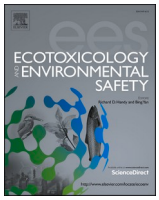
Ecotoxicology and Environmental Safety 209 (2021) 111778

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

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



Impact of 2,4-D and fipronil on the tropical midge *Chironomus sancticaroli* (Diptera: Chironomidae)

Thandy Junio da Silva Pinto [a,*], Raquel Aparecida Moreira [a], Laís Conceição Menezes da Silva [a], Maria Paula Cardoso Yoshii [a], Bianca Veloso Goulart [b], Priscille Dreux Fraga [a], Cassiana Carolina Montagner [b], Michiel Adriaan Daam [c], Evaldo Luiz Gaeta Espindola [a]

[a] PPG-SEA and NEEA/CRHEA/SHS, São Carlos School of Engineering, University of São Paulo, Av. Trabalhador São Carlense, 400, São Carlos 13560-970, Brazil
[b] Analytical Chemistry Department, Institute of Chemistry, University of Campinas, Campinas, São Paulo, Brazil
[c] CENSE, Department of Environmental Sciences and Engineering, Faculty of Sciences and Technology, New University of Lisbon, Quinta da Torre, 2829-516 Caparica, Portugal

ARTICLE INFO

Edited by: Dr R Pereira

Keywords:
Pesticides
Sublethal effects
Mentum deformity
Synergism
Larval growth
Larval development

ABSTRACT

Increased use of pesticides in conventional agriculture implies potential risks to the environment. In aquatic ecosystems, benthic organisms may be exposed to pesticides via contaminated water and sediment, leading to several potential cascading effects on the food web. The aim of this study was to assess the functional implications of environmental realistic concentrations of the herbicide 2,4-D and the insecticide fipronil (alone and in combination) to the native tropical chironomid *Chironomus sancticaroli.* These two pesticides are widely applied to different crops and have frequently been detected (together) in surface water bodies in Brazil and elsewhere. Commercial products containing fipronil (Regent® 800WG) and 2,4-D (DMA® 806BR) were evaluated in 8-day toxicity tests for their effects on larval survival, growth (body length and biomass), head capsule width, development, and mentum deformities. Fipronil decreased the larval survival at the highest test concentration and the effective concentrations (EC) after eight days of exposure were: EC_{10} = 0.48 μg L^{-1} (0.395–0.565), EC_{20} = 1.06 μg L^{-1} (0.607–1.513), and EC_{50} = 3.70 μg L^{-1} (1.664–5.736). All sublethal test concentrations of fipronil decreased the larval growth, causing reductions in biomass up to 72%. The two highest test concentrations of fipronil decreased the head capsule width and after exposure to 3.7 μg fipronil L^{-1}, only half of the larvae reached the fourth instar. The incidence of deformities was increased by fipronil in a concentration dependent manner with an increase ranging from 23% to 75%. The highest test concentration of 2.4-D (426 μg L^{-1}) decreased the head capsule width, but larval development was unaffected at all concentrations evaluated. In the mixture tests, antagonism was observed at lower fipronil concentrations and synergism at higher fipronil concentrations for growth. The incidence of deformities rose with increasing fipronil concentrations. The results showed that environmental realistic concentrations of fipronil may have serious ecological implications for *C. sancticaroli* populations and that a mixture with the herbicide 2,4-D can have synergistic effects, potentiating the risks to the aquatic ecosystem.

1. Introduction

Freshwater pollution has become a global problem and its origin lies with industrial activities, agriculture and urban areas (Gage et al., 2004; Gunkel et al., 2007; Hua and Relyea, 2014). In agriculture, pesticides are considered the most problematic chemicals, especially in tropical regions due to a greater number of pests affecting production, which increases the amount of pesticides applied in conventional agriculture (Printes et al., 2011). In these regions, the application of pesticides is intensified during the rainy season (summer), and the intensive precipitation in this season and subsequent runoff leads to a transfer of these contaminants to adjacent aquatic ecosystems (Gripp et al., 2017; Taniwaki et al., 2017).

Several pesticides can be applied at different stages of agricultural plant development, alone or in combination, at the same time or in short time intervals (Vale et al., 2019). The herbicide 2,4-D and the insecticide

* Corresponding author.
E-mail address: thandyjuniosilva@usp.br (T.J.S. Pinto).

https://doi.org/10.1016/j.ecoenv.2020.111778
Received 14 May 2020; Received in revised form 1 December 2020; Accepted 6 December 2020
Available online 15 December 2020

fipronil are incorporated in 37 and 55 formulated products that are registered for use in Brazil, respectively. Both pesticides are licensed for use in sugarcane, rice, corn, and soybean crops, among five other minor crops (MAPA, 2019). Thus, both pesticides may occur together in terrestrial and aquatic environments in these crop areas. Concentrations ranging from 0.1 to 465 μg fipronil L^{-1} and 0.4–366 μg 2,4-D L^{-1} have previously been reported in Brazilian surface waters (Albuquerque et al., 2016; CETESB, 2018; Grützmacher et al., 2008; Marchesan et al., 2010; Vieira et al., 2016), in addition to the simultaneous occurrence of both compounds in areas with predominance of sugarcane crops (CETESB, 2018).

The systemic herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) was developed as a growth inhibitor of dicotyledonous weeds and is one of the most used herbicides worldwide (Islam et al., 2018; Tomlin, 1994). The herbicide is applied directly to the soil or over the plant foliage. Due to its intensive use, persistence in the soil (half-life 17–39 days), high water solubility (23.2 g L^{-1} at 22 °C, pH 7) and low adsorption to soil particles (Koc ranging from 20 to 280), its acids and amines have high mobility in water, and as such easily reach aquatic ecosystems (Barriuso et al., 1992; Boivin et al., 2005; Bolan and Baskaran, 1996; Laganà et al., 2002).

Fipronil is a phenylpyrazole insecticide developed to block the GABA-regulated chloride channels in insect pest species (Tomlin, 1994). Its use has increased with the ban and restrictions on the use of organochlorine and organophosphate insecticides. Fipronil has been used in agricultural and urban areas, mainly in underdeveloped countries where it is still allowed (Brennan et al., 2009; Weston and Lydy, 2014). Fipronil has high persistence in soil (half-life 8.78–33 days), low water solubility (1.9 mg L^{-1} at pH 5, 20 °C), and high affinity with organic matter (Koc = 825). Thus, in agricultural areas this insecticide is transported to edge of field waterbodies though adsorption to soil organic material (Mize et al., 2008; Tomlin, 1994; Ying and Kookana, 2001; Zhu et al., 2004). In aquatic ecosystems, fipronil may occur in both the water column and the sediment, while 2,4-D remains mainly in the dissolved phase (Islam et al., 2018; Mize et al., 2008; Peret et al., 2010). The half-life in water ranges from 13.2 to 87.9 days for fipronil (Peret et al., 2010; Thuyet et al., 2013) and 18.6–90 days for 2,4-D (EFSA, 2014; Girardi et al., 2013). In the sediment, the values range from 6.9 to 91.2 and 15–25 days for fipronil and 2,4-D, respectively (Chinalia and Killham, 2006; Lin et al., 2008; Peret et al., 2010).

Benthic organisms, such as the chironomids, may be exposed to chemicals both via contaminated water and sediment (Brennan et al., 2009). Species belonging to the Chironomidae family are widely used in ecotoxicological studies due to its importance in aquatic systems (Fonseca and Rocha, 2004; Park and Kwak, 2008). *Chironomus sancticaroli* Trivinho-Strixino & Strixino, 1982 (Chironomidae: Chironominae) is a common midge in South America and synonym of *C. xantus* Rempel, 1939, and *C. domizzi* Paggi, 1977 (Fonseca and Rocha, 2004; Trivinho-Strixino, 2011). This insect species was successfully used in toxicity tests that include the evaluation of survival, larval growth, and alterations in the development and occurrence of deformities (e.g. Morais et al., 2019; Palacio-Cortés et al., 2017; Richardi et al., 2018).

Toxic effects of sole exposure to fipronil and 2,4-D on some species of midges other than *C. sancticaroli* have been reported in the literature (e.g. Maul et al., 2008; Monteiro et al., 2019; Park et al., 2010). In addition, recent studies have demonstrated the combined effects of these compounds on organisms from different trophic levels of aquatic biota, such as algae (Moreira et al., 2020a) and cladocerans (Moreira et al., 2020b; Silva et al., 2020). However, there is still a lack of information about the single toxicity of fipronil and 2,4-D to tropical macroinvertebrate taxa, besides the responses caused by their combination to benthic organisms exposed at low concentrations likely to occur in their natural environments. Thus, the aim of this study was to evaluate the adverse effects of environmentally relevant concentrations of the herbicide 2,4-D and the insecticide fipronil, alone and in combination, on the survival, growth, development and occurrence of mentum deformity of *C. sancticaroli*. We hypothesize that (*i*) fipronil alone will have more deleterious effects on *C. sancticaroli* than 2,4-D due to its neurotoxicity to insects; (*ii*) exposure to 2,4-D will not decrease the survival, but prolonged exposure to the herbicide may stress the organisms and subsequently decrease the growth and development and increase the occurrence of mentum deformities; (*iii*) the effects will occur in a concentration-dependent manner; and (*iv*) the pesticide mixture will potentiate effects on survival and growth of *C. sancticaroli* when compared to the effects of the compounds alone.

2. Methods

2.1. Test organisms

Chironomus sancticaroli was obtained from cultures kept at the Nucleus of Ecotoxicology and Applied Ecology (NEEA), in the Center for Water Resources and Environmental Studies (CRHEA), São Carlos School of Engineering, at the University of São Paulo, located in the municipality of Itirapina, São Paulo state, Brazil. In accordance with Fonseca and Rocha (2004), the insects were maintained in non-toxic plastic trays containing 1 kg sterilized sediment (fine sand washed and burned at 550 °C for 2 h) and 4 L culture water (well water, pH: 7.0–7.5, conductivity: 50.5 ± 1.4 μS cm^{-1} and hardness: 12–16 mg $CaCO_3$ L^{-1}), with constant aeration, temperature 25 ± 1 °C and a daily cycle of 12:12 light: dark. Larvae were fed ad libitum three times per week with a Tetramin® suspension (5 g L^{-1}). The water was renewed weekly and the cultures were reinitiated monthly. For the toxicity tests, newly hatched larvae were maintained for four days in new trays until the start of the test.

2.2. Toxicity tests

The pesticide stock solutions were prepared by diluting the commercial formulations Regent 800WG® (BASF S.A.) a.i. fipronil (80% w/w) and DMA 806BR® (Dow AgroSciences Industrial Ltda) a.i. 2,4-D (67% w/v of acid equivalent) with distilled water to final concentrations of 0.8 mg L^{-1} and 1000 mg L^{-1} for fipronil and 2,4-D, respectively. Static subchronic tests were performed at the same temperature and light conditions as described above for the cultures, in non-toxic plastic vessels containing 200 mL of the test solutions and 60 g of sterilized fine sand. The sand was previously burned (550 °C) and had no organic matter content. Eighty larvae (4-day old) were used for each treatment, divided over eight replicates (10 larvae/replicate). Based on acute preliminary tests, five nominal test concentrations were selected for fipronil (0.2, 0.4, 0.8, 1.6 and 3.2 μg a.i. L^{-1}) and 2,4-D (22.5, 45, 90, 180 and 360 μg a.i. L^{-1}). The highest fipronil test concentration (3.2 μg a.i. L^{-1}) was chosen based on the 96 h-LC_{10} toxicity value obtained in these preliminary tests. The test concentrations were also selected based on the environmental concentrations of these compounds measured in Brazilian surface water, as presented in the introduction section. The exposure period was eight days to avoid metamorphosis according to the life circle described in Fonseca and Rocha (2004). The larvae were fed every 48 h (1.24 mg Tetramin® $larva^{-1}$). The test solution was completely renewed once at the middle of the test (four days post start test). At the end of the test, the living larvae were counted and preserved with 70% ethanol. The tests with single exposure to both pesticides were repeated three times under the same conditions but with larvae originating from different egg masses. Tests were considered valid if survival in control was higher than 80%. The water parameters pH (micronal B374), dissolved oxygen and temperature (YSI55-25 ft), and electrical conductivity (Oriom 145) were measured in the beginning, middle, and at the end of the tests and water samples were taken for hardness and ammonium analysis at the beginning and end of the tests (APHA, 2018; Hansen and Koroleff, 2007). The preserved larvae were photographed, and the body length was measured using the free Kinovea 0.8.15 software (https://www.kinovea.org), which was calibrated using graph

paper. The body length was determined as a line from the top of the head to the anal papillae.

The mixture toxicity test was performed under the same conditions as described above for the single pesticide toxicity tests, but with four replicates containing ten individuals (40 animals/treatment) for each concentration instead of eight. Five concentrations of fipronil and 2,4-D on isolation were prepared (the same concentrations as those described above), as well as the 25 combinations of both pesticides, selected according to the full factorial modeling design (see Freitas et al., 2014), in addition to an untreated control. Thus, 36 concentrations were prepared in the mixture toxicity test. All concentration combinations are presented in Table S1. At the end of the test, the living larvae were counted, preserved in 70% ethanol and photographed, after which the body length was measured as described above.

2.3. Mentum deformities and development stage

Post-photographed, head capsules from the larvae were removed from the body and placed on semi-permanent blades with the Hoyer medium for the buccal structure observation on an optic microscope at 200-fold magnification. Four types of deformities were considered based on Kuhlmann et al. (2000): missing-teeth, extra-teeth, Köehn *gap* and bifurcation of central median teeth. Moreover, massive deformities such as deviation from the normal mentum configuration and teeth absence were considered as deformities. Worn teeth were registered as an alteration of non-deformed larvae but broken teeth were not considered as deformity because this could have happened due to the sample treatment. Examples of the deformities and wear are presented in Fig. 3. The head capsule width was measured on a stereoscopic microscopic and applied for the determination of the instar of development according to Fonseca and Rocha (2004). According to the life cycle of *C. sancticaroli*, it may be expected that after eight days of exposure most larvae would reach the fourth instar (see Fonseca and Rocha, 2004).

2.4. Body length-biomass ratio estimation

One hundred larvae were obtained from cultures maintained in the laboratory for the body length-biomass ratio estimation. The larvae were selected according to the body length and the development stages (16-second, 42-third, and 42-fourth-instar larvae) and maintained for 24 h in clean culture water to allow gut clearing. After that, the larvae were anesthetized and killed with phenoxyethanol, weighed immediately (± 0.01 mg) for fresh biomass determination and photographed to measure the body length. Then, the larvae were dried at 60 °C for 24 h, weighed and burned at 550 °C for 2 h to allow determination of the ash free dry weight (AFDW) in accordance with USEPA (2000). The relationship between body length and biomass of *C. sancticaroli* was determined by linear regression. In this way, the biomass could be calculated from the body length of the larvae as measured in the toxicity tests.

2.5. Chemical analyses

All chemical pesticide analyses were carried out at the Environmental Chemistry Laboratory of the Institute of Chemistry at the State University of Campinas (UNICAMP), according methodology described in Goulart et al. (2020). The nominal concentrations in the tests were verified by chemical analyses using an Agilent 1200 Liquid Chromatograph coupled with an Agilent 6410B Mass Spectrometer (QqQ) (Agilent Technologies – Santa Clara, USA) with an electrospray ionization source (ESI) in negative mode. Chromatographic separation was performed using a Zorbax SB-C18 column (2.1 ×30 mm, particle size of 3.5 μm) and Poroshehell 120 EC-C18 column (3.0 ×50 mm, particle size of 2,7 μm) at 30 °C, injection volume of 10 μL and flow rate of the mobile phase of 0.3 mL min^{-1}. The mobile phase was aqueous 0.01% ammonium hydroxide and methanol in gradient elution mode. The preparation of the 2,4-D samples was done by diluting the sample 1.25 times (800 μL of the sample and 200 μL of methanol) which was then filtered through a syringe filter (PTFE 0.22 μm). The fipronil sample preparation was carried out by online Solid Phase Extraction (SPE) after filtration (syringe PTFE filter 0.22 μm).

The determination coefficients (R^2) and method sensitivity for 2,4-D were 0.996 and 384.6, respectively, and corresponding values for fipronil were 0.998 and 463.5. The LOD and LOQ were obtained by the signal-to-noise method comparing the analytical signal of samples in low concentrations of the compound with the noise in the baseline. The concentration was considered in which the signal-to-noise ratio was observed in the proportion 10:1 and 3:1 for the LOQ and LOD, respectively. The LOD and LOQ of the method were 0.5 and 1.0 μg/L for 2,4-D and 0.001 and 0.005 μg/L for fipronil, respectively.

2.6. Mixture synergism/antagonism interaction

The assessment of the mixture interaction was based on Gottardi et al. (2017) by applying the model of Independent Action (IA), because the pesticides have different modes of action (Jonker et al., 2005; Loureiro et al., 2010). The existence of synergism or antagonism was estimated by the relationship between the predicted and observed effects of the mixture (Gottardi et al., 2017). The predicted effect was estimated by:

$$Predictedeffect = \frac{D}{C} \quad * \quad \frac{F}{C}$$

Where D, F, and C are the values of the response (e.g. body length) evaluated for the larvae exposed to either 2,4-D (D) or fipronil (F) alone, relative to the untreated control (C). The observed effect was calculated considering the ratio of the effect on the mixture (M) and the control ($Observed\ effect = M/C$). The predicted and observed effects were calculated only when the effect in the mixture was statistically different from the control or at least one compound alone (Gottardi et al., 2017).

2.7. Data analyses

All analyses were performed in R version 3.6.0 (2009) with the application of RStudio version 1.2.1335 (2019). All comparisons of treatments with the control grouping were carried out with a confidence level of 95% ($p = 0.05$) using Generalized Linear Models (GLM) (Figueirêdo et al., 2020; Lopes et al., 2018; Scherer et al., 2020). Our data met the assumption of independence between treatments. The effects on survival were analysed by GLM with the Binomial family with the logit-link function. The effective concentrations (EC) for 8 days of exposure for 10% (EC_{10}), 20% (EC_{20}), and 50% (EC_{50}) of the population were determined by a non-linear estimation method using the logistic model. Effects on growth (body length and biomass) and head capsule width were determined by GLM with the Gaussian family and the identity-link function. Statistical analysis and the estimation of the effect concentrations were performed for the three repetitions separately, and results are presented as the overall mean of the three tests. In the mixture experiment, GLM comparisons were made between the control and all pesticide mixture treatments and each pesticide mixture combination was compared with the corresponding treatment containing the sole concentration of fipronil and 2,4-D.

3. Results

3.1. Chemical analysis

The stock solution concentrations of fipronil and 2,4-D quantified by LC-MS/MS analysis were 536 μg L^{-1} (nominal 800 μg L^{-1}) and 0.9 g L^{-1} (nominal 1.0 g L^{-1}), respectively. The initial fipronil concentrations in the bioassays were 0.3, 0.4, 0.7, 1.3, and 3.7 μg L^{-1} as compared to the respective nominal concentrations of 0.2, 0.4, 0.8, 1.6, and 3.2 μg a.i.

L^{-1}. For 2,4-D, the initial concentrations were 29, 66, 112, 221, and 426 μg L^{-1}, and the nominal concentrations 22.5, 45, 90, 180, and 360 μg a.i. L^{-1}. Since the difference between nominal and initial concentrations was more than 20% in some treatments, the mean measured concentrations were reported as the test concentrations and used for the calculation of the toxicity values (OECD, 2011).

3.2. Single toxicity tests

3.2.1. Larval survival

Over the eight day exposure period, the larval survival was higher than 90% in the controls of all tests, thus validating the tests. At the end of the tests the values of pH ranged between 6.5 and 7.5, hardness between 12 and 16 mg $CaCO_3$ L^{-1}, ammonium between 1.5 and 1.8 mg L^{-1}, temperature 24 ± 1 °C and oxygen was always above 6 mg L^{-1}. No differences in water parameters between treatments and between the beginning and end of the tests were denoted ($p > 0.05$). No effects were observed on survival for larvae exposed to any of the 2,4-D concentrations tested (Fig. 1, $p > 0.05$). For fipronil, however, larval survival was significantly decreased ($p < 0.05$) at 0.7 μg L^{-1} (79 ± 7%), 1.3 μg L^{-1} (59 ± 12%) and 3.7 μg L^{-1} (42 ± 10%) when compared to the control (98 ± 0.70%). The effective concentrations for larval survival after eight days of exposure were: EC_{10} = 0.48 μg L^{-1} (0.40–0.57), EC_{20} = 1.1 μg L^{-1} (0.61–1.5), and EC_{50} = 3.7 μg L^{-1} (1.7–5.7).

3.2.2. Growth: body length, fresh biomass and ash free dry weight (AFDW)

The equations to estimate the biomass from the larval body length indicated a significant correlation between these two test parameters (Fig. S1). In controls of the tests with the insecticide fipronil, the body length was 11.08 ± 0.48 mm (mean ± SE) and the fresh biomass and AFDW were 3.7 ± 0.26 mg and 0.50 ± 0.037 mg, respectively (Fig. 2a and b). The lowest fipronil concentration (0.3 μg L^{-1}) slightly decreased ($p < 0.05$) the body length (10.24 ± 0.50 mm), the fresh biomass (3.3 ± 0.28 mg) and AFDW (0.44 ± 0.040 mg). The intermediate concentrations of fipronil (0.4, 0.7, and 1.3 μg L^{-1}) had a comparable growth, which was lower than that in controls ($p < 0.05$). At the highest fipronil concentration (3.7 μg L^{-1}), the body length (6.4 ± 0.74 mm) was 42% lower than that in the control ($p < 0.05$, Fig. 2a). The reduction in this treatment reached 69% for fresh biomass (1.2 ± 0.41 mg) and 72% for AFDW (0.14 ± 0.060 mg, Fig. 2b). The larvae exposed to the herbicide had values for body length and biomass similar to those in controls at all concentrations tested (Fig. 2b and c, $p > 0.05$).

3.2.3. Head capsule width and development stage

Table 1 presents the mean head capsule width of *C. sancticaroli* larvae exposed to the two pesticides. The herbicide 2,4-D decreased the head width of chironomids when exposed to the highest test concentration of 426 μg L^{-1} (0.57 ± 0.010 mm) in comparison to control (0.586 ± 0.004 mm, $p < 0.05$). In the lower 2,4-D concentration, the head capsule width was similar to the control ($p > 0.05$). The percentage of fourth instar larvae at the end of the experiment in all 2,4-D treatments was similar to that in the control ($p > 0.05$, Table 1). Fipronil significantly decreased the head capsule width at concentrations of 1.3 μg L^{-1} (0.51 ± 0.0070 mm) and 3.7 μg L^{-1} (0.41 ± 0.040 mm) compared to the control (0.55 ± 0.0070 mm, $p < 0.05$, Table 1). Despite the head width reduction of chironomids exposed to 1.3 μg L^{-1}, the development of these animals was similar to the control ($p > 0.05$). On the other hand, the highest fipronil concentration (3.7 μg L^{-1}) delayed the larval development and only 46 ± 22% of these larvae reached the fourth instar (Table 1, $p < 0.05$), with the remaining larvae all reaching their third instar at the end of the tests. In the three lowest fipronil treatments, the head capsule and development were similar as those in the control ($p > 0.05$).

3.2.4. Deformities

Larvae of *C. sancticaroli* from the controls presented a deformity percentage of 5.9% and 5.2% in the single exposure tests with 2,4-D and fipronil, respectively. Fig. 3 presents examples of the deformities observed. Bifurcations of central median teeth and deviations from the normal mentum configuration were not observed in any treatment. Extra and missing teeth were the only deformities registered in both controls (Table 2). The herbicide 2,4-D slightly increased the deformity frequency, but the occurrence was not dose-level dependent and not significant ($p > 0.05$). At 66 and 426 μg 2,4-D L^{-1}, the frequencies were around 12%, and extra- and missing-teeth were observed. The non-deformed larvae presented normal teeth and no wear was observed at any 2,4-D test concentration (Table 2).

Regarding fipronil, the deformity occurrence in chironomids was dose-level dependent, and even the lower test concentrations (0.3, 0.4 and 0.7 μg L^{-1}) presented high deformity rates ($p < 0.05$), and absent and missing teeth were the most frequent observed deformities. At 1.3 and 3.7 μg fipronil L^{-1}, deformities reached 40% and 71%, respectively, with the absence of teeth as the most frequent deformity observed. The larvae also presented an increase in the occurrence of worn teeth ranging from 15% in the lowest fipronil concentration to 39% in the highest fipronil concentration ($p < 0.05$, Table 2).

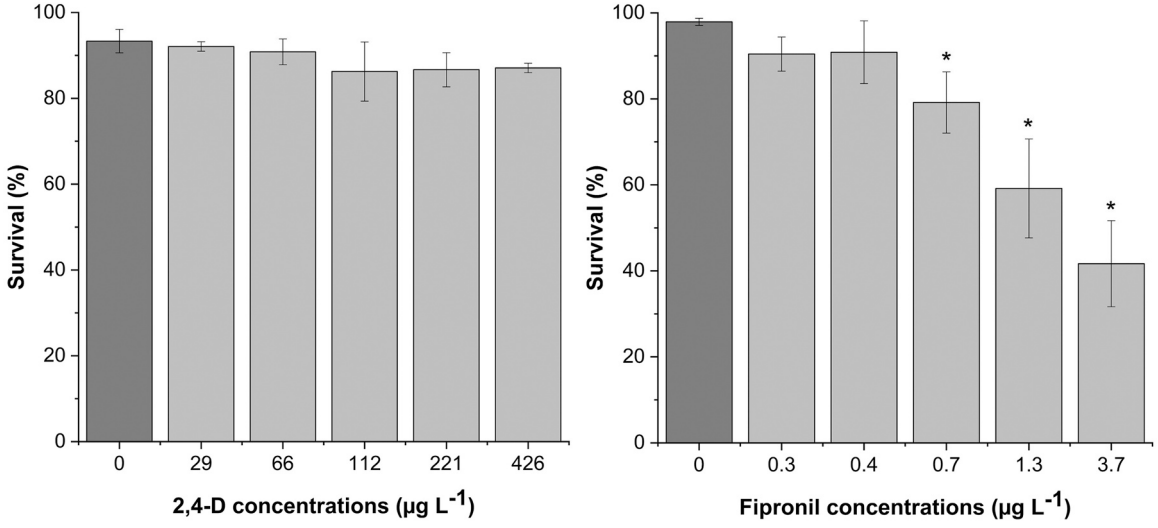


Fig. 1. Survival (mean ± SE) of *C. sancticaroli* exposed to the herbicide 2,4-D and the insecticide fipronil. Asterisks (*) indicate values statistically different from control ($p < 0.05$).

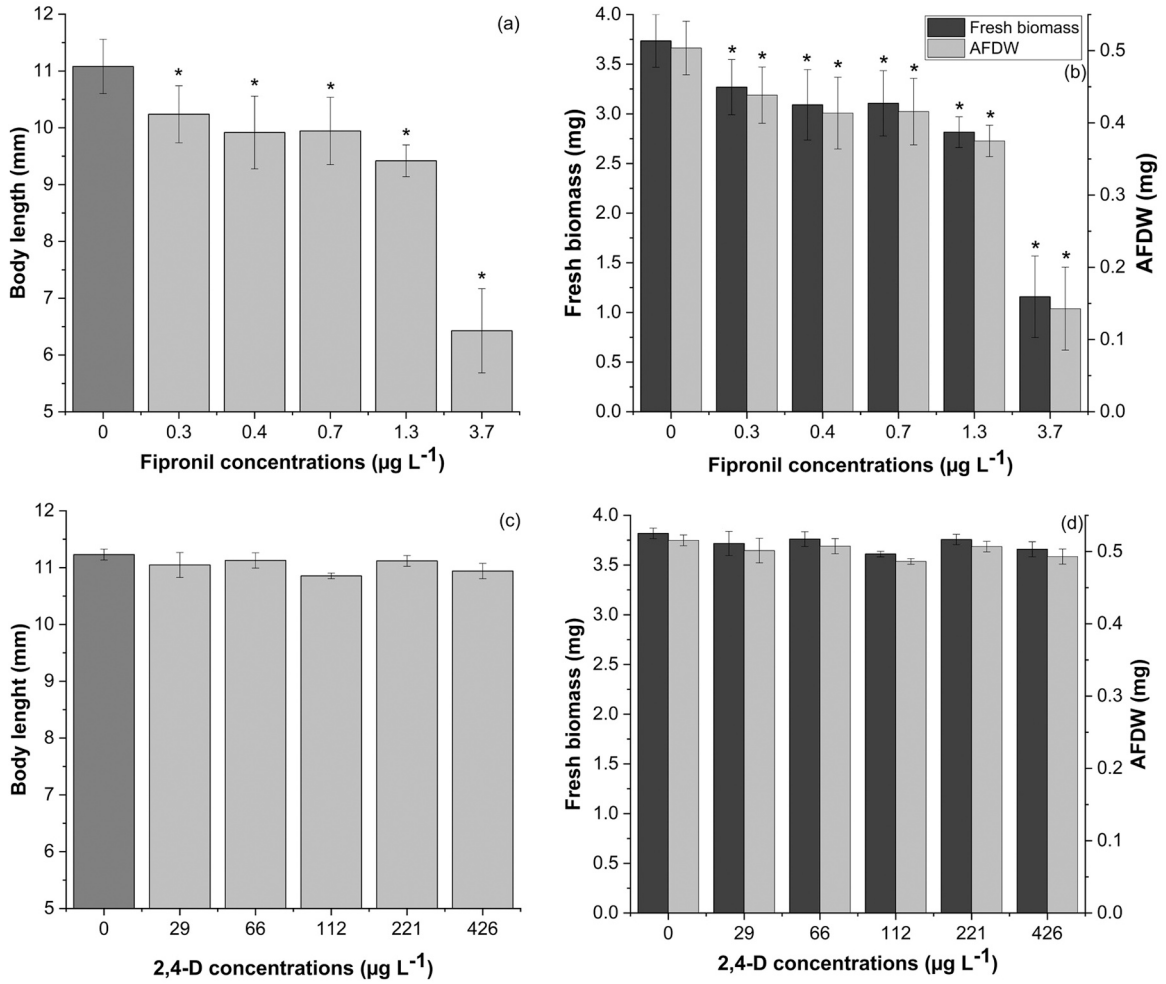


Fig. 2. Body length, fresh biomass, and ash free dry weight (AFDW; mean ± SE) of *C. sancticaroli* exposed to the insecticide fipronil (a) and (b); and the herbicide 2,4-D (c) and (d). Asterisks (*) indicate values statistically different from control ($p < 0.05$).

Table 1
Head capsule width and frequency of the fourth instar larvae (mean ± SE) of *C. sancticaroli* exposed to 2,4-D and fipronil.

2,4-D			**Fipronil**		
Concentration (µg L^{-1})	Head capsule (mm)	Fourth instar (%)[a]	Concentration (µg L^{-1})	Head capsule (mm)	Fourth instar (%)[a]
0	0.586 ± 0.004	99.1 ± 0.9	0	0.550 ± 0.007	100
29	0580 ± 0.011	99.5 ± 0.5	0.3	0.534 ± 0.005	98.4 ± 1.6
66	0.575 ± 0.007	97.4 ± 1.9	0.4	0.531 ± 0.013	98.0 ± 2
112	0.572 ± 0.010	96.2 ± 2.6	0.7	0.534 ± 0.005	100
221	0.576 ± 0.009	98.2 ± 1.1	1.3	**0.512 ± 0.007***	97.0 ± 3
426	**0.570 ± 0.010***	97.9 ± 0.5	3.7	**0.410 ± 0.040***	**45.7 ± 22.4***

[a] Percentual of fourth instar larvae at the end of the experiment.
* Values statistically different from control ($p < 0.05$).

3.3. Mixture toxicity tests

In the mixture toxicity test, no larvae mortality occurred in the controls, and survival in all single 2,4-D treatments were similar to control ($p > 0.05$). After single fipronil exposure, only the 3.7 µg L^{-1} treatment significantly decreased survival (54 ± 14%, $p < 0.05$). Regarding the mixture treatments, only the highest fipronil concentration (3.7 µg L^{-1}) in the mixture with 112 µg 2,4-D L^{-1} (F5D3) and 426 µg 2,4-D L^{-1} (F5D5) decreased survival rates (50 ± 14% and 63 ± 8%; $p < 0.05$). Fig. S2 presents the survival rates for all mixture combinations.

Regarding growth, the treatments containing 2,4-D and fipronil alone in the mixture test showed the same pattern as that noted in the single exposure toxicity tests. Table 3 summarizes the values estimated for the predicted and observed effects, as well as the indication of synergism and antagonism for body length and AFDW. Fig. S3 also visualizes these parameter values for all mixture combinations. The fresh biomass had the same pattern as AFDW and is therefore not presented. The combinations with the lower concentrations of fipronil (0.3–0.7 µg L^{-1}) caused antagonistic effects in *C. sancticaroli*, and only F2D1 caused synergism. Mixtures containing higher concentrations of fipronil decreased the growth of the chironomids as compared to the control and single 2,4-D treatments ($p < 0.05$), in which combinations F4D2 to F4D4, F5D1, and F5D5 demonstrated synergistic effects (Table 3). The other combinations were antagonistic since observed values for growth of the chironomids were higher than the expected

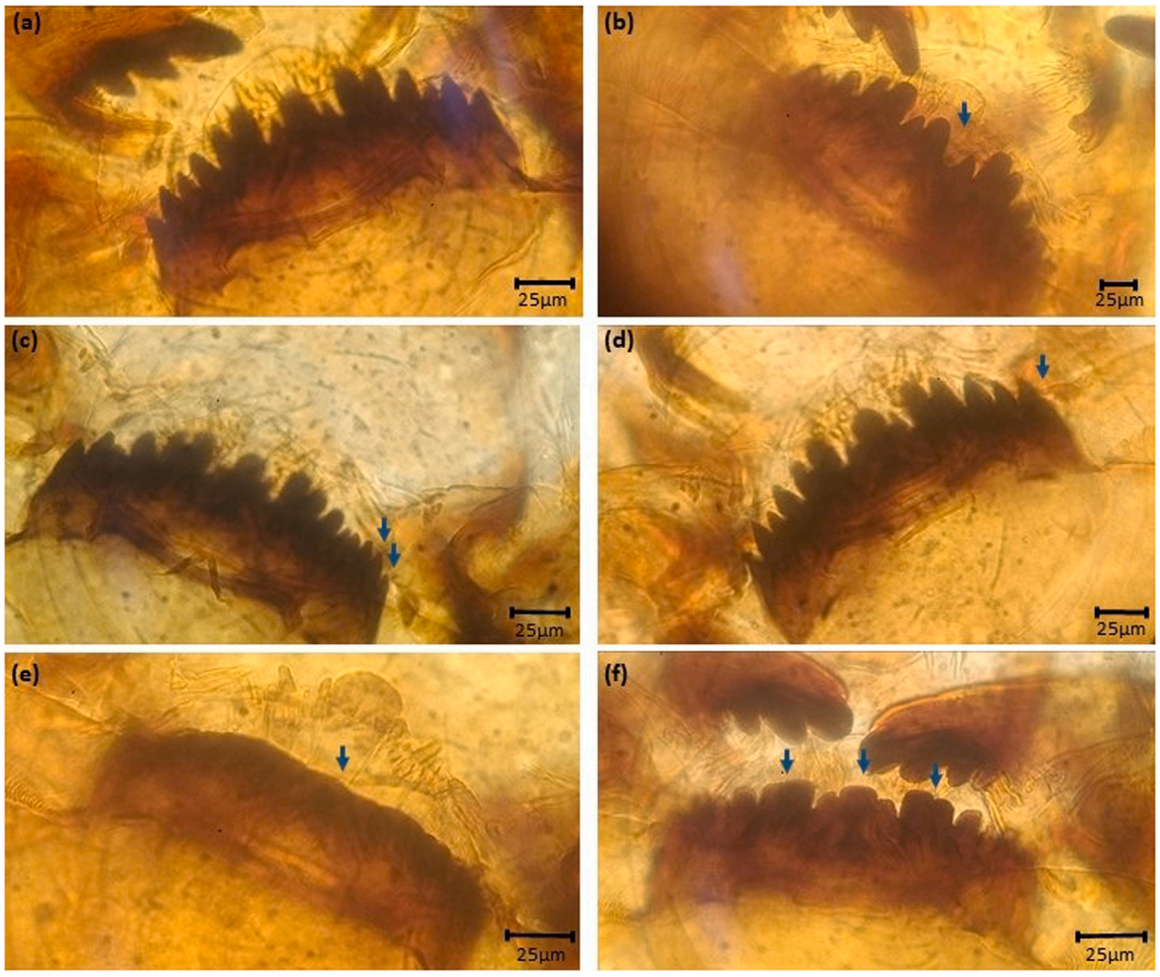


Fig. 3. Examples of mentum deformities and worn teeth observed on *C. sancticaroli* in the present study: (a) normal mentum configuration; (b) Köehn gap; (c) extra-teeth; (d) missing-teeth; (e) teeth-absence, (f) worn teeth.

Table 2
Frequency of mentum deformities and alterations of the health larvae with wear teeth.

2,4-D													
Concentration (µg L^{-1})	*n*	Specific deformities								Total of deformed larvae		Worn teeth frequency	
		Köehn gap		Missing-teeth		Extra-teeth		Absent teeth					
		n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
0	204	0	0	0	0	7	3.4	5	2.5	12	5.9	0	0
29	199	1	0.5	6	3.0	10	5.0	0	0	17	8.5	0	0
66	206	0	0	13	6.3	11	5.4	0	0	24	11.7	0	0
112	202	0	0	11	5.4	7	3.5	0	0	18	8.9	0	0
221	191	0	0	4	2.1	15	7.9	0	0	19	10.0	0	0
426	197	1	0.5	8	4.1	15	7.6	0	0	24	12.2	0	0
Fipronil													
Concentration (µg L^{-1})	*n*	Specific deformities								Total of deformed larvae		Worn teeth frequency	
		Köehn gap		Missing-teeth		Extra-teeth		Absent teeth					
		n	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
0	192	0	0	5	2.6	5	2.6	0	0	10	5.2	2	1.1
0.3	196	2	1.0	13	6.6	3	1.5	21	10.7	39	**19.9***	23	**14.6***
0.4	182	0	0	17	9.3	4	2.2	18	9.9	39	**21.4***	20	**14.0***
0.7	158	0	0	6	3.8	2	1.3	30	18.9	38	**24.0***	24	**20.0***
1.3	133	0	0	8	6.0	2	1.5	43	32.3	53	**39.8***	21	**26.2***
3.7	80	2	2.5	4	5.0	6	7.5	45	56.3	57	**71.3***	9	**39.0***

* Values statistically different from control ($p < 0.05$).

Table 3
Body length and AFDW for 2,4-D (D), fipronil (F), and the mixture of both pesticides (M). For control, the body length was 12.46 ± 0.28 mm, and AFDW was 0.61 ± 0.02 mg. The predicted and observed effects were calculated only to the combinations statistically different from control or any pesticide alone.

	Body length (mm)						AFDW (mg)					
	D	F	M	Pred.	Obs.	S/A	D	F	M	Pred.	Obs.	S/A
F_1D_4	11.72 ± 0.41	10.99 ± 0.43	10.59 ± 0.48	0.83	0.85	A	0.55 ± 0.03	0.50 ± 0.03	0.47 ± 0.04	0.74	0.77	A
F_2D_1	12.10 ± 0.43	11.20 ± 0.38	10.64 ± 0.53	0.87	0.85	S	0.58 ± 0.03	0.51 ± 0.03	0.47 ± 0.04	0.80	0.77	S
F_2D_2	12.20 ± 0.32		11.26 ± 0.51	0.88	0.90	A	0.59 ± 0.02		0.52 ± 0.04	0.81	0.85	A
F_2D_3	11.76 ± 0.25		11.32 ± 0.43	0.86	0.91	A	0.56 ± 0.02		0.52 ± 0.03	0.77	0.85	A
F_2D_4	11.72 ± 0.35		10.86 ± 0.39	0.84	0.87	A	0.55 ± 0.03		0.49 ± 0.03	0.75	0.80	A
F_2D_5	11.66 ± 0.41		11.00 ± 0.51	0.84	0.88	A	0.55 ± 0.03		0.50 ± 0.04	0.75	0.82	A
F_3D_1	12.10 ± 0.43	10.60 ± 0.52	10.34 ± 0.34	0.82	0.83	A	0.58 ± 0.03	0.46 ± 0.04	0.45 ± 0.03	0.72	0.74	A
F_3D_2	12.20 ± 0.32		10.87 ± 0.56	0.83	0.87	A	0.59 ± 0.02		0.49 ± 0.04	0.73	0.80	A
F_3D_4	11.72 ± 0.35		10.78 ± 0.64	0.80	0.86	A	0.55 ± 0.03		0.48 ± 0.05	0.68	0.79	A
F_4D_1	12.10 ± 0.43	11.15 ± 0.31	10.88 ± 0.57	0.86	0.87	A	0.58 ± 0.03	0.51 ± 0.02	0.49 ± 0.04	0.79	0.80	A
F_4D_2	12.20 ± 0.32		10.26 ± 0.55	0.88	0.82	S	0.59 ± 0.02		0.44 ± 0.04	0.81	0.72	S
F_4D_3	11.76 ± 0.25		10.45 ± 0.52	0.84	0.83	S	0.56 ± 0.02		0.45 ± 0.04	0.77	0.74	S
F_4D_4	11.72 ± 0.35		10.48 ± 0.36	0.84	0.84	S	0.56 ± 0.03		0.46 ± 0.03	0.77	0.75	S
F_4D_5	11.66 ± 0.41		10.64 ± 0.51	0.84	0.85	A	0.55 ± 0.03		0.47 ± 0.04	0.75	0.77	A
F_5D_1	12.10 ± 0.43	10.22 ± 0.64	9.82 ± 0.52	0.80	0.79	S	0.58 ± 0.03	0.44 ± 0.05	0.41 ± 0.04	0.69	0.67	S
F_5D_2	12.20 ± 0.32		10.28 ± 0.45	0.80	0.82	A	0.59 ± 0.02		0.44 ± 0.03	0.70	0.72	A
F_5D_3	11.76 ± 0.25		10.48 ± 0.49	0.77	0.84	A	0.56 ± 0.02		0.46 ± 0.04	0.66	0.75	A
F_5D_4	11.72 ± 0.35		10.38 ± 0.30	0.77	0.83	A	0.55 ± 0.03		0.45 ± 0.02	0.65	0.73	A
F_5D_5	11.66 ± 0.41		7.89 ± 0.50	0.77	0.63	S	0.55 ± 0.03		0.26 ± 0.04	0.65	0.43	S

A = antagonism; S = synergism, AFDW (Ash Free Dry Weight), Pred. (predicted effect), and Obs. (observed effect). F indicate the concentrations (μg L^{-1}) of fipronil (F_1 = 0.3, F_2 = 0.4, F_3 = 0.7, F_4 = 1.3, and F_5 = 3.7), and D indicate the concentrations (μg L^{-1}) of 2,4-D (D_1 = 29, D_2 = 66, D_3 = 112, D_4 = 221, and D_5 = 426).

values. Regarding the head capsule width, only F4D2 (0.48 ± 0.020 mm) and F5D5 (0.49 ± 0.030 mm) showed decreased values when compared to the control (0.55 ± 0.010 mm, $p < 0.05$). For F1D1 (0.57 ± 0.0050 mm) and F1D2 (0.56 ± 0.0050 mm), the head capsule width was higher than in F1 (0.53 ± 0.067 mm, $p < 0.05$). For the other combinations, no differences were observed when compared to the control, or single fipronil and 2,4-D treatments (Fig. S4, $p > 0.05$). Regarding the development stage, all mixture combinations had more than 80% of their larvae in the fourth instar, except for F5D5 (77%; Fig. S4).

Fig. 4 presents the alterations of the mentum of *C. sancticaroli*, indicating the occurrence of mentum deformities and worn teeth in the different treatments. No occurrence of mentum deformity was observed in the control group, nor in F2D3 and F2D4. The occurrences of deformed larvae, as well as the number of worn teeth, presented an increase with increasing fipronil concentrations (Fig. 4). In the combinations between F1D1 and F4D5, the missing teeth deformity was more frequent, whereas from F5D1 to F5D5, teeth absence had a higher occurrence. The higher incidence of deformed larvae occurred for F5D5 (29%) and F4D4 (33%). Regarding the number of larvae with worn teeth, the higher values were counted for F5D2 (43%) and F5D1 (57%). When considering the cumulative number larvae with either deformed or worn mentum, F5D1 had the highest incidence (83%), followed by the other combinations of 2,4-D with 3.7 μg fipronil L^{-1}.

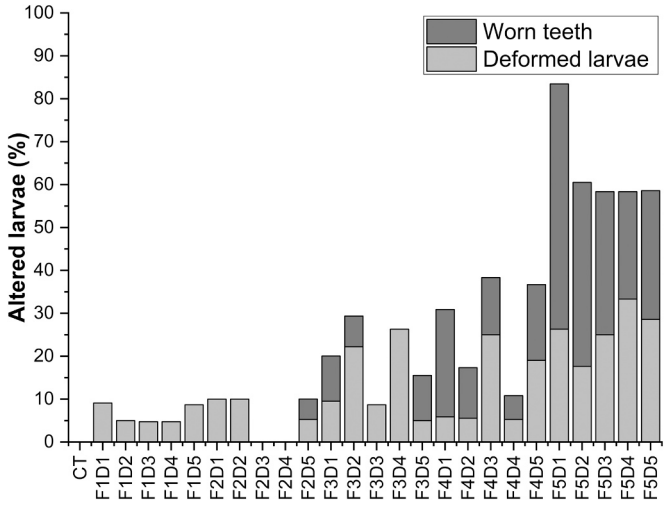


Fig. 4. Percentual of altered larvae with occurrence of mentum deformities and health larvae with worn teeth. F indicate the concentrations (μg L^{-1}) of fipronil (F_1 = 0.3, F_2 = 0.4, F_3 = 0.7, F_4 = 1.3, and F_5 = 3.7), and D indicate the concentrations (μg L^{-1}) of 2,4-D (D_1 = 29, D_2 = 66, D_3 = 112, D_4 = 221, and D_5 = 426), applied at the mixture.

4. Discussion

4.1. Toxicity of single pesticides

The herbicide 2,4-D did not exhibit detrimental effects to the midge *C. sancticaroli* at the concentrations tested in the present study. In line with this, lethal toxicity values reported in the literature demonstrate a relative resistance of dipteran larvae to this herbicide, with lethal concentrations in the mg L^{-1} range (Al-Shami et al., 2006; Farah et al., 2004; Shim and Self, 1973). On the other hand, *C. sancticaroli* larvae exhibited a high sensitivity to fipronil, as anticipated in the first hypothesis (see introduction section). In line with this, low values of LC_{50}, ranging from 0.42 to 1.74 μg fipronil L^{-1}, have been reported for dipterans (Ali et al., 1998; Monteiro et al., 2019; Stevens et al., 2011; Stratman et al., 2013).

In accordance with our results, Monteiro et al. (2019) reported a decrease in growth of *C. riparius* after 10 days of exposure to fipronil. These authors reported a lowest observed effect concentration (LOEC) of 0.081 μg L^{-1} and a 40% larval length reduction at 0.162 μg L^{-1}. This thus indicates that *C. sancticaroli* was less sensitive to fipronil in terms of larval length than *C. riparius*. Monteiro et al. (2019) explained the larval length reduction with a feeding impairment caused by the neurotoxic action of fipronil and with the reallocation of energy as a compensatory mechanism for physiological alterations.

Chironomids are one of the dominant freshwater insect groups in tropical regions around the world (Jacobsen et al., 2008). In Brazilian streams, their dominance in the macroinvertebrate community ranges from 30% to 70% (Corbi and Trivinho-Strixino, 2008; Kleine et al., 2011; Saulino et al., 2014). According to Berg and Hellenthal (1992), the chironomid biomass may contribute to 80% of the secondary production of insects in aquatic systems. Thus, the biomass reduction due to reduced growth, in addition to increased mortality caused by the exposure to

fipronil, lead to ecological implications to aquatic ecosystems.

Small decreases in biomass, as observed for the lower fipronil test concentrations, may have implications for adult size and reproduction of midges. For chironomids, most of the consumed food in the larval stage is applied to growth, and the energy is allocated to the development of somatic and gametic tissue (Péry et al., 2005; Sibley et al., 1997). In species with non-feeding adults, such as *C. sancticaroli*, the larvae have to expend much of the energy in developing gametic tissue, thus alteration in normal growth during the larval stage will have severe implications on adult size and reproductive success (Ball and Baker, 1995). In line with this, *C. riparius* showed decreased dry weight of adults of both males and females after exposure to sublethal concentrations of fipronil (Monteiro et al., 2019). In dipterans, smaller males and females have been associated with lower sperm and egg production, respectively (Ponlawat and Harrington, 2007; Sibley et al., 2001). High biomass reduction, as observed for the higher fipronil concentrations, may have implications for the emergence of *C. sancticaroli*. For *C. tentans,* decreases in larval biomass from 64% to 74% reduced emergence rates with 88% (Liber et al., 1996). The *C. riparius* larval body length reduction observed by Monteiro et al. (2019) discussed above was even accompanied with a complete absence of adult emergence.

Contrary to the initial hypothesis (see introduction), no effects on growth were observed for larvae exposed to 2,4-D. However, the herbicide may have caused other effects that were not evaluated in the present study. For *C. riparius,* for example, exposure of larvae to low concentrations of 2,4-D (0.1–10 μg L^{-1}) caused oxidative damage and alterations in the emergence of males and females (Park et al., 2010).

Similar to the results observed for fipronil in the present study, exposure of *C. sancticaroli* larvae to antimony led to decreased head capsule lengths, which was accompanied with a reduction in larvae attaining their fourth instar and (subsequently) their emergence rates (Morais et al., 2019). *C. sancticaroli* also showed retarded development after eight days of exposure to higher test concentrations of phenanthrene hydrocarbon (Richardi et al., 2018). The delay observed in the development of larvae exposed to fipronil may be attributed to two main reasons. Firstly, larvae could have decreased their food uptake because of the fipronil neurotoxicity, delaying growth and development (Dias et al., 2008). The second hypothesis is associated with the possible activity of fipronil as an endocrine disruptor (Goff et al., 2017; Leemans et al., 2019). Gaertner et al. (2012) demonstrated that fipronil may alter the expression of the ecdysone receptor in the harpacticoid copepod *Amphiascus tenuiremi*. In insects, ecdysones are responsible for controlling the molting process throughout the different larval developmental stages (Gilbert, 2004; Muñiz-González and Martínez-Guitarte, 2018). Delays in molting after exposure to endocrine disrupting chemicals (EDCs) have indeed previously been reported for the *Chironomus* genus (Watts et al., 2003).

The herbicide 2,4-D did not alter the incidences of mentum deformities in *C. sancticaroli* larvae at any of the concentrations tested. In contrary to our study, the exposure of *C. riparius* to 2,4-D increased deformities by 30% compared to controls at 0.1 μg L^{-1} (Park et al., 2010). Fipronil presented a clear dose-response relationship for the incidence of deformities, partially confirming the third hypothesis of our study (see introduction section). A similar pattern was registered for chironomids exposed to DDT and heavy metals (Di Veroli et al., 2012; Madden et al., 1992). The lower concentrations of fipronil also caused less severe deformations compared to the higher concentrations tested. Martinez et al. (2006) found a pattern in the deformity type and arsenic concentrations, with Köehn *gaps* occurring in the lower test concentrations and fused teeth dominating the median and higher test concentrations. To the best of our knowledge, no effects of fipronil on mouthpart deformities have previously been reported in the literature.

The main hypothesis for the high occurrence of deformities after fipronil exposure was an alteration in the hormonal system of *C. sancticaroli*, as discussed above. Since mouthpart deformities occur during the molting process through physiological disturbances, effects on molting caused by EDCs can lead to increased deformity occurrences (Bisthoven et al., 1992). Previous studies have indeed attributed increased deformity rates of midges to endocrine disruption (Kwak and Lee, 2005; Watts et al., 2003). Similar to deformities, the occurrence of worn teeth was increased by fipronil in a dose-level dependent manner. Mechanical teeth wear is a natural process that is directly related to the larval age and may have no relationship with different substrates (Bird, 1997; Bisthoven et al., 1992). Our results demonstrate that the fine sand substratum and larval age had no influence on worn teeth occurrence given the low values (or even absence) noted in the controls and larvae exposed to 2,4-D. The results thus point to an increase in mentum wear of *C. sancticaroli* induced by fipronil exposure. Additional studies are needed to evaluate the underlying mechanisms involved in this process.

4.2. Toxicity of pesticide mixtures

The mixture of substances may result in an alteration in the bioavailability of the mixture components, as well as their chemical uptake, transport, metabolization, excretion or binding to the target site (Cedergreen, 2014). In our study, an antagonistic interaction was observed between fipronil and 2,4-D for several mixture combinations. It is hypothesized that this was caused by an increase in the metabolization and excretion of fipronil through the activation of detoxification mechanisms by 2,4-D. In line with this hypothesis, increases in glutathione S-transferases (GSTs) levels after exposure to 2,4-D have previously been reported for *C. riparius* (Park et al., 2010). In addition, increases in phase I and phase II detoxification enzymes have previously been observed in several aquatic and terrestrial species exposed to 2,4-D (Arcaute et al., 2019; Banaoui et al., 2015; Gaaied et al., 2019; Hattab et al., 2015). Oruc et al. (2004) reported that the mixture of 2,4-D and the insecticide azinphos-methyl induced significant increases in GST activity for two fish species. Some studies have also previously demonstrated an antagonistic interaction of 2,4-D with other pesticides (Oruç and Üner, 2000; Van Meter et al., 2018).

Several synergetic interactions between fipronil and 2,4-D were also observed in the present study, especially for mixtures containing higher fipronil concentrations. This may be related with increases in cellular stress resulting from the detoxification of the two compounds, and/or a depletion of the energy reserves. Monteiro et al. (2019), for example, demonstrated that fipronil increased the consumed oxygen levels through alterations in the metabolism of *C. riparius*, besides a decrease in the GST and catalase (CAT) activities and an increase in indicatives of oxidative stress.

Silva et al. (2020) found a mixture effect pattern similar to our study in an acute toxicity test with the cladoceran *C. silvestrii* exposed to the mixture of fipronil and 2,4-D, with antagonism at low and synergism at high mixture combinations. Moreira et al. (2020a) also observed increased effects to the algae *Raphidocelis subcapitata* when exposed to the mixture of fipronil and 2,4-D as compared to its single mixture constituents. Synergic interactions are reported in mixtures composed of several other pesticides (Arcaute et al., 2019; Carvalho et al., 2020; Levchenko and Silivanova, 2019; Raimets et al., 2018; Taillebois and Thany, 2016). To the best of our knowledge, however, no data is available for the mixture of 2,4-D and fipronil to aquatic insects, including chironomids. Finally, the mixture experiment was conducted with formulated products and the interaction of compounds present at the formulation other than the active ingredients can influence the final response to (non-target) organisms (see Nagy et al., 2020 and references therein).

Most experiments available in the literature have demonstrated synergism at high doses of pesticides (ranging from μg L^{-1} to mg L^{-1}) at concentrations with low environmental relevance (Cedergreen, 2014). Our results demonstrate a synergistic interaction at environmentally relevant concentrations of fipronil (0.1–465 μg L^{-1}) and 2,4-D 0.4–366 μg L^{-1}) that have previously been reported in Brazilian water bodies located in sugar cane and rice crop areas (CETESB, 2018;

Grützmacher et al., 2008; Marchesan et al., 2010; Vieira et al., 2016). Considering these detected concentrations, our results point to a realistic scenario of risks of both the individual compounds and especially their mixture to edge of field aquatic ecosystems in these areas. Future studies should be conducted to elucidate the mechanistic interaction underlying the 2,4-D and fipronil mixture toxicity.

5. Conclusion

The single fipronil treatment was more toxic to *C. sancticaroli* than 2,4-D, which did not exert any effects on survival, growth, and development of larvae at any of the concentrations tested. However, results suggest that 2,4-D can alter deformity rates but that this is not dose-dependent. Fipronil increased deformities at all test concentrations with greater alterations at increasing doses. The mixture presented antagonism for body length and AFDW parameters especially in mixtures with low fipronil concentrations and synergism in mixtures with higher fipronil concentrations for these parameters. The synergism was observed at environmentally relevant concentrations, implying an increased risk scenario at a real-world setting since the two compounds are often applied to the same cultures and their co-occurrence has already been registered in Brazilian water bodies located in areas with a predominance of sugarcane crops.

Funding

The work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, Brazil, grant no. 2015/18790-3). T.J.S.P., L.C.M.S., M.P.C.Y., and B.V.G. have a Ph.D. scholarship from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). R.A.M. has a pos doctoral grant from FAPESP (2017/24126-4) and P.D.F has a scientific initiation grant (FAPESP 2019/04198-6). Financial support was also provided to M.A.D. by the Portuguese government (Fundação para a Ciência e Tecnologia; FCT) through the research unit UIDB/04085/2020 (CENSE).

CRediT authorship contribution statement

Thandy Junio da Silva Pinto: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Original Draft. **Raquel Aparecida Moreira**: Writing - Review & Editing. **Laís Conceiç ão Menezes da Silva**: Investigation. **Maria Paula Cardoso Yoshii**: Investigation. **Bianca Veloso Goulart**: Resources, Investigation. **Cassiana Carolina Montagner**: Resources; Writing - Review & Editing. **Michiel Adriaan Daam**: Conceptualization, Methodology, Writing - Review & Editing. **Evaldo Luiz Gaeta Espindola**: Conceptualization; Methodology, Writing - Review & Editing; Project administration; Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2020.111778.

References

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. Process. Impacts 18, 779–787. https://doi.org/10.1039/C6EM00268D.

Ali, A., Nayar, J.K., Gu, W.D., 1998. Toxicity of a phenyl pyrazole insecticide, fipronil, to mosquito and chironomid midge larvae in the laboratory. J. Am. Mosq. Control Assoc. 14, 216–218.

Al-Shami, S.A., Che Salmah, M.R., Siti Azizah, M.N., Abu Hassan, A., Azmi, M., 2006. Toxicity of two herbicides 2,4-D dimethylamine and bensulfuron methyl to rice field Chironomus kiiensis (Tokunaga) (Diptera: Chironomidae). Wetl. Sci. 4, 241–246.

APHA, 2018. 2340 Hardness. In: Standard Methods For the Examination of Water and Wastewater, Standard Methods for the Examination of Water and Wastewater. American Public Health Association. https://doi.org/10.2105/SMWW.2882.025.

Arcaute, C.R., Ossana, N.A., Pérez-Iglesias, J.M., Soloneski, S., Larramendy, M.L., 2019. Auxinic herbicides induce oxidative stress on Cnesterodon decemmaculatus (Pisces: Poeciliidae). Environ. Sci. Pollut. Res. 26, 20485–20498. https://doi.org/10.1007/s11356-019-05169-z.

Ball, S.L., Baker, R.L., 1995. The non-lethal effects of predators and the influence of food availability on life history of adult Chironomus tentans (Diptera: Chironomidae). Freshw. Biol. 34, 1–12. https://doi.org/10.1111/j.1365-2427.1995.tb00417.x.

Banaoui, A., El Hamidi, F., Kaaya, A., Bouhaimi, A., Zekhnini, A., Moukrim, A., 2015. Assessment of multimarker responses in Perna perna, Mytilus galloprovincialis and Donax trunculus bivalves exposed to malathion and 2,4-dichlorophenoxyacetic acid pesticides. J. Mater. Environ. Sci. 6, 1678–1683.

Barriuso, E., Feller, Ch, Calvet, R., Cerri, C., 1992. Sorption of atrazine, terbutryn and 2,4-D herbicides in two Brazilian Oxisols. Geoderma 53, 155–167. https://doi.org/10.1016/0016-7061(92)90028-6.

Berg, M.B., Hellenthal, R.A., 1992. The role of Chironomidae in energy flow of a lotic ecosystem. Neth. J. Aquat. Ecol. 26, 471–476. https://doi.org/10.1007/BF02255277.

Bird, G.A., 1997. Deformities in cultured Chironomus tentans larvae and the influence of substrate on growth, survival and mentum wear. Environ. Monit. Assess. 45, 273–283. https://doi.org/10.1023/A:1005782803930.

Bisthoven, L.G.J., Timmermans, K.R., Ollevier, F., 1992. The concentration of cadmium, lead, copper and zinc in Chironomus grthummi larvae (Diptera, Chironomidae) with deformed versus normal menta. Hydrobiologia 239, 141–149. https://doi.org/10.1007/BF00007671.

Boivin, A., Amellal, S., Schiavon, M., van Genuchten, M.Th, 2005. 2,4-Dichlorophenoxyacetic acid (2,4-D) sorption and degradation dynamics in three agricultural soils. Environ. Pollut. 138, 92–99. https://doi.org/10.1016/j.envpol.2005.02.016.

Bolan, N.S., Baskaran, S., 1996. Biodegradation of 2,4-D herbicide as affected by Its adsorption-desorption behaviour and microbial activity of soils. Soil Res. 34, 1041–1053. https://doi.org/10.1071/sr9961041.

Brennan, A.A., Harwood, A.D., You, J., Landrum, P.F., Lydy, M.J., 2009. Degradation of fipronil in anaerobic sediments and the effect on porewater concentrations. Chemosphere 77, 22–28. https://doi.org/10.1016/j.chemosphere.2009.06.019.

Carvalho, W.F., Ruiz de Arcaute, C., Torres, L., de Melo e Silva, D., Soloneski, S., Larramendy, M.L., 2020. Genotoxicity of mixtures of glyphosate with 2,4-dichlorophenoxyacetic acid chemical forms towards Cnesterodon decemmaculatus (Pisces, Poeciliidae). Environ. Sci. Pollut. Res. 27, 6515–6525. https://doi.org/10.1007/s11356-019-07379-x.

Cedergreen, N., 2014. Quantifying synergy: a systematic review of mixture toxicity studies within environmental toxicology. PLoS One 9, e96580. https://doi.org/10.1371/journal.pone.0096580.

CETESB, 2018. Qualidade das águas interiores no estado de São Paulo 2017, Relatórios. Série Relatórios / CETESB,, São Paulo.

Chinalia, F.A., Killham, K.S., 2006. 2,4-Dichlorophenoxyacetic acid (2,4-D) biodegradation in river sediments of Northeast-Scotland and its effect on the microbial communities (PLFA and DGGE). Chemosphere 64, 1675–1683. https://doi.org/10.1016/j.chemosphere.2006.01.022.

Corbi, J.J., Trivinho-Strixino, S., 2008. Relationship between sugar cane cultivation and stream macroinvertebrate communities. Braz. Arch. Biol. Technol. 51, 569–579.

Di Veroli, A., Goretti, E., Paumen, M.L., Kraak, M.H.S., Admiraal, W., 2012. Induction of mouthpart deformities in chironomid larvae exposed to contaminated sediments. Environ. Pollut. 166, 212–217. https://doi.org/10.1016/j.envpol.2012.03.029.

Dias, V., Vasseur, C., Bonzom, J.-M., 2008. Exposure of Chironomus riparius larvae to uranium: effects on survival, development time, growth, and mouthpart deformities. Chemosphere 71, 574–581. https://doi.org/10.1016/j.chemosphere.2007.09.029.

European Food Safety Authority, 2014. Conclusion regarding the peer review of the pesticide risk assessment of the active substance 2,4-D. EFSA J. 12 (9), 3812. https://doi.org/10.2903/j.efsa.2014.3812.

Farah, M.A., Ateeq, B., Ali, M.N., Sabir, R., Ahmad, W., 2004. Studies on lethal concentrations and toxicity stress of some xenobiotics on aquatic organisms. Chemosphere 55, 257–265. https://doi.org/10.1016/j.chemosphere.2003.10.063.

Figueirêdo, L.P., Athayde, D.B., Daam, M.A., van Gestel, C.A.M., Guerra, G., da, S., Duarte-Neto, P.J., Espíndola, E.L.G., 2020. Impact of temperature on the toxicity of Kraft 36 EC® (a.s. abamectin) and Score 250 EC® (a.s. difenoconazole) to soil organisms under realistic environmental exposure scenarios. Ecotoxicol. Environ. Saf. 194, 110446 https://doi.org/10.1016/j.ecoenv.2020.110446.

Fonseca, A.L., Rocha, O., 2004. Laboratory cultures of the native species Chironomus xanthus Rempel, 1939 (Diptera-Chironomidae). Acta Limnol. Bras. 2, 153–161.

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. https://doi.org/10.1016/j.hal.2013.11.004.

Gaaied, S., Oliveira, M., Le Bihanic, F., Cachot, J., Banni, M., 2019. Gene expression patterns and related enzymatic activities of detoxification and oxidative stress systems in zebrafish larvae exposed to the 2,4-dichlorophenoxyacetic acid herbicide. Chemosphere 224, 289–297. https://doi.org/10.1016/j.chemosphere.2019.02.125.

Gaertner, K., Chandler, G.T., Quattro, J., Ferguson, P.L., Sabo-Attwood, T., 2012. Identification and expression of the ecdysone receptor in the harpacticoid copepod, Amphiascus tenuiremis, in response to fipronil. Special Issue Section: SETAC North America 31st Annual Meeting Ecotoxicol. Environ. Saf. 76, 39–45. https://doi.org/10.1016/j.ecoenv.2011.09.008.

Gage, M.S., Spivak, A., Paradise, C.J., 2004. Effects of land use and disturbance on benthic insects in headwater streams draining small watersheds North of Charlotte, NC. Southeast. Nat. 3, 345–358.

Gilbert, L.I., 2004. Halloween genes encode P450 enzymes that mediate steroid hormone biosynthesis in *Drosophila melanogaster*. Mol. Cell. Endocrinol. 215 (1–2), 1–10. https://doi.org/10.1016/j.mce.2003.11.003.

Girardi, C., Nowak, K.M., Carranza-Diaz, O., Lewkow, B., Miltner, A., Gehre, M., Schäffer, A., Kästner, M., 2013. Microbial degradation of the pharmaceutical ibuprofen and the herbicide 2,4-D in water and soil — use and limits of data obtained from aqueous systems for predicting their fate in soil. Sci. Total Environ. 444, 32–42. https://doi.org/10.1016/j.scitotenv.2012.11.051.

Goff, A.D., Saranjampour, P., Ryan, L.M., Hladik, M.L., Covi, J.A., Armbrust, K.L., Brander, S.M., 2017. The effects of fipronil and the photodegradation product fipronil desulfinyl on growth and gene expression in juvenile blue crabs, Callinectes sapidus, at different salinities. Aquat. Toxicol. 186, 96–104. https://doi.org/10.1016/j.aquatox.2017.02.027.

Gottardi, M., Birch, M.R., Dalhoff, K., Cedergreen, N., 2017. The effects of epoxiconazole and α-cypermethrin on Daphnia magna growth, reproduction, and offspring size. Environ. Toxicol. Chem. 36, 2155–2166. https://doi.org/10.1002/etc.3752.

Goulart, B.V., Vizioli, B.D.C., Espindola, E.L.G., Montagner, C.C., 2020. Matrix effect challenges to quantify 2,4-D and fipronil in aquatic systems. Environ. Monit. Assess. 192, 797. https://doi.org/10.1007/s10661-020-08776-3.

Gripp, H.S., Freitas, J.S., Almeida, E.A., Bisinoti, M.C., Moreira, A.B., 2017. Biochemical effects of fipronil and its metabolites on lipid peroxidation and enzymatic antioxidant defense in tadpoles (Eupemphix nattereri: Leiuperidae). Ecotoxicol. Environ. Saf. 136, 173–179. https://doi.org/10.1016/j.ecoenv.2016.10.027.

Grützmacher, D., Grützmacher, A., Agostinetto, D., Loeck, A., Roman, R., Peixoto, S., Zanella, R., 2008. Monitoring of pesticides in two water sources in southern Brazil. Rev. Bras. Eng. Agríc. E Ambient. 12, 632–637.

Gunkel, G., Kosmol, J., Sobral, M., Rohn, H., Montenegro, S., Aureliano, J., 2007. Sugar cane industry as a source of water pollution – Case study on the situation in Ipojuca River, Pernambuco, Brazil. Water Air Soil Pollut. 180, 261–269. https://doi.org/10.1007/s11270-006-9268-x.

Hansen, H.P., Koroleff, F., 2007. Determination of Nutrients. In: Methods of Seawater Analysis. John Wiley & Sons, Ltd, pp. 159–228. https://doi.org/10.1002/9783527613984.ch10.

Hattab, S., Boughattas, I., Boussetta, H., Viarengo, A., Banni, M., Sforzini, S., 2015. Transcriptional expression levels and biochemical markers of oxidative stress in the earthworm Eisenia andrei after exposure to 2,4-dichlorophenoxyacetic acid (2,4-D). Ecotoxicol. Environ. Saf. 122, 76–82. https://doi.org/10.1016/j.ecoenv.2015.07.014.

Hua, J., Relyea, R., 2014. Chemical cocktails in aquatic systems: pesticide effects on the response and recovery of > 20 animal taxa. Environ. Pollut. 189, 18–26. https://doi.org/10.1016/j.envpol.2014.02.007.

Islam, F., Wang, J., Farooq, M.A., Khan, M.S.S., Xu, L., Zhu, J., Zhao, M., Muños, S., Li, Q. X., Zhou, W., 2018. Potential impact of the herbicide 2,4-dichlorophenoxyacetic acid on human and ecosystems. Environ. Int. 111, 332–351. https://doi.org/10.1016/j.envint.2017.10.020.

Jacobsen, D., Cressa, C., Mathooko, J.M., Dudgeon, D., 2008. 4 - Macroinvertebrates: Composition, Life Histories And Production. In: Dudgeon, D. (Ed.), Tropical Stream Ecology. Academic Press, London, pp. 65–105. https://doi.org/10.1016/B978-012088449-0.50006-6.

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. https://doi.org/10.1897/04-431R.1.

Kleine, P., Trivinho-Strixino, S., Corbi, J.J., 2011. Relationship between banana plant cultivation and stream macroinvertebrate communities. Acta Limnol. Bras. 23, 344–352.

Kuhlmann, M., Y. Hayashida, C., P. A. Araújo, R., 2000. Using Chironomus (Chironomidae: Diptera) mentum deformities in environmental assessment. Acta Limnol. Bras. 12, 55–61.

Kwak, I.-S., Lee, W., 2005. Mouthpart deformity and developmental retardation exposure of Chironomus plumosus (Diptera: Chironomidae) to tebufenozide. Bull. Environ. Contam. Toxicol. 75, 859–865. https://doi.org/10.1007/s00128-005-0829-2.

Laganà, A., Bacaloni, A., De Leva, I., Faberi, A., Fago, G., Marino, A., 2002. Occurrence and determination of herbicides and their major transformation products in environmental waters. Anal. Chim. Acta 462, 187–198. https://doi.org/10.1016/S0003-2670(02)00351-3.

Leemans, M., Couderq, S., Demeneix, B., Fini, J.-B., 2019. Pesticides with potential thyroid hormone-disrupting effects: a review of recent data. Front. Endocrinol. 10 https://doi.org/10.3389/fendo.2019.00743.

Levchenko, M.A., Silivanova, E.A., 2019. Synergistic and antagonistic effects of insecticide binary mixtures against house flies (Musca domestica). Regul. Mech. Biosyst. 10, 75–82. https://doi.org/10.15421/021912.

Liber, K., Call, D.J., Dawson, T.D., Whiteman, F.W., Dillon, T.M., 1996. Effects of Chironomus tentans larval growth retardation on adult emergence and ovipositing success: implications for interpreting freshwater sediment bioassays. Hydrobiologia 323, 155–167. https://doi.org/10.1007/BF00007844.

Lin, K., Haver, D., Oki, L., Gan, J., 2008. Transformation and sorption of fipronil in urban stream sediments. J. Agric. Food Chem. 56, 8594–8600. https://doi.org/10.1021/jf8018886.

Lopes, L.F., de, P., Agostini, V.O., Guimarães, S.S., Muxagata, E., 2018. Evaluation of the effect of antimicrobials in marine cultures, using the copepod Acartia tonsa as a bioindicator. Chem. Ecol. 34, 747–761. https://doi.org/10.1080/02757540.2018.1482886.

Loureiro, S., Svendsen, C., Ferreira, A.L.G., Pinheiro, C., Ribeiro, F., Soares, A.M.V.M., 2010. Toxicity of three binary mixtures to Daphnia magna: comparing chemical modes of action and deviations from conceptual models. Environ. Toxicol. Chem. 29, 1716–1726. https://doi.org/10.1002/etc.198.

Madden, C., Suter, P., Nicholson, B., Austin, A., 1992. Deformities in chironomid larvae as indicators of pollution (pesticide) stress. Neth. J. Aquat. Ecol. 26, 551–557. https://doi.org/10.1007/BF02255289.

MAPA, 2019. AGROFIT [WWW Document]. Braz. Minist. Agric. Livest. Food Supply. URL http://agrofit.agricultura.gov.br/agrofit_cons/principal_agrofit_cons. (Accessed 29 June 2019).

Marchesan, E., Sartori, G.M.S., Avila, L.A. de, Machado, S.L. de O., Zanella, R., Primel, E. G., Macedo, V.R.M., Marchezan, M.G., 2010. Residues of pesticides in the water of the Depression Central rivers in the State of Rio Grande do Sul, Brazil. Ciênc. Rural 40, 1053–1059.

Martinez, E.A., Wold, L., Moore, B.C., Schaumloffel, J., Dasgupta, N., 2006. Morphologic and growth responses in Chironomus tentans to arsenic exposure. Arch. Environ. Contam. Toxicol. 51, 529–536. https://doi.org/10.1007/s00244-005-0308-0.

Maul, J.D., Brennan, A.A., Harwood, A.D., Lydy, M.J., 2008. Effect of sediment-associated pyrethroids, fipronil, and metabolites on Chironomus tentans growth rate, body mass, condition index, immobilization, and survival. Environ. Toxicol. Chem. 27, 2582–2590. https://doi.org/10.1897/08-185.1.

Mize, S.V., Porter, S.D., Demcheck, D.K., 2008. Influence of fipronil compounds and rice-cultivation land-use intensity on macroinvertebrate communities in streams of southwestern Louisiana, USA. Environ. Pollut. 152, 491–503. https://doi.org/10.1016/j.envpol.2007.03.021.

Monteiro, H.R., Pestana, J.L.T., Novais, S.C., Leston, S., Ramos, F., Soares, A.M.V.M., Devreese, B., Lemos, M.F.L., 2019. Assessment of fipronil toxicity to the freshwater midge Chironomus riparius: molecular, biochemical, and organismal responses. Aquat. Toxicol. 216, 105292 https://doi.org/10.1016/j.aquatox.2019.105292.

Morais, G. dos S., Vieira, T.B., Santos, G.S., Baika, L.M., Cestari, M.M., Grassi, M.T., Navarro da Silva, M.A., 2019. Biological, biochemical and genotoxic effects of Sb in the midge *Chironomus sancticaroli* Strixino and Strixino, 1981 (Diptera: Chironomidae). Ecotoxicol. Environ. Saf. 176, 196–203. https://doi.org/10.1016/j.ecoenv.2019.03.080.

Moreira, R.A., Rocha, G.S., da Silva, L.C.M., Goulart, B.V., Montagner, C.C., Melão, M. da G.G., Espindola, E.L.G., 2020a. Exposure to environmental concentrations of fipronil and 2,4-D mixtures causes physiological, morphological and biochemical changes in Raphidocelis subcapitata. Ecotoxicol. Environ. Saf. 206, 111180 https://doi.org/10.1016/j.ecoenv.2020.111180.

Moreira, R.A., Rocha, O., Pinto, T.J., da, S., da Silva, L.C.M., Goulart, B.V., Montagner, C. C., Espindola, E.L.G., 2020b. Life-history traits response to effects of fish predation (kairomones), fipronil and 2,4-D on neotropical cladoceran Ceriodaphnia silvestrii. Arch. Environ. Contam. Toxicol. 79, 298–309. https://doi.org/10.1007/s00244-020-00754-7.

Muñiz-González, A.-B., Martínez-Guitarte, J.-L., 2018. Effects of single exposure and binary mixtures of ultraviolet filters octocrylene and 2-ethylhexyl 4-(dimethylamino) benzoate on gene expression in the freshwater insect Chironomus riparius. Environ. Sci. Pollut. Res. 25, 35501–35514. https://doi.org/10.1007/s11356-018-3516-7.

Nagy, K., Duca, R.C., Lovas, S., Creta, M., Scheepers, P.T.J., Godderis, L., Ádám, B., 2020. Systematic review of comparative studies assessing the toxicity of pesticide active ingredients and their product formulations. Environ. Res. 181, 108926 https://doi.org/10.1016/j.envres.2019.108926.

OECD, 2011. Test No. 235: Chironomus sp., Acute Immobilisation Test. https://doi.org/10.1787/9789264122383-en.

Oruc, E.O., Sevgiler, Y., Uner, N., 2004. Tissue-specific oxidative stress responses in fish exposed to 2,4-D and azinphosmethyl. Comp. Biochem. Physiol. Part C. Toxicol. Pharmacol. 137, 43–51. https://doi.org/10.1016/j.cca.2003.11.006.

Oruç, E.Ö., Üner, N., 2000. Combined effects of 2,4-D and azinphosmethyl on antioxidant enzymes and lipid peroxidation in liver of Oreochromis niloticus. Comp. Biochem. Physiol. C. Pharmacol. Toxicol. Endocrinol. 127, 291–296. https://doi.org/10.1016/S0742-8413(00)00159-6.

Palacio-Cortés, A.M., Signorini-Souza, I. de L., Yoshio Hara, E.L., Disner, R.G., Rebechi, D., Grassi, M.T., Cestari, M.M., Navarro-Silva, M.A., 2017. Polybrominated diphenyl ethers (PBDEs) effects on *Chironomus sancticaroli* larvae after short-term exposure. Ecotoxicol. Environ. Saf. 139, 308–315. https://doi.org/10.1016/j.ecoenv.2017.01.052.

Park, K., Kwak, I.-S., 2008. Characterization of heat shock protein 40 and 90 in Chironomus riparius larvae: effects of di(2-ethylhexyl) phthalate exposure on gene expressions and mouthpart deformities. Chemosphere 74, 89–95. https://doi.org/10.1016/j.chemosphere.2008.09.041.

Park, K., Park, J., Kim, J., Kwak, I.-S., 2010. Biological and molecular responses of Chironomus riparius (Diptera, Chironomidae) to herbicide 2,4-D (2,4-dichlorophenoxyacetic acid). Comp. Biochem. Physiol. C Toxicol. Pharmacol. 151, 439–446. https://doi.org/10.1016/j.cbpc.2010.01.009.

Peret, A.M., Oliveira, L.F., Bianchini, I., Seleghim, M.H.R., Peret, A.C., Mozeto, A.A., 2010. Dynamics of fipronil in Óleo Lagoon in Jataí Ecological Station, São Paulo-Brazil. Chemosphere 78, 1225–1229. https://doi.org/10.1016/j.chemosphere.2009.12.060.

Péry, A.R.R., Mons, R., Garric, J., 2005. Modelling of the life cycle of Chironomus species using an energy-based model. Chemosphere 59, 247–253. https://doi.org/10.1016/j.chemosphere.2004.11.083.

Ponlawat, A., Harrington, L., 2007. Age and body size influence male sperm capacity of the dengue vector Aedes aegypti (Diptera: Culicidae). J. Med. Entomol. 44, 422–426. https://doi.org/10.1603/0022-2585(2007)44[422:AABSIM]2.0.CO;2.

Printes, L.B., Fernandes, M.N., Espíndola, E.L.G., 2011. Laboratory measurements of biomarkers and individual performances in Chironomus xanthus to evaluate pesticide contamination of sediments in a river of southeastern Brazil. Ecotoxicol. Environ. Saf. 74, 424–430. https://doi.org/10.1016/j.ecoenv.2010.10.033.

Raimets, R., Karise, R., Mänd, M., Kaart, T., Ponting, S., Song, J., Cresswell, J.E., 2018. Synergistic interactions between a variety of insecticides and an ergosterol biosynthesis inhibitor fungicide in dietary exposures of bumble bees (Bombus terrestris L.). Pest Manag. Sci. 74, 541–546. https://doi.org/10.1002/ps.4756.

Richardi, V.S., Vicentini, M., Morais, G.S., Rebechi, D., da Silva, T.A., Fávaro, L.F., Navarro-Silva, M.A., 2018. Effects of phenanthrene on different levels of biological organization in larvae of the sediment-dwelling invertebrate *Chironomus sancticaroli* (Diptera: Chironomidae). Environ. Pollut. 242, 277–287. https://doi.org/10.1016/j.envpol.2018.06.091.

Saulino, H., Corbi, J., Trivinho-Strixino, S., 2014. Aquatic insect community structure under the influence of small dams in a stream of the Mogi-Guaçu river basin, state of São Paulo. Braz. J. Biol. 74, 79–88.

Scherer, C., Wolf, R., Völker, J., Stock, F., Brennhold, N., Reifferscheid, G., Wagner, M., 2020. Toxicity of microplastics and natural particles in the freshwater dipteran Chironomus riparius: same same but different? Sci. Total Environ. 711, 134604 https://doi.org/10.1016/j.scitotenv.2019.134604.

Shim, J.C., Self, L.S., 1973. Toxicity of agricultural chemicals to larvivorous fish in korean rice fields.

Sibley, P.K., Ankley, G.T., Benoit, D.A., 2001. Factors affecting reproduction and the importance of adult size on reproductive output of the midge Chironomus tentans. Environ. Toxicol. Chem. 20, 1296–1303. https://doi.org/10.1002/etc.5620200618.

Sibley, P.K., Benoit, D.A., Ankley, G.T., 1997. The significance of growth in Chironomus tentans sediment toxicity tests: relationship to reproduction and demographic endpoints. Environ. Toxicol. Chem. 16, 336–345. https://doi.org/10.1002/etc.5620160232.

Silva, L.C.M., Moreira, R.A., Pinto, T.J.S., Ogura, A.P., Yoshii, M.P.C., Lopes, L.F.P., Montagner, C.C., Goulart, B.V., Daam, M.A., Espindola, E.L.G., 2020. Acute and chronic toxicity of 2,4-D and fipronil formulations (individually and in mixture) to the Neotropical cladoceran Ceriodaphnia silvestrii. Ecotoxicology 29, 1462–1475. https://doi.org/10.1007/s10646-020-02275-4.

Stevens, M.M., Burdett, A.S., Mudford, E.M., Helliwell, S., Doran, G., 2011. The acute toxicity of fipronil to two non-target invertebrates associated with mosquito breeding sites in Australia. Acta Trop. 117, 125–130. https://doi.org/10.1016/j.actatropica.2010.11.002.

Stratman, K.N., Wilson, P.C., Overholt, W.A., Cuda, J.P., Netherland, M.D., 2013. Toxicity of fipronil to the midge, Cricotopus lebetis Sublette. J. Toxicol. Environ. Health A 76, 716–722. https://doi.org/10.1080/15287394.2013.802266.

Taillebois, E., Thany, S.H., 2016. The differential effect of low-dose mixtures of four pesticides on the pea aphid Acyrthosiphon pisum. Insects 7, 53. https://doi.org/10.3390/insects7040053.

Taniwaki, R.H., Cassiano, C.C., Filoso, S., Ferraz, S.F., de, B., Camargo, P.B., de, Martinelli, L.A., 2017. Impacts of converting low-intensity pastureland to high-intensity bioenergy cropland on the water quality of tropical streams in Brazil. Sci. Total Environ. 584, 339–347. https://doi.org/10.1016/j.scitotenv.2016.12.150.

Thuyet, D.Q., Watanabe, H., Ok, J., 2013. Effect of pH on the degradation of imidacloprid and fipronil in paddy water. J. Pestic. Sci. 38, 223–227. https://doi.org/10.1584/jpestics.D12-080.

Tomlin, C., 1994. The Pesticide Manual, 10th ed. The Royal Society Of Chemistry, Cambridge.

Trivinho-Strixino, S., 2011. Chironomidae (Insecta, Diptera, Nematocera) do Estado de São Paulo, Sudeste do Brasil. Biota Neotrop. 11, 675–684. https://doi.org/10.1590/S1676-06032011000500032.

USEPA, 2000. Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates, 2nd ed. Office of Research and Development, U.S. Environmental Protection Agency, Washington, DC.

Vale, R.L., Netto, A.M., Toríbio de Lima Xavier, B., de Lâvor Paes Barreto, M., Siqueira da Silva, J.P., 2019. Assessment of the gray water footprint of the pesticide mixture in a soil cultivated with sugarcane in the northern area of the State of Pernambuco, Brazil. J. Clean. Prod. 234, 925–932. https://doi.org/10.1016/j.jclepro.2019.06.282.

Van Meter, R.J., Glinski, D.A., Purucker, S.T., Henderson, W.M., 2018. Influence of exposure to pesticide mixtures on the metabolomic profile in post-metamorphic green frogs (Lithobates clamitans). Sci. Total Environ. 624, 1348–1359. https://doi.org/10.1016/j.scitotenv.2017.12.175.

Vieira, D.C., Noldin, J.A., Deschamps, F.C., Resgalla, C., 2016. Ecological risk analysis of pesticides used on irrigated rice crops in southern Brazil. Chemosphere 162, 48–54. https://doi.org/10.1016/j.chemosphere.2016.07.046.

Watts, M.M., Pascoe, D., Carroll, K., 2003. Exposure to 17α-ethinylestradiol and bisphenol A - effects on larval moulting and mouthpart structure of Chironomus riparius. Ecotoxicol. Environ. Saf. 54, 207–215. https://doi.org/10.1016/S0147-6513(02)00029-5.

Weston, D.P., Lydy, M.J., 2014. Toxicity of the insecticide fipronil and its degradates to benthic macroinvertebrates of urban streams. Environ. Sci. Technol. 48, 1290–1297. https://doi.org/10.1021/es4045874.

Ying, G.G., Kookana, R.S., 2001. Sorption of fipronil and its metabolites on soils from South Australia. J. Environ. Sci. Health B 36, 545–558. https://doi.org/10.1081/PFC-100106184.

Zhu, G., Wu, H., Guo, J., Kimaro, F.M.E., 2004. Microbial degradation of fipronil in clay loam soil. Water Air Soil Pollut. 153, 35–44. https://doi.org/10.1023/B:WATE.0000019928.67686.b1.