UNIVERSIDADE FEDERAL DO PAMPA

MAURICIO TAVARES JACQUES

REPROTOXICIDADE DE FORMULAÇÃO À BASE DE GLIFOSATO EM *Caenorhabditis elegans* NÃO SE DEVE APENAS AO INGREDIENTE ATIVO

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Dissertação apresentada ao programa de Pós-graduação em Bioquímica da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Mestre em Bioquímica.

Orientador: Profa. Dra. Daiana Silva Ávila

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Área de concentração: Bioprospecção molecular.

Dissertação defendida e aprovada em: 08 de fevereiro de 2018

Banca examinadora:

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Dedico este trabalho a meus pais, meu irmão e meus avós por terem deixado esse deserto menos árido.

AGRADECIMENTO

Agradeço a minha família por tudo.

Agradeço aos meus amigos, aos bons colegas e professores que fizeram e fazem parte dessa jornada. Aos amigos do GBToxCe, praticamente os únicos que tenho e que acham graça das minhas piadas, dão caronas, dão companhia e dão ouvidos.

Agradeço a minha orientadora por todo ensino social e científico desses anos, sendo o mais longo relacionamento que tive com uma mulher.

Agradeço a banca que podia estar festejando carnaval, mas preferiu ler sobre pesticidas.

Por fim, agradeço a UNIPAMPA e as agências de fomento a pesquisa.

"Ver um mundo num grão de areia,
E um céu numa flor do campo,
Capturar o infinito na palma da mão
E a eternidade numa hora."

William Blake

RESUMO

Os pesticidas nos garantem uma alta produtividade na agricultura, mas os custos a longo prazo se mostraram muito altos. A intoxicação aguda e crônica de seres humanos e animais, a contaminação do solo, da água e dos alimentos são consequências da demanda atual e das vendas desses produtos. Sucessos comerciais, com altas vendas globais, como o glifosato, ilustram esse cenário. Além disso, pesticidas, como o glifosato, são vendidos em formulações comerciais que possuem ingredientes inertes, substâncias com composição e proporção desconhecida. Diante desse cenário, são necessários estudos toxicológicos que investigam a composição e a interação entre o princípio ativo e os inertes. O seguinte trabalho propôs estudos de toxicologia comparativa entre o glifosato e sua formulação comercial usando o modelo alternativo *Caenorhabditis elegans*. Vermes foram primeiro expostos em meio líquido durante 30 minutos a diferentes concentrações do ingrediente ativo (glifosato em sal de monoisopropilamina) e sua formulação comercial. No final deste período, os vermes foram transferidos para um meio sólido, onde a exposição continuou por mais 48 horas. A capacidade reprodutiva foi avaliada através do tamanho da ninhada, análise morfológica de ovócitos e através da cepa MD701 (bcIs39), que permite a visualização de células germinativas na apoptose. Além disso, a composição de metais potencialmente tóxicos na formulação comercial também foi analisada por ICP-MS. Apenas a formulação comercial de glifosato apresentou efeitos negativos significativos sobre o tamanho da ninhada, comprimento do corpo, tamanho do ovócito e o número de células apoptóticas. A análise de metais mostrou a presença de Hg, Fe, Mn, Cu, Zn, As, Cd e Pb na formulação comercial. Em conjunto, esses resultados trazem luz ao desconhecido, que é a composição de metais das formulações comerciais de pesticidas. Por fim, demonstramos no modelo *C. elegans* que a adição de ingredientes inertes aumentou o perfil tóxico do ingrediente ativo glifosato.

Palavras-Chave: *C. elegans*; Glifosato; Formulação comercial; Ingredientes Inertes; Reprodução; Desenvolvimento; Metais.

ABSTRACT

Pesticides guarantee 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. Commercial successes, with high global sales, like glyphosate illustrate this scenario. In addition, pesticides such as glyphosate are sold in commercial formulations which have inert ingredients, substances with unknown composition and proportion. Faced with this scenario, toxicological studies that investigate the composition and interaction between the active principle and the inerts are necessary. The following work proposed comparative toxicology studies between glyphosate and its commercial formulation using the alternative model *Caenorhabditis elegans*. Worms were first exposed in liquid medium for 30 minutes at different concentrations of the active ingredient (glyphosate in monoisopropylamine salt) and its commercial formulation. At the end of this period, worms were transferred to a solid medium, where exposure continued for another 48 hours. 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 also 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. Taken together, these results bring light to the unknown, which is the metal composition of commercial pesticide formulations. Finally, we demonstrated within the C. elegans model that the addition of inert ingredients increased the toxic profile of the active ingredient glyphosate.

Key words: *C. elegans;* Glyphosate; Commercial formulation; Inert ingredients; Reproduction; Development; Metals.

APRESENTAÇÃO

A presente dissertação foi desenvolvida em três partes:

CAPÍTULO 1

Contém as seções Introdução, Revisão Bibliográfica, Justificativa e Objetivos.

CAPÍTULO 2

Os resultados que fazem parte desta dissertação estão apresentados sob a forma de artigo científico. As seções Materiais e Métodos, Resultados e Discussão encontram-se no próprio manuscrito. O manuscrito está apresentado da mesma forma que será submetido à revista ***Archives of Toxicology.***

CAPÍTULO 3

Contêm as conclusões, perspectivas e referências bibliográficas.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

C. elegans - Caenorhabditis elegans

ICP-MS - Inductively coupled plasma mass spectrometry

GBCF - Glyphosate based commercial formulation

GMIPA salt - Glyphosate in monoisopropylamine salt solution

EPSPS - 5-enolpyruvylshikimate-3-phosphate synthase

GFP - green fluorescent protein

OGM - Organismo geneticamente modificado

DNA - Deoxyribonucleicacid

CED-1; CED-3; CED-4 - Cell Death abnormality

CEP-1 - C. *elegans* P-53-like protein

SUMÁRIO

1. INTRODUÇÃO

Defensivos agrícolas ou pesticidas são compostos químicos usados com a intenção de prevenir, destruir ou controlar qualquer peste. Seu uso destaca-se na agricultura, onde se diferem, principalmente, em herbicidas, fungicidas e inseticidas. A utilização de pesticidas na agricultura aumenta a cada ano, e proporcionalmente elevam-se os níveis de contaminações aos solos e rios próximos às plantações, bem como, o número de intoxicações agudas e crônicas de seres humanos e animais, e todos os problemas decorrentes dessas exposições, dentre os pesticidas mais usados destaca-se o glifosato (Boyd et al. 2009; Duke and Powles 2008; Peruzzo et al. 2008; Samsel and Seneff 2015).

O glifosato é o ingrediente ativo de herbicida mais vendido mundialmente, seu grande volume de uso e aplicação, juntamente com a utilização de culturas geneticamente modificadas, agrava o quadro de contaminação ambiental. Pesticidas são aplicados em suas formulações comerciais, das quais a maior proporção, em média, é de ingredientes inertes não especificados nos rótulos, uma vez que não há regulamentação sobre estes componentes. Estudos sugerem que estes adjuvantes desconhecidos são fundamentais para a elevada toxicidade da formulação final (Barron and Woodburn 1995; Gasnier et al. 2009; Mesnage et al. 2013a; Mesnage et al. 2014; Negga et al. 2011).

Tendo em vista tal realidade, estudos acerca da toxicologia e composição dos pesticidas tornam-se imprescindíveis para uma maior compreensão e avaliação dos riscos gerados por sua utilização. Porém, estudos em animais superiores, como por exemplo, mamíferos, tornam-se uma questão problemática, principalmente em termos bioéticos e pelo uso racional de animais na experimentação científica. Além disso, o nível de complexidade do organismo adiciona ao estudo muitas variáveis, tornando-se um problema nas primeiras fases da pesquisa. Como solução, propõe-se a utilização de um modelo alternativo para pesquisa, o nematódeo *Caenorhabditis elegans* (*C. elegans*), o qual possui uma menor complexidade e grande homologia genética com os humanos, não possuindo restrições éticas.

2. REVISÃO BIBLIOGRÁFICA

2.1. Glifosato

O glifosato (N-(fosfonometil) glicina), uma glicina substituída (figura 1) é o herbicida mais produzido e vendido mundialmente e está no mercado desde 1974 (Duke and Powles 2008). Seu sucesso de vendas deve-se principalmente a sua alegada baixa toxicidade e ao seu mecanismo de ação, que consiste no bloqueio da via do ácido chiquímico, pela inibição reversível da enzima 5-enolpiruvil chiquimato 3-fosfato sintase (figura 2), a qual é ausente em mamíferos (Duke and Powles 2008; Schönbrunn et al. 2001). Essa via está relacionada com a síntese de aminoácidos aromáticos e seu bloqueio impede que a planta produza proteínas funcionais e metabólitos essenciais, levando a sua morte (Dor et al. 2017). Particularmente, esta via não existe em insetos e animais e, por este motivo, o impacto ambiental do glifosato seria baixo (Duke and Powles 2008).

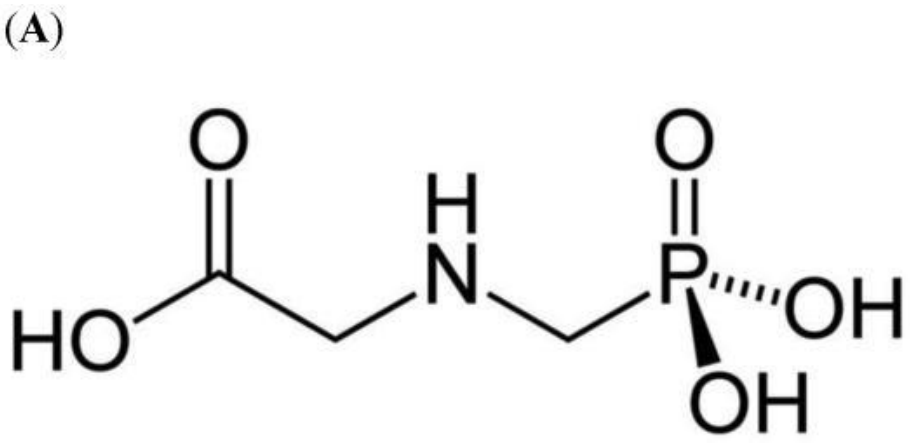

Figura 1. Fórmula estrutural do glifosato. FONTE: (Negga et al. 2011)

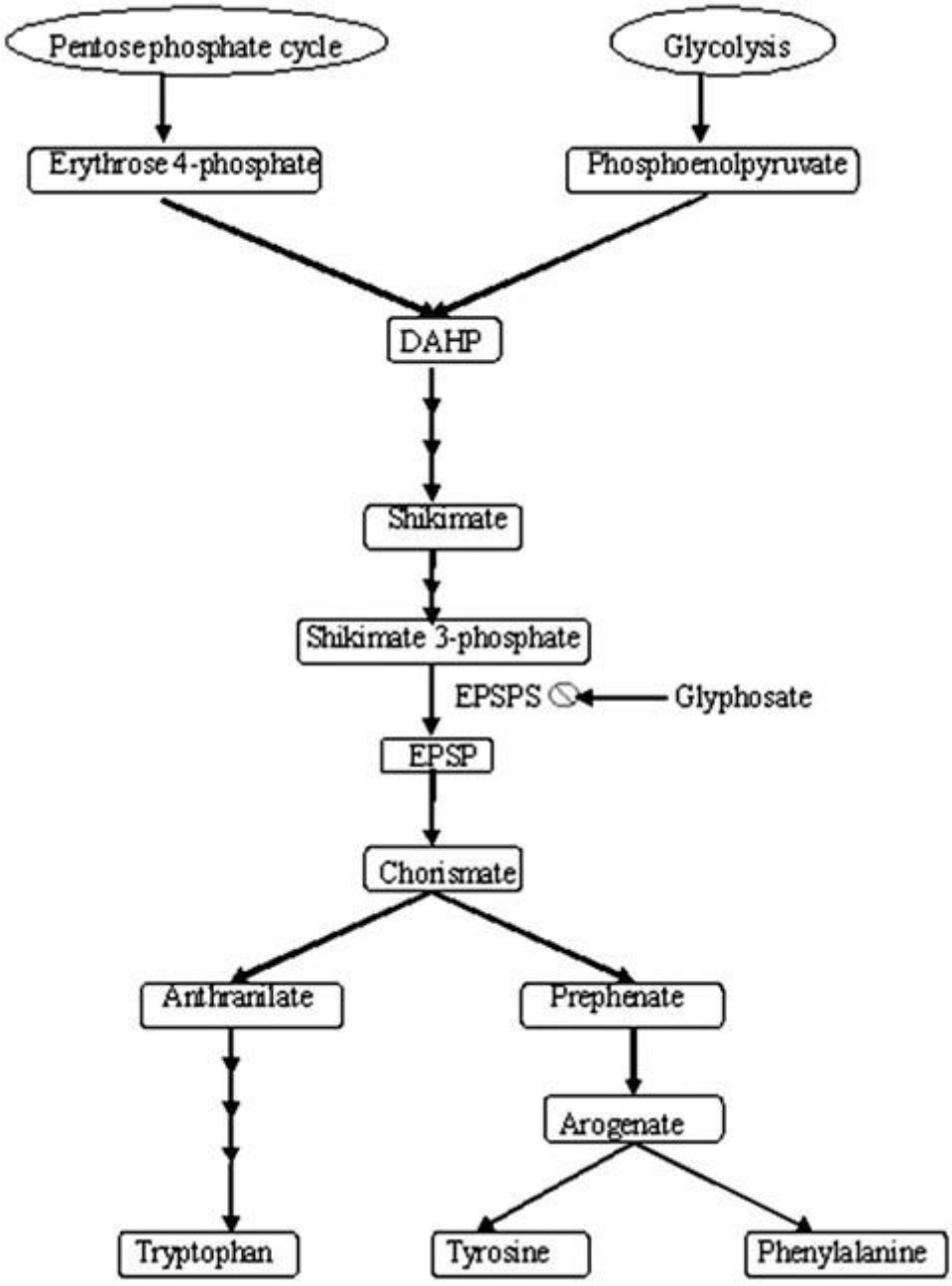

Figura 2. Via do Ácido Chiquímico e sua interação com glifosato. FONTE: (Vats 2015)

Outro fator para o sucesso comercial do glifosato é a possibilidade da utilização de plantas transgênicas resistentes ao herbicida como, por exemplo, soja e milho. O glifosato age de maneira não seletiva, eliminando qualquer planta que entre em contato com ele, com exceção das geneticamente modificadas. Em função da constante aplicação ao longo do tempo, as culturas se tornaram tolerantes ao glifosato e houve a necessidade do aumento no volume necessário deste princípio

ativo na agricultura (Benbrook 2012; Powles 2008). Logo, a utilização de organismos geneticamente modificados (OGM) e o desenvolvimento de resistência ao princípio ativo, contribuíram para o maior uso e venda de glifosato, sendo um ciclo que se retroalimenta, no qual em seu núcleo reside a alta demanda por produtividade. O OGM tolerante a glifosato não metaboliza o glifosato, apenas o acumula durante seu crescimento, fato que contribui para o aparecimento de resíduos de glifosato nos produtos que consumimos (Arregui et al. 2004; Kovacova et al. 2014; Samsel and Seneff 2015). Estudos mostram a presença de glifosato na urina de fazendeiros, na água potável engarrafada, em órgãos do gado e em maior concentração na urina de doentes crônicos (Krüger M 2014; Rendón-von Osten and Dzul-Caamal 2017). Também foi reportada maior presença de glifosato na urina de gado bovino onde há utilização de OGM, quando comparado a lugares livres dessas culturas (Krüger M 2014).

Apesar do fato de este herbicida não apresentar mecanismo de ação tóxico a animais, diversos são os estudos destacando o seu potencial carcinogênico: efeitos teratogênicos em linhagens de células humanas (Paganelli et al. 2010), crescimento de células do câncer de mama (Thongprakaisang et al. 2013), além de recentemente ser considerado pela Organização Mundial da Saúde como provável cancerígeno a humanos (Guyton et al. 2015). Ainda, pode ser considerado um disruptor endócrino com uma dose resposta invertida, isto é, há mais efeitos tóxicos agudos com baixas doses do que com altas (Gasnier et al. 2009). Um estudo reportou correlações com o aumento do uso de glifosato e neuropatologias como autismo, doença de Alzheimer, doença de Parkinson e depressão (Samsel and Seneff 2015).

Dentre os diversos estudos, a área de reprotoxicidade se destaca, com estudos *in vitro* e *in vivo* apontando os efeitos negativos do glifosato e suas formulações comerciais. Em modelo de células isoladas de testículos de ratos adultos, foi realizado estudo comparativo entre as toxicidades de glifosato e sua formulação comercial. Ambos causaram a diminuição (37%) da produção de testosterona nas células expostas por 24h à concentração equivalente a presente na urina de humanos (1 ppm) (Clair et al. 2012). Ainda no mesmo estudo foi demonstrada significativa degradação da membrana de células de Leydig apenas pela formulação comercial a 0.1%, sendo que tal concentração é 10 vezes menor que a recomendada para uso agrícola (Clair et al. 2012). Também foi relatado dano nas células de Sertoli com 0.05%, durante 24 h, da formulação comercial de glifosato

(Clair et al. 2012). Por fim, durante o mesmo período foi observado apoptose nas células de Leydig, com 1% e 0.05% de glifosato e sua formulação comercial, respectivamente (Clair et al. 2012). Em investigação semelhante, utilizando ratos sexualmente maduros, foi administrado glifosato por gavagem durante 5 semanas, e na dose de 500 mg/kg houve diminuição do peso da vesícula seminal e do número total de espermatozóides (Dai et al. 2016). A utilização de modelos alternativos também é viável para estudos relativos à toxicologia reprodutiva. Após exposição subletal (4.2 ppm) à formulação comercial de glifosato durante 3 semanas, o caramujo *Biomphalaria alexandrina* apresentou deformação de ovócitos, azoospermia e redução dos níveis de testosterona (Omran and Salama 2016). Por fim, foi demonstrado que 10 mg/L de glifosato, durante 21 dias, causou a diminuição da produção de ovos no peixe *Danio rerio* (Uren Webster et al. 2014).

2.2. Ingredientes inertes

Os pesticidas são apresentados e comercializados para uso agrícola em formulações comerciais, as quais apresentam em grande quantidade os chamados ingredientes inertes. Estes componentes não são devidamente especificados nos rótulos, pois são considerados segredo comercial. Isso é possível, pois o registro de um pesticida envolve apenas testes com seu princípio ativo, não com a formulação final que contêm inertes (Cox and Surgan 2006). Os ingredientes inertes tem função de adjuvantes, otimizando a solubilidade, ação e estabilidade do ativo e da formulação final (Cox and Surgan 2006; Mesnage et al. 2013b). Apesar do seu nome, não são necessariamente inertes, e podem ser biologicamente ativos (Cox and Surgan 2006).

Estudos reportaram que os inertes contribuem ainda mais para a toxicidade final da formulação, podendo ser, em alguns casos, os principais fatores para determinada toxicidade (Mesnage et al. 2014; Negga et al. 2011). Devemos considerar que a maioria dos estudos avalia a toxicidade da formulação comercial, pouco é relatado sobre a composição e interação dos inertes com seu princípio ativo (Bøhn et al. 2014; Ingaramo et al. 2016; Mesnage et al. 2015; Myers et al. 2016; Negga et al. 2012; Peruzzo et al. 2008; Samsel and Seneff 2015). Um estudo indica que uma porção dos inertes constitui-se de compostos etoxilados, os quais possuem função de adjuvante (Mesnage et al. 2013b). Tais compostos, presentes na

formulação comercial de glifosato reduziram a sobrevivência de linhagens celulares de maneira mais significativa que o princípio ativo da formulação (Mesnage et al. 2013a). Um estudo recente encontrou arsênio, cromo, cobalto, chumbo e níquel em 11 formulações comerciais de glifosato, ao avaliar a atividade herbicida e a alteração endócrina em linhagens celulares foi concluído que a principal causa eram as formulações comerciais e não o glifosato sozinho (Defarge et al. 2018). Ainda, foi reportado a presença de alquilpoliglicosídeos, polioxietilenoamina e compostos de amônia quaternária em formulações comerciais de glifosato (Defarge et al. 2018). Levando em consideração a grande proporção de ingredientes inertes utilizados na maioria das formulações comerciais que são amplamente vendidas e a carência de estudos sobre a composição dos mesmos juntamente com a sua interação com o princípio ativo, tornam-se necessárias investigações acerca da constituição e toxicologia dos ingredientes inertes.

2.3. *Caenorhabditis elegans*

Estudos toxicológicos *in vivo* ou *ex vivo* com mamíferos tem sido bastante discutidos em razão do grande número de animais utilizados e sacrificados nestas avaliações e, por este motivo, o uso de invertebrados ou modelos biológicos mais simples tem crescido na toxicologia. Modelos experimentais que produzam informações biológicas relevantes em estágios iniciais de pesquisa, a baixos custos, são necessários. Assim, este estudo visa à utilização de um modelo animal mais simples para o estudo de pesticidas e seus inertes em formulações comerciais: o nematódeo *Caenorhabditis elegans* (*C. elegans*). A utilidade e relevância do modelo experimental *C. elegans* é estendida a áreas como a biologia, medicina, química e ciência de materiais, sendo um modelo intermediário entre os ensaios *in vitro* e *in vivo* com mamíferos complexos, complementando ambos (Figura 3) (Gonzalez-Moragas et al. 2015).

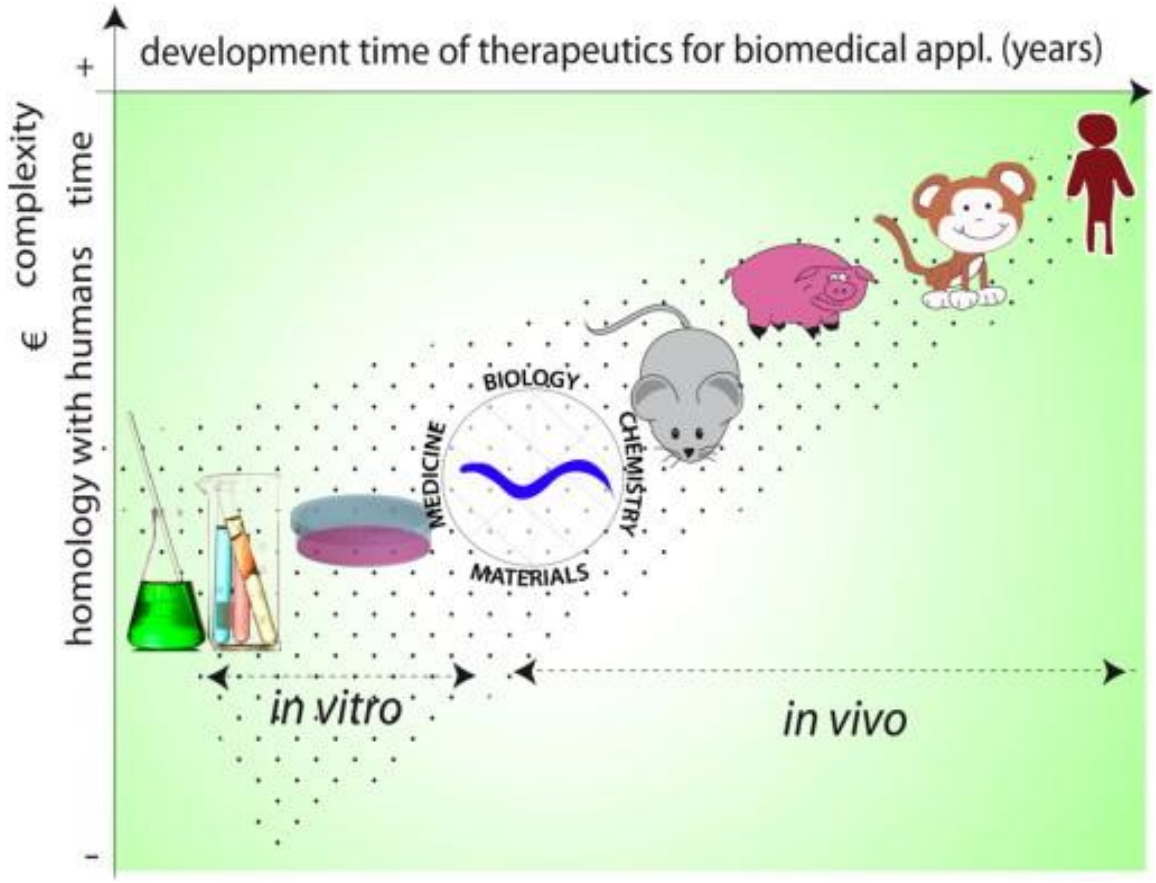

Figura 3. Relevância e posição do C. elegans dentre os demais modelos.
FONTE:(Gonzalez-Moragas et al. 2015)

Este verme tem sido utilizado como uma ferramenta útil em toxicologia experimental devido ao alto grau (60-80%) de homologia entre o genoma humano (Kaletta and Hengartner 2006). A sua fácil manutenção, manipulação, rápido ciclo de vida (Figura 4), curta longevidade (aproximadamente 20 dias), corpo transparente, geração facilitada de cepas com mutações tipo deleção em genes de interesse, e a existência de diversas cepas transgênicas expressando a proteína verde fluorescente combinada a genes promotores de proteínas de interesse, tornam o *C.elegans* um modelo experimental de grande relevância na toxicologia (Chalfie et al. 1994).

T	Time (h after hatch)				
	L1/L2 molt	L2/L3 molt	L3/L4 molt	L4/Adult molt	Egg-laying
16°C	24	38	55	74	94
20°C	15	24	34	48	58
25°C	11	18	26	35	45

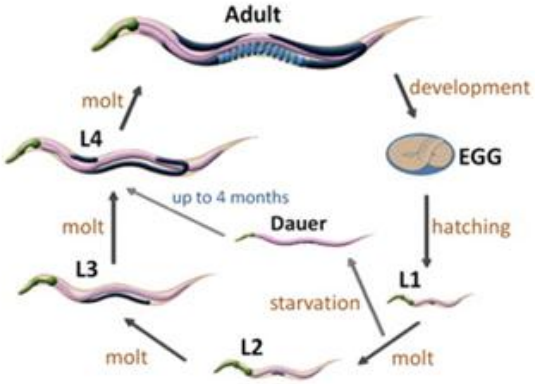

Figura 4. Ciclo de vida do *C. elegans* em determinadas temperaturas. FONTE: (Gonzalez-Moragas et al. 2015)

Particularmente, para estudos de toxicologia reprodutiva, especificamente quando se estuda a linhagem germinativa, esse modelo oferece a possibilidade de diversos ensaios. No C. *elegans* esse tecido é único e se diferencia basicamente dos tecidos somáticos, por ser pluripotente e imortal; ser constantemente mantido por células tronco; e ser o único tecido onde aparentemente a apoptose é uma parte intrínseca da oogênese (Gartner A 2005). Há dois tipos de apoptose nas células germinativas, uma fisiológica, que ocorre como suporte para os ovócitos em desenvolvimento e outra, caracterizada por ser desencadeada por dano ao DNA, estressores ambientais e por infecções causadas por determinados tipos de bactéria (Gartner A 2005). A figura 5 destaca os principais pontos de regulação da via apoptótica em C. *elegans,* muitos mecanismos ainda são desconhecidos, mas podemos citar que a apoptose fisiológica depende de CED-3 e CED-4, e independe de cep-1 (Gartner A 2005).

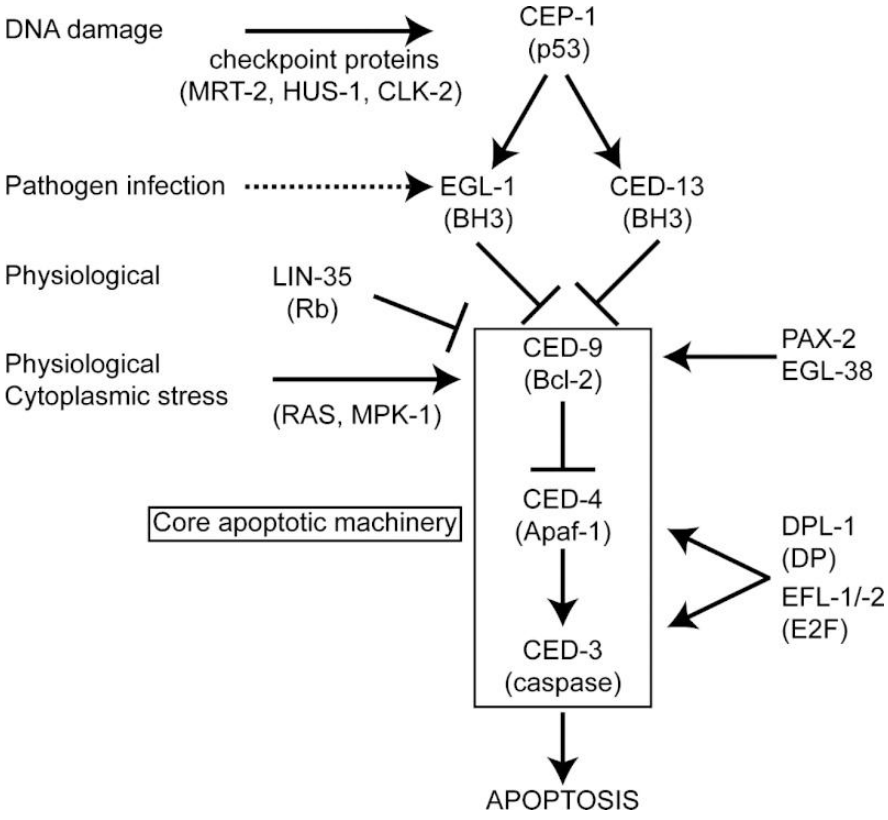

Figura 5. Principais pontos de regulação da apoptose em C. elegans. ***cep-1*** (encodes an ortholog of the human tumor suppressor p53)**;** ***mrt-2*** (encodes a highly conserved DNA-damage checkpoint protein homologous to the RAD1 protein found in S. pombe, Drosophila, and mammals); **hus-1** (encodes the C. elegans ortholog of the Schizosaccharomyces pombe Hus1 DNA damage checkpoint protein); **clk-2** (conserved DNA damage checkpoint protein); **egl-1** (encodes a novel protein that contains a region similar to the BH3, Bcl-2 homology region 3, domain of mammalian cell death activators); **ced-13** (encodes a 98-residue protein with a single BH3 domain, overexpression promotes CED-3/4-dependent apoptosis); **lin-35** (encodes the C. elegans retinoblastoma protein (Rb) ortholog); **pax-2** (Pax proteins, directly induce *ced-9* transcription); **ced-9** (Bcl2 homolog and general apoptosis inhibitor); **ced-4** (required for the initiation of programmed cell death); **ced-3** (encodes a caspase, a cysteine-aspartate protease; CED-3 activity is required for execution of apoptosis). FONTE-ADAPTADO: (Gartner A 2005)

Outra característica notável, em particular para estudos de reprotoxicologia, é a facilidade da visualização da apoptose em células germinativas. Através da utilização de uma cepa que possui fusão de GFP com a bainha de células somáticas que envolvem as células germinativas, é possível visualizar em microscópio de fluorescência o engolfamento da célula morta (Figura 6 B) (Ruan et al. 2012a). O estudo da oogênese também é facilitado, pois o ovócito desenvolve-se da parte distal para a proximal no braço da gônada do *C. elegans*, passando por fases características da prófase I meiótica (figura 7) (Hubbard and Greenstein 2000).Devido a todas essas características únicas, este modelo já vem sendo utilizado em toxicologia de pesticidas.Por exemplo, estudos já relacionam a exposição de glifosato com a neurodegeneração e a reprotoxicidade de clorpirifós, (Negga et al. 2011; Negga et al. 2012; Ruan et al. 2012a). Estudos investigando a toxicidade reprodutiva de glifosato e metais potencialmente tóxicos em C. *elegans* também são reportados (Ruan et al. 2009; Wang et al. 2017)

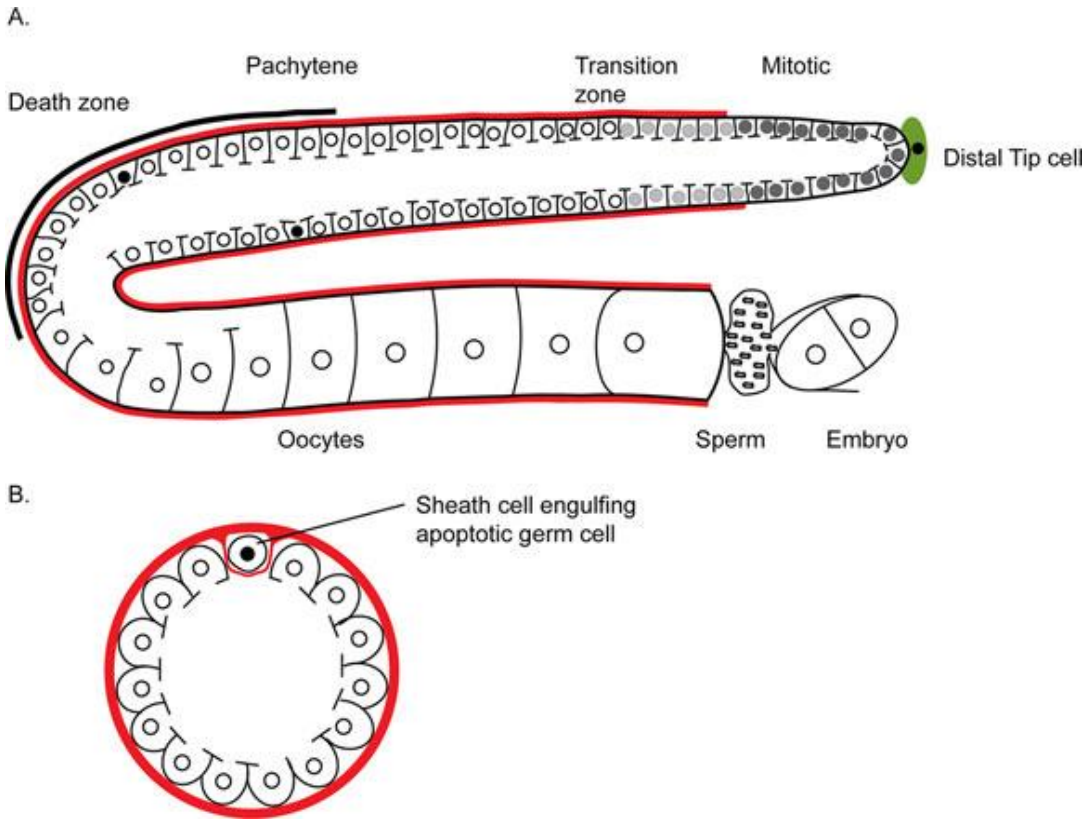

Figura 6. Composição da gônada do *C. elegans (A)* e apoptose (B) nas células germinativas. FONTE: (Gumienny et al. 1999)

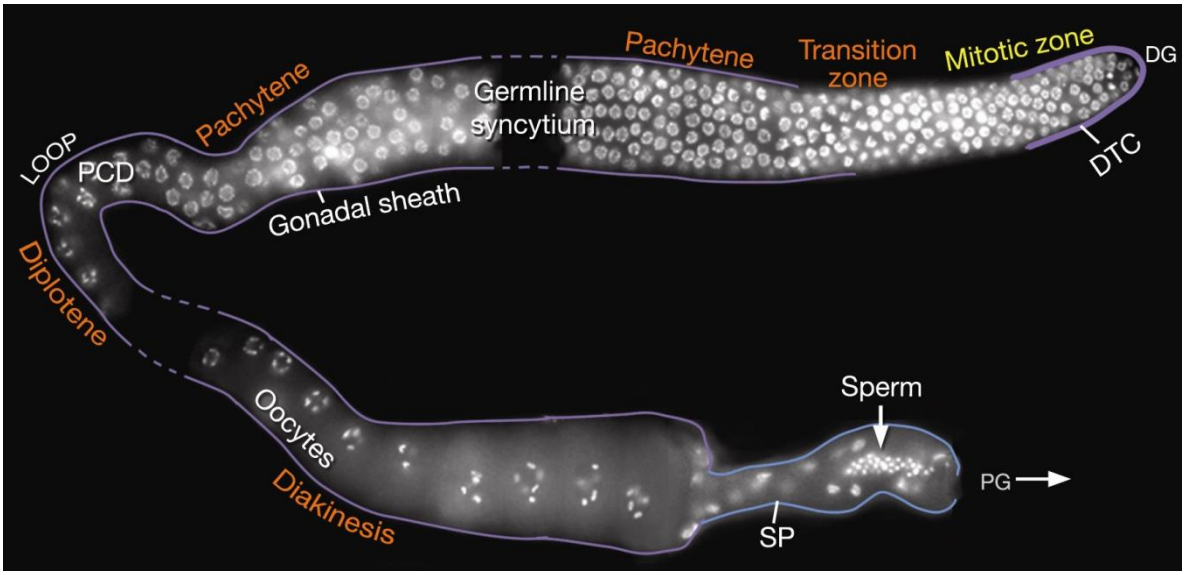

Figura 7. Estágios da prófase I meiótica em *C. elegans*. Fonte: (Hubbard and Greenstein 2000)

3. JUSTIFICATIVA

As vendas de pesticidas aumentam a cada ano e, do mesmo modo, cresce a necessidade por maiores quantidades de alimentos. Consequentemente, para uma alta produtividade na agricultura, agroquímicos (pesticidas, por exemplo) são necessários. Estudos que se dediquem a investigar a toxicologia de pesticidas serão úteis principalmente aos principais alvos de exposição, trabalhadores que aplicam os agroquímicos e a população em geral que consome os produtos. Já podemos reportar estudos que avaliam os efeitos negativos da exposição de pesticidas em fazendeiros, a presença de glifosato em produtos alimentícios industrializados e na urina de famílias próximas as áreas de aplicação (Acquavella et al. 2004; Kovacova et al. 2014; Ogut et al. 2015). Devido à proporção majoritária dos inertes nas formulações comerciais, é esperado que juntamente com os resíduos contaminantes de glifosato, ocorra também, a presença de resíduos de ingredientes inertes. Tendo em vista esse cenário, no qual pesticidas são amplamente utilizados, principalmente o glifosato, estudos acerca da composição de metais e a toxicologia de formulações comerciais de pesticidas são necessários, visando à segurança do trabalhador, qualidade e saúde dos consumidores e uma maior regulamentação destes produtos.

4. OBJETIVOS

4.1 Geral

Avaliar comparativamente a toxicidade de glifosato contra uma formulação comercial à base de glifosato em *C. elegans,* visando definir diferentes perfis toxicológicos.

4.2 Específicos

1) Identificar os perfis de letalidade do sal de glifosato versus a formulação à base de glifosato;

2) Comparar o prejuízo à capacidade reprodutiva e a alteração do desenvolvimento após as exposições;

3) Investigar a presença de metais potencialmente tóxicos na formulação comercial de glifosato;

4) Relacionar a toxicidade da formulação comercial à base de glifosato com a presença de inertes

5. MANUSCRITO

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

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Abstract

Pesticides guarantee 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. Commercial successes, with high global sales, like glyphosate illustrate this scenario. 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 first exposed in liquid medium for 30 minutes at different concentrations of the active ingredient (glyphosate in monoisopropylamine salt) and its commercial formulation. At the end of this period, worms were transferred to a solid medium, where exposure continued for another 48 hours. 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 also 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. Taken together, these results bring light to the unknown, which is the composition of commercial pesticide formulations. Finally, we demonstrated within the C. *elegans* model that the addition of inert ingredients increased the toxic profile of the active ingredient glyphosate.

Key words: *C. elegans;* Glyphosate; Commercial formulation; Inert ingredients; Reproduction; Development; Metals.

1. Introduction

Glyphosate is the most widely produced and sold herbicide worldwide and has been on the market since 1974 (Duke and Powles 2008). Its 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 the commercial success of glyphosate 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 plant that comes in contact with it, except for those genetically modified. Due to the constant application over time, there was the emergence of unwanted cultures tolerant to glyphosate (Benbrook 2012; Powles 2008).

The tripod alleged low toxicity, GMOs and resistance to glyphosate were essential for glyphosate-based herbicides to be heavily applied worldwide. In fact, glyphosate use increases worldwide approximately 20% by year (Benbrook 2012; Cai et al. 2017; Mesnage et al. 2015). In the US the herbicide-resistant crop technology moved the use of 239 million kilogram between 1996 and 2011 (Benbrook 2012; Mesnage et al. 2015). 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 and 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 et al. 2013b; 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 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 its unknown inert components, studies in the areas presented, aiming the comparison and interaction of the inert components with the active principle are necessary, since there are deficiencies of these studies especially 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) and no need for submission to an ethics committee make *C. elegans* an experimental model of great relevance in toxicology (Chalfie et al. 1994). The main premise of this work is that inert ingredients potentiate the toxic effect of the active ingredient. This hypothesis has been supported and investigated by several studies (Benachour and Séralini 2009; Gasnier et al. 2009; Mesnage et al. 2013b; Mesnage et al. 2014). Therefore, the objective of this study was focused on the inert ingredients, a part of its composition and the difference of its presence or absence in the commercial formulation.

2. Materials and methods

2.1. Chemicals

The commercial formulation of glyphosate was obtained from local commerce, and the isolated form of glyphosate (N-Phosphonomethyl glycine, monoisopropylamine salt solution) was obtained from Sigma-Aldrich, which is commonly used in commercial formulations (Adcock et al. 2004; Lipok et al. 2010; Lozano et al. 2018). The concentration of glyphosate based commercial formulation (GBCF) was 1% (active ingredient), same as that obtained for glyphosate in monoisopropylamine salt solution (GMIPA salt). All treatment dilutions started from 1%.

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 were N2 (wild type) and MD701 (bcIs39), which has expression of 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. 2012a). 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 hours of the synchronization process, freshly hatched larvae were treated with different concentrations of pesticides in commercial glyphosate form (GBCF) or glyphosate in monoisopropylamine salt (GMIPA salt). Concentrations were calculated as percentage of active ingredient (0.010 to 0.015%). First, 1500 L1 worms were exposed for 30 minutes, in liquid medium; subsequently, the whole treatment 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 paradigm

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 were used), lasting also 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. 2012a). 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 in 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 LC50 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 hours to GBCF or GMIPA salt was analyzed. Briefly, worms were fixed in microscope slides (with levamisole) 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, size and oocytes 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 paradigm of item 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 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), and 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. 2012b).

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 in the previous item. The circular GFP bright, characterizing apoptotic corpses of germ cells were scored in a fluorescence microscopy (EVOS FLoid Cell Imaging Station) (Ruan et al. 2012b; Yang et al. 2013).

2.7. Metal analysis

GBCF was analyzed through ICP-MS in order to investigate the presence of heavy metals in the formulation.

2.8. Statistical analysis

All experiments were done in duplicate and repeated at least 3 times. Data were expressed as mean ± standard error and were analyzed statistically by one-way ANOVA and Tukey's post-hoc. Comparisons between groups of equal concentrations were analyzed with two-way ANOVA. All statistical analysis was performed with Graph Pad Prism 6 software.

3. Results

3.1. GBCF induced decrease in the length of worms

Worms (L1) exposed to GBCF or GMIPA salt chronically according to paradigm 2.3.1, showed different responses when compared to each other and to the untreated group. The difference is evident in Figure 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 LC50 (Figure S1-B) for worms in larval stage L1. On the other hand, in the comparative concentrations of GMIPA salt there was no significant change in the length of the worms.

3.2. GBCF was more lethal than 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 figure 2. The LC50 of GBCF was 0.01937% (figure 2A), which is approximately 85 times lower than the LC50 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 figure 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 when compared to control and GMIPA salt groups.

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

Figure 4 shows the results obtained after exposure to pesticides, in L4 worms for 48h. Figure 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 (figure 4D). The rounded and less developed 2-oocyte shape in Figure 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. Figure 5A and B depict this. It was possible to notice a significant increase in the number of apoptotic cells in worms treated with GBCF (Figure 5 B and C). The exposure to GMIPA salt did not induce a significant difference when compared to the control (Figure 5 C).

3.6. GBCF contains heavy metals not specified on 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 discrimination of the presence of these metals on the product label.

4. Discussion

To better understand the impact of the findings of this work, we must first acknowledge that agriculture follows the global food need, and obviously must increase its production due to population growth. Facing challenges such as climate change, pest control 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 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 (not specified on the label) in the composition of a GBCF (table 1). 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 contaminants a long with their active principle, we could evidence the toxicological consequences of their presence.

The reduction of the length of worms exposed to GBCF (figure 1) in contrast to the lack of alterations in the same parameter in worms exposed to GMIPA salt group, at the same concentrations, shows that the inert ingredients potentiate the toxic effect of the active principle. The same has been observed when lethality endpoint was assessed (figure 2). It has been reported growth retardation in worms exposed to 0.3 mM of $CuSO_4$ and 0.03 mM of $CdCl_2$ (Harada et al. 2007). The same was observed in worms treated with iron sulphate solution (2.5 µmol/L, 75 µmol/L, and 200 µmol/L) (Hu et al. 2008). Trace levels of non-essential metals, or excess of essential metals are toxic to cells due to the fact that interaction of metal ions with cells may inactivate or denature proteins and also lead to the formation of reactive oxygen species (ROS) (Zhou et al. 2011). Worms exposed to Mn (200 µmol/L) for 48 h showed abnormal body morphology and abnormal body development at concentrations of 75 and 200 µmol/L for 48 h (Xiao et al. 2009). In the same study, the observed effects were seen as a response to oxidative stress, as expression levels of sod-1, sod-2, sod-3, and sod-4 decreased after exposure to Mn (75 and 200 µmol/L for 48 h) (Xiao et al. 2009). Concentrations as low as 1 mg/L of Cu, Zn, Cd and Cr inhibited in a concentration-dependent manner the activity of superoxide dismutase (SOD), which was associated to a decrease of worms length (Jiang et al. 2016). Consequences of oxidative stress have been reported in a study that analyzed the effects of copper *C. elegans* cuticle (Song et al. 2014). It was observed after exposure the appearance of wrinkles from vulva to tail and a decrease in vulva size, approximately 47%, following Cu exposure to 800 µmol/L for 24 h (Song et al. 2014). Wrinkles are associated with aging, which can be caused by ROS (Baumann 2007; Song et al. 2014). Regarding body size, shorter worms have been observed from 200 µmol/L of $CuSO_4$ (Song et al. 2014). Finally, exposure to Cd induced a decrease in C. *elegans* development (Popham and Webster 1979). This effect was related to the morphological alteration of cytosomes in the intestinal cells and to the appearance of inclusion bodies in the nucleus of the esophageal cells, interfering in the absorption of nutrients, and consequently in the normal development of the worm (Popham and Webster 1979)

Reproduction was severily affected by GBCF, once worms exposed to non-lethal concentrations depicted decreased brood size when compared to control and GMIPA salt groups. Negative effects on reproduction have already been observed in worms exposed to Cu, Zn, Cd and Cr, in a concentration dependent manner (Jiang et al. 2016). Similarly, worms at the larval stage L4, exposed for 3 days toPb, Hg, Cd and Cr (2.5, 50 and 100 µM) presented decrease in brood size (Guo et al. 2009). In addition, adult worms exposed for 24 h to As (13.05 mg/L), Cu (8.51 mg/L) and As + glyphosate (1.31 mg/L and 0.19 mg/L, respectively) showed decrease in brood size, producing, according to the authors, a synergistic interaction between glyphosate and As (Wang et al. 2017).In view of these studies and the results shown in figure 3, we investigated the possible mechanisms behind these effectsby analyzing the maturation of the oocytes and then the cell death in germline.

For a complete maturation of oocytes, the 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 (figure 4) indicate that the presence of the inert ingredients in GBCF induced incomplete oocyte maturation (by length reduction). 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 (figure 5). We believe that together these endpoints (-2 oocyte

length and apoptotic germ cells) had repercussions on the effect observed in figure 3, the reduction of brood size, only in worms exposed to GBCF.

It is necessary to emphasize that in any of the tests that evaluated the reproductive capacity, the GMIPA salt showed a significant difference when compared to the control group, only when compared with GBCF. This leads us to believe, that at the concentrations used, the active principle glyphosate alone was insufficient to induce any effect, showing toxicity only when within a commercial formulation, with inert ingredients potentializing its effect. Corroborating to this theory, a recent study found arsenic, chromium, cobalt, lead and nickel in 11 GBCF, besides the main adjuvant (polyethoxylated tallow amine), interestingly, the herbicidal activity and endocrine disruption in cell lines was caused mainly by the commercial formulation and not by glyphosate alone (Defarge et al. 2018)

5. Conclusion

To date, no studies have found to demonstrate the toxicity of GBCF and GMIPA salt in *C. elegans*. Here we show that the presence of inert ingredients has potentiated glyphosate, increasing its lethality and reprotoxicity. We also found heavy metals in GBCF, constituents not specified in pesticide labels. Metals are not the majority composition of inert ingredients, hence further studies will be necessary to evaluate the toxicity of the metals in proportion to the composition of the commercial formulation.

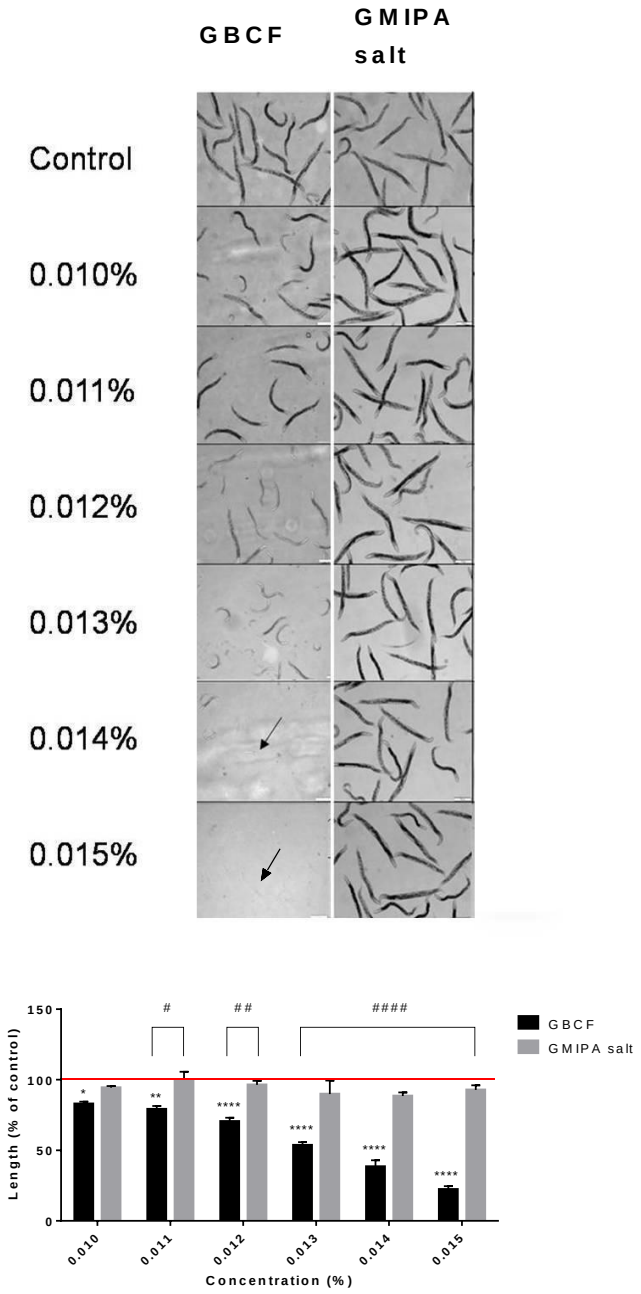

Figure 1. Body length. Worms exposed to different concentrations of commercial glyphosate formulation showed significant decreases in body length when compared to control and GMIPA salt groups (A). Arrow shows worms affected dramatically in terms of length and development. (B) Quantitative representation 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 concentrations. #p < 0.05; ##p < 0.01; ####p < 0.0001.

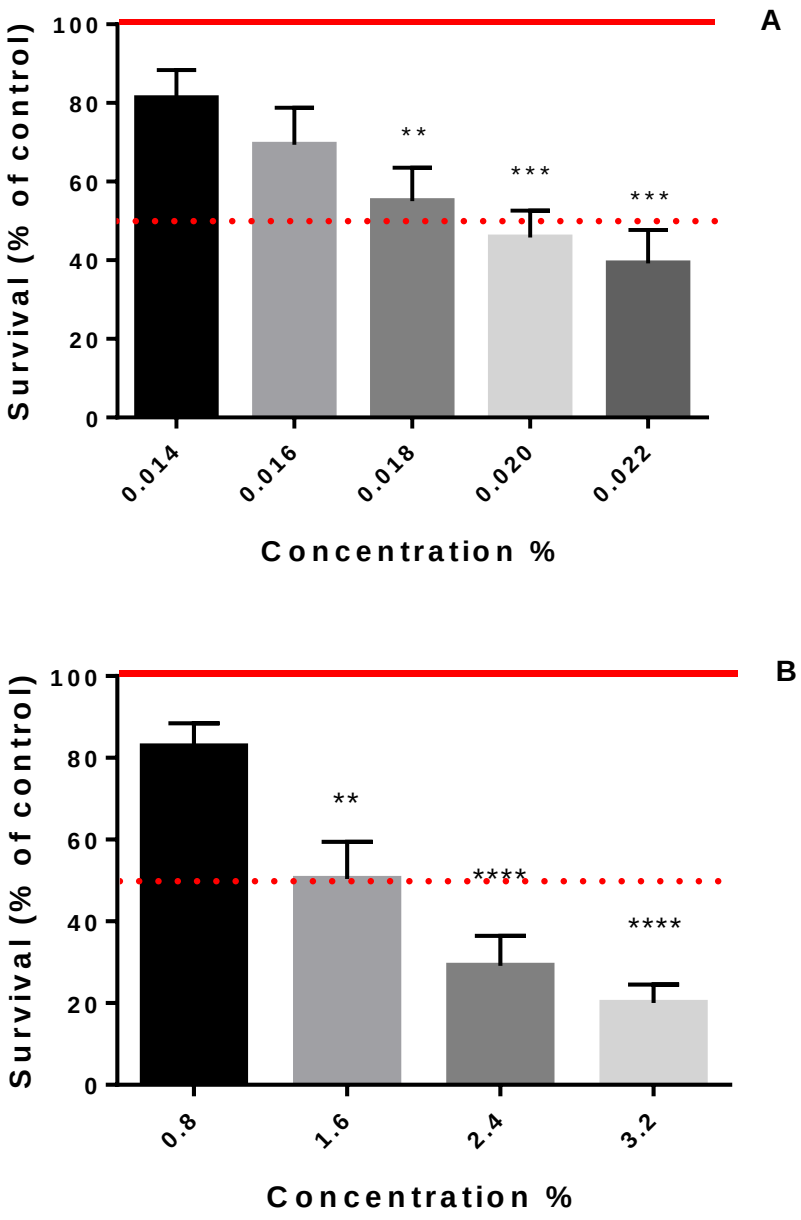

Figure 2. Survival rate. Worms exposed to different concentrations of commercial glyphosate (A) and GMIPA salt (B). Worms treated with commercial glyphosate showed a lower LC_{50} (0.01937%) when compared to worms treated with GMIPA salt (1.662%). A (n=4); B (n=3). Pointed line indicates the LC_{50}.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

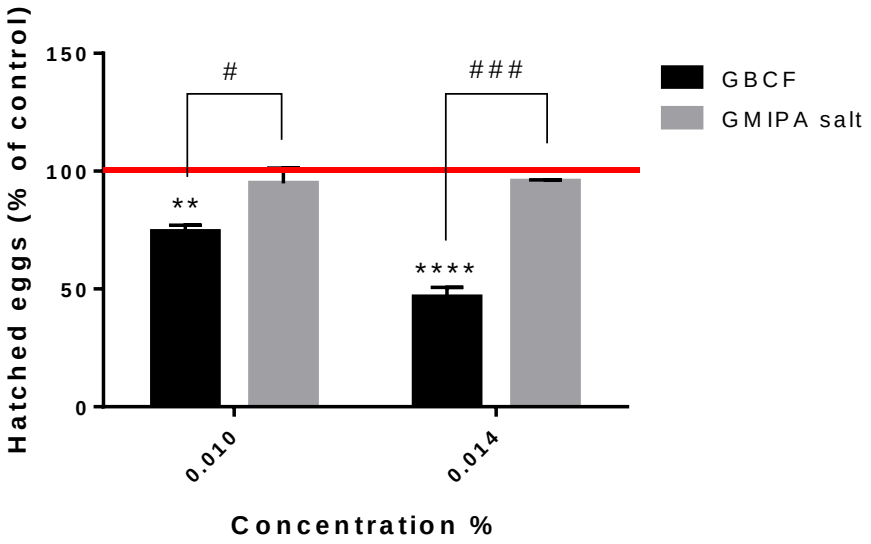

Figure 3. Brood size. Worms exposed to commercial glyphosate showed a significant reduction in the viability of the eggs and brood size in both concentrations when compared to control and GMIPA salt groups. n= 3. * indicates statistical significance in comparison with control group (untreated=100%).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

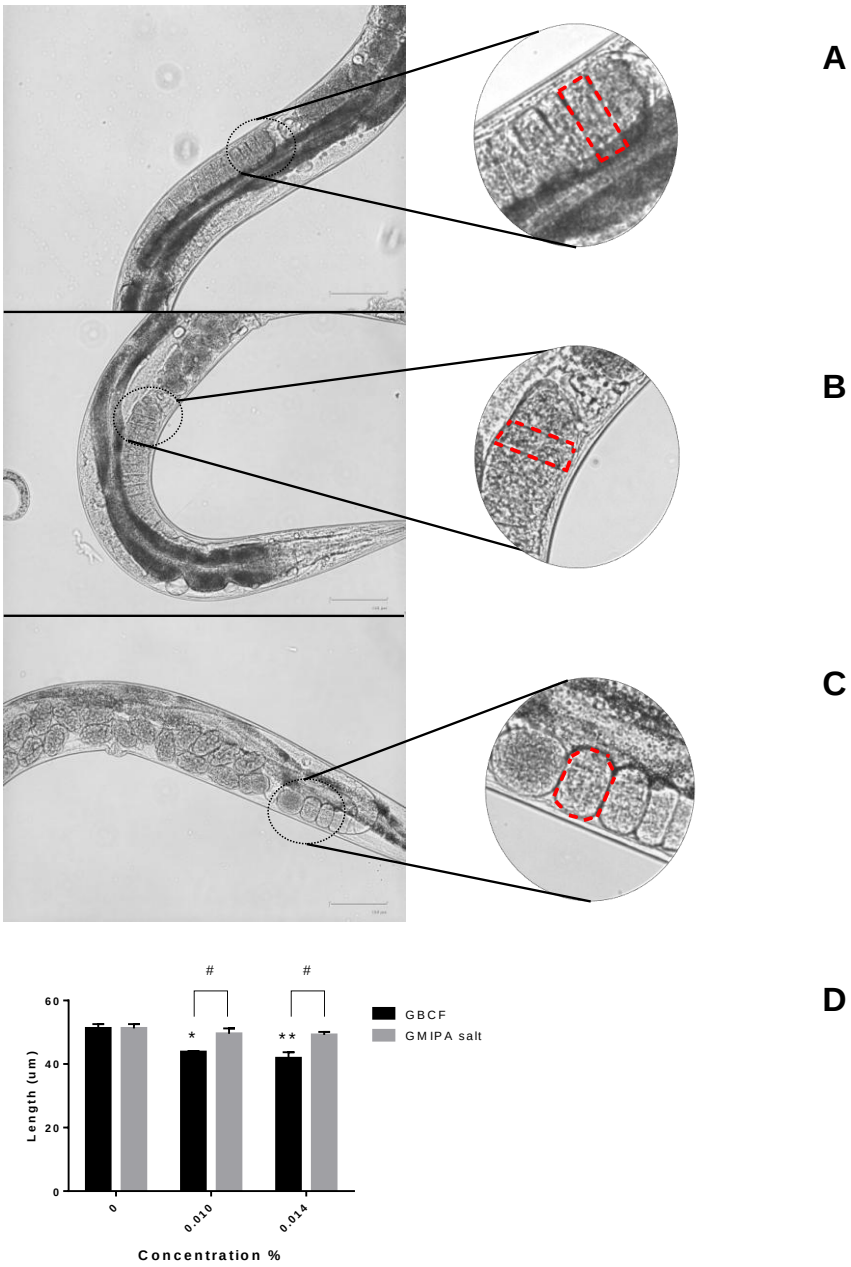

Figure 4. Length of -2 oocyte. Length of the second oocyte from the spermathecae was measured. Figure C shows the morphological alteration of oocytes in commercial glyphosate group (0.010%) when compared to control (A) and GMIPA salt 0.010% (B). The length decreased significantly in worms exposed to 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. #$p < 0.05$.

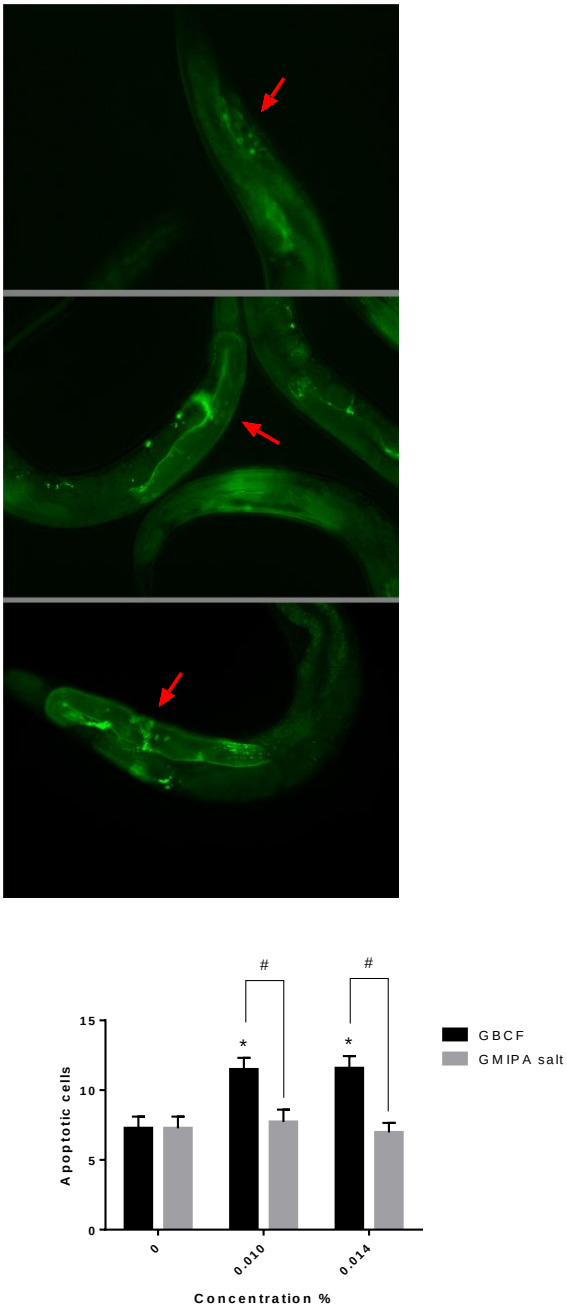

Figure 5. Apoptotic cells. MD701 strain showing germ cells in apoptosis (arrows). Worms exposed to the commercial formulation of glyphosate 0.010% (C) showed higher numbers of apoptotic cells when compared to control (A) and GMIPA salt (B). The significant differences remained in both concentrations 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.

Table 1. Metals found in GBCF

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**

Supplementary Material

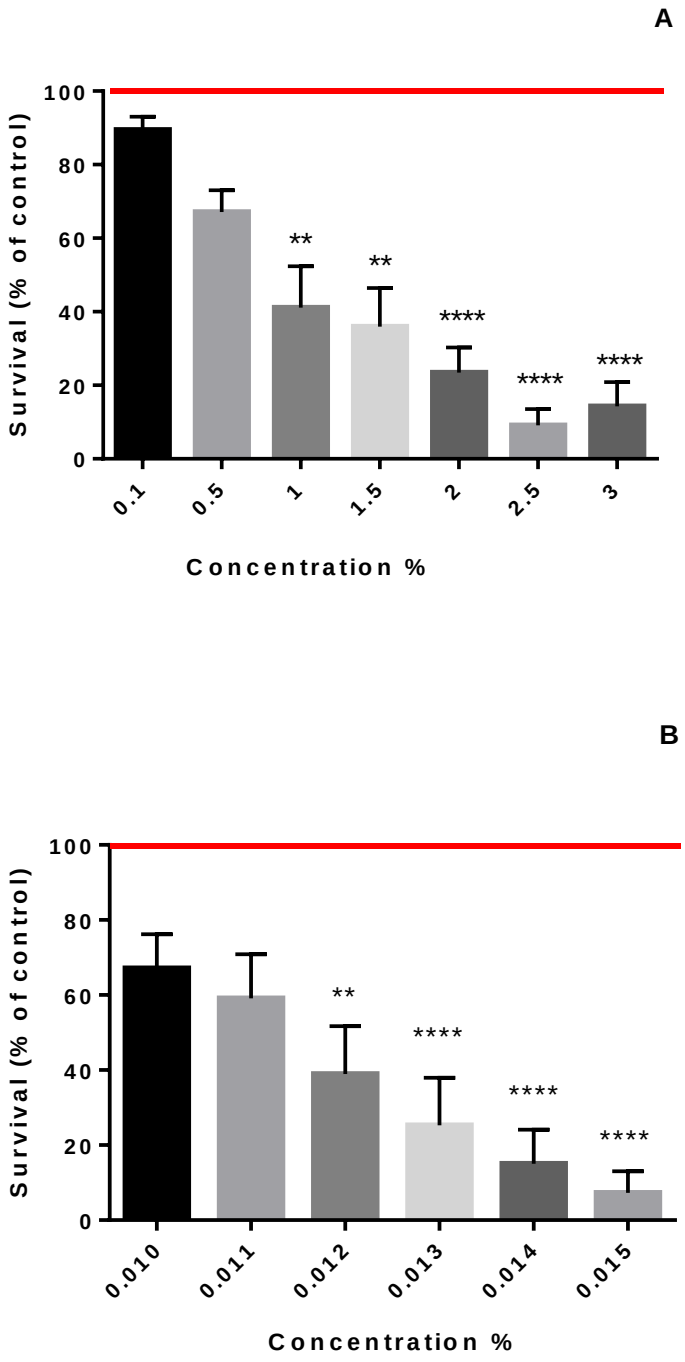

Figure S1. Survival rate. Worms (L1) exposed to different concentrations of GMIPA salt (A) and GBCF (B). Worms treated with commercial glyphosate showed a lower LC_{50} (0.0112%) when compared to worms treated with GMIPA salt (0.8045%). A (n=3); B (n=6). * indicates statistical significance in comparison with control group (untreated=100%) **p < 0.01; ***p < 0.001; ****p < 0.0001.

6. CONCLUSOES

Nesse trabalho a utilização do modelo alternativo C. *elegans* mostrou-se fundamental para a obtenção de diferentes perfis toxicológicos para exposições com glifosato e sua formulação comercial. Foi demonstrado que a presença dos inertes afetou negativamente o comprimento dos vermes e a capacidade reprodutiva, além de induzir a apoptose em células germinativas. Metais potencialmente tóxicos foram identificados na formulação comercial, denotando a omissão de sua presença no rótulo da formulação comercial. Espera-se que o conjunto de dados gerado por este trabalho, sirva como base cientifica para o desenvolvimento de regulamentações e políticas públicas que contribuam para a regulamentação da produção e comércio de pesticidas, visando maior segurança para humanos e o meio ambiente. Além disso, considerando o desenvolvimento de nanotecnologias, espera-se que nanomateriais que possam carrear pesticidas sejam avaliados e que possam reduzir a aplicação de pesticidas, bem como aplicá-los sem a necessidade de adjuvantes que contribuem para toxicidade de pesticidas.

7. PERSPECTIVAS

Metais são uma parte minoritária da composição total dos ingredientes inertes. Como perspectiva, devemos investigar a toxicidade dos metais isolados ou em conjunto com o princípio ativo, para de fato determinar se sua proporção é responsável pelos efeitos observados, ou isso é determinado por outros ingredientes inertes.

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