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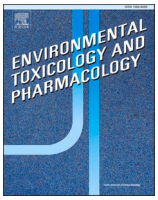

Impaired physiological responses and neurotoxicity induced by a chlorpyrifos-based formulation in *Caenorhabditis elegans* are not solely dependent on the active ingredient

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ABSTRACT

The current massive and indiscriminate agrochemicals usage, which is inexorably linked to the toxic consequences to the environment and people, represents a great concern. Our work aimed to compare the toxicity induced by chlorpyrifos in its pure form (CPF) with that of a commercial formulation containing allegedly inert ingredients (CBCF) using *Caenorhabditis elegans* as *in vivo* model. After a 48 h exposure period, CBCF was 14 times more lethal than CPF; Hatching, brood size, body length and motor-related behavioral parameters were decreased, but these effects were significantly higher in CBCF-exposed worms. Additionally, CBCF induced significant morphological changes in cholinergic neurons, which are associated with the motor-related behavioral parameters. Finally, by analyzing the CBCF, we detected the presence of potentially-toxic metals that were not specified in the label. The presented results highlight the toxicological relevance of components present in the commercial formulations of pesticides, which have been claimed as inert compounds.

1. Introduction

It is estimated that the world population will reach 9.8 billion in 2050, which brings an unprecedented logistical problem since agriculture may not be able to provide enough food for everyone (Bailey-Serres et al., 2019; Kopittke et al., 2019). Agricultural activities provide 98.8 % of food production (Kopittke et al., 2021), however, food crops are vulnerable to pathogens and pests that can threaten their quality and yields (Savary et al., 2019). To fulfill food demand and increase productivity, pest management is the main strategy. Chlorpyrifos (CPF) is an organophosphate insecticide that causes acute neurotoxic action by inhibiting acetylcholinesterase (AChE), leading to cholinergic hyperstimulation and consequent death of the insects attacking the crops (Silva et al., 2017). However, the long-term use of these products imposes environmental consequences and also health issues to farmers and to the subjects that can be non-occupationally exposed by living nearby farms (Dereumeaux et al., 2020).

The use of CPF by agricultural workers has been implicated in several disease-related events, such as genotoxicity (Li et al., 2015); carcinogenicity (Andreotti et al., 2020); reprotoxicity (Alaa-Eldin et al., 2017); endocrine disruption (Otenio et al., 2019), and neurological damage (Abreu-Villaca and Levin, 2017). Neurotoxic effects have been mainly described as neurobehavioral changes such as motor dysfunctions, deficits in attention, information processing, learning and memory, as well as psychiatric symptoms (Freire and Koifman, 2013). Some authors also associate acetylcholinesterase (AchE) activity as a biomarker (Naughton and Terry, 2018), however, there is evidence of non-enzymatic involvement in neurotoxicity induced by organophosphate insecticides

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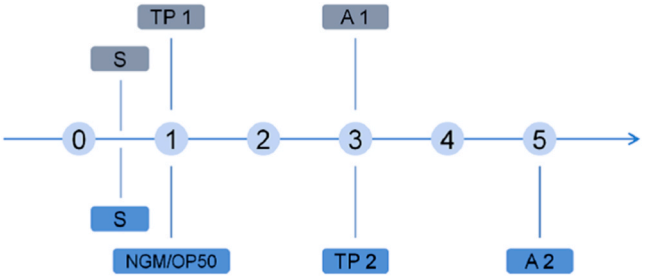


Fig. 1. Experimental design. Our study evaluated the effects of chlorpyrifos formulations in two stages of the life cycle of C. *elegans*. After the synchronization process (S) exposure to pesticides occurred in L1 (TP 1 - Treatment Paradigm 1, item 2.4) or in L4 (TP 2 - Treatment Paradigm 2, item 2.5). The analyses performed in L1 worms (A 1) were: lethality, brood size and body length. The analyses performed in L4 worms (A 2) were: neurobehavioral tests, abnormal neuron morphology and AChE levels. The arrow represents the passage of time in days.

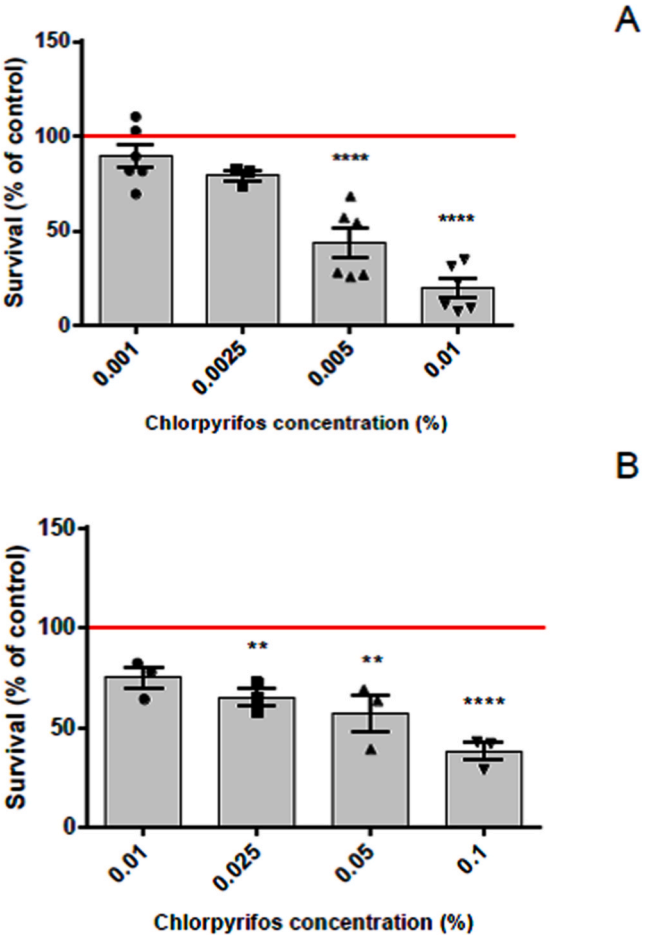


Fig. 2. Survival rate. Chlorpyrifos is 14 times more lethal when in a commercial formulation (CBCF - A) than its active ingredient (CPF - B). The LC_{50} found for CBCF and CPF were 0.004 % and 0.060 %, respectively. Data are expressed as mean ± SEM and analyzed by one-way ANOVA followed by Tukey's post-hoc test. * indicates statistical significance in comparison with the control group (untreated = 100%). * *p < 0.01; * ** *p < 0.0001.

(OP), such as CPF (Rohlman et al., 2011). Non-occupationally exposed individuals have also demonstrated neurological dysfunctions, decline in planning abilities, executive functions, and motor speed and coordination (Ramirez-Santana et al., 2020). Therefore, CPF has been banned in California and Hawaii (USA), and in countries like Denmark and

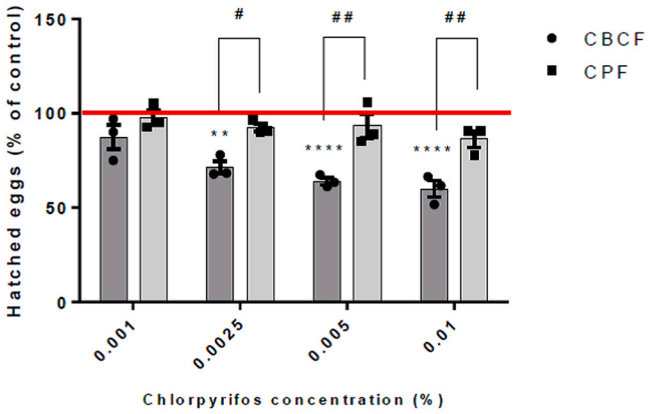


Fig. 3. Brood size. CBCF significantly decreased the number of hatched eggs at concentrations of 0.0025%, 0.005% and 0.01%. None of the CPF concentrations were able to significantly change the number of hatched eggs when compared to the control group. However, when compared to CBCF, they showed a statistical difference. Data are expressed as mean ± SEM and analyzed by two-way ANOVA followed by Tukey's post-hoc test. * indicates statistical significance in comparison with the control group (untreated = 100%). * *p < 0.01; * ** *p < 0.0001. # indicates statistical significance between formulations at the same concentrations. #p < 0.05; ##p < 0.01.

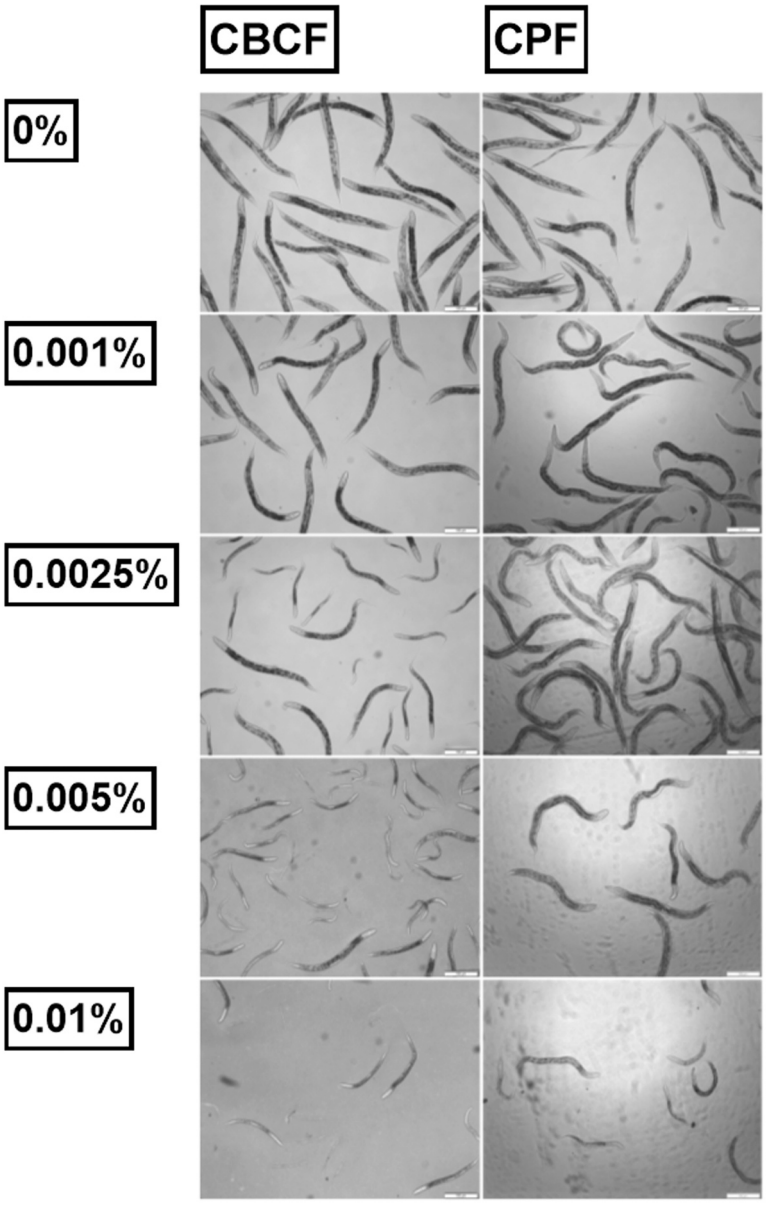
China (Hites, 2021; Sang et al., 2020). However, it is still widely used in Brazil, Argentina and India, countries known for producing and exporting global commodities (Disner et al., 2020; Tames et al., 2020; Verma et al., 2020).

Most of the toxicological studies on CPF have used animal models based on the consumption of the active molecule, thus disregarding the massive environmental and human exposure to the other ingredients of commercial-based CPF formulations. Usually, the composition of commercial pesticide formulations is described in two parts on the label: active ingredient and inert or other ingredients. These "inert" ingredients have no declared composition and may have an adjuvant role in the formulation, therefore contributing to the final toxicity of the formulation (Jacques et al., 2019; Mesnage and Antoniou, 2018; Mesnage et al., 2014). Consequently, the wide application of pesticides combined with the lack of knowledge of certain components of a commercial formulation results in a scenario that aggravates the risks to human and environmental health.

In order to fulfill this gap, the use of the invertebrate model *Caenorhabditis elegans (C. elegans)* has been one the most useful tools for toxicological investigations. This nematode combines the advantages of presenting a high homology to mammals (relevant for translational studies) and the fact that one of its habitat is the soil (relevant for environmental impact studies). *C. elegans* has a short life cycle, can be easily manipulated and allows uncountable possibilities for toxicological screening (Gao et al., 2018; Hunt, 2017). Within the context of neurotoxicological research, the hermaphrodite nematode presents 302 neurons, and the major neurotransmitter systems are conserved with mammals such as serotonergic, GABAergic, dopaminergic, glutamatergic and cholinergic (Ruszkiewicz et al., 2018). Besides, their body transparency enables the visualization of neurons tagged with GFP *in vivo* (McVey et al., 2016; Negga et al., 2012). Organophosphates, such as CPF, display particular toxicities toward the cholinergic system, which has been reported to control countless events related to the worm's development, survival and homeostasis (Treinin and Jin, 2021). Of note, *C. elegans* has 160 cholinergic neurons distributed in head, tail, pharynx and ventral and dorsal nerve body. Besides, the biosynthesis (choline acetyltransferase), catabolism (AChE) and vesicular transport/storage (vesicular acetylcholine transporter) processes are very similar with mammals (Loer and Rand, 2010; Pereira et al., 2015).

Considering that (i) the massive application of CPF pollutes and disturbs the environmental balance, (ii) threatens human and animal

A



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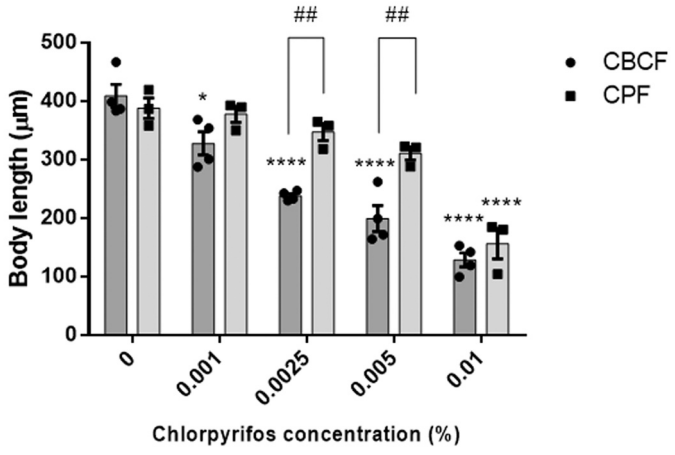

Fig. 4. Body length. CBCF significantly reduced the length of worms at all concentrations tested (B), when compared to the control group. When the comparison was made between the formulations, CBCF was different from CPF in the concentrations of 0.0025 % and 0.005 %. CPF decreases the length of worms only at the highest concentration (0.01 %), when compared to the control group. In A, the representative images of worms length from each treatment are shown. Data are expressed as mean ± SEM and analyzed by two-way ANOVA followed by Tukey's post-hoc test. * indicates statistical significance in comparison with the control group (0%). *$p < 0.05$; * ** *$p < 0.0001$. # indicates statistical significance between formulations at the same concentrations. ##$p < 0.01$.

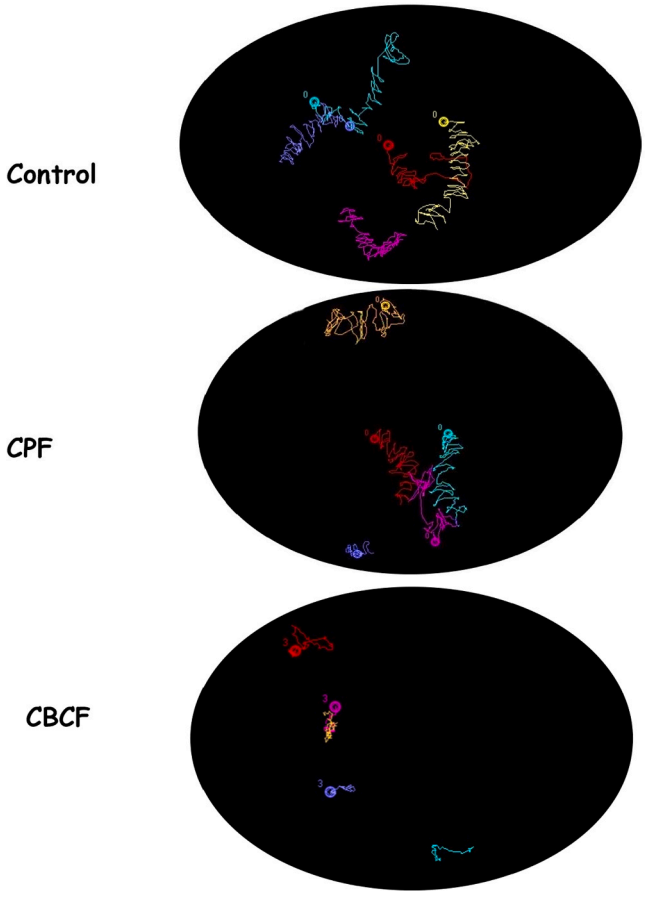

Fig. 5. Path length assay. CBCF significantly reduced the distance traveled by the worms, representative paths images of control; CPF and CBCF groups, respectively. Each color line represents one nematode.

health, and (iii) has commercial versions with unknown ingredients, our study aimed to compare the toxicity induced by chlorpyrifos (a widely-used insecticide) in its pure form (CPF) with a commercial formulation containing inert ingredients (CBCF) using *C. elegans* as *in vivo* experimental model. We hypothesized that the addition of unspecified ingredients to the commercial formulation results in greater toxicity in our model. The mentioned comparison was based on the evaluation of physiological endpoints (reproductive, developmental and behavioral parameters), as well as in the evaluation of cholinergic neurons and acetylcholinesterase activity. Once confirmed, such hypothesis could highlight concerns related to environmental and human impacts.

2. Materials and methods

2.1. Chemicals/reagents

The chlorpyrifos analytical standard (PESTANAL® - Sigma-Aldrich) was used as an isolated form of chlorpyrifos (CPF). The final 4.8,% stock solution was made with chlorpyrifos in acetone (99.5,% purity). Lorsban® 480 BR (Dow AgroSciences) was used in this work as a chlorpyrifos-based commercial formulation (CBCF) and was locally acquired. Its label describes the concentration of 48% (m/v) chlorpyrifos and approximately 10,% of "other ingredients" (inert ingredients). The remaining percentage of the product consists of a "mixture of heavy aromatic hydrocarbons", including the solvent naphtha (petroleum). CBCF was diluted in acetone to a 4.8,% stock solution. All other reagents, if not specified, were of the highest grade commercially available.

2.2. Strains and maintenance

Worms were purchased from the Caenorhabditis Genetics Center, Minnesota, USA. The strains used were N2 (wild type) and LX929 (*vsls48 [unc-17::gfp]*, which has GFP expressed in all cholinergic neurons. All worms were kept in plastic dishes containing NGM (nematode growth media) coated with *Escherichia coli* OP50. The animals were grown and handled at a temperature of 20 °C.

2.3. Synchronization process

Animals were submitted to a synchronization process to obtain a population at the first larval stage (L1) (Soares et al., 2020). Briefly, gravid adults were treated with a lysis solution composed of 1 mL 10 M NaOH (0.25 %), 4 mL NaOCl (1 %) and 5 mL distilled H_2O. The eggs obtained were kept in M9 buffer at 20 ºC. After 14 h, a L1 age-synchronized population was obtained.

2.4. Exposure paradigm 1: protocol and toxic endpoints

Exposure to different concentrations of CPF and CBCF formulations at the L1 larval stage was named as paradigm 1. The protocol started with the synchronization process (item 2.3). The animals (1500 L1 worms) were exposed for 30 min in liquid medium (0.5 % NaCl) to the formulations or to the vehicle (control). Afterwards, the liquid medium with the respective treatments were transferred to NGM plates with E. *coli* OP50 (Jacques et al., 2019). After 48 h exposure, lethality, reproduction and growth of the animals were analyzed through survival, brood size and body length (Fig. 1A).

2.4.1. Survival and lethal concentration 50 % (LC_{50})

The survival analysis of worms challenged by different concentrations of CPF and CBCF formulations within paradigm 1 was performed to obtain the LC_{50}. Briefly, a transparent grid was placed underneath the plate and the live worms (responsive to touch) in 25 quadrants were counted. All data obtained here were plotted in a sigmoidal dose-response model, with a top constraint at 100% for calculating the LC_{50} with Graphpad Prism 6 (Jacques et al., 2019).

2.4.2. Brood size

Worms exposed to paradigm 1 were transferred individually and daily to NGM/OP50 plates until the end of their reproductive period. Then, the number of hatched eggs per worm was used for the brood size analysis. This assessment was repeated 3 times and every experiment was done in duplicates.

2.4.3. Body length

Worms exposed to pesticides according to paradigm 1 were transferred to microscope slides with levamisole (1 mM). Images were obtained for further analysis of the head-tail length using the ImageJ software. A total of 10 worms per group were used for each independent experiment (30 animals total in three independent experiments).

2.5. Exposure paradigm 2: exposure protocol and neurotoxicity endpoints

Paradigm 2 consisted of exposing L4 worms to a non-lethal concentration of 0.0025% CPF or CBCF for 48 h. The use of an older larval stage facilitates the morphological analysis of neurons in the LX929 strain, as well as the investigations involving neurobehavioral and acetylcholinesterase (AChE) activity determination. The protocol of paradigm 2 started with synchronization (described in Section 2.3) and after the eggs hatched, the worms were transferred to NGM/*E. coli* OP50 plates for 48 h until reaching L4 stage. Exposure to the concentration 0.0025 % of CPF or CBCF was conducted for 30 min in a liquid medium and subsequently in NGM/OP50 for 48 h (Fig. 1B).

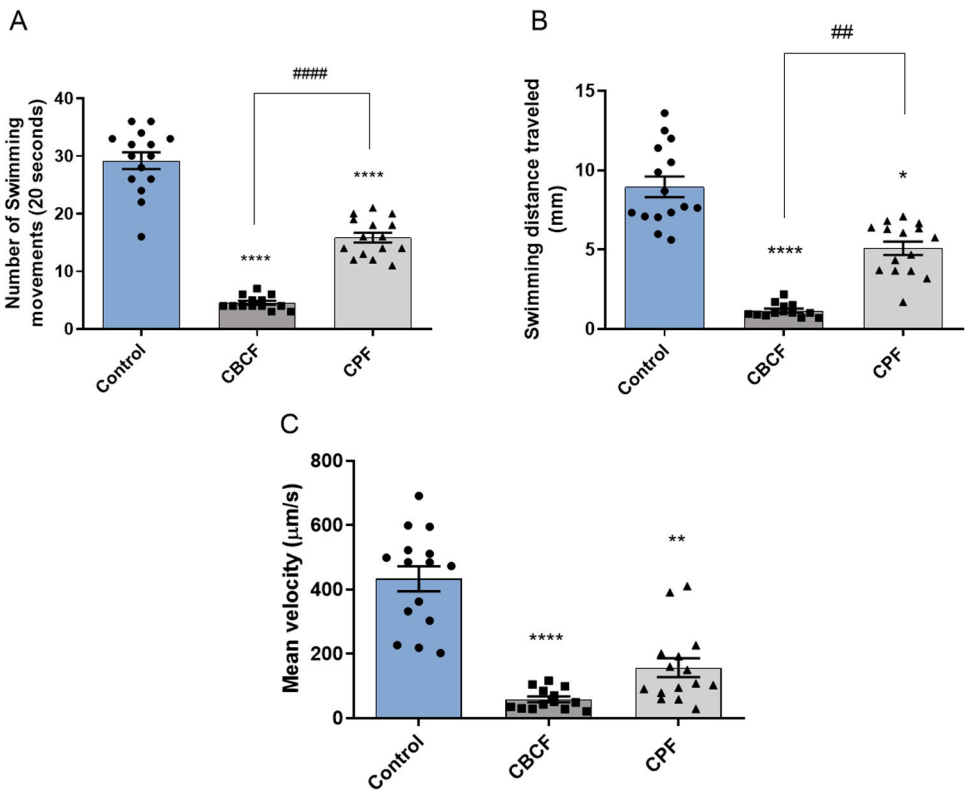

Fig. 6. Behavioral tests. CBCF significantly reduced the worm's locomotion, represented by swimming movements (A); distance traveled (B) and velocity (C). Graphs A and B were analyzed by one-way ANOVA followed by Tukey's post-hoc test. Graph C was analyzed by Kruskal-Wallis followed by Dunn's *post hoc* test. All data are expressed as mean ± SEM. * indicates statistical significance in comparison with the control group. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$. # indicates statistical significance between formulations at the same concentrations. ##$p < 0.01$; ####$p < 0.0001$.

2.5.1. Neurobehavioral tests

Locomotor parameters were performed according to Soares et al. (2020), with minor modifications. Movement of five exposed worms and the control group as described in Section 2.5 were recorded for 20 s (15 frames per second). Average speed, swimming movements and path length were calculated. At least three independent experiments were performed (n = 15 worms per group).

2.5.2. Morphological analysis of cholinergic neurons

The investigation of abnormal neuronal morphology (ANM) induced by chlorpyrifos formulations was performed in the LX929 strain. This transgenic strain has a GFP reporter in the *unc-17* gene, which encodes a vesicular acetylcholine transporter, allowing fluorescent visualization of all cholinergic neurons (Alfonso et al., 1993; Haque and Nazir, 2016). At the end of the treatment, worms were photographed in the EVOS FLoid Cell Imaging Station microscope (Thermo Fisher Scientific). Ten worms per group were randomly analyzed for each individual experiment. The conduction of this protocol and the criteria for morphological abnormalities were established according to a previous study (Soares et al., 2020). The ANMs recorded were "Y" shaped commissures and neuronal blebbing.

2.5.3. Acetylcholinesterase activity

Acetylcholinesterase activity was investigated and performed according to Ellman's method (Ellman et al., 1961). Briefly, a homogenate of 500 worms per group (paradigm exposure 2) was obtained through sonication (3 times of 15 s, on ice), with cooling intervals. The homogenate was centrifuged at 10,000 rpm. The supernatant was mixed with DTNB (10 mM) and acetylthiocholine iodide (8 mM) in 96-well plates, and were immediately monitored in a microplate reader at 405 nm (Spectramax M5 Molecular Devices) for 5 min, with readings at every 30 s at a temperature of 25 °C (Cole et al., 2004; Joshi et al., 2018). The results were normalized by protein, which was quantified from the supernatant by the Bradford method (Bradford, 1976; Joshi et al., 2018). The data were first calculated as µmol/h/mg protein and then normalized as percentage of control.

2.6. Metal analysis in CBCF

The concentration of Mn, Fe, Cu, Zn, As, Cd, Pb and Hg was determined using ICP-MS/MS (Agilent 8800 ICP-QQQ). The nebulizer gas flow and parameters of lenses, Q1, collision cell and Q2 were tuned daily on a daily basis for maximum sensitivity. Mn, Fe, Cu, Zn, As, Cd and Pb were measured as described in (Meyer et al., 2018). The Hg measurement was performed as previously described (Lohren et al., 2015).

2.7. Statistical analysis

Data were submitted to the Shapiro-Wilk normality test for verification of normal or non-normal distribution. All tests were performed in duplicate, repeated at least 3 times and expressed as mean ± SEM or SD, depending on the type of assay. The individual variation of each experiment (n) is indicated in the graphics through the scatter dot plot configuration. All data obtained in the analysis were subjected to statistical treatment using the GraphPad Prism 6 software. Data with Gaussian distribution were analyzed by one- or two-way ANOVA, depending on the experimental design, followed by Tukey's *post hoc* test. For the behavioral assays (non-parametric data) we used Kruskal-Wallis, followed by Dunn's *post hoc* test.

3. Results

3.1. CBCF is more lethal than CPF

Worms were exposed to different concentrations of CBCF or CPF and their LC_{50} were calculated. CBCF was not lethal at concentrations of 0.001 % and 0.0025 %, but caused significant lethality at 0.005 % and 0.01 % (Fig. 2A). The LC_{50} of CBCF (0.004%) was approximately 14 times lower as compared to CPF. Of note, CPF was not lethal at 0.01 %, whereas this concentration caused an average survival of 19.8 % in

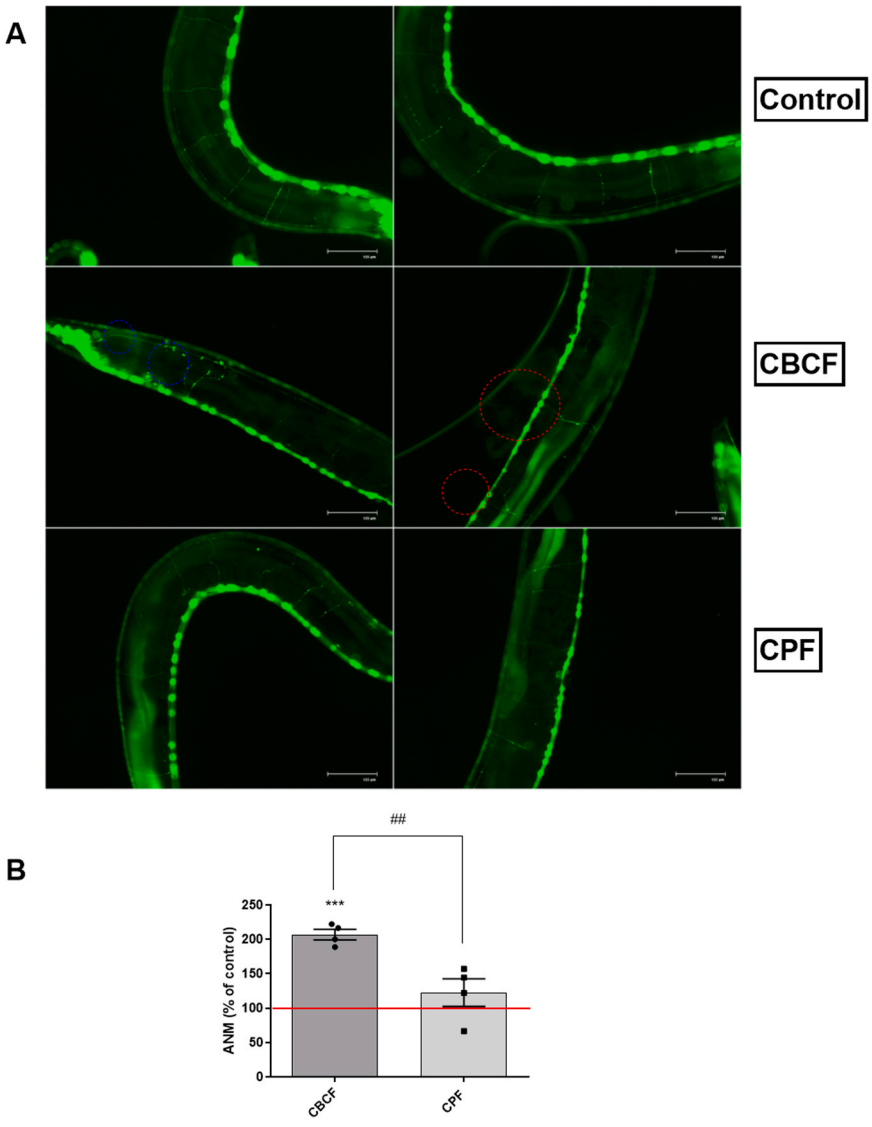

Fig. 7. ANM analysis. CBCF induced abnormal formations in cholinergic neurons when compared to control group and CPF, at the concentration of 0.0025%, representative images of neurons (A) and quantification of ANM (B). Blue circle shows the neuronal blebbing. Red circle shows "Y" shaped commissures. Data are expressed as mean ± SEM and analyzed by one-way ANOVA followed by Tukey's post-hoc test. * indicates statistical significance in comparison with the control group (untreated = 100 %). * **$p < 0.001$. # indicates statistical significance between formulations at the same concentrations. ##$p < 0.01$.

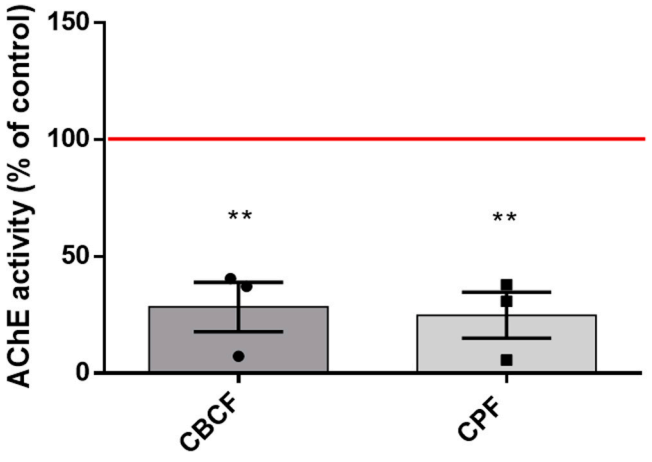

Fig. 8. AChE activity. Both CPF and CBCF at a non-lethal concentration of 0.0025% were able to significantly inhibit acetylcholinesterase when compared to the control group. Data are expressed as mean ± SEM and analyzed by one-way ANOVA followed by Tukey's post-hoc test. * indicates statistical significance in comparison with the control group (untreated = 100%). * *$p < 0.01$.

Table 1
Metals quantified in CBCF by ICP-MS.

Metal	Concentration (mg/L)
Zn	1.16
Mn	2.16
Fe	1497.76
Hg	$< 4.7 \times 10^{-5}$
Cu	< 0.00142
As	$< 6.7 \times 10^{-5}$
Cd	$< 2.7 \times 10^{-5}$
Pb	< 0.000145
Al	< 0.00194

CBCF (Fig. 2A and B, note the different X-axis concentrations).

3.2. CBCF decreased brood size

The number of hatched eggs was not affected at the concentration of 0.001 % for both formulations. However, there was a significant reduction in brood size at 0.0025 %, 0.005 % and 0.01 % of CBCF compared to the control group (Fig. 3). The 0.0025 % concentration of CBCF which wasn't lethal (Fig. 2A) was sufficient to cause a significant decrease in brood size. On the other hand, all tested sub-toxic CPF

concentrations were not able to reduce the number of hatched eggs when compared to the control group (Fig. 3).

3.3. CBCF decreased the length of the worms at all concentrations

CBCF caused significant decreases in worm length (at all concentrations tested) compared to the control group (Fig. 4A and B). This event was observed even at the low concentration (0.001 %), which did not cause significant effects on lethality and brood size. CBCF exposure significantly decreased the length of worms at concentrations of 0.0025 % and 0.005 % compared to CPF (Fig. 4B). The decrease in the length of worms exposed to CPF was observed only at the highest concentration (0.01 %), showing again that the commercial formulation induced significant toxic effects at lower concentrations when compared to the active ingredient (CPF).

3.4. CBCF affects locomotion behaviors

The exposure to the commercial formulation significantly affected the locomotion pattern of nematodes in comparison to control group (untreated) and to CPF at the same concentration (0.0025 %). As shown in Fig. 6, there were significant decrease in the number of swimming movements (Fig. 6A) and speed (Fig. 6C). In addition, CBCF-exposed worms exhibited a reduction of path length, as demonstrated in Fig. 6B and representative images (Fig. 5).

3.5. CBCF induced abnormal morphology in cholinergic neurons

The morphology of cholinergic neurons was observed in LX929 transgenic animals and compared to untreated animals. Exposure to a non-lethal concentration of 0.0025 % of CPF did not significantly alter the neurons morphology (Fig. 7A). However, this concentration of CBCF induced the appearance of abnormal neuronal morphology, such as "Y" shaped commissures and neuronal blebbing in exposed worms (Fig. 7A). The number of morphological changes were statistically significant when compared to the control group and CPF (Fig. 7B).

3.6. CBCF and CPF inhibited AChE at the same level

Chlorpyrifos is an organophosphate that irreversibly inhibits AChE (Colović et al., 2013). We initially expected differences in the intensity of these inhibitions when using a commercial formulation. However, both formulations, at the same non-lethal concentration of 0.0025 %, similarly inhibited 50 % of AChE activity when compared to the control group (Fig. 8).

3.7. CBCF has metals as components of the inert ingredients

The chlorpyrifos commercial formulation analyzed in this study disclosed in its label the presence of chlorpyrifos at the concentration of 48 %. The remaining percentage is mostly composed of "a mixture of heavy aromatic hydrocarbons"; however, approximately 10% of the composition is unknown, because it was classified as "other ingredients" on the label. The chemical variety that covers this trade secret makes it difficult to analyze and fully understand all the components of the formulation. However, studies report the presence of metals in the inert portion of commercial pesticide formulations (Defarge et al., 2018; Jacques et al., 2019). Therefore, the presence of metals was investigated at the CBCF. Quantifiable amounts of Zn, Mn and Fe were found, as depicted in Table 1.

4. Discussion

In this study, we presented a toxicological comparison between CPF (pure active ingredient) and a commercially-available formulation using the *in vivo* model *C. elegans*. We found that worms exposed to a non-lethal (0.0025%) concentration of CBCF displayed impairment in all physiological parameters evaluated, when compared to animals that were exposed to the same concentration of CPF only. In particular, worms exposed to 0.0025% CBCF reduced their viable egg laying and body length by approximately 40%. The data gathered in this work demonstrate the potential of the *C. elegans* model to evidence interactions with complex substances, such as pesticide formulations. These observations, unlike *in vitro* models, have the advantages of a whole animal, *i.e.*, the presence of specialized tissues such as the digestive, reproductive, neuromuscular and sensory systems (Wittkowski et al., 2019). The *C. elegans* model is a useful tool that can be used to screen molecules of interest, contributing to the rational and reduced use of mammals, in addition to having a reported ability to predict and replicate the toxic effects of various substances in mammals (Cole et al., 2004; Harlow et al., 2016; Li et al., 2013).

It is known that neurobehavioral alterations can predict neuronal damage (Legradi et al., 2018; Leung et al., 2008). Particularly in *C. elegans*, behaviors associated with specific classes of neurons have been highly correlated with their morphological alterations (Schetinger et al., 2019; Xu et al., 2017). One example is the characteristic sinusoidal movement of nematodes, which is mediated by cholinergic and GABAergic neurotransmission for muscular contraction and relaxation, respectively, thus being crucial for the normal function of motor neurons that are connected by commissures from the dorsal to the ventral cords (Gjorgjieva et al., 2014). Neurotoxic effects induced by OPs have been extensively reported in the literature (Jokanović, 2018; Naughton and Terry, 2018), but additional toxicodynamics mechanisms besides AChE inhibition and the role of the inert ingredients remain unknown. Although both formulations inhibited AChE at the same level, only CBCF exposure caused significant impairments in physiological endpoints such as survival rate, brood size, development and morphology of cholinergic neurons. Such observations indicate that AChE inhibition alone is not sufficient to cause the mentioned deleterious effects, once the active ingredient CPF, at the same concentrations, was not able to reduce survival rate, brood size, body length, nor was able to generate morphological abnormalities in cholinergic neurons, although inhibiting AChE at the same level as CBCF (Fig. 8). Therefore, our results indicate that the other ingredients present in CBCF, also euphemistically called "inert ingredients", offer an important additional toxicity. Although our results do not provide specific mechanisms involved in such additional toxicity, it is possible to state that they are not exclusively related to the inhibition of AChE. This is in line with recent literature data indicating that the toxicity induced by organophosphate pesticides, including CPF, represents the consequence of toxic events that is beyond AChE inhibition (Naime et al., 2020). Fig. 8.

Upon occupational and non-occupational exposure to CBCF, subjects demonstrated neurobehavioral deficits as evidenced in neurobehavioral tests such as learning focus (Ismail et al., 2017) and locomotor tests in farmers and in residents that live nearby farms (M Khan et al., 2019). An *in vivo* study with mice also demonstrated that animals exposed to a CBCF showed a decrease in the number of hippocampal neuronal cells (Mitra et al., 2009). A recent study reinforced that exposure to CBCF promotes a significant impairment in neurological tests of exploratory behavior and muscle coordination (Kudavidanage et al., 2020). In C. *elegans,* some authors have shown that nematodes exposed to pure CPF exhibited locomotion deficits, as evidenced by reduction in the number of body bends, in path length (Ju et al., 2014) and head thrash frequency (Ruan et al., 2009). Notably, in these studies, only the behavioral alterations were assessed, but no observation of the neuronal morphology was performed. Indeed, there are few *in vivo* studies correlating neurobehavioral changes with cholinergic neuronal damage (Anderson et al., 2004; Boyd et al., 2010; Ju et al., 2014; Ruan et al., 2009), and no studies reporting neurotoxic effects when comparing commercial formulation *versus* active ingredient in *in vivo* models (Nagy et al., 2020).

Considering the above, the unknown composition of the commercial pesticide formulations is a limitation. For example, the CBCF analyzed in

our work has a constitution declared as "a mixture of heavy aromatic hydrocarbons" and approximately 10% of other ingredients. Therefore, if there is no knowledge of the real identity of the toxic elements, their individual and comparative analysis becomes more complex, adding a higher degree of experimental difficulty when attributing a hypothesis and an effect to a given constituent. The lack of complete and proper information concerning the chemical composition of commercial formulations is still supported by toxicological studies that evaluate active ingredients and other ingredients alone and not associated, which can generate toxicological profiles that do not compromise with the reality of the final commercial formulation (Nagy et al., 2020). A recent review using database search on studies that evaluated the toxicity of commercial pesticide formulations compared to their declared active ingredient(s) showed that 24 of 36 of them reported increased toxicity of the commercial formulation due to the addition of adjuvants or inert ingredients (Nagy et al., 2020). Of note, these studies used several *in vitro* and *in vivo* models, focusing mainly on cytotoxicity, embryo toxicity and lethality, and no studies with *in vivo* models investigating neurotoxic effects with C. *elegans* were described (Nagy et al., 2020). Interestingly, we have found significant levels of neurotoxic metals as Zn, Mn and Fe, metals that are not declared on the CBCF label and which are part of the "inert" ingredient portion, as already reported for commercial glyphosate formulations (Defarge et al., 2018; Jacques et al., 2019).

The investigation of undeclared heavy metals in commercial pesticide formulations is relatively recent. Our group has previously reported the presence of Hg, Fe, Mn, Cu, Zn, As, Cd and Pb in a commercial formulation of glyphosate (Jacques et al., 2019). The toxic effects observed in physiological, behavioral and cellular endpoints may be a consequence of the interaction of these metals alone or associated with the active ingredient and/or other components of the CBCF. Notably, detrimental effects on longevity, body size, brood size and behavior have been observed in worms exposed to Fe, Mn or Zn alone (Hu et al., 2008; Lin et al., 2006; Valentini et al., 2012; Wang et al., 2007; Xiao et al., 2009). However, the concentration of these metals may be relatively insufficient to generate the observed effects. In our previous study, we evaluated such probability, where a mixture of Fe, Mn, Cu, Zn, As, Cd and Pb, at concentrations higher than those found in this work (except for Fe) was unable to reduce worms brood size (Jacques et al., 2019). Hence, we believe that the association of the several chemicals (organic and inorganic) presented in the formulations are responsible for the potentiation of the neurotoxic effects of CPF.

Despite the difficulty in evaluating undeclared constituents of unknown identity, there is growing literature on this topic and the results obtained in this work contribute to this field (for a review see Nagy et al., 2020). Regardless of the hypotheses, *i.e.*, whether there is a synergy mechanism between active and inert ingredients, or distinct toxicological mechanisms (or both), the interaction between other ingredients and the active ingredients in the commercial formulation should not be disregarded in toxicological terms. This notion has already started to be assimilated in some countries, resulting in restrictions of certain inert ingredients, such as polyethoxylated tallowamine (POEA), a surfactant widely used in glyphosate formulations, which was banned by the European Commission in 2016 due to its greater toxicity than the active ingredient itself (Nagy et al., 2020). Thus, we expect that our work, together with the literature cited, will reinforce the need for further investigation into the constituents of commercial pesticide formulations and updates in the regulation of these products.

5. Conclusion

Our work presented a toxicological comparison between a CBCF and its active ingredient CPF using the experimental model *C. elegans*. We have shown that at an equimolar concentration range, only CBCF was able to reduce survival, brood size and body length, and caused greater behavioral impairments and neuronal morphological anomalies. Since the addition of adjuvants and the so called inert ingredients significantly increases the final toxicity of the formulation, the omission of the identity of these constituents in the labels must be reconsidered.

CRediT authorship contribution statement

Mauricio Tavares: Performed the experiments, analyzed the data, and Writing- Original draft preparation. **Marcell Valandro:** Conduced some experiments and reviewing and editing the final version of the manuscript**. Julia Bornhorst and Tanja Schwerdtle:** Conducted the ICP-MS experiments of metals quantification in worms. **Marcelo Farina and Daiana Ávila:** Supervision, reviewing and editing the final manuscript.

Declaration of Competing Interest

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

Data availability

No data was used for the research described in the article.

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