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Reprotoxicity of glyphosate-based formulation in *Caenorhabditis elegans* is not due to the active ingredient only☆

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ABSTRACT

Pesticides guarantee us high productivity in agriculture, but the long-term costs have proved too high. Acute and chronic intoxication of humans and animals, contamination of soil, water and food are the consequences of the current demand and sales of these products. In addition, pesticides such as glyphosate are sold in commercial formulations which have inert ingredients, substances with unknown composition and proportion. Facing this scenario, toxicological studies that investigate the interaction between the active principle and the inert ingredients are necessary. The following work proposed comparative toxicology studies between glyphosate and its commercial formulation using the alternative model *Caenorhabditis elegans*. Worms were exposed to different concentrations of the active ingredient (glyphosate in monoisopropylamine salt) and its commercial formulation. Reproductive capacity was evaluated through brood size, morphological analysis of oocytes and through the MD701 strain (bcIs39), which allows the visualization of germ cells in apoptosis. In addition, the metal composition in the commercial formulation was analyzed by ICP-MS. Only the commercial formulation of glyphosate showed significant negative effects on brood size, body length, oocyte size, and the number of apoptotic cells. Metal analysis showed the presence of Hg, Fe, Mn, Cu, Zn, As, Cd and Pb in the commercial formulation, which did not cause reprotoxicity at the concentrations found. However, metals can bioaccumulate in soil and water and cause environmental impacts. Finally, we demonstrated that the addition of inert ingredients increased the toxic profile of the active ingredient glyphosate in C. elegans, which reinforces the need of components description in the product labels.

1. Introduction

Glyphosate is the most widely produced and sold herbicide worldwide and has been on the market since 1974 (Duke and Powles, 2008). The sales success is mainly due to its supposed low toxicity due to its mechanism of action: blocking the shikimic acid pathway by the inhibition of the enzyme 5-enolpyruvylshikimate 3-phosphate synthase, which is absent in mammals (Duke and Powles, 2008). Another factor for glyphosate commercial success is the possibility of using transgenic plants resistant to the herbicide, for example, soybean and corn. Glyphosate acts in a non-selective manner, eliminating any plants that come in contact with it, except for those genetically modified. Due to the constant application over time, the emergence of unwanted cultures tolerant to glyphosate has occurred (Benbrook, 2012; Powles, 2008).

The use of GMOs (Genetically Modified Organisms) was essential for glyphosate-based herbicides to be heavily applied

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worldwide. In the U.S., it is estimated that over 1.6 billion kilograms of glyphosate have been applied (between 1974 and 2014) and two-thirds of this amount were sprayed in the last 10 years, a proportional increase resulting from the introduction of genetically engineered glyphosate-tolerant crops in 1996 (Benbrook, 2016). It is worth mentioning that glyphosate-tolerant GMOs do not metabolize or excrete this active principle, only accumulate during their growth (Arregui et al., 2004; Mesnage et al., 2015). Even GMO-free crops are subject to exposure to glyphosate during pre-harvest crop desiccation (Mesnage et al., 2015). The long history of use of glyphosate, added to these factors brings us to the obvious scenario, a self-fed cycle of drinking water, soils and food contamination, causing exposure to humans and animals (Bøhn et al., 2014; Mesnage et al., 2015; Myers et al., 2016; Peruzzo et al., 2008; Samsel and Seneff, 2015).

However, another variable must be added to the equation: Pesticides are used in the form of commercial formulations, which have large quantities of the so-called inert ingredients. These components are not specified in the labels, but some studies have investigated their identity. In commercial formulations of glyphosate were found co-formulants such as polyoxyethylenamines (polyethoxylated tallowamine), alkyl polyglucoside, quaternary ammonium compounds and heavy metals (Defarge et al., 2018; Defarge et al., 2016; Mesnage et al., 2013). Interestingly, studies have shown that they contribute even more to the final toxicity of the formulation and may in some cases be the main factors for certain toxicity (Mesnage and Antoniou, 2018; Mesnage et al., 2013; Mesnage et al., 2014; Negga et al., 2011; Richard et al., 2005). There are few studies analyzing the constitution and interaction of the inert ingredients with their active principle in *in vivo* models. However, toxicological studies of glyphosate or its commercial formulation are common, particularly toxicity to reproduction and development. Necrosis and apoptosis in rat testicular cells and decreased weight of seminal vesicle gland in rats have been reported (Clair et al., 2012; Dai et al., 2016; Ingaramo et al., 2016). Studies in less common models are also described: glyphosate caused DNA damage on *Caiman latirostris* embryos and reduced reproduction of the earthworm *Lumbricus terrestris* (Burella et al., 2017; Gaupp-Berghausen et al., 2015). Furthermore, studies on apoptosis and necrosis of human umbilical, embryonic and placental cells induced by glyphosate have been reported (Benachour and Séralini, 2009; Benachour et al., 2007). Finally, aquatic species such as frogs are the targets of toxicological studies, where glyphosate impairs the normal development of the species (Bach et al., 2016; Lanctôt et al., 2014; Wagner et al., 2017). It is worth mentioning that glyphosate obtained a 2A (probably carcinogenic to humans) classification by the International Agency for Research on Cancer (the specialized cancer agency of the World Health Organization) (Guyton et al., 2015).

Considering the increasing and global application of glyphosate and consequently the unknown composition of the formulations, studies aiming the comparison and interaction of the inert components with the active principle are necessary. Nevertheless, toxicology studies with vertebrates have been widely criticized, but there is a need to study toxicants in *in vivo* models. Hence, for this study we proposed the use of the *Caenorhabditis elegans* (C. *elegans*) as animal model. This worm has been used as a useful tool in experimental toxicology due to the high degree (60–80%) of homology between the genome of humans. Its easy maintenance, handling, fast lifecycle, short longevity (approximately 20 days), transparent body, facilitated generation of deletion-type mutations in genes of interest, the existence of several transgenic strains expressing green fluorescent protein (GFP) (Chalfie et al., 1994) and is in agreement to the 3R policy. Additionally, it is an experimental model of great relevance in reproductive toxicology. C. *elegans* has a short reproductive cycle, it is possible in about three days to obtain a physiological endpoint, particularly sensitive to heavy metals, chemicals and pesticides (Allard et al., 2013; Daniela A et al., 2015; Du et al., 2015; Jiang et al., 2016; Wang et al., 2014).

In summary, a commercial pesticide formulation will be composed of its active ingredient and inert ingredients. Due to solubility problems the most commonly used form of glyphosate in commercial formulations (including Roundup Original®) is the monoisopropylamine salt (Adcock et al., 2004; Lanctôt et al., 2014). With the growing literature indicating the significant role of inert ingredients in the high toxicity of GBCFs, the aim of this study was to compare the reproductive toxicity of the glyphosate salt versus a glyphosate-based commercial formulation commonly used in Brazil (Benachour and Séralini, 2009; Gasnier et al., 2009; Mesnage et al., 2013; Mesnage et al., 2014). In addition, due to a recent study identifying the presence of heavy metals in pesticides commercial formulations, the need to reproduce and uncover the other constituents of pesticide formulations and their impact on the environment, we investigated the presence of heavy metals in a glyphosate-based commercial formulation (Defarge et al., 2018).

2. Materials and methods

2.1. Chemicals

2.1.1. Glyphosate

The isolated form of glyphosate (N-Phosphonomethyl glycine, monoisopropylamine salt solution- GMIPA) was obtained from Sigma-Aldrich, with a concentration of 40 wt% solution in water, the same used in several toxicological and analytical studies which also used this very same salt without further purification (Adcock et al., 2004; Cuhra et al., 2013; Diaz Kirmser et al., 2010; Hansen and Roslev, 2016; Hassoon and Schechter, 2000; Lipok et al., 2010). This solution was diluted to 1% (m/v) (all dilutions were made with deionized water), the same concentration of the commercial formulation.

2.1.2. Glyphosate-based commercial formulation

The glyphosate-based commercial formulation (GBCF) chosen for this study was Termifin - Dexter Latina, a common formulation used in Brazil. This formulation has the same percentage of active ingredient as Roundup® Ready-To-Use Extended Control, also containing 1% glyphosate in the form of monoisopropylamine salt.

2.2. Maintenance of the worms

C. elegans were obtained from *Caenorhabitis* Genetics Center, Minnesota, USA and maintained in Petri dishes containing NGM (nematode growth media) with *E. coli* OP50 and kept in incubators at 20 °C. The strains used include N2 (wild type) and MD701 (bcIs39). MD701 expresses functional CED-1:: GFP fusion protein in the sheath cells, which facilitates the visualization of apoptotic corpses of germ cells (Cheng et al., 2014; Ruan et al., 2012). The worms at the first larval stage (L1) were obtained by a synchronization process (Brenner, 1974).

2.3. Pesticides exposure

2.3.1. Development paradigm

After 14 h of the synchronization process, hatched larvae were treated with different concentrations of pesticides in the commercial glyphosate form (GBCF) or glyphosate in monoisopropylamine salt (GMIPA salt). Concentrations were calculated as

percentage of active ingredient (0.010–0.015%). These concentrations were chosen based on the lethality test, ranging from non-lethal to almost LC_{100}. First, 1500 L1 worms were exposed for 30 min, in liquid medium to GBCF or GMIPA salt; subsequently, the liquid medium with treatments of each group, was transferred to Petri dishes with NGM and *E. coli* OP50 for 48 h (Jacques et al., 2017; Negga et al., 2011). The analyses were performed after 48 h exposure.

2.3.2. Reprotoxicity endpoints

For these assays, the time of exposure, handling and treatments were all the same as described in 2.3.1, with the exception that worms were, at the beginning of exposure, at the L4 larval stage (500 worms), exposed as well for 48 h. This difference is justified for further directing the reprotoxicity (reproductive maturation of the worm) and better use of the strain MD701 following, with few changes, protocols with similar studies (Cheng et al., 2014; Ruan et al., 2012). All concentrations used in this paradigm were non-lethal to L4 worms (0.010% and 0.014%).

2.4. Lethal concentration 50% (LC_{50})

The lethality curve and LC_{50} were determined for both paradigms (worms exposed from stages L1 and L4). After the end of the exposure to both pesticides forms, the survival rate was counted: a transparent grid was placed beneath the NGM plate and 25 quadrants were analyzed under a dissection microscope, where the live worms were differentiated from dead worms (motionless and with no reaction to a platinum wire touch). A sigmoidal dose-response model, with a top constraint at 100%, was used to draw the curves and obtain the LC_{50} with Graphpad Prism 6 (Jacques et al., 2017).

2.5. Body length

Using the paradigm exposure described in 2.3.1, the length of L1 worms exposed for 48 h to GBCF or GMIPA salt was analyzed. Briefly, worms were fixed in microscope slides (with levamisole 1 mM) and photos were obtained. Then, these photos were analyzed through ImageJ software, where the length from the head to tail was measured.

2.6. Reproductive capacity analysis

The reproductive capacity was assessed through three assays: brood size, oocytes size and morphology and the number of germ cells in apoptosis (MD701 strain). All analysis described below and related to reproductive capacity were made at the end of the exposure paradigm described in 2.3.2.

2.6.1. Brood size

Treated worms were transferred individually to a NGM/OP50 plate. The number of hatched eggs was counted daily, and the P0 worm daily transferred to another plate to facilitate counting.

2.6.2. Size and morphology of oocytes

Treated worms (10 for each group) were fixed on microscope slides (with levamisole 1 mM). Two worms were chosen per field, in a total of five random visual fields. The second oocyte (−2 oocyte) from the spermatheca of each worm was photographed. Using ImageJ software, the length and morphological changes of the −2 oocytes were analyzed (Ruan et al., 2012).

2.6.3. Number of apoptotic cells

The MD701 strain highlights the somatic cell sheath around the germ cells. The process of fixing the worms (10 for each group) was the same as 2.6.2. The circular bright GFP, characterizing apoptotic corpses of germ cells, were scored in a fluorescence microscopy (EVOS FLoid Cell Imaging Station) (Ruan et al., 2012; Yang et al., 2013).

2.7. Metal analysis by inductively coupled plasma mass spectrometry (ICP-MS)

The concentration of Mn, Fe, Cu, Zn, As, Cd, Pb and Hg was determined using 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 (an oxide ratio of <1.0% ($^{140}Ce1^{60}+/^{140}Ce+$) and a double charged ratio of <1.5% ($^{140}Ce++/^{140}Ce+$) with background counts <0.1 cps). 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).

2.8. Effects of metals on reprotoxicity

To investigate whether metals found in GBCF contribute to the reprotoxic effects, all the metals found (Table 1) were mixed in their respective concentrations without GMIPA salt (metals mix) or with the GMIPA salt (0.010% and 0.014%). Then, worms were exposed using the same treatment paradigm described in 2.3.2. Brood Size was conducted as described in 2.6.1.

2.9. Statistical analysis

All experiments were done in duplicate and repeated at least 3 times. All figures show the variation of each independent experiment (n) in a scatter dot plot. Data were expressed as ± standard error mean and were analyzed statistically by one-way ANOVA and Tukey's post-hoc. Comparisons between groups of equal concentrations were analyzed by two-way ANOVA. All statistical analysis was performed with Graph Pad Prism 6 software.

3. Results

3.1. GBCF induced decrease in the worms length

Worms (L1) exposed to GBCF or GMIPA salt chronically according to paradigm 2.3.1 showed different responses compared to each other and to the untreated group. The difference is evident in Fig. 1, where all concentrations of GBCF significantly decreased the length (head-to-tail) of the worms. Exposure to GBCF decreased the length of worms even below the LC_{50} (Figs. S1–B) for worms in larval stage L1. On the other hand, GMIPA salt, did not cause any significant change in the length of the worms at ay of the tested concentrations.

Table 1
Metals quantified in GBCF by ICP-MS.

Metal	Concentration (mg/L)
Hg	**0.170**
Mn	**45.68**
Fe	**193.7**
Cu	**220.8**
Zn	**193.1**
As	**0.034**
Cd	**0.069**
Pb	**0.021**

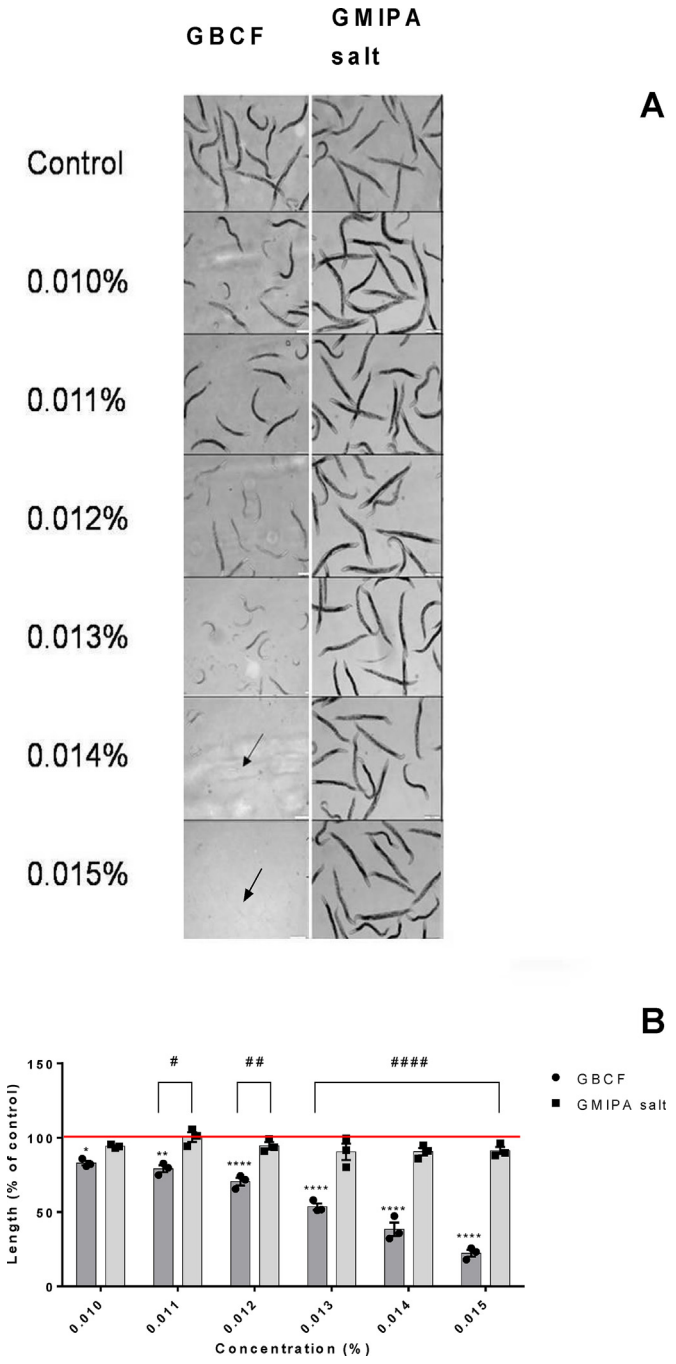

Fig. 1. Body length. Worms exposed to different concentrations of commercial glyphosate formulation showed significant decreases in body length when compared to control and GMIPA salt groups (A). Arrow shows worms affected dramatically in terms of length and development. (B) Quantitative representation of worms length measurements in relation to untreated worms (red line, as data were normalized to percentage of control) n = 3. * indicates statistical significance in comparison with control group (untreated = 100%). *p < 0.05; **p < 0.01; ****p < 0.0001. # indicates statistical significance between formulations at the same concentrations. #p < 0.05; ##p < 0.01; ####p < 0.0001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

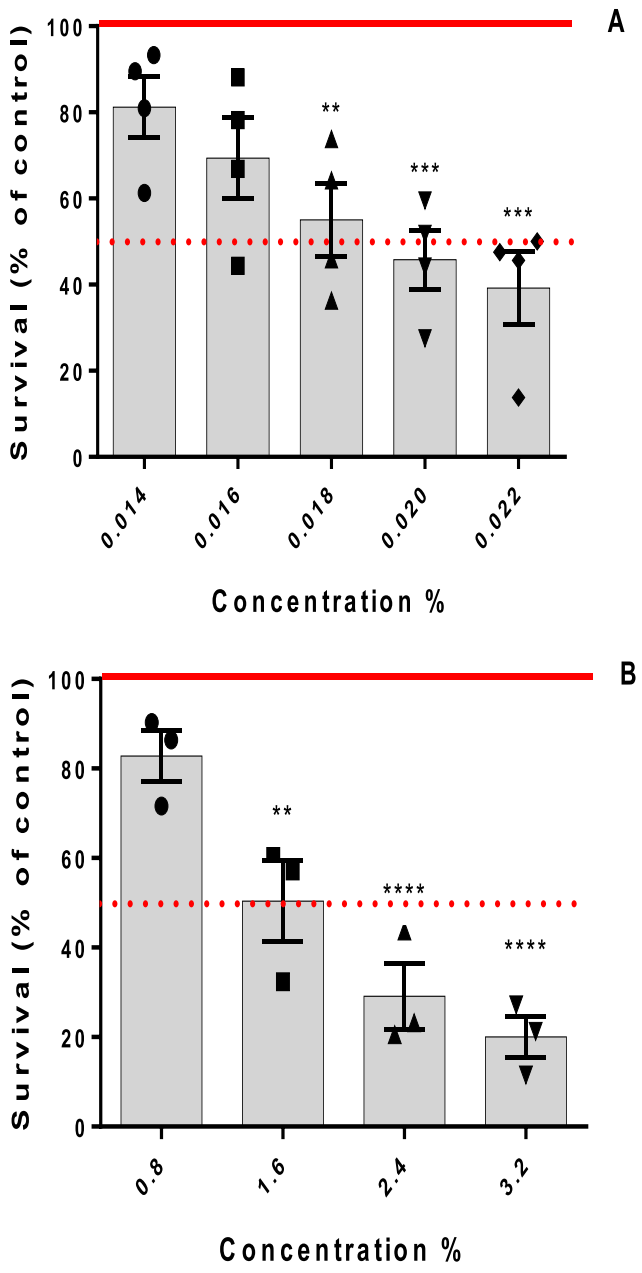

Fig. 2. Survival rate. Worms exposed to different concentrations of commercial glyphosate (A) and GMIPA salt (B). Worms treated with commercial glyphosate showed a lower LC_{50} (0.01937%) when compared to worms treated with GMIPA salt (1.662%). A (n = 4); B (n = 3). Pointed line indicates 50% mortality. * indicates statistical significance in comparison with control group (untreated = 100%) **p < 0.01; ***p < 0.001; ****p < 0.0001.

3.2. GBCF was more lethal as compared to GMIPA salt

The lethality assay presented here follows item 2.3.2, that is, 500 L4 worms were exposed for 48 h to GBCF or GMIPA salt. We can observe the results in Fig. 2. The NOAEL (No Observed Adverse Effect Level) for the GBCF was 0.014%. The LC_{50} of GBCF was 0.01937% (Fig. 2A), which is approximately 85 times lower than the LC_{50} of GMIPA salt (1.662%). This result shows that the presence of inert ingredients dramatically increased the lethality of GBCF.

3.3. GBCF decreased total brood size

The analysis of reproductive capacity described below, and the next two items (3.4 and 3.5), were performed according to item 2.3.2. As shown in Fig. 3 only the worms exposed to GBCF had a decrease in brood size. Even at non-lethal concentrations, GBCF showed a significant difference in the rate of hatched eggs

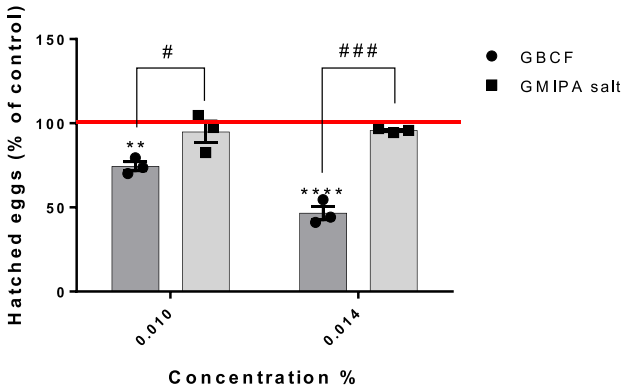

Fig. 3. Brood size. Worms exposed to commercial glyphosate showed a significant reduction in the viability of the eggs and brood size in both concentrations when compared to control and GMIPA salt groups. n = 3. * indicates statistical significance in comparison with control group (untreated = 100%). **p < 0.01; ****p < 0.0001. # indicates statistical significance between concentrations. #p < 0.05; ###p < 0.001.

compared to control and GMIPA salt groups.

3.4. GBCF induced shortening and morphological changes in the −2 oocyte

Fig. 4 shows the results obtained after exposure to pesticides, in L4 worms for 48 h. Fig. 4 A, B and C highlight the position and the shape of the 2-oocyte, in control, GMIPA and GBCF salt treated worms, respectively. Significant change in length was observed only in worms treated with GBCF, when compared to the control and GMIPA salt groups (Fig. 4D). The rounded and less developed 2-oocyte shape in Fig. 4C (probably due to the shorter gonad arm and fewer oocytes) was an observable characteristic in some worms exposed to GBCF.

3.5. GBCF increased the number of apoptotic cells

The MD701 strain allows the visualization of apoptotic corpses of germ cells as depticted in Fig. 5A and B. It was possible to notice a significant increase in the number of apoptotic cells in worms treated with GBCF (Fig. 5 B and C). The exposure to GMIPA salt did not induce a significant difference compared to the control (Fig. 5 C).

3.6. GBCF contains metals which are not specified on the label

The presence of metals was investigated in order to uncover the identity of a portion of the inert ingredients. Table 1 shows that Hg, Fe, Mn, Cu, Zn, As, Cd and Pb were found in GBCF. However, there is no description of the presence of these metals on the product label.

3.7. Metals are not responsible for the observed reprotoxicity

The mixture of metals alone or with GMIPA salt did not cause lethal effects (Fig. S2) and showed no reproductive damage. As observed in Fig. 6, there were no significant changes in brood size.

4. Discussion

To better understand the impact of the findings of this work, we must first acknowledge that agriculture follows the global food needs, and obviously must increase its production due to population growth. Facing challenges such as climate change, pest control

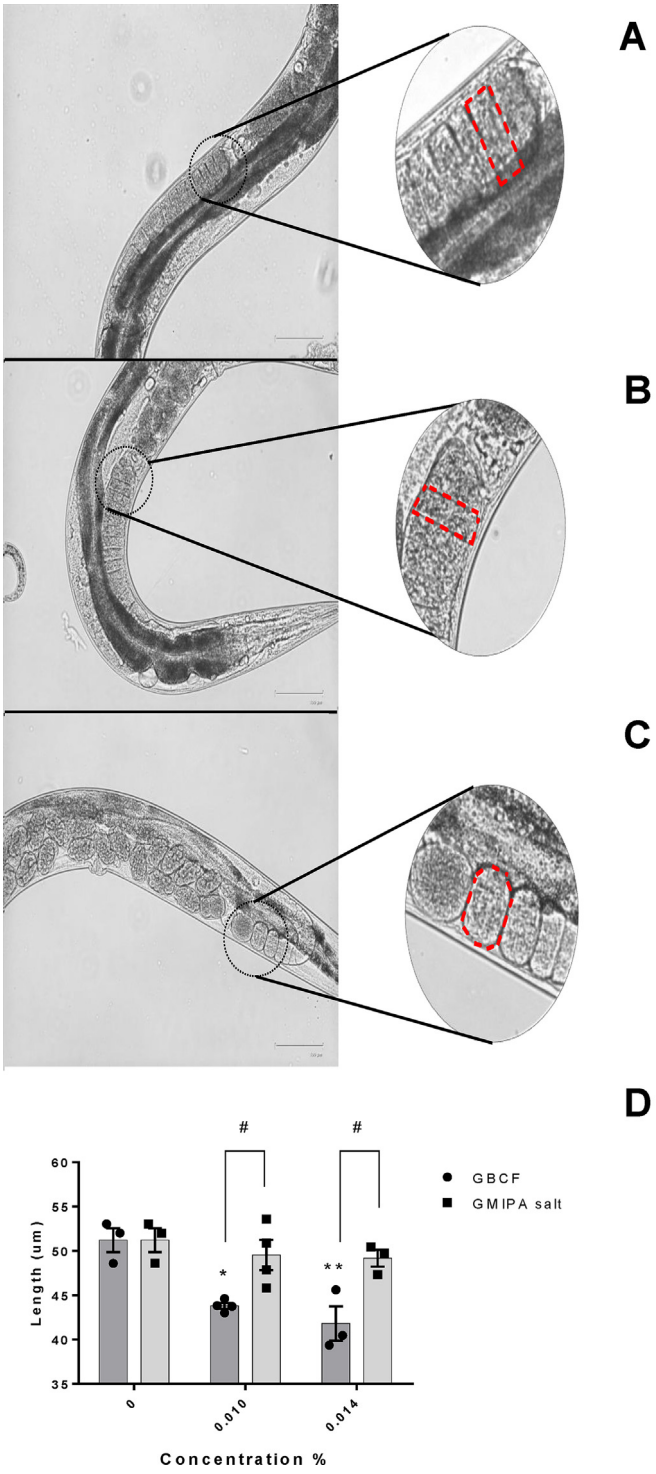

Fig. 4. Length of -2 oocyte. Length of the second oocyte from the spermathecae was measured. Figure C shows the morphological alteration of oocytes in commercial glyphosate group (0.010%) when compared to control (A) and GMIPA salt 0.010% (B). The length decreased significantly in worms exposed to the commercial formulation of glyphosate, in both concentrations, when compared to control and GMIPA salt groups (D). n = 4. * indicates statistical significance in comparison with control group (untreated = 100%). *p < 0.05; **p < 0.01. # indicates statistical significance between concentrations. #p < 0.05.

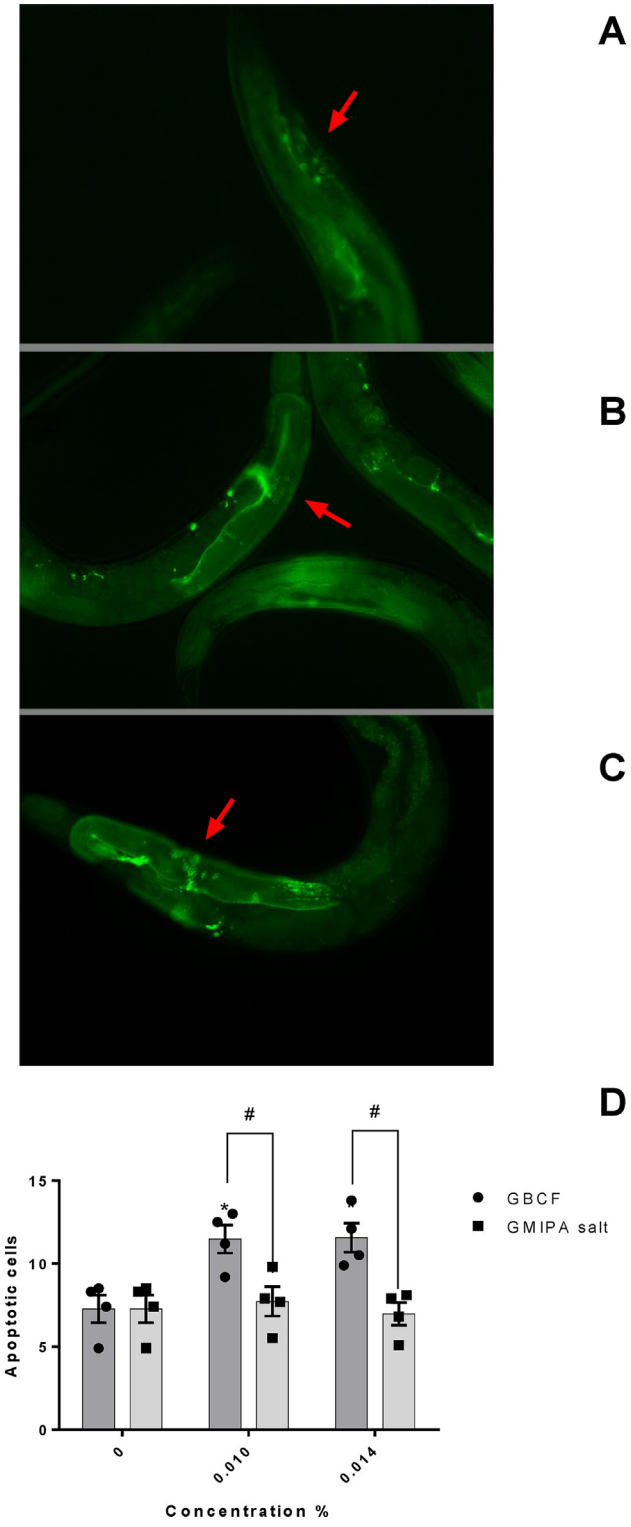

Fig. 5. Apoptotic cells. MD701 strain showing germ cells in apoptosis (arrows). Worms exposed to the commercial formulation of glyphosate 0.010% (C) showed higher numbers of apoptotic cells when compared to control (A) and GMIPA salt (B). The significant differences remained in both concentrations when compared to the control group and between concentrations (D). n = 4. * indicates statistical significance in comparison with control group. *p < 0.05. # indicates statistical significance between concentrations. #p < 0.05.

and the very high demand resulting from population growth, agriculture must adapt focusing on high productivity, relying on the use of agrochemicals, e.g. pesticides (Godfray and Garnett, 2014; Jacques et al., 2017; Lu et al., 2014). The fact that the annual global consumption of pesticides is approximately 2 million tons demonstrates the strong position in the market and the dependence on pesticides (De et al., 2014). Glyphosate, the world's best-selling pesticide, occupies a large portion of the world market, so the use of different commercial formulation with variable inert ingredients is widespread. Therefore, studies that consider contamination of water, soil and food by glyphosate do not take into account contamination by inert ingredients (Bøhn et al., 2014; Mesnage et al., 2015; Myers et al., 2016; Peruzzo et al., 2008; Samsel and Seneff, 2015). In this work, we find metals in the composition of a GBCF (Table 1), which were not specified in the label. Similarly, arsenic, chromium, cobalt, lead and nickel were found in 11 commercial formulations of glyphosate (Defarge et al., 2018). Based on the premise that metals are added to the active principle in a commercial formulation, we can emphasize that all environmental contamination and consequent human and animal intoxication is not caused only by a single component, but by a set of constituents of the commercial formulation. The origin of heavy metals in commercial formulations is unknown. They could be added intentionally as "inert" components, with adjuvant functions, or could be contaminants from the manufacturing process (Defarge et al., 2018). Notably, no reprotoxic effects were observed by the addition of metals mix to glyphosate salt (Fig. 6), probably due to their low concentrations and by the absence of other major inert ingredients present in the formulation. However, the presence of metals in various commercial pesticide formulations, not only GBCF, is a phenomenon already described (Defarge et al., 2018). This becomes problematic with long-term use, as heavy metals can be biomagnified, bioaccumulated and enter the food chain (Kumar et al., 2017; Rainbow, 2007; Real et al., 2017). Even at low levels, exposure to heavy metals can be harmful (Real et al., 2017). Progressive degenerative diseases of the musculoskeletal and nervous systems, gastrointestinal cancer, disabilities due to malnutrition, reduced immunity and intrauterine growth retardation may be caused by exposure to heavy metals (Iyengar and Nair, 2000; Jaishankar et al., 2014; Khan et al., 2008; Real et al., 2017; Türkdoğ;an et al., 2003). Pesticides are not the only sources of contamination by heavy metals in the environment, but their use in excess has already been described as depicting a significant role in the incorporation of these pollutants (Deng et al., 2017; Micó et al., 2006). Taiwan and China are among the world's largest consumers of pesticides, and evidences of the impacts of heavy metal contamination in these countries can be easily found (Yadav et al., 2015). Bioaccumulation and high levels of heavy metals in fish, plants and soils irrigated with wastewater have been reported in China (Cui et al., 2015; Deng et al., 2017; Khan et al., 2008). Interestingly, the source of soil contamination by Zn and Cu was attributed to agrochemicals (Khan et al., 2008) and such metals were found at high concentrations in our study (Table 1) (Khan et al., 2008). Similarly, studies report the contamination of rivers, soils and fish by heavy metals in Taiwan (Chen et al., 2007; Römkens et al., 2009; Vu et al., 2017). One study analyzed the presence of heavy metals in the blood of Taiwanese individuals, higher levels of Hg and Cd were found in Taiwan blood samples when compared to western populations (Liu et al., 2017). Even developed countries are not free of this reality, as one report relates the contamination of food by pesticides and heavy metals in the European Union (Nasreddine and Parent-Massin, 2002). Few studies have directly analyzed the metal constitution of a commercial pesticide formulation; therefore we believe that our

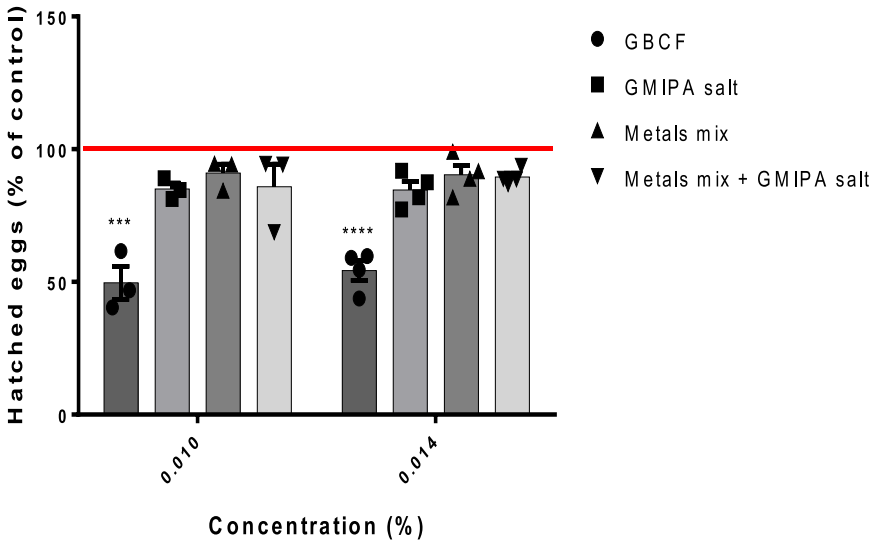

Fig. 6. Effects of metals on reprotoxicity. Worms exposed to the mixture of metals without GMIPA salt or the mixture of metals with the GMIPA salt did not cause brood size reduction as GBCF. n = 3–4. * indicates statistical significance in comparison with control group (untreated = 100%). ***p < 0.001; ****p < 0.0001.

findings may instigate other researchers and even regulatory agencies to include this analysis for these formulations.

Reproduction was severely affected by GBCF, as worms exposed to non-lethal concentrations depicted decreased brood size compared to control and GMIPA salt groups. Considering the results shown in Fig. 3, we investigated the possible mechanisms behind these effects by analyzing the maturation of the oocytes and the cell death in germline. For a complete maturation of oocytes, meiotic cells need to develop in the extension of the gonad arm. In this anatomical portion, it is possible to observe phases characteristic of the progression of meiotic prophase I, such as pachytene, diplotene and diakinesis (Hubbard and Greenstein, 2000). The general organization of the gonad has mitotic cells in its distal arm, and in its extension, up to the proximal arm, meiotic cells in different stages of meiotic prophase I (Hubbard and Greenstein, 2000). It is in the "loop" of the gonad, a transition between pachytene and diplotene for developing oocytes, which occurs the physiological germ cell apoptosis (Gumienny et al., 1999). It is hypothesized that this physiological cell death has the role of providing proteins and other cytoplasmic components to developing oocytes, thus acting as "nurse cells", (Gumienny et al., 1999; Hengartner, 1997). Therefore, it is clear that the oocyte increases in volume and length as it develops (from the distal to the proximal arm of the gonad) (Wolke et al., 2007). Consequently, any change in size when compared to a control group indicates abnormal maturation, an impaired oogenesis.

Our findings indicate that the presence of inert ingredients in GBCF induced incomplete oocyte maturation by length reduction (Fig. 4). Finally, we observed cell death in the germline, the MD701 strain has CED1:: GFP, a fusion that allows the visualization of early apoptotic corpses of germinative cells, being considered a sensitive method of observation (Schumacher et al.). Exposure to GBCF induced an increase in the number of germ cells in apoptosis, when compared to the control group (Fig. 5). We believe that together these endpoints (−2 oocyte length and apoptotic germ cells) had repercussions on the effect observed in Fig. 3, the reduction of brood size, only in worms exposed to GBCF.

The total composition of GBCFs is confidential and their components may vary depending on the brand, but co-formulants such as polyethoxylated tallow amine and alkyl polyglucoside have already been identified in GBCFs (Defarge et al., 2016; Mesnage et al., 2013). Studies (*in vitro* and *in vivo*) have shown that glyphosate salt is less toxic than glyphosate-based formulations. For instance, commercial formulation and adjuvants exhibited toxicity at concentrations below the recommended dilution; on the other hand, glyphosate alone was not toxic at the same concentrations (Chłopecka et al., 2017; Defarge et al., 2016; Howe et al., 2004; Mesnage and Antoniou, 2018; Mesnage et al., 2013; Mesnage et al., 2014; Séralini, 2015). In fact, the agricultural recommended dilutions of GBCFs have glyphosate at concentrations that do not have herbicidal activity *per se*, therefore the addition of other compounds is necessary for their effect (Defarge et al., 2018; Séralini, 2015). In our study, reprotoxicity was not observed with GMIPA salt alone when directly compared to GBCF. This points out that at the concentrations used, the active principle glyphosate alone was insufficient to induce any toxic effect, depicting toxicity only when associated to a commercial formulation. Hence, it is speculated that the high toxicity of GBCFs is a result of the addition of inert ingredients (adjuvants). We hypothesize that one or more GBCF co-formulants, interacting with each other or with the active ingredient were responsible for the observed effects. The reduction of the length of worms exposed to GBCF (Fig. 1) in contrast to the lack of alterations in the same parameter in worms exposed to GMIPA salt group, at the same concentrations, and a LC50 85 times lower for the GBCF (Fig. 2), evidences our hypothesis. Similarly, two published studies have found that exposing C. elegans to a glyphosate commercial formulation is lethal in a concentration-dependent manner, and adversely affects reproduction and growth (García-Espiñeira et al., 2018; Kronberg et al., 2018). These effects were related to an oxidative imbalance, with increases in the expression of genes such as *ctl-1, sod-1, sod-4 and gpx-4*, and activation of DAF-16, a transcription factor involved in the response to stress, reproduction and longevity (García-Espiñeira et al., 2018; Kronberg et al., 2018).

5. Conclusion

To date, no studies have demonstrated the comparative toxicity

of GBCF and GMIPA salt in *C. elegans*. Here we show that the presence of inert ingredients plays a decisive role in the GBCF, increasing its lethality and reprotoxicity. We also found metals in GBCF, constituents not specified in pesticide labels. Our work, together with the growing scientific literature on inert ingredients, emphasize the need for more specific regulations on the production and sale of pesticides, discriminating the composition of inert ingredients in the labels, considering the safety of consumers and the environment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.06.099.

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