curve; EC_{50} is the median effect concentration; *a*, *b*DR and *b*DL are parameters of the function; SS is the sum of squared residuals; r^2 is the regression coefficient; χ^2 or *F* test is the test statistic; df is the degrees of freedom; and p (χ^2/*F*) is the significance level of the test statistic. CA is concentration addition model and IA is independent action model, S/A is synergism or antagonism deviation, DR is dose-ratio dependent deviation and DL is dose-level deviation

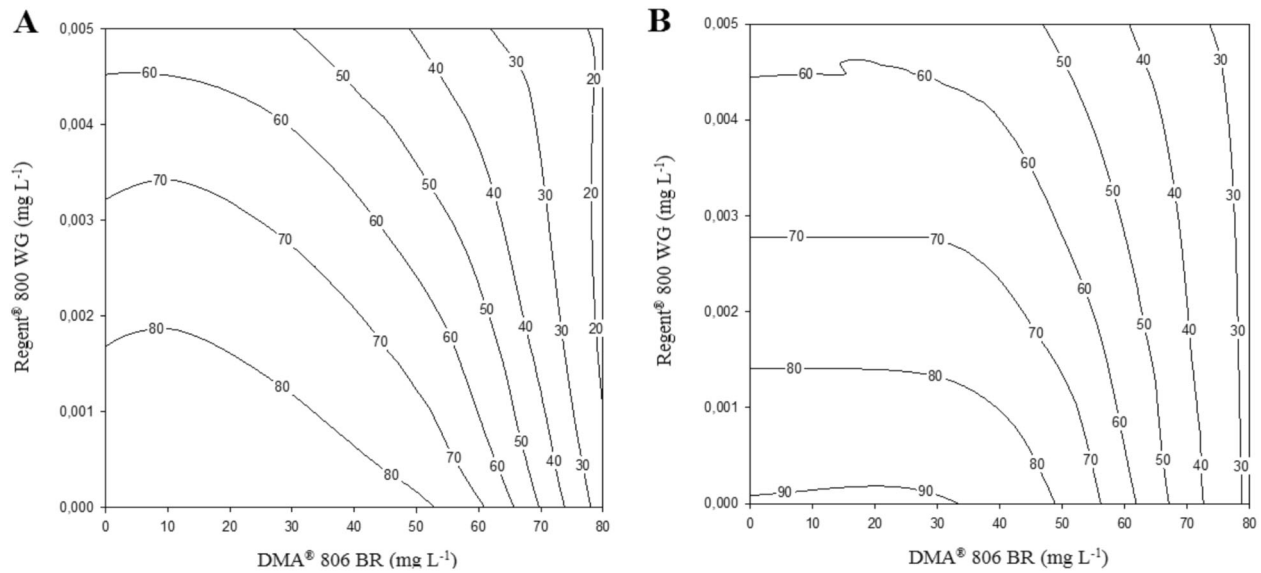

Fig. 4 Isobolograms of the pesticide mixture effects on reproduction of *Ceriodaphnia silvestrii*. **a** antagonism from the concentration addition model (CA) and **b** antagonism from the independent action model (IA)

All parameters and significance test results obtained by fitting the MIXTOX tool to the chronic data are shown in Table 3. The derived chronic mixture test data also fitted both the IA and CA models. The model that best described the effects on reproduction of *C. silvestrii* was also the CA model (Fig. 4a), which yielded a sum of squared residuals (SS) of 2771.85 ($p < 0.05$; $r^2 = 0.69$). After adding parameter "a" to the model in order to describe the S/A deviation, the SS value decreased to 2438.4 and was statistically significant ($p < 0.05$; $r^2 = 0.73$) showing antagonism as a result of the interaction of pesticide concentrations (Fig. 4). The dose-ratio dependent (DR) deviation and dose-level dependent (DL) deviation were not statistically significant ($p = 0.09$ and 0.10, respectively). In the case of IA (Fig. 4b), the sum of residues was 2558.02 ($p < 0.05$; $r^2 = 0.72$). After adding parameter "a"

to the model to describe the S/A deviation, the SS value decreased to 2469.03 but was not significant ($p > 0.05$; $r^2 = 0.73$) as well as for DR and DL ($p = 0.33$ and 0.31) respectively.

As in our study, in other mixture toxicity assessments the effects were better interpreted by CA (Gazonato Neto et al. 2019), even for mixture compounds with different mechanisms of action (Barata et al. 2012). In the case of herbicides, for example, that have a mechanism of action for plant cells, it is difficult to define direct action at the molecular level for other living things, because there are several direct and indirect mechanisms that can be activated upon exposure (Bukowska 2006). Neurotoxicity, genotoxicity, histopathology, biochemical damage and endocrine disruption (Martinez-Tabche et al. 2004; Xie et al. 2005; Lajmanovich et al. 2013; Menezes et al. 2015; Benli et al. 2016) are examples of effects of 2,4-D found in aquatic organisms other than primary producers. Nevertheless, Barata et al. (2012) evaluated the effect of simple and ternary mixtures of nine compounds on the growth rate of *C. dubia* and observed that the ecotoxicological mode of action is more effective at predicting effects than the substance's mode of action.

Both 2,4 D and fipronil are commonly present in complex mixtures in the environment, even in areas where they are not used (Donald et al. 2001). Near agricultural areas, the pesticide product and its metabolites may persist for a certain period with peaks of the active ingredient during application periods (Gilliom et al. 2006). In the aquatic environment, 2,4-D has a half-life between 38 and 90 days. The DT_{50} (period required for 50 percent disappearance) is 9.4 days (EFSA 2014) and in clear surface water this value can be up to 4 weeks (PPDB: Pesticide Properties Database 2020). In the case of fipronil, the study by Belayneh (1998) indicates that fipronil has a half-life in water of about 3.6 h when exposed to sunlight. The half-life of the major metabolites is 3.6 h for fipronil sulfide, 13 h for fipronil sulfone and 38.9 h for fipronil desulfinyl, which is considered to be in addition extremely stable and more orally toxic than fipronil (EFSA 2006). An important point raised by Belayneh (1998) is that fipronil degrades faster under tropical than temperate conditions having as its main metabolite in this process fipronil desulfinyl.

Marchesan et al. (2010) reported concentrations between 3 to 3.4 µg/L of 2,4-D and 0.05 to 26.2 µg/L of fipronil in rivers near agricultural crops from the south of the country. In the state of São Paulo, a monitoring conducted in 2017 detected the active ingredient fipronil in 75% of the samples while 2,4-D was present in 14% (CETESB 2018). This evaluation was performed at seven sampling points, in six different water bodies with 176 samples collected for pesticide analysis. The maximum concentrations found for the two pesticides were from 2.4 to 465 µg/L for fipronil and 143.1 to 366.6 µg/L for 2,4-D. Both studies, Marchesan et al. (2010) and CETESB (2018), present concentrations exceeding the environmental quality standards of 0.2 µg/L for 2,4-D and 0.012 µg/L for fipronil (Albuquerque et al. 2016).

The maximum concentrations of fipronil and 2,4-D obtained by CETESB (2018) are from predominant sugarcane cultivation sites. In addition, the reported 465 µg/L concentration exceeds the fipronil LC_{50} and EC_{50} value of a variety of species contained in the EPA research platform (US EPA 2019), such insect larvae (Chaton et al. 2002), molluscs (Overmyer et al. 2007), larvae and adult fish (Baird et al. 2013; Wang et al. 2010) and algae (US EPA 1992). For crustaceans, the most sensitive organisms are marine species *Americamysis bahia* $LC_{50} = 0.14$ µg/L (US EPA 1992), *Palaemonetes pugio* $LC_{50} = 0.3$ µg/L (Key et al. 2003) and *Amphiascus tenuiremis* $LC_{50} = 1.68$ µg/L (Bejarano et al. 2005) followed by freshwater species, *Diaptomus castor* $LC_{50} = 3.4$ µg/L (Chaton et al. 2002) and *Macrobrachium nipponense* $LC_{50} = 4.3$ µg/L (Shan et al. 2003). *C. silvestrii* according to our results will be greatly affected in agricultural fields because most of the environmental concentrations reported are higher than concentrations that cause effects on the different endpoints evaluated in the present study: acute (immobility) and chronic (first reproduction, fecundity and intrinsic rate of population increase).

Furthermore, bad agricultural practices, such as using pesticides above the recommended dose (overuse) and applying mixtures pesticides, may also make even higher concentrations of these products available in natural aquatic environments (Nunes 2010; Gazziero 2015).

Conclusions

In this study, we showed that exposure to 2,4-D based herbicide and fipronil based insecticide, commonly used pesticides in sugarcane crops, can affect vulnerable organisms in the aquatic environment. *Ceriodaphnia silvestrii* exposure to DMA® 806 BR (a.i. 2.4-D) caused immobility at concentrations above 100 mg a.i./L and reproduction and intrinsic rate of population increase decreased above 60 mg a.i./L. In addition, reproduction has been practically inhibited above 70 mg a.i./L. Regent® 800 WG (a.i. fipronil) presented high toxicity for the species at concentrations often found in aquatic ecosystems (e.g. caused immobility in 2 µg a.i./L and decrease in reproduction rate in 0.8 µg a.i./L). The acute mixture toxicity revealed a dose level dependent deviation (DL) of the concentration addition model (CA), with antagonism at low and synergism at high pesticide mixture concentrations. And for chronic mixture test occurred antagonism as a result of the interaction of

pesticide concentrations also of the CA model. Although there has been a great increase in research on mixture toxicity over the past years, additional information is required to develop practical criteria for selecting pesticide mixtures that require additional attention such as 2,4-D and fipronil which can be found in mixtures in natural environments. Therefore, suggestions for future studies would be to evaluate complex mixtures of pesticides, using native representatives from different trophic levels with different endpoints. On that note, it would also be important to focus on molecular mechanisms behind the mode of action of pesticides, in an attempt to better understand its ecological implications on populations dynamics.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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