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Lethal and sublethal responses in the fish, *Odontesthes bonariensis*, exposed to chlorpyrifos alone or under mixtures with endosulfán and lambda-cyhalothrin

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Abstract

Need for ecotoxicological information on local species has been recently highlighted as a priority issue in Latin America. In addition, little information has been found on concentration distances between lethal and sublethal effects, and the effect of mixtures at these two levels of analysis. Chlorpyrifos (CPF) is an organophosphate insecticide broadly used in soybean crops which has dramatically expanded in Latin America and other regions of the world. The aim of the present study was to evaluate lethal and sublethal effects of CPF, singly or in mixtures, on the inland "Pejerrey" (*Odontesthes bonariensis*) under laboratory conditions. Bioassays were performed using 15–30 d post hatch Pejerrey larvae. Six toxicity tests were run for estimating the average inter-assay dose–response curve of CPF and other six for assessing the effects of mixtures of CPF with endosulfan (EN) or lambda-cyhalothrin (LC), at three toxic units (TU) proportions (25:75, 50:50, 75:25). In addition, four assays were performed to describe the average inter-assay dose–response inhibition curve of acetylcholinesterase (AchE) for CPF alone and two for assessing the mixtures. The estimated 96 h-LC_{50} for CPF was 2.26 ± 1.11 μg/L and the incipiency value was 0.048 ± 0.012 μg/L, placing this Neotropical species among the 13% of worldwide fish more sensitive to CPF. In addition, the 96 h-LC_{50} for EN and LC were 0.30 ± 0.012 μg/L and 0.043 ± 0.031 μg/L, respectively. Therefore, relative toxicity of the three soybean insecticides for *O. bonariensis* was LC > EN > CPF. Effects of mixtures with EN and LC were variable, but in general fitted to both, independent action (IA) and concentration addition (CA) models. Slight antagonism was found when CPF TU proportions were above 50%. Therefore, from the regulatory point of view, the use of both mixture models, CA or IA, would be precautionary. Differential sensitivity to CPF was found for AchE inhibition at the head (96 h-$IC_{50} = 0.065 \pm 0.058$ μg/L) and the body (96 h-$IC_{50} = 0.48 \pm 0.17$ μg/L). In addition, whereas no significant effects induced by mixtures was observed in body AchE activity, antagonism was induced in head AchE inhibition in presence of both, EN and LC in the mixture. The lethal to sublethal ratio was close to 25.2 and 3.4 when comparing the CPF-LC_{50} and IC_{50}s for head and body AchE activity, respectively. However, considerable overlapping was observed between concentration–response curves, indicating that the use of AchE as biomarker for environmental monitoring would be limited.

Keywords Ecotoxicology · Pesticides · Mextures · *O. bonariensis* · LC_{50} · AchE

Introduction

Pesticides were defined by the FAO as "substances (or mixtures of substances) intended for preventing, destroying, or controlling any pest…" (FAO 2002). Despite the acknowledged benefits of pesticides, concern exists about potential adverse effects of these compounds on non-target organisms. In particular, the introduction of genetically modified soybean has dramatically expanded the agricultural frontier in developing countries and has greatly increased the use of agriculture pesticides. To address that

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issue, a workshop on the "Aquatic Risk Assessment of Pesticides in Latin America", was organized by the Society of Environmental Toxicology and Chemistry, and the need for research on local species was remarked among the main conclusions (Carriquiriborde et al. 2014). Increasing the knowledge on the response of local species to environmentally relevant pollutants was also among the 20 priority questions identified in the Latin American task group of the Global Horizon Scanning Prioritization Project (Furley et al. 2018).

Chlorpyrifos (O,O-diethyl O-(3,5,6-trichlor-2-pyridyl) phosphorothioate; CPF) is a broad spectrum organophosphate insecticide, and since its initial production in 1962, has been broadly used around the world for pest control at residential, industrial and agricultural sites (Racke 1993). In particular, CPF is one of the major soybean insecticide, used alone or, more commonly, in combination with the herbicide glyphosate, and other insecticides like pyrethroids and organochlorines (Ronco et al. 2008; Di Marzio et al. 2010). The pyrethroid cypermethrin is currently the most important soybean pesticide, but its replacement by lambda-cyhalothrin (LC) is becoming everyday more frequent. The organochlorine endosulfan (EN) used to be the second soybean insecticide, but it started to be replaced by CPF in those countries were its production or importations was banned. However, legal or illegal use of EN is still frequent in several countries. In particular, mixtures of insecticide are typically used at late stages of the crop when pest infestation is severe (e.g., for green stink bug control) (Ronco et al. 2008).

The toxicology of CPF for aquatic and terrestrial organisms has been broadly studied and extensively reviewed by Barron and Woodburn (1995). Toxic action of CPF is mainly due to its metabolic activation to CPF-oxon, which specifically inhibit the acetylcholinesterase (AChE) at the synaptic junction. Enzyme inhibition occurs by phosphorylation of the active site and is rapidly reversible. Although the mode of action (MOA) is basically the same, a wide variation in sensitivity has been observed across fish species. That has been explained mainly as consequence of CPF metabolization rates and specificity of AChE inactivation, but also owing to pharmacokinetics, behavior, feeding ecology, and ecological relationships (competitor, predator effects).

Odontesthes bonariensis, is a freshwater fish characteristic of the Pampas Region, highly appreciated because of the quality of the flesh and as a game fish (Somoza et al. 2008). In addition, this Neotropical teleost has shown to be highly sensitive to environmental pollutants (Carriquiriborde and Ronco 2006). In the Pampas, this fish inhabits shallow lakes and rivers, where concentrations of CPF has been reported at up to 10 µg/L (Marino and Ronco 2005). The 96 h-LC_{50} of CPF for other the Neotropical fish, *Cnesterodon decemmaculatus*, has been reported between 104 and 108 µg/L (Paracampo et al. 2014). However, under field studies using caged and wild fish, lethal effects have been observed for *C. decemmaculatus* during acute exposure scenarios after CPF spraying in farm plots close to small streams (Ronco et al. 2008). On the other hand, less studied have been the potential sublethal effects induced by soybean insecticides on fish, and the reported results were weakly conclusive (Di Marzio et al. 2010; Brodeur et al. 2017).

CPF in surface waters has not usually detected alone, but in co-occurrence with other insecticides (Jergentz et al. 2005; Marino and Ronco 2005). Maximum reported concentrations of EN in rivers and streams across soybean agriculture areas were among 4.26 and 20.0 µg/L (Di Marzio et al. 2010; Etchegoyen et al. 2017). Less studied were the concentration of LC in surface waters of the region, but up to 40% occurrence and maximum concentrations of 5, 6.09, and 16.57 µg/kg were found in sediments of soybean regions of Brazil, Argentina, and Paraguay, respectively (Miranda et al. 2008; Hunt et al. 2016). Therefore, understanding toxicity of CPF in the presence of other insecticides would help to better explain or predict potential adverse effects in the field.

According with the general theoretical framework for binary mixtures proposed by Hewlett (1969), toxicants may act independently or jointly and may or may not interact, and therefore, three models can be derived: concentration addition (CA), independent action (IA), or simple interaction (SI) (Belden et al. 2007). CA model assumes that toxicants in the mixture have the same MOA, whereas IA model assumes each toxicant acts completely independently within the organism. SI model assumes that one substance in the mixture, at a none toxic concentration, is able to influence the toxicity of other substance through an indirect mechanism. The three models have been successfully used to predict toxicity of mixtures of pesticides depending on the MOAs and the existence, or not, of interactions (Belden et al. 2007). CPF toxicity for fish under mixtures has been usually assessed in combination with other insecticides with the same MOA (Belden and Lydy 2006; Chen et al. 2014). Less studied were mixtures with insecticides having different MOA (Pérez et al. 2013), a more realistic scenario according the spraying cocktails used in soybean crops.

The aim of the present study was to assess the toxicity of CPF on the Neotropical fish *O. bonariensis*, both at lethal and sublethal levels, and in relation with other soybean insecticides of relevance, assessed alone or under binary mixtures.

Materials and methods

Chemicals

Insecticides active ingredients: CPF (IUPAC Name: O,O-Diethyl O-3,5,6-trichloropyridin-2-yl phosphorothioate, CAS: 2921-88-2, Purity: 98 % w/w), EN (IUPAC Name: 6,7,8,9,10,10-Hexachloro-1,5,5a,6,9,9 a-hexahydro- 6,9-methano-2,4,3-benzodioxathiepine-3-oxide, CAS: 115-29-7, Purity: 97% w/w) and LC (IUPAC Name: 3-(2-chloro-3,3,3-trifluoro-1-propenyl)-2,2-dimethyl-cyano (3-phenoxyphenyl)methyl cyclopropanecarboxylate, CAS: 68085-85-8, Purity: 95% w/w) were kindly donated by Gleba S.A. Argentina. Dimethyl sulfoxide (DMSO) analytical grade was Biopack, dichloromethane, and 1-octanol were Merck. Tris base and sucrose were from Sigma, mono, and di-basic potassium phosphate and hydrochloric acid were from Anedra. Reagents for AchE analysis: acetylthiocholine iodide (98%) and 5,5′-Dithiobis (2-nitrobenzoic acid (98%) were purchased from Sigma.

Test organisms

Newly borne inland Pejerrey, *O. bonariensis* (Valenciennes 1835), larvae were kindly provided by the Hydro-biological Station of Chascomús (Ministry of Agriculture Affairs of the Buenos Aires Province). Fish were carried to the laboratory in polyethylene bags with oxygen supply and reared during 2 weeks. Fish were maintained in a 2000 L closed circulating system with dechlorinated and charcoal-filtered La Plata tap water (hardness 250 mg $CaCO_3$/L; pH 7,8; dissolved oxygen; 9.0 mg/L). Temperature was set at 25 ± 1 °C and photoperiod 16 h light: 8 h darkness. Larvae were fed with *Artemia sp*. and fish commercial food for larvae BB Shulet® four-time a day.

Experiments

Two type of experiments were conducted in the present study: (i) one aimed to evaluate lethal effects of CPF, EN, and LC, and sublethal effects of CPF under single exposure, and (ii) the other aimed to evaluate lethal and sublethal effects of mixtures of CPF in combinations with the other two insecticides.

In both cases, 96-h static-renewal toxicity tests were conducted following general guidelines established in the protocol for *Menidia beryllina* (USEPA 2002), but adapted to the objectives and species requirements of the present study (Carriquiriborde and Ronco 2002). In order to assesses inter-assay variability, all tests (single compound and mixtures) were repeated six times given a total number of 30 toxicity tests (CPFx6, ENx6, LCx6, CPF-ENx6, CPF-LCx6). For each test, at least five treatments (concentrations or mixture proportions) were evaluated by quadruplicate, using 10 fish per replicate. Fifteen to 30 day-old post hatch *O. bonariensis* larvae (14.55 ± 2.93 mg) were used as test organisms. Three-L-glass aquaria filled with 2 L testing solutions were used as test chambers. Charcoal-filtered “La Plata” tap water was used for preparing test media. DMSO was used as solvent vehicle at 0.01% in all concentrations, including the control group. Fish were fed 0.5 ml of 24-h *Artemia sp*. nauplii concentrate at the 48 h, 1 h before whole testing media volume was renewed. As *O. bonariensis* is extremely sensitive to handling, media renewal was performed by siphoning the test solutions up to 1 cm above the test chamber bottom and immediately refilled with 2 L fresh media. The siphoning devise was cleaned between treatments and always the procedure was performed from controls to higher concentrations to avoid contamination. Temperature and illumination conditions were the same as the described in the holding conditions.

To evaluate lethal effects of CPF, EN, and LC under single exposure, a minimum of five concentrations levels were assessed including the solvent control group. In four of the six repeated experiments with CPF, sublethal effects (AchE activity inhibition) were also assessed in the surviving fish after 96 h of exposure. To evaluate lethal effects of CPF in binary mixtures with LC and EN, five treatments were assessed according with the following toxic units (TU) proportions of CPF and LC or EN, respectively: 1–0, 1/4–3/4, 1/2–1/2, 3/4–1/4, and 0–1. Therefore, one TU would be expected in all treatments assuming additive effect. The sublethal effects of the binary mixtures on AchE activity were assessed in surviving fish of two of the six repeated experiments.

Chemical analysis

For the chemical analyses, water samples (250 ml) were collected immediately after (0 h) and before (48 h) test media renewal from treatments with concentrations approximately three orders of magnitude above the detection limits of the method (5 µg/L of CPF, 0.5 µg/L of EN and 0.1 µg/L of LC). Samples were extracted using liquid-liquid extraction with dichloromethane. The pyrethroid bifenthrin (BF) was added (100 µg/L) as internal standard. Extracts were concentrated using a rotary evaporator and the final volume blown-up to dryness under gentle nitrogen stream using toluene as keeper. Samples were then resuspended in toluene (250 µl) and analyzed by CG-MS using a Perkin Elmer Clarus 600 gas chromatograph equipped with a single quadrupole mass detector. Quantification and conformation ions were CPF *m/z* 197/314, LC *m/z* 181/197 and α and β-EN *m/z* 195/241. The recovery and the limits of detection of the method were 96.7 ± 9.7% and 5 ng/L for

CPF, 89.5% ± 1.5% and 1 ng/L for EN, 112.3 ± 5.6% and 0.5 ng/L for LC, and 116.6 ± 12.2% and 0.1 ng/L, for BF.

AchE analysis

Surviving fish from different treatments of toxicity tests with CPF or the binary mixtures were ice-cold euthanized and the body dissected in two portions, the head and the trunk. Each portion was separately homogenized in 250 µl of buffer pH 7.8 (Tris–HCl 20 mM, sucrose 250 mM, EDTA 1 mM, KCl 150 mM) at 15,000 rpm using a PRO Bio-Gen PRO200 Homogenizer (PRO Scientific Inc). Homogenates were then centrifuged at 10,000 g during 20 min at 4 °C in a SIGMA2-16PK (Sigma Laborzentrifugen GmbH) centrifuge and supernatant stored at −80 °C until analysis. AchE activity was assessed following the method of Ellman et al. (1961) and adapted to microtiter plate reader using 10 µl of homogenate and reading absorbance (412 nm) of each sample by triplicate at 25 °C during 5 min. In addition, total protein was measured using the method of Lowry et al. (1951), also adapted to microplate reader according with Akins and Tuan (1995), using 25 µl of homogenate and reading absorbance (660 nm) of each sample by triplicate against a bovine serum albumin calibration curve. In both cases absorbance was measured using a Multi-Mode Micro Plate Reader Synergy HT (BioTeck Instruments Inc.) with optic path length correction to 1 cm.

Data analysis

The acute lethal toxicity concentration–response curve was described for CPF, LC, and EN and the LC_{50} at 24, 48, 72, and 96 h estimated using the Probit Method (Finney 1971). Toxicity tests were accepted when mortality in the control group was below 10%. The incipient LC_{50} for CPF was estimated using the equation based in the theoretical bioconcentration model toxicity (Verhaar et al. 1999; Carriquiriborde and Ronco 2006). The 96 h-LC_{50} of *O. bonariensis* to CPF was ranked in comparison with the one for other freshwater fish of the world acquired from the USEPA ECOTOX Database (accession date September 2017). Data from experiments assessing acute lethal toxicity of CPF in mixtures were compared with the estimated values obtained from the "CA" and "IA" models using the "Model Deviation Ratio (MDR)" following the procedures discussed in previous studies (Faust et al. 2003; Belden et al. 2007). In addition, the Chi-squared test (χ^2) was used to assess the differences between observed and expected responses in mixtures. The expected response at each tested concentration in the mixtures was estimated using the inverse Probit function (Faust et al. 2003). For the experiments assessing the effect of mixtures on CPF inhibition of the AchE activity was assessed assuming the "SI Model".

ANOVA was used for assessing the statistical significance of the effects induced by CPF on AchE. Dunnett analysis was then used to obtain the NOEC and LOEC for AchE inhibition. In addition, the concentration–response curve between the concentration of CPF, alone or in the mixtures, and AchE inhibition was described fitting a 4-parameter logistic (4PL) model (Sebaugh 2011; Crupkin et al. 2013). The model was used therefore to estimate the 50% inhibitory concentration (IC_{50}) for each of the six independent experiments. Then the average IC_{50}, the standard error and 95% confidence limits were obtained. The lethal to sublethal ratio (LSR) was calculated as LC_{50}/IC_{50}.

Results

Actual concentrations of CPF, EN, and LC measured in the testing solutions immediately after start or media renewal were 101.6 ± 17.3%, 113.6 ± 16.2%, and 96.5 ± 3.4% of the nominal value, respectively. Actual concentrations dropped to 78.2 ± 1.9 and 33.9 ± 3.7% of the initial values for CPF and EN, or even below the detection level for LC after 48 h.

The concentration–response curve of CPF on *O. bonariensis* mortality after 96 h exposure is shown in Fig. 1a. The parameters of the Probit regression line were: slope = 1.027 and intercept = 1.690. The estimated 96 h-LC_{50} was 1.68 µg/L, and the 96 h-LC25, and 96 h-LC10, usually used as LOEC and NOEC, were 0.37 and 0.09 µg/L, respectively. The time-response curve is shown in Fig. 1b. The incipient LC_{50} for CPF obtained from the theoretical bioconcentration model was 0.048 µg/L. The average LC_{50} from the six acute lethal toxicity tests for CPF at 24, 48, and 96 h are shown in comparison with the values for END and LC (Table 1). Results indicate that under laboratory conditions, the CLF has the lowest toxicity for *O. bonariensis* in comparison with the other two soybean insecticides. The LC_{50} for CPF was one and two order of magnitude higher that the LC_{50} for EN and LC, respectively. In comparison with other freshwater fish species of the world, *O. bonariensis* was ranked among the 13% more sensitive species to CPF (Fig. 2), but it could rank lower if the flow-through tests are excluded from the analysis.

The average values of the MDR for the six experiments assessing the acute lethal toxicity of CPF in combination with LC and EN are shown in Fig. 3. Although variable among experiments, in general, both the IA and CA models fitted well to empirical data when CPF was half or less of the mixture proportion. More in detail, CA seems to fit data a little better than IA, which tends to overestimate toxicity. In particular, both models overestimated the toxicity for those mixtures in which CPF was at higher proportions, indicating that some antagonist interaction could be acting.

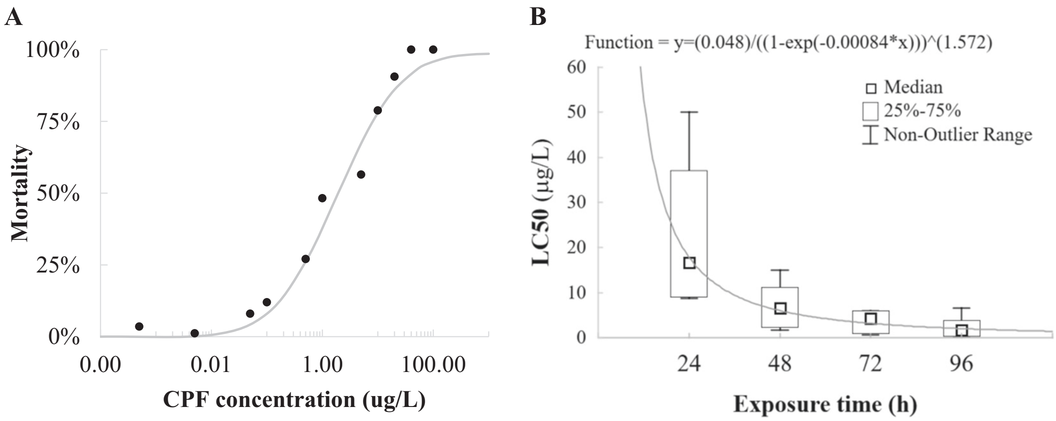

Fig. 1 Acute lethal effects of CPF on *O. bonariensis*. **a** Concentration–response curve for after 96 h exposure and **b** time–response curve from 24 to 96 h exposure

Table 1 LC_{50} (µg/L) of chlorpyrifos, endosulfan, and lambda-cyhalothrin for *Odontesthes bonariensis*[a]

	Chlorpyrifos		Endosulfan		Lambda-cyhalothrin	
Exp. time (h)	LC_{50}	95% CI	LC_{50}	95% CI	LC_{50}	95% CI
24	22.99	4.03–41.95	0.91	0.54–1.27	0.167	0.063–0.271
48	7.34	2.31–12.36	0.82	0.54–1.10	0.134	0.062–0.205
72	5.14	0.99–9.29	0.52	0.31–0.72	0.060	0.033–0.087
96	2.26	0.53–3.99	0.30	0.28–0.31	0.043	0.015–0.071

[a]Average values and 95% confidence intervals (CI) from six independent experiments

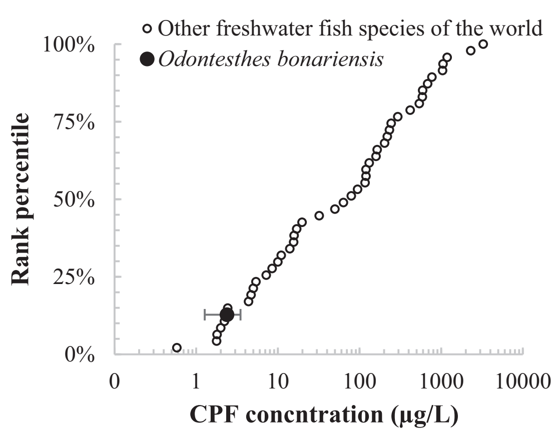

Fig. 2 Sensitivity of *O. bonariensis* to CPF in comparison with the other freshwater fish species of the world. Data gathered from USEPA Ecotox Database

Toxicity of CPF at sublethal level, assessed by the AchE activity, showed a clear concentration–response relationship, both in the head and in the trunk (Fig. 4). The activity values of the enzyme and percentage of inhibition induced by CPF in the four conducted experiments are shown in Table 2. The LOEC in the head AchE activity were between 0.005 to 1.2 µg/L and in the trunk between 0.5 and 5 µg/L. The parameters of the concentration–response curve estimated by the 4PL model together with the estimated 96 h IC_{50} are shown in Table 3. The AchE IC_{50} for CPF varied from 0.022 to 0.820 µg/L in the head and from 0.38 to 0.69 µg/L in the trunk.

The 4PL model parameters and the IC_{50} for experiments 3 and 4 assessing the AchE inhibition by the CPF alone and in mixtures with EN and LC are shown in Table 3. A clear reduction of the CPF inhibitory effect was observed in the tested mixtures on the head, but not in the trunk (Fig. 5). In experiment 3, the induced antagonism was similar in the both mixtures, but in experiment 4, it was greater in the mixture with EN than with LC (Fig. 5).

The concentration-dependent curves obtained for lethal and sublethal effects showed not only differences in the EC_{50}, but also different slopes, with greater differences among both kind of effects at lower concentrations, and converging as the concentrations were increased (Fig. 6). The EC_{50} for mortality and AchE inhibition in the head and the trunk were 1.64, 0.48 and 0.065 µg/L, respectively. Therefore, the LSRs were 3.4 and 25.2 for those concentrations that induced mortality and inhibition of AchE in the trunk and the head, respectively. It is worthy to note, that the IC_{50} in the head and the trunk were, respectively, close to the LC_{10} and LC_{30}. On the other hand, the LC_{50} was near the IC_{80} and IC_{70} estimated for each tissue, respectively.

Discussion

The need for ecotoxicological data on local species from Latin America has been highlighted as a priority issue in recent workshops, as inputs for ecological risk assessment (Carriquiriborde et al. 2014; Furley et al. accepted). In addition, soybean agriculture expansion and the associated increase of pesticide volumes used represents a major

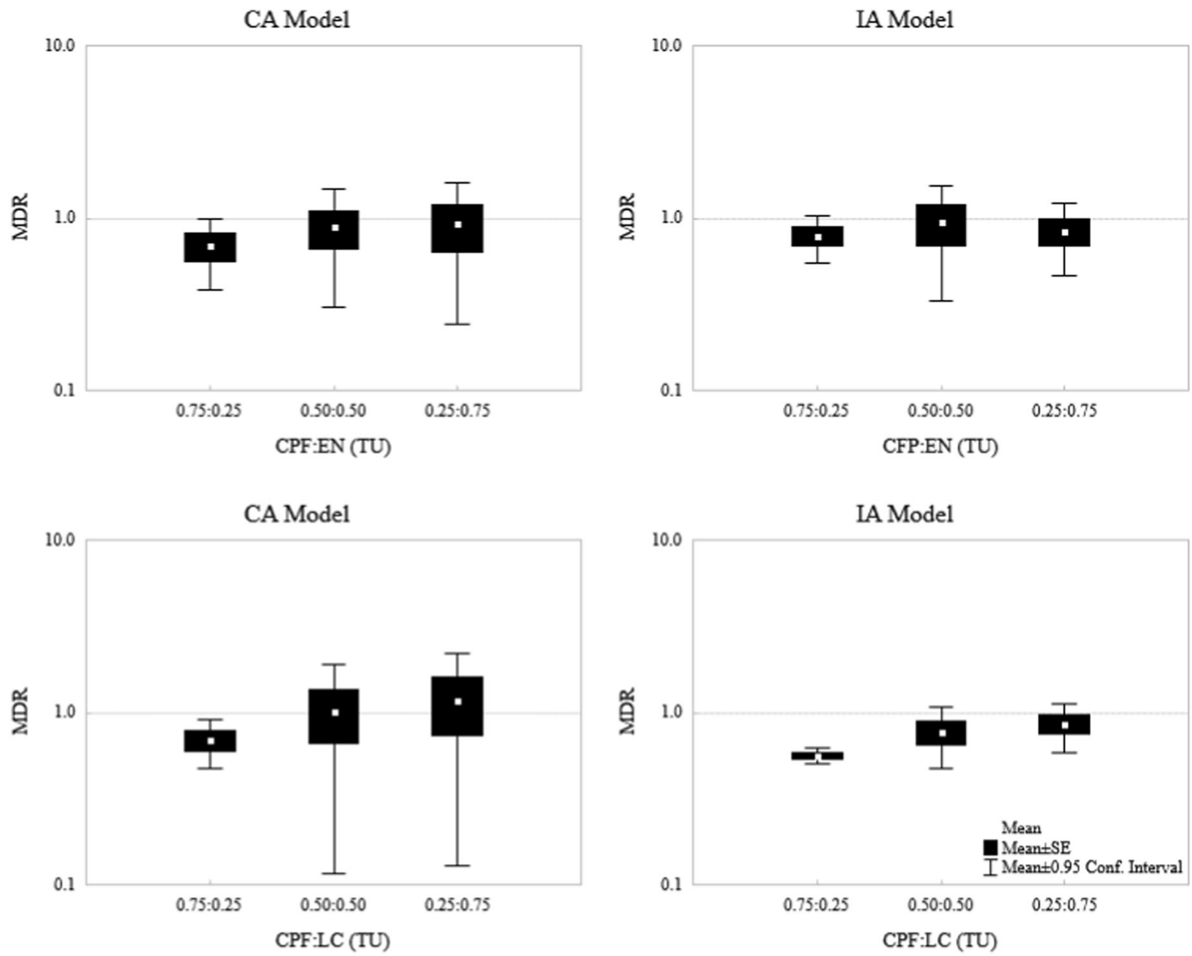

Fig. 3 Acute lethal effects of CPF to *O. bonariensis* under equitoxic and nonequitoxic mixtures with EN and LC, assessed by the MDR for the IA and CA. MDR model deviation ratio, IA independent action model, CA concentration addition model, CPF chlorpyrifos, EN endosulfan, LC lambda-cyhalothrin. Data are the mean, standard error (SE) and 95% confidence interval from six independent experiments

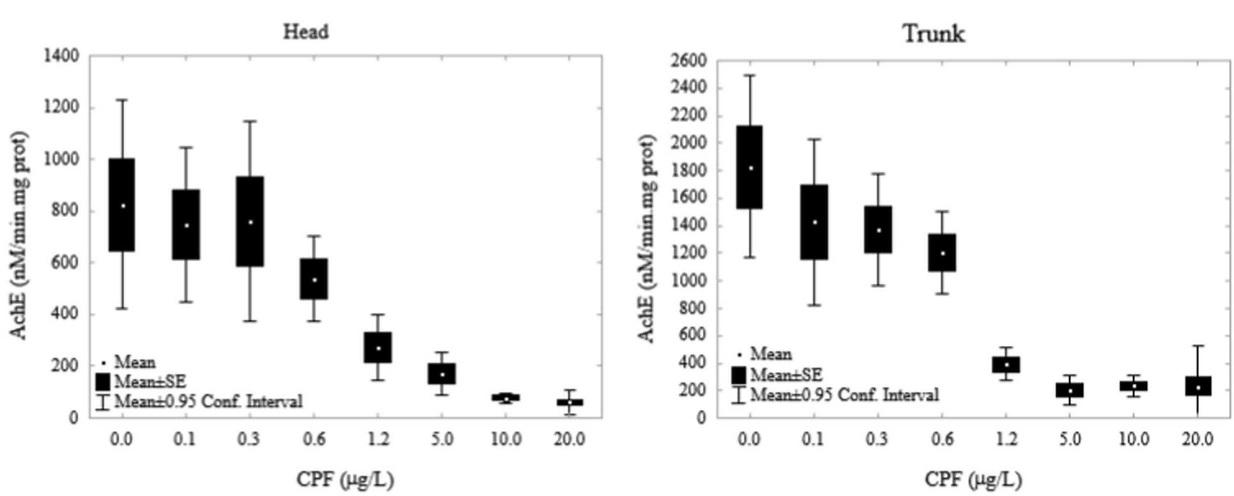

Fig. 4 Response of AchE activity in *O. bonariensis* after acute exposure to CPF for 96 h. Results from experiment 4. **a** AchE activity in the head and **b** AchE activity in the trunk

environmental issue in the region. In the present study, lethal and sublethal toxicity of the organophosphate insecticide CPF on the Neotropical fish *O. bonariensis* was assessed under single and combined exposures with other two commonly used insecticides for pest control in soybean crops, EN and LC.

Table 2 Response of AchE activity in *O. bonariensis* after 96 h exposure to CPF

	Head				Trunk			
	CPF	AchE (nM/min.mg prot.)		Inhibition	AchE (nM/min.mg prot.)			Inhibition
	(μg/L)	Average SE	(*N*)	(%)	Average	SE	(*N*)	(%)
Exp. 1	Ctrl	374.5 ± 35.2	(3)	0.0	1684.2 ± 244.9		(3)	0.0
	0.005	265.0 ± 11.4	(3)	29.2	1469.4 ± 239.0		(3)	23.3
	0.050	157.7 ± 16.9	(3)	57.9*	1309.6 ± 265.9		(3)	31.7
	0.500	148.7 ± 0.4	(3)	60.3*	1509.6 ± 184.1		(3)	21.2
	5.000	95.3 ± 10.4	(3)	74.6*	102.2 ± 30.9		(3)	94.7*
Exp. 2	Ctrl	377.5 ± 53.6	(3)	0.0	1562.5 ± 673.6		(3)	0.0
	0.005	381.5 ± 31.9	(3)	1.1	1401.5 ± 243.1		(3)	10.3
	0.050	221.9 ± 9.6	(3)	41.2*	1142.5 ± 13.5		(3)	26.9
	0.500	88.9 ± 7.3	(3)	76.5*	826.7 ± 311.2		(3)	47.1
	5.000	56.0 ± 9.1	(3)	85.2*	166.1 ± 19.3		(3)	89.4*
Exp. 3	Ctrl	300.6 ± 2.3	(3)	0.0	1590.1 ± 267.1		(3)	0.0
	0.005	176.9 ± 13.6	(3)	41.2*	1684.4 ± 117.3		(3)	−5.9
	0.050	142.1 ± 19.5	(3)	52.7*	1254.2 ± 111.5		(3)	21.1
	0.500	81.3 ± 2.2	(3)	73.0*	805.9 ± 190.4		(3)	49.3*
	5.000	37.7 ± 35.2	(3)	87.4*	191.8 ± 23.1		(3)	87.9*
Exp. 4	Ctrl	825.6 ± 178.4	(10)	0.0	1826.8 ± 300.7		(10)	0.0
	0.1	816.5 ± 117.6	(10)	1.1	1771.5 ± 640.8		(10)	3.0
	0.3	760.9 ± 170.3	(10)	7.8	1373.4 ± 170.7		(10)	24.8
	0.6	539.5 ± 73.6	(10)	34.6	1205.3 ± 129.4		(10)	34.0*
	1.2	271.7 ± 56.4	(10)	67.1*	391.0 ± 53.1		(10)	78.6*
	5.0	132.5 ± 17.0	(10)	84.0*	206.4 ± 47.3		(10)	88.7*
	10.0	78.1 ± 9.0	(10)	90.5*	234.1 ± 33.3		(10)	87.2*
	20.0	60.4 ± 10.8	(3)	92.7*	233.6 ± 83.2		(3)	87.2*

* Treatments statistically different from the control group (ANOVA, followed by the Dunnett *post hoc* analysis, $p < 0.05$)

Table 3 4PL model parameters and the IC_{50} after 96 h exposure obtained from the four conducted experiments assessing AchE inhibition induced by CPF alone, or combined with LC and EN

Experiment number	1	2			3			4
Toxicant	CPF	CPF	CPF-EN	CPF-LC	CPF	CPF-EN	CPF-LC	CPF
Head								
Parameters								
b (min)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
a (max)	0.72	0.95	0.97	0.88	1.00	1.00	1.00	0.90
c (1/2 I_{max})	0.008	0.078	0.446	0.523	0.022	5.612	0.169	0.747
d (slope)	−0.626	−0.923	−1.187	−1.174	−0.318	−0.771	−0.405	−2.322
IC_{50}	0.030	0.087	0.460	0.650	0.022	5.500	0.160	0.820
Trunk								
Parameters								
b (min)	NA	0.00	0.00	0.00	0.00	0.00	0.00	0.00
a (max)	NA	1.00	1.00	1.00	1.00	1.00	1.00	0.89
c (1/2 I_{max})	NA	0.369	0.127	0.253	0.437	1.290	0.740	0.611
d (slope)	NA	−0.591	−0.630	−0.687	−0.721	−0.502	−0.924	−2.016
IC_{50}	NA	0.380	0.130	0.260	0.430	1.300	0.750	0.690

Parameters constrains: $a \geq 0$, $b \leq 1$. NA: no assessed

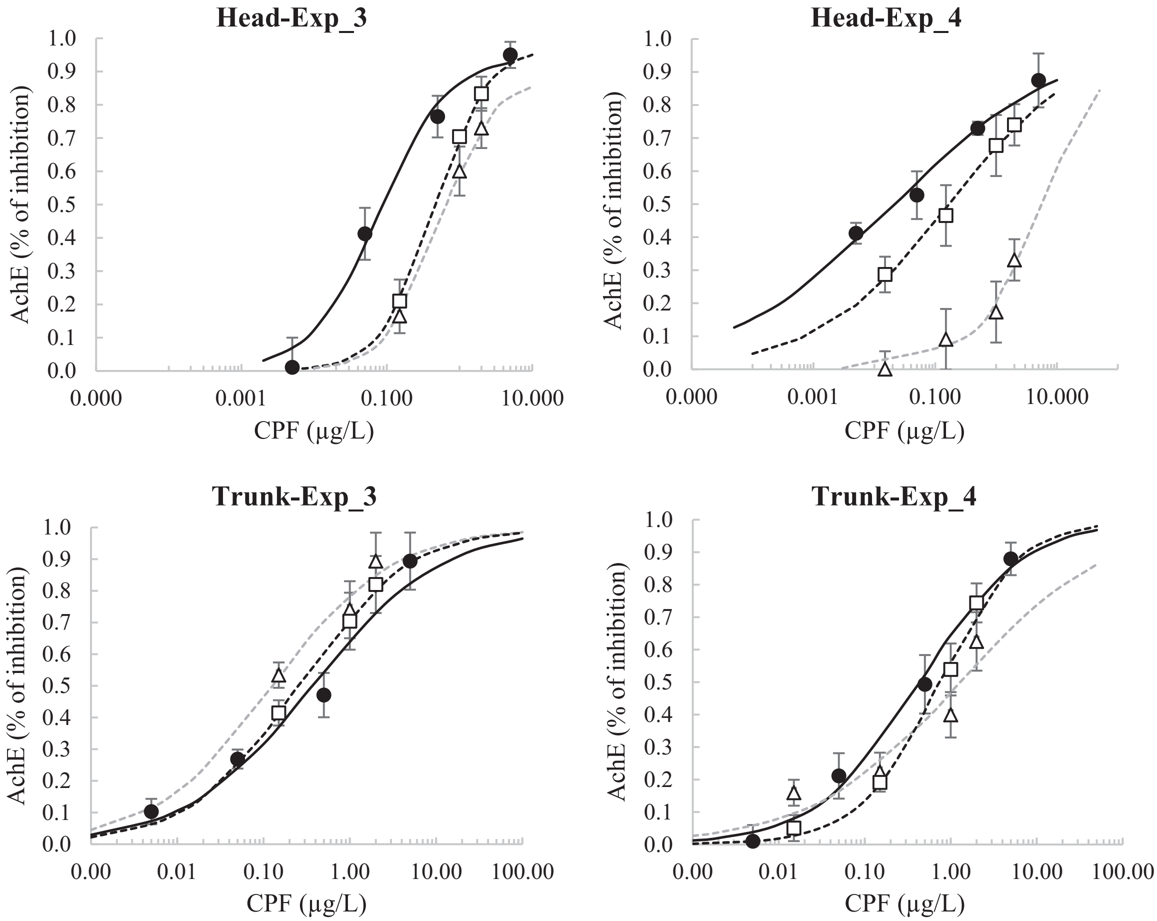

Fig. 5 Effects of mixtures on AchE inhibition by CPF alone or combined with LC or EN. **a**, **b** head AchE, **c**, **d** trunk AchE, **a**, **c** experiment 3, **b**, **d** experiment 4. Circles: CPF, squares: CPF + LC and triangles: CPF + EN. Curves correspond with the respective 4PL model fitted to each set of data, black solid line: CPF, black dotted line: CPF + LC and gray dotted line: CPF + EN

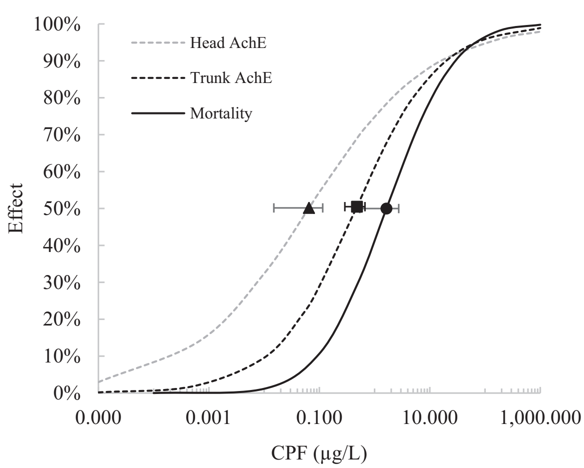

Fig. 6 Comparison among concentration–response curves of mortality and AchE inhibition in the head and trunk of *O. bonariensis* exposed to CPF. Curves were built with the average data from six and four experiments assessing acute lethal and sublethal effects (AchE), respectively. Black solid line: mortality, black dotted line: head AchE and gray dotted line: trunk AchE. Markers indicate the EC_{50} with the 95%-confidence intervals, circle: LC_{50}, square: IC_{50} (head) and triangle: IC_{50} (trunk)

Based on the obtained LC_{50}s, the relative toxicity among the three assessed insecticides was LC > EN > CPF, showing the organophosphate CPF as the lesser toxic of the three. Anyway, according to chemical toxicity classification (GESAMP 2002), CPF should be considered still extremely toxic to *O. bonariensis*. When the sensitivity of *O. bonariensis* to CPF was compared with other fish species, a broad range of sensitivities was observed in early-stage organisms, showing 96 h-LC_{50} values ranging from 170 µg/L in 1 d-old fathead minnow (Jarvinen and Tanner 1982) to 0.40 µg/L in 14 d-old tidewater silverside (Borthwick et al. 1985). Therefore, important differences in sensitivity would exist among fish species depending on their own biological traits, and *O. bonariensis* would be placed among the most sensitive of the world. In this study, *O. bonariensis* was ranked among the 13% most sensitive fish of the word, though, that rank value could be lower if flow-through test would be used. A dissipation of 22.8% of the CPF concentrations was measured between media renewal (48 h) in this study, and changes of 70% (from 1.8 to 0.4 µg/L) in the CPF LC_{50} values for *Menidia peninsulae* were reported when assessed by static or flow-through test (Borthwick et al. 1985). In spite of that, the sensitivity of *O. bonariensis* was among the reported for other atherinid species, like *Leuresthes tenuis*, *Menidia menidia* and *M. peninsulae* (Borthwick et al. 1985), showing this family as highly sensitive to CPF. On the other hand, early-life stages of *O. bonariensis* were almost 50-fold more sensitive than the adult stages of *Cnesterodon decemmaculatus*, other Neotropical fish broadly used in toxicity tests (Paracampo et al. 2014). These results support the previous concept that *O. bonariensis* is highly sensitivity to environmental pollutants (Carriquiriborde and Ronco 2002, 2006), and show this specie as a suitable Neotropical fish for toxicity tests. It is also important to note, that the dissipation kinetic observed for tested pesticides would be indicating the need of flow-through system, or more frequent media renewal, to assess the lethal effects of LC and EN minimizing the underestimation of their toxicity.

Under field conditions, fish are rarely exposed to single pesticides, but to mixtures of them, and therefore it is important to understand how mixtures affect their toxicity. The study of mixtures can pursuits different objectives depending on the scope of the environmental study (Altenburger et al. 2003). Here, the attention was mainly focused on the effect assessment, with the intention of generating information for the identification of potential hazard of the joint occurrence of mentioned soybean pesticides for sensitive Neotropical fish species. According to the proposed models to describe the joint action of pesticides: CA, IA, and SI (Belden et al. 2007), the IA model would be the one expected for the binary mixtures of CPF and EN or LC, as different MOAs have been described for each of the pesticides in the mixture. Whereas CPF acts on AchE enzyme inhibiting it and altering the nerve impulse transmission at the synapsis, EN and LC act mainly interfering with the ion channels (Na, K, Ca, Cl), affecting the nerve impulse conduction through the axon (Ecobichon 2001). Results were also contrasted against the CA model, assuming it would not fit suitable to experimental data. At the lethal level, the observed responses in the tested binary mixtures were variable among experiments, but in average, they fitted well to both models (IA and CA) when CPF TUs were < 50% of the mixture. Otherwise mixture toxicity was overestimated by models, indicating an antagonistic interaction. Therefore, none of the models was able to explain the behavior for all mixture proportions. Such results were different from those obtained in most studies testing binary mixtures of organophosphates and pyrethroids. Synergic interactions were observed for mixtures of dimethoate and cypermethrin or deltamethrin in *Orechromis niloticus* (Fai et al. 2017), diazinon and esfenvalerate (Denton et al. 2003), and CPF and esfenvalerate (Belden and Lydy 2006) in *Pimephales promelas*. It is known, that interactions among toxicants occur at toxicokinetic (absorption, distribution, biotransformation, or excretion) and toxicodynamic (physiological mechanisms) level (Hernández et al. 2017). In particular, the synergic interactions among organophosphates and pyrethroids have been explained mainly by carboxylesterases inhibition and the associated reduction in the pyrethroid detoxification, or the CYP system induction and the associated increase of the oxon metabolite synthesis. However, a few studies have been demonstrated that antagonism can be also occur at toxicodynamic level. For example, such type of interaction was observed in the inhibition of voltage-gated calcium channels of PC12 cells exposed to ternary mixtures of organophosphates, organochlorine and pyrethroids pesticides (Meijer et al. 2014). Although for some tested mixture proportions lethal effects were overestimated by tested models, from a regulatory point of view, both models would result precautionary for the studied species. Despite IA model should be more suitable for explaining effects induced by tested mixtures, it was more prone to overestimate toxicity than the CA model.

Regarding the sublethal effects, a clear concentration–response relationship was observed between CPF concentration and AchE inhibition. AchE activity in the trunk was always higher than in the head, though, the last was always more sensitive to the CPF exposure. Higher activity in the muscle than in the brain was also observed the Neotropical fish, *Jenynsia multidentata*, but for that species AchE in the muscle was more sensitive to CPF than in the brain (Bonansea et al. 2016). The LOEC in the mentioned study for muscle AchE was 4 µg/L, whereas the LOECs observed in head AchE for *O. bonariensis* in the different experiments ranged from 0.05 to 1.2 µg/L, showing again *O. bonariensis* as a sensitive species. Inter-experiment variations showed that LOECs were more variable than IC_{50}s (ranging from 0.022 to 0.82 µg/L), and therefore the last comes up as a more accurate endpoint to be assessed.

Neither EN nor LC have been described as specific AchE inhibitors (Ecobichon 2001), but they could interact with CPF, modulating its inhibitory effect on the AchE activity. Thus, SI model was assumed for assessing sublethal effect of tested mixtures. Differential effects were observed in the head and trunk. Although, in the first, no effects were induced, in the last, antagonism was observed for both insecticides. The antagonistic effect in the head was consistent with, and could help to explain, the effect observed at the lethal level when the CPF proportion in the mixture was increased. Results obtained in other studies about the inhibitory effects of binary mixtures of organophosphates and pyrethroid pesticides on AchE activity are not conclusive. Strong synergic interaction has been reported *in vitro* on brain AchE of house fly treated with a binary mixture of CPF and deltamethrin or cypermethrin (Arora et al. 2017). Conversely, as in *O. bonariensis*, antagonism has been reported in brain AchE of rats exposed *in vivo* to binary mixtures of CPF and deltamethrin (Tüzmen et al. 2007). Accordingly, results obtained for different species and exposure conditions would suggest that different interactions would occur at toxicodynamic and toxicokinetic level, and therefore further studies would be still necessary to understand and predict the effects of these mixtures on AchE activity *in vivo*.

Biomarkers have been defined as early warning signals, that alert about adverse effects before they were irreversible (Van Gestel and Van Brummelen 1996), and AchE has been broadly accepted as a relevant one for environmental biomonitoring (Lionetto et al. 2011). In the present study, when the AchE IC_{50} for CPF was compared with its LC_{50}, it was possible to establish that approximately one order of magnitude separates both endpoints. However, due to the relatively smooth slope of the obtained concentration–response curves it was also possible to note a considerable overlapping between lethal and AchE responses. Therefore, the usefulness of AchE as a biomarker in *O. bonariensis* to evaluate CPF sublethal exposure/effect under field conditions would be limited.

Detectable concentrations of CPF in surface waters within the soybean crop region of the Pampas were between 0.4 and 10.8 µg/L in highly impacted streams (Marino and Ronco 2005) and between 0.01 to 0.48 µg/L in large rivers, like the Paraná River (Etchegoyen et al. 2017). In addition, the reported range of detectable concentration of EN were 0.01–4.26 µg/L in the Paraná River (Etchegoyen et al. 2017) and 0.8–20.0 µg/L for smaller streams within soybean crop areas (Di Marzio et al. 2010). When those concentrations were compared with the effective concentrations obtained for CPF and EN, alone or in mixtures, not only sublethal, but also lethal effects would be expected for early-life stage of *O. bonariensis* inhabiting mentioned agriculture districts. Particularly relevant is the fact that, as most of other fish species of the region, *O. bonariensis* reproductive season extensively overlap with the soybean crop season. Therefore, the risk of exposure for early-life-stages is high. In the case of LC, reported environmental concentrations were only available for sediments. They would be indicating that the insecticide is been broadly used in the region, though, data about surface water concentrations would still be lacking to be able to evaluate more accurately the potential risk of this pesticide on fish and other water column organisms.

Major outcomes from the present study were: (i) *O. bonariensis* is highly sensitive to CPF, and other tested soybean pesticides, ranking it among the most sensitive fish species of the world, (ii) Relative toxicity among tested soybean pesticides for *O. bonariensis* was LC > EN > CPF, (iii) AchE activity was more sensitive to CPF inhibition in the head than in the trunk, (iv) effects of binary mixtures of CPF and LC or EN at the lethal level were explained by either CA or IA models, but were slight antagonistic when CPF TUs in the mixture were above 50%; (v) mixture effects on the AchE activity were different among tissues, without effects at the trunk or antagonism at the head; vi) the obtained lethal and AchE concentration–response curves were considerably overlapped, limiting the use of AchE as a good biomarker for assessing sublethal exposure/effects for environmental biomonitoring, vii) the LC_{50}s for CPF and EN were clearly in the same range of the reported surface water concentrations for soybean crop regions, indicating that substantial risk would exist for the species, (viii) lack of information exist on LC surface water concentrations for accurately risk assessment.

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Compliance with ethical standards

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

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Protocols for animal handing and testing were approved by the Animal Welfare Committee of the Facultad de Ciencias Exactas, UNLP. In addition, this article does not contain any studies with human participants performed by any of the authors.

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