



Cytogenotoxicity of 2,4-D and Glyphosate Herbicides: Effects of Isolated and Combined Environmental Concentrations on Onion Root Tips (*Allium Cepa*)

Tatiana da Silva Souza · Gabriele Bitencourt Tavares · Victor Ventura de Souza

Received: 1 November 2023 / Accepted: 18 June 2024 / Published online: 13 July 2024
© The Author(s), under exclusive licence to Springer Nature Switzerland AG 2024

Abstract Glyphosate and 2,4-dichlorophenoxyacetic acid (2,4-D) are the most widely used pesticides in the world. In this work, the cytogenotoxic potential of these herbicides and their binary mixtures was investigated using *A. cepa* test. 2,4-D was assayed at 0.4 – 400 $\mu\text{g L}^{-1}$ and glyphosate at 0.65 – 650 $\mu\text{g L}^{-1}$ concentration range. The concentrations evaluated cover those permitted by Brazilian legislation for the protection of aquatic life and human health. Sixteen binary mixtures were examined to replicate different contamination scenarios. When analyzed separately, 2,4-D at 400 $\mu\text{g L}^{-1}$ inhibited the mitotic index, while glyphosate at 0.65 and 65 $\mu\text{g L}^{-1}$ increased it. In addition, the herbicides increased the frequency of chromosomal aberrations (2,4-D at 0.4, 4, and 40 $\mu\text{g L}^{-1}$ and glyphosate at 0.65, 65, and 650 $\mu\text{g L}^{-1}$) and micronuclei (2,4-D at 400 $\mu\text{g L}^{-1}$ and glyphosate at 650 $\mu\text{g L}^{-1}$). Regarding the mixtures, six of them were cytotoxic while eight were genotoxic. Additive, synergistic, or potentiated effects were observed. However, none of the mixtures tested

increased the frequency of micronuclei. The results showed that even concentrations allowed by Brazilian legislation pose a potential risk to exposed organisms. There is little research on the cytogenotoxic effects of 2,4-D + glyphosate mixtures, so this is the first study to analyze a large number of combinations. The detection of additive, synergistic, or potentiated toxicities highlights the need for better pesticide legislation and discussions among regulators and environmental managers regarding pesticide mixtures in water.

Keywords Herbicide Mixtures · Mitotic Index · Chromosomal Aberrations · Genotoxicity

1 Introduction

Water contamination by pesticides is a global concern, with a particular focus on Brazil because it ranks among the leading consumers of pesticides in the world (Brovini et al., 2023; Panis et al., 2022). According to the latest data from the Brazilian Institute of the Environment and Renewable Natural Resources, the number of active ingredients sold in the country doubled in a decade, rising from 425 thousand tons in 2012 to 800 thousand tons in 2022 (IBAMA, 2012, 2022). The widespread use of pesticides in Brazil has been defended based on the favorable climate that promotes large-scale agricultural output centered on monocultures and the production

T. da Silva Souza (✉) · G. B. Tavares
Departamento de Biologia, Centro de Ciências Exatas,
Naturais e da Saúde, Universidade Federal do Espírito
Santo, Alto Universitário, S/nº, Guararema, Alegre,
Espírito Santo 29500-000, Brasil
e-mail: tatianas.souza@hotmail.com; tatiana.s.souza@ufes.br

V. V. de Souza
Departamento de Morfologia, Universidade Federal de
Minas Gerais, Belo Horizonte, Minas Gerais, Brasil

of agrofuels (Brovini et al., 2021a; Gaboardi et al., 2023). Moreover, the country has permissive legislation that favors the production and registration of pesticides while neglecting inspection (Gaboardi et al., 2023; Souza et al., 2023a).

The herbicides glyphosate [N-(phosphonomethyl) glycine] and 2,4-dichlorophenoxyacetic acid (2,4-D) are the most widely used pesticides in Brazil and worldwide (Brovini et al., 2021a). Growers frequently combine these herbicides in tanks to achieve more efficient control of difficult-to-control weeds (Palma-Bautista et al., 2021; Takano et al., 2013). Heavily contaminated agricultural soils with glyphosate and 2,4-D lead to runoff and leaching (Carles et al., 2021; Lima et al., 2023), causing adverse effects on non-target organisms.

Considering this aspect, most of the knowledge on the toxic and genotoxic effects of glyphosate and 2,4-D on non-target organisms is derived from the effects of each compound separately. In this sense, both herbicides caused acute and chronic toxicity (Brovini et al., 2021a; Martins et al., 2021; Rodrigues et al., 2019), oxidative stress, and the induction of DNA damage (Arcaute et al., 2018; Gaaied et al., 2019; Nwani et al., 2013; Rodrigues et al., 2019) and micronuclei and chromosomal mutations (Marcato et al., 2017; Mercado & Caleño, 2020; Montero-Montoya et al., 2023; Truta et al., 2011; Vieira et al., 2024) in different organisms.

The International Agency for Research on Cancer categorizes glyphosate as probably carcinogen and 2,4-D (IARC, 2017) as possibly carcinogen to humans (IARC, 2018). In their review study, Panis et al. (2022) demonstrated the risks linked to widespread contamination of drinking water with proven, probable, or potentially carcinogenic pesticides in the second-largest grain-producing state in Brazil. In fact, in contrast to other countries with more restrictive legislation, pesticides with mutagenic, carcinogenic, and reproductive risks have been widely registered in Brazil, in an evident conflict of interests between the agribusiness industry and the scientific community (Rocha & Grisolia, 2019).

Since there is no safe exposure limit for genotoxic substances, and genotoxicity and mutagenicity are predictive factors for several human diseases, including cancer (Marchetti et al., 2023), the understanding the effects of 2,4-D and glyphosate in combination is particularly important because their interaction may

result in unexpected genotoxic damage to non-target organisms.

However, there have been limited studies on the cytogenotoxic effects of mixtures containing 2,4-D and glyphosate. Additionally, most of these studies did not evaluate concentrations that reflect those found in the water, or only a limited number of mixtures were examined. For instance, Bernardi et al. (2022) only associated one high concentration for the aquatic environment (5 mg L^{-1} of 2,4-D + 10 mg L^{-1} of glyphosate) and reported increased DNA damage using the comet assay on fish. Finkler et al. (2022) assessed the highest concentrations applied in the agricultural fields (1.56 mg mL^{-1} for glyphosate and 0.28 mg mL^{-1} for 2,4-D) and the acute reference doses (11.66 mg mL^{-1} for glyphosate and 1.75 mg mL^{-1} for 2,4-D). This study found that these mixtures decreased the mitotic index and increased the frequency of chromosomal aberrations on *Allium cepa*.

Carvalho et al. (2020) evaluated the effects of several commercial formulations containing 2,4-D and glyphosate on fish. The mixtures consisted of 5% + 5% or 10% + 10% of the LC50 for each substance: 4.58 and 9.17 mg L^{-1} glyphosate, 0.023 and 0.046 mg L^{-1} 2,4-D-acid, 33.90 and 67.80 mg L^{-1} 2,4-D-amine and 0.13 and 0.26 mg L^{-1} 2,4-D-ester. The results showed that the mixtures increased the genotoxic damage index, as measured by the comet assay.

Finally, Pavan et al. (2021) assessed five mixtures containing 2,4-D and glyphosate in concentrations representative of the aquatic environment. The lowest concentrations associated were 2,4-D at $4 \text{ } \mu\text{g L}^{-1}$ + glyphosate at $65 \text{ } \mu\text{g L}^{-1}$, which represent the maximum values allowed in Brazilian freshwater (Brasil, 2005; Table 1). The highest mixture assessed was 2,4-D at $74.5 \text{ } \mu\text{g L}^{-1}$ + glyphosate at $1000 \text{ } \mu\text{g L}^{-1}$. The mixtures increased the frequency of micronuclei and other erythrocyte nuclear abnormalities, as well as caused morphological and behavioral changes in tadpoles. It is important to note that this study only evaluated a limited number of mixtures. In addition, the authors did not compare the results of the mixtures regarding individual herbicide concentrations.

To address these gaps, we assessed the cytogenotoxic potential of 16 mixtures containing realistic environmental concentrations set by Brazilian legislation to protect aquatic life and human health. The concentrations tested ranged from 0.4 to $400 \text{ } \mu\text{g L}^{-1}$ for 2,4-D and 0.65 to $650 \text{ } \mu\text{g L}^{-1}$ for glyphosate.

Table 1 Maximum values allowed for 2,4-D and glyphosate herbicides ($\mu\text{g L}^{-1}$) in fresh and potable water in Brazil and other countries

Regulatory council	2,4-D ($\mu\text{g L}^{-1}$)	Glyphosate ($\mu\text{g L}^{-1}$)	References
Brazil	4 (class 2 and potable water); 30 (class 3)	65 (class 2); 280 (class 3); 500 (potable water)	Brazil (2005; 2011)
Canada	100	280	Health Canada (2019)
United States	70	700	US EPA (2024)
Argentina	100	*	Argentina (2019)
China	30	700	China (2006)
India	30	*	India (2012)
Japan	20	2000	Japan (2006)
European Union	0.1	0.1	EU (2020)
World Health Organization	30	*	WHO (2018)

*The Regulatory Council has not established a maximum value

The mixtures were formulated to replicate various scenarios, ranging from a less severe to the most extreme contamination scenario. Additionally, the study compared the effects of the mixtures concerning the individual herbicides. This is because chemical mixtures can result in either enhanced (additive or synergistic effect) or reduced (antagonistic effect) levels of toxicity compared to those of each herbicide when used in isolation (Ilyushina et al., 2020).

In this study, the cytogenotoxic assay was conducted on *A. cepa* root tip cells. This plant model is commonly used to evaluate environmental contaminants, including pesticides (Felisbino et al., 2018; Liman et al., 2015; Souza et al., 2016, 2022, 2023a, b; Türkoğlu, 2012; Verma & Srivastava, 2018). The *A. cepa* test has several advantages. Firstly, it assesses different endpoints such as cytotoxicity, genotoxicity, and mutagenicity. Secondly, it has a high correlation with carcinogenesis tests in rodents. Thirdly, it can detect chemical toxins even at low concentrations. Finally, it can indicate the mechanisms of action of the tested agents, whether pure substances or complex mixtures (Rank & Nielsen, 1994). Camilo-Cotrim et al. (2022) stated that the *A. cepa* bioassay is an alternative method to animal research based on the 3Rs (Replace Reduce Refine) principle due to its strong correlation with the carcinogenesis assay in rodents (Rank & Nielsen, 1994).

We hypothesize that the combination of 2,4-D and glyphosate may result in mixtures with increased cytogenotoxic effects, which are precursors of genetic instability.

2 Materials and Methods

2.1 Pesticides

Two commercial herbicide formulations were used in this study: (1) Roundup Original DI® Monsanto, containing 445 g L⁻¹ (44.5% w/v) diammonium salt of [N-(phosphonomethyl) glycine], 370 g L⁻¹ (37% w/v) acid equivalent of [N-(phosphonomethyl) glycine] and 751 g L⁻¹ (75% w/v) other ingredients; and (2) U-46 BR, containing 806 g L⁻¹ (80.6% w/v) 2,4- 2,4-dichloro phenoxy acetic acid (2,4-D amine) dimethylamine salt, 670 g L⁻¹ (67% w/v) acid equivalent and 380.9 g L⁻¹ (38.09% w/v) other ingredients.

The concentrations of 2,4-D and glyphosate were determined based on the acid equivalent concentration found in the commercial formulations. Stock solutions of 670 $\mu\text{g L}^{-1}$ 2,4-D and 740 $\mu\text{g L}^{-1}$ glyphosate were prepared, as well as test solutions of 0.4, 4, 40 and 400 $\mu\text{g L}^{-1}$ 2,4-D and 0.65, 6.5, 65 and 650 $\mu\text{g L}^{-1}$ glyphosate. The solutions were prepared using distilled water as the dilution medium.

The concentrations of individual herbicides were chosen based on the maximum values allowed by Resolution No. 357 of the National Council for the Environment (CONAMA) (Brasil, 2005). This resolution provides guidelines for the classification of water bodies. In class I waters (suitable for human consumption after simplified treatment and for the protection of aquatic communities, recreation, and irrigation) and class II waters (suitable for human consumption after conventional treatment and for the protection of aquatic communities, recreation,

irrigation, sport, leisure, aquaculture, and fishing), the maximum permitted concentrations of 2,4-D and glyphosate are 4 and 65 $\mu\text{g L}^{-1}$, respectively (Table 1).

The concentrations tested also included a) the maximum values allowed for class III waters (suitable for human consumption after conventional or advanced treatment and for recreation, irrigation, fishing and watering of animals) which can contain up to 30 $\mu\text{g L}^{-1}$ 2,4-D and 280 $\mu\text{g L}^{-1}$ glyphosate (Brasil, 2005); b) the maximum values established by Ordinance No. 2914 of the Ministry of Health (Brasil, 2011) legislating potable water, which regulates the levels of pesticides in water intended for human consumption (30 $\mu\text{g L}^{-1}$ for 2,4-D and 500 $\mu\text{g L}^{-1}$ for glyphosate) (Table 1); and c) the values detected in tap water according to data from the Information System for the Surveillance of the Quality of Water for Human Consumption (SISAGUA), coordinated by the Ministry of Health: up to 30 $\mu\text{g L}^{-1}$ for 2,4-D and up to 200 $\mu\text{g L}^{-1}$ for glyphosate (Aranha & Rocha, 2019).

The concentrations tested in this study also included the concentrations of 2,4-D and glyphosate allowed by the legislation of other countries, as presented in Table 1.

The binary mixtures were prepared by combining each concentration of 2,4-D with each concentration of glyphosate. Table 2 provides a summary of the individual concentrations and mixtures tested.

2.2 Bioassay with *A. Cepa*

Seeds of *A. cepa* (variety Baia Periforme) that had not been treated with pesticides were used. Distilled water was used as a negative control. Initially, the

seeds were germinated in Petri dishes lined with filter paper moistened with distilled water. When the roots reached approximately 1.5 cm, they were treated with the herbicides and mixtures for 24 h. Next, the roots were fixed in Carnoy I [ethanol+acetic acid (3:1, v/v)] and stored in a refrigerator. To prepare the slides, the roots were washed three times with distilled water for five minutes and then hydrolyzed in 5 N HCl for 20 min. The apical root meristem was sectioned, stained with 2% acetic orcein, and crushed between a slide and coverslip. The slides were analyzed under a light microscope (Leica DM750, Germany) equipped with an eyepiece camera (Leica ICC50, Germany) at $\times 400$ magnification. A total of 5,000 cells per treatment (500 cells/10 slides) were analyzed.

The effects of the herbicides and mixtures on cell cycle progression were evaluated using the phase index and the mitotic index. The phase index is determined by the frequency of cells in a particular phase of mitosis, while the mitotic index is characterized by the proportion of dividing cells. Any changes in these parameters compared to the control indicate cytotoxicity. The genotoxicity of the treatments was inferred by calculating the frequency of aberrant cells, while the mutagenic potential was assessed by determining the frequency of micronucleated cells (Leme & Marin-Morales, 2009). The following formulas have applied to calculate the mitotic index (MI), phases index (PI) and chromosomal aberrations (AC) and micronuclei (MN) frequencies:

$$MI(\%) = \frac{\text{number of cells in division}}{\text{total number of cells observed}} \times 100$$

$$PI(\%) = \frac{\text{particular phase of mitosis}}{\text{number of cells in division}} \times 100$$

Table 2 Concentrations of herbicides and binary mixtures

Concentration ($\mu\text{g L}^{-1}$)		2,4-D	0.4	4	40	400
Glyphosate	0.65		M1	M5	M9	M13
	6.5		M2	M6	M10	M14
	65		M3	M7	M11	M15
	650		M4	M8	M12	M16

$$AC(\%) = \frac{\text{number of cells with mitotic and chromosomal abnormalities}}{\text{total number of cells observed}} \times 100$$

$$MN(\%) = \frac{\text{number of cells with micronuclei}}{\text{total number of cells observed}} \times 100$$

2.3 Statistical Analysis

The *software* used for the statistical analysis was GraphPad Prism version 9.4. The normality of the collected data was tested using the Shapiro–Wilk test, and the homogeneity of variances was tested using the Brown–Forsythe test. As the data were not normally distributed and showed inequality of variance, comparisons between the experimental groups were performed using the Kruskal–Wallis test followed by Dunn’s test ($p < 0.05$). Comparisons were performed between a) the concentrations of each herbicide and the control group; b) the mixtures and the control group; and c) the mixture and each individual concentration of pesticide included in the mixture. The results are presented as the mean \pm standard deviation.

3 Results and Discussion

3.1 Cytogenotoxic Effect of Isolated Herbicide: 2,4-D

Table 3 displays the results obtained for the 2,4-D which only significantly inhibited the mitotic index at

a concentration of 400 $\mu\text{g L}^{-1}$ ($p = 0.0063$, $H = 28.23$; Kruskal–Wallis test). The reduction in mitotic activity is a cytotoxicity marker for all living organisms. It can delay normal development and growth of exposed organisms (Camilo-Cotrim et al., 2022). Antimitotic agents can damage DNA and impair the functioning of essential enzymes involved in cell cycle progression, including polymerases and kinases (Türkoğlu, 2012). Consequently, the cell cycle is transiently blocked due the activation of checkpoints, providing time for damage to be repaired before the transition from G1 to S and from G2 to M (Fioresi et al., 2020; Miranda et al., 2023; Moreira et al., 2021; Souza et al., 2023b).

Considering the genotoxicity index, 2,4-D at 0.4, 4 and 40 $\mu\text{g L}^{-1}$ significantly increased the frequency of aberrant cells compared to the control ($p < 0.0001$, $p = 0.0247$ and $p = 0.0225$, respectively, $H = 29.40$; Kruskal–Wallis test). According to Han et al. (2023), genotoxic agents can cause harm to DNA and cellular components responsible for maintaining the integrity of the genome, such as the spindle apparatus, topoisomerases, DNA repair systems, and DNA polymerases. Cells that are damaged and escape the repair processes activated during interphase contribute to an increased frequency of aberrant cells observed during mitosis (Fioresi et al., 2020).

On the contrary, the concentration of 400 $\mu\text{g L}^{-1}$ did not induce any mitotic and chromosomal

Table 3 Phase index, mitotic index, chromosomal aberrations and micronuclei (mean \pm standard deviation) in meristematic cells of *A. cepa* treated with the isolated herbicides

	Concentra- tions ($\mu\text{g L}^{-1}$)	Interfase	Prophase	Metaphase	Anaphase	Telophase	MI	CA	MN
Control	0	310.20 \pm 34.87	120.4 \pm 4.18	24.1 \pm 12.22	17.10 \pm 7.70	25.1 \pm 11.99	186.70 \pm 32.20	1.00 \pm 1.00	1.30 \pm 1.15
2,4-D	0.4	267.9 \pm 57.40	172.40 \pm 59.55	22.70 \pm 5.94	12.30 \pm 4.62	20.20 \pm 5.34	227.60 \pm 56.18	15.80 \pm 5.07	3.50 \pm 4.00
	4	317.6 \pm 37.26	120.90 \pm 28.66	28.40 \pm 11.48	10.10 \pm 3.90	18.8 \pm 8.03	178.20 \pm 37.18	10.5 \pm 7.38	4.20 \pm 2.34
	40	255.20 \pm 69.64	173.70 \pm 55.59	29.60 \pm 14.90	13.20 \pm 5.67	21.8 \pm 10.58	238.30 \pm 66.17	8.50 \pm 8.34	4.81 \pm 5.15
	400	471.50 \pm 10.10	5.00 \pm 2.35	2.70 \pm 2.71	2.10 \pm 2.28	5.10 \pm 4.53	14.90 \pm 9.68	2.10 \pm 2.76	13.9 \pm 4.70
Glyphosate	0.65	221.60 \pm 9.97	265.8 \pm 61.13	5.10 \pm 7.91	3.50 \pm 3.98	5.50 \pm 4.76	279.90 \pm 65.94	5.40 \pm 3.37	4.40 \pm 2.91
	6.5	315.00 \pm 45.46	139.80 \pm 5.83	16.20 \pm 8.71	11.30 \pm 6.97	18.00 \pm 6.25	185.3 \pm 46.24	6.90 \pm 4.67	0.6 \pm 1.07
	65	201.0 \pm 68.310	216.8 \pm 70.37	22.50 \pm 9.93	10.40 \pm 4.83	27.20 \pm 1.65	276.90 \pm 66.42	9.20 \pm 3.55	4.10 \pm 2.55
	650	247.10 \pm 46.83	182.10 \pm 4.83	23.40 \pm 6.89	13.60 \pm 5.48	24.80 \pm 6.37	243.90 \pm 44.77	17.60 \pm 7.01	9.20 \pm 4.63

MI: mitotic index. CA: chromosomal aberrations. MN: micronuclei. Values in bold denote statistical significance in relation to the control ($p < 0.05$)

changes above basal levels ($p > 0.9999$, $H = 29.40$; Kruskal–Wallis test). The low genotoxic damage index observed at this concentration can be attributed to its strong mitodepressive action. According to Dhyèvre et al. (2014), the reduction in mitotic activity can prevent the observation of aberrant cells, i.e., the genotoxic effect can be overshadowed by the cytotoxic effect. The limited number of cells that completed mitotic division inherited damage induced in the parental cells. As a result, the frequency of micronucleated cells was higher in the group treated with $400 \mu\text{g L}^{-1}$ 2,4-D than in the control group ($p < 0.0001$, $H = 24.68$; Kruskal–Wallis test). Micronuclei are among the main biomarkers of DNA damage and chromosomal instability. They arise from chromosome fragments (clastogenesis) or whole chromosomes (aneuploidy) that fail to segregate into daughter nuclei during mitosis (Fenech et al., 2020).

3.2 Cytogenotoxic Effect of Isolated Herbicide: Glyphosate

The results obtained for glyphosate are shown in Table 3. Glyphosate at 0.65 and $65 \mu\text{g L}^{-1}$ significantly increased the proportion of dividing cells ($p = 0.0054$ and $p = 0.0068$, respectively, $H = 20.25$; Kruskal–Wallis test). Additionally, both concentrations also increased the frequency of cells in prophase compared to the control ($p < 0.0001$ and $p = 0.0076$, respectively, $H = 28.50$; Kruskal–Wallis test). An increased mitotic index is also a cytotoxicity marker and can lead to disordered cell proliferation (Leme & Marin-Morales, 2009). Prophase arrest indicates the interference of chemical agents in crucial events that ensure the correct segregation of genetic material during mitosis, such as chromatin condensation and the assembly of the mitotic spindle. Seong et al. (2002) reported that prophase-arrested mammalian cells exhibited randomly oriented spindle structures. Therefore, prophase delay may result in chromosomal abnormalities.

Marc et al. (2004) demonstrated that glyphosate inhibits the activation of the CDK1/cyclin B, a regulator of the M-phase of the cell cycle, including the main events in prophase (Champion et al., 2017; Schatten, 2013). In addition, Díaz-Martín et al. (2021) reported that glyphosate decreased

levels of acetylated α -tubulin and caused microtubule instability.

In this study, glyphosate at concentrations of 6.5 , 65 , and $650 \mu\text{g L}^{-1}$ significantly increased the frequency of aberrant cells compared to untreated cells ($p = 0.0157$, $p = 0.0022$ and $p < 0.0001$ respectively, $H = 30.86$; Kruskal–Wallis test). Regarding micronuclei, only the concentration of $650 \mu\text{g L}^{-1}$ induced a higher damage rate than the control ($p = 0.0002$, $H = 30.05$; Kruskal–Wallis test).

3.3 Cytogenotoxic Effect of Mixtures: 2,4-D + Glyphosate

The *A. cepa* test was also employed to assess the cytogenotoxic effects of the mixtures 2,4-D + glyphosate compared to the control group (Table 4). To better understand these results, we performed a comparative analysis of each mixture with respect to the effects of the individual herbicides (Table 5).

Six mixtures, M8, M10, M12, M13, M15 and M16, inhibited the mitotic index compared to the control ($p = 0.0002$, $p = 0.0139$, $p = 0.0002$, $p = 0.0256$, $p = 0.0034$ and $p < 0.0001$, respectively, $H = 135.0$; Kruskal–Wallis-test) (Table 4). When comparing these effects with those of each herbicide, two distinct responses were obtained: (a) mixtures that showed a lower mitotic index than both the herbicides (M8, M10, and M12), which in turn did not differ statistically from each other (M8 vs. $4 \mu\text{g L}^{-1}$: $p = 0.0175$, M8 vs. $650 \mu\text{g L}^{-1}$: $p < 0.0001$, $4 \mu\text{g L}^{-1}$ vs. $650 \mu\text{g L}^{-1}$: $p = 0.1263$, $H = 52.43$; M10 vs. $40 \mu\text{g L}^{-1}$: $p < 0.0001$, M10 vs. $6.5 \mu\text{g L}^{-1}$: $p = 0.0092$, $40 \mu\text{g L}^{-1}$ vs. $6.5 \mu\text{g L}^{-1}$: $p = 0.6394$, $H = 18.66$; M12 vs. $40 \mu\text{g L}^{-1}$: $p = 0.0006$, M12 vs. $650 \mu\text{g L}^{-1}$: $p = 0.0003$, $40 \mu\text{g L}^{-1}$ vs. $650 \mu\text{g L}^{-1}$: $p > 0.9999$, $H = 19.40$; Kruskal–Wallis test (Table 5); and (b) mixtures that significantly inhibited the mitotic index compared to glyphosate and showed a higher mitotic index in relation to 2,4-D (M13, M15 and M16). In this case, the herbicides differed statistically from each other (M13 vs. $400 \mu\text{g L}^{-1}$: $p = 0.0333$, M13 vs. $0.65 \mu\text{g L}^{-1}$: $p = 0.0333$, $400 \mu\text{g L}^{-1}$ vs. $0.65 \mu\text{g L}^{-1}$: $p < 0.0001$, $H = 25.81$; M15 vs. $400 \mu\text{g L}^{-1}$: $p = 0.0332$, M15 vs. $65 \mu\text{g L}^{-1}$: $p = 0.0332$, $400 \mu\text{g L}^{-1}$ vs. $65 \mu\text{g L}^{-1}$: $p < 0.0001$, $H = 25.81$; M16 vs. $400 \mu\text{g L}^{-1}$: $p = 0.0333$, M16 vs. $650 \mu\text{g L}^{-1}$: $p = 0.0333$, $400 \mu\text{g L}^{-1}$ vs. $650 \mu\text{g L}^{-1}$: $p < 0.0001$, $H = 25.81$; Kruskal–Wallis test) (Table 5).

Table 4 Phase index, mitotic index, chromosomal aberrations, and micronuclei (mean \pm standard deviation) in meristematic cells of *A. cepa* treated with mixtures of herbicides 2,4-D + glyphosate ($\mu\text{g L}^{-1}$)

	Interfase	Prophase	Metaphase	Anaphase	Telophase	MI	CA	MN
Control	310.20 \pm 34.87	120.4 \pm 24.18	24.10 \pm 12.22	17.10 \pm 7.70	25.10 \pm 11.99	186.70 \pm 32.20	1.00 \pm 1.00	1.30 \pm 1.15
Mixtures								
M1 (0.4+0.65)	215.30 \pm 41.87	235.40 \pm 31.58	25.10 \pm 11.17	12.30 \pm 4.76	17.40 \pm 9.94	290.20 \pm 33.36	13.10 \pm 6.72	6.30 \pm 5.20
M2 (0.4+0.65)	188.20 \pm 34.21	251.20 \pm 36.73	28.90 \pm 7.85	17.60 \pm 5.71	22.50 \pm 6.68	320.20 \pm 26.99	11.70 \pm 6.4	3.10 \pm 2.76
M3 (0.4+65)	272.5 \pm 28.40	167.80 \pm 32.04	22.60 \pm 4.74	16.10 \pm 6.43	19.00 \pm 3.29	225.50 \pm 28.92	6.30 \pm 8.3	2.30 \pm 1.76
M4 (0.4+650)	298.10 \pm 40.73	167.70 \pm 39.52	12.70 \pm 7.68	6.00 \pm 5.61	11.30 \pm 6.34	197.00 \pm 37.21	10.20 \pm 10.20	5.80 \pm 4.54
M5 (4+0.65)	366.10 \pm 26.93	94.10 \pm 23.83	15.20 \pm 5.45	11.00 \pm 3.52	10.90 \pm 3.98	131.20 \pm 25.82	8.40 \pm 5.2	3.20 \pm 2.93
M6 (4+6.5)	375.90 \pm 32.58	109.30 \pm 34.26	18.10 \pm 6.57	12.10 \pm 4.17	13.10 \pm 4.97	152.60 \pm 33.40	10.90 \pm 5.7	6.20 \pm .86
M7 (4+65)	391.90 \pm 93.06	100.10 \pm 38.03	14.60 \pm 6.75	8.00 \pm 5.03	9.20 \pm 4.28	131.90 \pm 40.22	4.60 \pm 3.65	3.60 \pm 4.55
M8 (4+650)	417.70 \pm 24.57	44.50 \pm 25.17	14.30 \pm 8.42	9.40 \pm 5.08	8.90 \pm 3.90	77.10 \pm 24.07	3.60 \pm 3.70	4.00 \pm 4.26
M9 (40+0.65)	358.20 \pm 41.87	85.60 \pm 33.46	24.10 \pm 9.42	13.20 \pm 5.11	14.10 \pm 4.01	137.00 \pm 40.67	12.30 \pm 11.16	5.50 \pm 4.30
M10 (40+6.5)	395.70 \pm 28.36	63.60 \pm 23.11	18.60 \pm 3.06	8.30 \pm 3.49	12.20 \pm 5.65	102.70 \pm 28.54	6.70 \pm 7.76	3.30 \pm 2.58
M11 (40+65)	348.00 \pm 31.74	95.40 \pm 27.01	23.70 \pm 5.73	14.80 \pm 5.57	17.50 \pm 7.56	151.40 \pm 36.20	5.20 \pm 3.39	4.20 \pm 3.67
M12 (4+650)	415.80 \pm 26.87	45.60 \pm 14.70	14.20 \pm 7.85	9.40 \pm 5.50	9.10 \pm 4.99	78.30 \pm 25.84	4.40 \pm 3.20	6.00 \pm 4.44
M13 (400+0.65)	390.20 \pm 19.78	57.10 \pm 16.16	20.80 \pm 5.49	9.70 \pm 3.56	17.40 \pm 5.84	105.00 \pm 19.07	7.80 \pm 5.34	5.50 \pm 5.56
M14 (400+6.5)	387.51 \pm 21.80	69.50 \pm 19.46	23.90 \pm 7.46	11.00 \pm 4.18	14.30 \pm 6.46	118.70 \pm 20.34	5.70 \pm 4.85	2.80 \pm 1.93
M15 (400+65)	358.50 \pm 56.57	49.30 \pm 11.84	19.60 \pm 4.88	9.70 \pm 6.44	16.20 \pm 4.41	94.80 \pm 12.70	5.20 \pm 3.79	5.70 \pm 3.88
M16 (400+650)	423.70 \pm 21.16	35.40 \pm 8.69	15.50 \pm 5.91	7.90 \pm 5.54	11.30 \pm 6.16	70.10 \pm 15.96	10.010.19	6.50 \pm 4.11

MI: mitotic index. AC: chromosomal aberrations. MN: micronuclei. Values in bold denote statistical significance in relation to the control ($p < 0.05$)

In summary, the cytotoxicity of mixtures M8, M10, and M12 was observed, while the individual herbicides did not show any cytotoxicity, indicating a synergistic effect. The mixtures M13 and M15 were found to be more cytotoxic than the isolated herbicides and showed additive or synergistic toxicities (Table 5). In general terms, an additive effect is the sum of the response of each substance. When the observed response exceeds the additive effect, this is assigned as a synergistic pattern (Carvalho et al., 2020; Finkler et al., 2022). The effect of M16 can be described as potentiation, in which the effect of only one of the constituents of the mixture is exerted (Felisbino et al., 2018).

Based on these results, it can be inferred that the cytotoxicity of the mixtures M8, M10, M12, M13, and M15 was due to the toxic action of the two herbicides, while only the M16 mixture ($400 \mu\text{g L}^{-1}$ 2,4-D + $650 \mu\text{g L}^{-1}$ glyphosate) exhibited an adverse effect attributed to 2,4-D.

Considering the mixtures that did not cause a change in the mitotic index compared to the

control, the findings obtained were as follows: (a) the response of M1, M3, M4, and M6 was equivalent to that of the individual herbicides (M1 vs. $0.4 \mu\text{g L}^{-1}$: $p = 0.0545$, M1 vs. $0.65 \mu\text{g L}^{-1}$: $p > 0.9999$, $0.4 \mu\text{g L}^{-1}$ vs. $0.65 \mu\text{g L}^{-1}$: $p = 0.1303$, $H = 6.52$; M3 vs. $0.4 \mu\text{g L}^{-1}$: $p > 0.9999$, M3 vs. $65 \mu\text{g L}^{-1}$: $p = 0.1383$, $0.4 \mu\text{g L}^{-1}$ vs. $65 \mu\text{g L}^{-1}$: $p = 0.1752$, $H = 5.05$; M4 vs. $0.4 \mu\text{g L}^{-1}$: $p = 0.8238$, M4 vs. $650 \mu\text{g L}^{-1}$: $p = 0.0582$, $0.4 \mu\text{g L}^{-1}$ vs. $650 \mu\text{g L}^{-1}$: $p = 0.6394$, $H = 5.47$; M6 vs. $4 \mu\text{g L}^{-1}$: $p = 0.3115$, M6 vs. $6.5 \mu\text{g L}^{-1}$: $p = 0.1801$, $4 \mu\text{g L}^{-1}$ vs. $6.5 \mu\text{g L}^{-1}$: $p > 0.9999$, $H = 4.165$; Kruskal–Wallis test); b) M2 showed a higher mitotic index than both the herbicides (M2 vs. $0.4 \mu\text{g L}^{-1}$: $p = 0.0162$, M2 vs. $6.5 \mu\text{g L}^{-1}$: $p > 0.0001$, $0.4 \mu\text{g L}^{-1}$ vs. $6.5 \mu\text{g L}^{-1}$: $p = 0.4427$, $H = 18.49$; Kruskal–Wallis test); (c) M5 and M7 exhibited equivalent mitotic index to that 2,4-D, but significantly lower than glyphosate (M5 vs. $4 \mu\text{g L}^{-1}$: $p = 0.1114$, M5 vs. $0.65 \mu\text{g L}^{-1}$: $p > 0.0001$, $4 \mu\text{g L}^{-1}$ vs. $0.65 \mu\text{g L}^{-1}$: $p = 0.0622$, $H = 19.35$; M7 vs. $4 \mu\text{g L}^{-1}$: $p = 0.1910$, M7 vs. $65 \mu\text{g L}^{-1}$: $p > 0.0001$, $4 \mu\text{g L}^{-1}$ vs. $65 \mu\text{g L}^{-1}$: $p = 0.0412$, $H = 18.77$; Kruskal–Wallis

Table 5 Cytogenotoxic effects of the mixtures in relation to that of the individual herbicides

	MI	AC	MN
Control	186.70±32.20	1.00±1.00	1.30±1.15
0.4	227.60±56.18a	15.80±5.07a	3.50±4.00a
0.65	279.90±65.94a	5.40±3.37b	4.40±2.91a
M1 (0.4 + 0.65)	290.20±33.36a	13.10±6.72a	6.30±5.20a
0.4	227.60±56.18a	15.80±5.07a	3.50±4.00a
6.5	185.3±46.24a	6.90±4.67bc	0.60±1.07a
M2 (0.4 + 6.5)	320.20±26.99b	11.70±6.4ac	3.10±2.76a
0.4	227.60±56.18a	15.80±5.07a	3.50±4.00a
65	276.90±66.42a	9.20±3.55ac	4.10±2.55a
M3 (0.4 + 65)	225.50±28.92a	6.30±8.3c	2.30±1.76a
0.4	227.60±56.18a	15.80±5.07a	3.50±4.00a
650	243.90±44.77a	17.60±7.01a	9.20±4.63b
M4 (0.4 + 650)	197.00±37.21a	10.20±10.20a	5.80±4.54ab
4	178.20±37.18ab	10.50±7.38a	4.20±2.34a
0.65	279.90±65.94a	5.40±3.37a	4.40±2.91a
M5 (4 + 0.65)	131.20±25.82b	8.40±5.20a	3.20±2.93a
4	178.20±37.18a	10.50±7.38a	4.20±2.34a
6.5	185.3±46.24a	6.90±4.67a	0.60±1.07b
M6 (4 + 6.5)	152.60±33.40a	10.90± 5.70a	6.20±5.86a
4	178.20±37.18a	10.5±7.38a	4.20±2.34a
65	276.90±66.42b	9.20±3.55a	4.10±2.55a
M7 (4 + 65)	131.90±40.22a	4.60±3.65a	3.60±4.55a
4	178.20±37.18a	10.50±7.38ab	4.20±2.34ab
650	243.90±44.77a	17.60±7.01a	9.20±4.63a
M8 (4 + 650)	77.10±24.07b	3.60±3.70b	4.00±4.26b

Table 5 (continued)

40	238.30±66.17a	8.50±8.34a	4.81±5.15a
0.65	279.90±65.94a	5.40±3.37a	4.40±2.91a
M9 (40 + 0.65)	137.00±40.67b	12.30±11.16a	5.50±4.30a
40	238.30±66.17a	8.50±8.34a	4.81±5.15a
6.5	185.30±46.24a	6.90±4.67a	0.60±1.07b
M10 (40 + 6.5)	102.70±28.54b	6.70±7.76a	3.30±2.58ab
40	238.30±66.17a	8.50±8.34a	4.81±5.15a
65	276.90±66.42a	9.20±3.55a	4.10±2.55a
M11 (40 + 65)	151.40±36.20b	5.20±3.39a	4.20±3.67a
40	238.30±66.17a	8.50±8.34ab	4.81±5.15a
650	243.90±44.77a	17.60±7.01a	9.20±4.63a
M12 (40 + 650)	78.30±25.84b	4.40±3.20b	6.00±4.44a
400	14.90±9.68a	2.10±2.76a	13.90±4.70a
0.65	279.90±65.94b	5.40±3.37ab	4.40±2.91b
M13 (400 + 0.65)	105.00±9.07c	7.80±5.34b	5.50±5.56b
400	14.90±9.68a	2.10±2.76a	13.90 ± 4.70a
6.5	185.30±46.24b	6.90±4.67b	0.60±1.07b
M14 (400 + 6.5)	118.70±20.34b	5.70±4.85ab	2.80±1.93b
400	14.90±9.68a	2.10±2.76a	13.90±4.70a
65	276.90±66.42b	9.20±3.55b	4.10±2.55b
M15 (400 + 65)	94.80±12.70c	5.20±3.79ab	5.70±3.88b
400	14.90±9.68a	2.10± 2.76a	13.90±4.70a
650	243.90±44.77b	17.60±7.01b	9.20±4.63ab
M16 (400 + 650)	70.10±15.96c	10.0±10.19ab	6.50± 4.11b

Concentrations of 2,4-D: 0.4, 4, 40 and 400 $\mu\text{g L}^{-1}$. Concentrations of glyphosate: 0.65, 6.5, 65 and 650 $\mu\text{g L}^{-1}$. MI: mitotic index. AC: chromosomal aberrations. MN: micronucleated cells. Values in bold denote statistical significance in relation to the control ($p < 0.05$). In the same column, values followed by different letters differ statistically from each other ($p < 0.05$). The colors indicate the possible effects of the mixtures: orange – additive or synergistic effect, pink – potentiation effect, yellow – antagonistic effect, red – the cytogenotoxicity was not increased in relation to each herbicide alone, brown – no interaction

test); (d) M9 and M11 showed a lower mitotic index than the both 2,4-D and glyphosate (M9 vs $40 \mu\text{g L}^{-1}$: $p=0.0092$, M9 vs. $0.65 \mu\text{g L}^{-1}$: $p=0.0002$, $40 \mu\text{g L}^{-1}$ vs. $0.65 \mu\text{g L}^{-1}$: $p=0.9286$, $H=17.07$; M11 vs. $40 \mu\text{g L}^{-1}$: $p=0.0156$, M11 vs. $65 \mu\text{g L}^{-1}$: $p=0.0005$, $40 \mu\text{g L}^{-1}$ vs $65 \mu\text{g L}^{-1}$: $p>0.999$, $H=15.25$; Kruskal–Wallis test); and (e) M14 exhibited equivalent mitotic index to that glyphosate, but significantly higher than 2,4-D (M14 vs. $400 \mu\text{g L}^{-1}$: $p=0.0175$, M14 vs. $6.5 \mu\text{g L}^{-1}$: $p=0.1049$, $400 \mu\text{g L}^{-1}$ vs. $6.5 \mu\text{g L}^{-1}$: $p<0.0001$, $H=23.81$; Kruskal–Wallis test) (Table 5).

In relation to non-cytotoxic mixtures, M5 and M7 showed a mitotic index equivalent to that of 2,4-D, but significantly lower than glyphosate (individually cytotoxic). Conversely, other hand, the cytotoxicity of 2,4-D was reduced in M14. As the toxicity of the herbicides was nullified in these mixtures, an antagonistic effect is suggested. According to Carvalho et al. (2020), antagonism occurs when the toxicity of the mixture is smaller than the effect caused by any of the components alone. Although M1 and M3 are similar to 2,4-D and glyphosate, they showed an antagonistic effect. Similarly, M9 and M11 were considered antagonist mixtures when compared to the separate herbicides. On the other hand, there were no significant interactions observed in the herbicide mixtures M2, M4, and M6 that would result in a discernible alteration in the mitotic index.

Regarding the chromosomal aberration index, M1, M2, M4, M5, M6, M9, M13 and M16 (half of the mixtures) induced greater damage than the control ($p<0.0001$, $p<0.0001$, $p=0.0036$, $p=0.0026$, $p<0.001$, $p=0.0004$, $p=0.0072$ and $p=0.0130$, respectively, $H=52.43$; Kruskal–Wallis test) (Table 4) and yielded the following results: a) mixture whose genotoxicity is equivalent to 2,4-D (M1) (M1 vs. $0.4 \mu\text{g L}^{-1}$: $p=0.9452$, M1 vs. $0.65 \mu\text{g L}^{-1}$: $p=0.0342$, $0.4 \mu\text{g L}^{-1}$ vs. $0.65 \mu\text{g L}^{-1}$: $p=0.0012$, $H=13.27$; Kruskal–Wallis test) (Table 5); b) mixture whose genotoxicity is equivalent to glyphosate (M13) (M13 vs. $400 \mu\text{g L}^{-1}$: $p=0.0246$, M13 vs. $0.65 \mu\text{g L}^{-1}$: $p>0.9999$, $400 \mu\text{g L}^{-1}$ vs. $0.65 \mu\text{g L}^{-1}$: $p=0.1881$, $H=7.38$; Kruskal–Wallis test) (Table 5); and mixtures whose genotoxicity is equivalent to both herbicides (M2, M4, M5, M6, M9 and M16) (M2 vs $0.4 \mu\text{g L}^{-1}$: $p=0.3706$, M2 vs. $6.5 \mu\text{g L}^{-1}$: $p=0.2940$, $0.4 \mu\text{g L}^{-1}$ vs. $6.5 \mu\text{g L}^{-1}$: $p=0.0042$, $H=10.21$; M4 vs $0.4 \mu\text{g L}^{-1}$: $p=0.2057$, M4 vs. $650 \mu\text{g L}^{-1}$: $p=0.0573$, $0.4 \mu\text{g L}^{-1}$ vs. $650 \mu\text{g L}^{-1}$: $p>0.9999$,

$H=6.06$; M5 vs. $4 \mu\text{g L}^{-1}$: $p>0.9999$, M5 vs. $0.65 \mu\text{g L}^{-1}$: $p=0.6198$, $4 \mu\text{g L}^{-1}$ vs. $0.65 \mu\text{g L}^{-1}$: $p=0.2995$, $H=2.97$; M6 vs. $4 \mu\text{g L}^{-1}$: $p>0.9999$, M6 vs. $6.5 \mu\text{g L}^{-1}$: $p=0.2617$, $4 \mu\text{g L}^{-1}$ vs. $6.5 \mu\text{g L}^{-1}$: $p=0.6472$, $H=3.12$; M9 vs $40 \mu\text{g L}^{-1}$: $p=0.9609$, M9 vs. $0.65 \mu\text{g L}^{-1}$: $p=0.3788$, $40 \mu\text{g L}^{-1}$ vs. $0.65 \mu\text{g L}^{-1}$: $p>0.9999$, $H=2.41$; M16 vs. $400 \mu\text{g L}^{-1}$: $p=0.1597$, M16 vs. $650 \mu\text{g L}^{-1}$: $p=0.2882$, $400 \mu\text{g L}^{-1}$ vs. $650 \mu\text{g L}^{-1}$: $p=0.0010$, $H=12.96$; Kruskal–Wallis test) (Table 5).

These findings indicate that M1 exhibited a potentiation effect due to 2,4-D. The effect of M5, M9 and M16 also suggests potentiation since only one herbicide in the combinations exerted genotoxic activity. The genotoxicity of the M2, M4, and M6 mixtures was similar for both herbicides, indicating no additive or synergistic effect. Finally, the response of M13 suggests a synergistic effect.

Regarding the nongenotoxic mixtures in relation to the control, the combination 2,4-D+glyphosate yielded: a) mixtures equivalent to the both the herbicides (M7, M10, M11, M14 and M15) (M7 vs. $4 \mu\text{g L}^{-1}$: $p=0.0910$, M7 vs. $65 \mu\text{g L}^{-1}$: $p=0.0774$, $4 \mu\text{g L}^{-1}$ vs. $65 \mu\text{g L}^{-1}$: $p=0.9999$, $H=6.44$; M10 vs. $40 \mu\text{g L}^{-1}$: $p>0.9999$, M10 vs. $6.5 \mu\text{g L}^{-1}$: $p=0.9601$, $40 \mu\text{g L}^{-1}$ vs. $6.5 \mu\text{g L}^{-1}$: $p>0.9999$, $H=1.37$; M11 vs. $40 \mu\text{g L}^{-1}$: $p>0.9999$, M11 vs. $65 \mu\text{g L}^{-1}$: $p=0.1001$, $40 \mu\text{g L}^{-1}$ vs. $65 \mu\text{g L}^{-1}$: $p=0.6496$, $H=4.57$; M14 vs. $400 \mu\text{g L}^{-1}$: $p=0.1818$, M14 vs. $6.5 \mu\text{g L}^{-1}$: $p>0.9999$, $400 \mu\text{g L}^{-1}$ vs. $6.5 \mu\text{g L}^{-1}$: $p=0.0283$, $H=7.19$; M15 vs. $400 \mu\text{g L}^{-1}$: $p=0.2320$, M15 vs. $65 \mu\text{g L}^{-1}$: $p=0.1959$, $400 \mu\text{g L}^{-1}$ vs. $65 \mu\text{g L}^{-1}$: $p=0.0009$, $H=13.03$; Kruskal–Wallis test) (Table 5); b) mixture statistically different to the 2,4-D (M3) (M3 vs. $0.4 \mu\text{g L}^{-1}$: $p=0.0021$, M3 vs. $65 \mu\text{g L}^{-1}$: $p=0.496$, $0.4 \mu\text{g L}^{-1}$ vs. $65 \mu\text{g L}^{-1}$: $p=0.1372$, $H=11.58$; Kruskal–Wallis test) (Table 5); c) mixture statistically different to the glyphosate (M8 and M12) (M8 vs. $4 \mu\text{g L}^{-1}$: $p=0.1040$, M8 vs. $650 \mu\text{g L}^{-1}$: $p=0.0004$, $4 \mu\text{g L}^{-1}$ vs. $650 \mu\text{g L}^{-1}$: $p=0.2506$, $H=14.81$; M12 vs. $40 \mu\text{g L}^{-1}$: $p=0.7882$, M12 vs. $65 \mu\text{g L}^{-1}$: $p=0.0016$, $40 \mu\text{g L}^{-1}$ vs. $650 \mu\text{g L}^{-1}$: $p=0.0576$, $H=12.48$; Kruskal–Wallis test) (Table 5). The ability of one or both herbicides to induce genotoxic damage was reduced in all these mixtures, characterizing an antagonistic effect. (Table 5).

Finally, no mixture increased the frequency of cells with micronuclei compared to the control

($p > 0.05$, $H = 25.42$; Kruskal–Wallis test) (Table 4). Therefore, it suggested that 2,4-D at $400 \mu\text{g L}^{-1}$ lost its mutagenic effect when combined with varying concentrations of glyphosate. The same occurred for glyphosate at $650 \mu\text{g L}^{-1}$ when mixed with different concentrations of 2,4-D, resulting in an antagonistic effect for M4, M8, M12, M13, M14, M15 and M16) (M4 vs. $0.4 \mu\text{g L}^{-1}$: $p = 0.7515$, M4 vs. $650 \mu\text{g L}^{-1}$: $p = 0.5037$, $0.4 \mu\text{g L}^{-1}$ vs. $650 \mu\text{g L}^{-1}$: $p = 0.0344$, $H = 6.41$; M8 vs. $4 \mu\text{g L}^{-1}$: $p > 0.9999$, M8 vs. $650 \mu\text{g L}^{-1}$: $p = 0.0145$, $4 \mu\text{g L}^{-1}$ vs. $650 \mu\text{g L}^{-1}$: $p = 0.0933$, $H = 8.69$; M12 vs. $40 \mu\text{g L}^{-1}$: $p > 0.9999$, M12 vs. $650 \mu\text{g L}^{-1}$: $p = 0.3969$, $40 \mu\text{g L}^{-1}$ vs. $650 \mu\text{g L}^{-1}$: $p = 0.0608$, $H = 5.55$; M13 vs. $400 \mu\text{g L}^{-1}$: $p = 0.0079$, M13 vs. $0.65 \mu\text{g L}^{-1}$: $p > 0.9999$, $400 \mu\text{g L}^{-1}$ vs. $0.65 \mu\text{g L}^{-1}$: $p = 0.0043$, $H = 12.81$; M14 vs. $400 \mu\text{g L}^{-1}$: $p = 0.0015$, M14 vs. $6.5 \mu\text{g L}^{-1}$: $p = 0.1923$, $400 \mu\text{g L}^{-1}$ vs. $6.5 \mu\text{g L}^{-1}$: $p < 0.0001$, $H = 22.87$; M15 vs. $400 \mu\text{g L}^{-1}$: $p = 0.0084$, M15 vs. $65 \mu\text{g L}^{-1}$: $p = > 0.99999$, $400 \mu\text{g L}^{-1}$ vs. $65 \mu\text{g L}^{-1}$: $p = 0.0002$, $H = 16.96$; M16 vs. $400 \mu\text{g L}^{-1}$: $p = 0.0072$, M16 vs. $650 \mu\text{g L}^{-1}$: $p = 0.7227$, $400 \mu\text{g L}^{-1}$ vs. $650 \mu\text{g L}^{-1}$: $p = 0.1882$, $H = 9.36$; Kruskal–Wallis test) (Table 5).

3.4 Representative Micrographs of Chromosomal Abnormalities and Micronuclei Observed

Figure 1 presents illustrative micrographs showing the main chromosomal abnormalities and micronuclei identified after exposure to the isolated herbicides and mixtures.

3.5 Final Considerations

Water contamination by pesticides has been studied by several researchers (Rocha & Grisolia, 2019; Daam et al., 2019; Moutinho et al., 2020; Brovini et al., 2021a; b; Mesnage et al., 2021; Panis et al., 2022; Gaboardi et al., 2023) due to its negative impact on human and environmental well-being.

Several studies have reported the presence of glyphosate and 2,4-D in Brazilian waters. However, it is important to note that the recorded concentrations vary depending on the study. In a broad review, Brovini et al. (2023) reported that the highest concentration of glyphosate found in freshwaters was $500 \mu\text{g L}^{-1}$, with an average of $56.7 \mu\text{g L}^{-1}$. Glyphosate-AMPA was also found at concentrations ranging from 51.88 to 117.07 and 23.46 to $41.25 \mu\text{g}$

L^{-1} , respectively (Gomes et al., 2022). In another study, Pires et al. (2023) found groundwater samples were contaminated with glyphosate (up to $1.5868 \mu\text{g L}^{-1}$) and AMPA (up to $0.2751 \mu\text{g L}^{-1}$), while surface water samples contained glyphosate (up to $0.0236 \mu\text{g L}^{-1}$). According to Brovini et al. (2023), the highest concentration of 2,4-D was found on freshwaters up to $30 \mu\text{g L}^{-1}$ (average = $1.1 \mu\text{g L}^{-1}$). Finally, Lima-Junior et al. (2024) recorded an average value of $0.122 \mu\text{g L}^{-1}$ for 2,4-D, and Pinheiro et al. (2010) reported 2,4-D at $74.50 \mu\text{g L}^{-1}$. According to data from SISAGUA, up to $30 \mu\text{g L}^{-1}$ for 2,4-D and up to $200 \mu\text{g L}^{-1}$ for glyphosate were recorded in drinking water from 2014 to 2017 (Aranha & Rocha, 2019).

The concentrations above cited are in accordance with the maximum concentration allowed by Brazilian laws. However, Brovini et al. (2021a) reviewed the surface freshwater concentrations of bestseller pesticides in Brazil and discussed the implications for the aquatic organisms. The highest value reported for glyphosate was $110 \mu\text{g L}^{-1}$ (median = $7.5 \mu\text{g L}^{-1}$). Toxicity analyses were performed on *Daphnia magna*, *Oncorhynchus mykiss*, and *Raphidocelis subcapitata*. The authors estimated that glyphosate concentrations below $0.1 \mu\text{g L}^{-1}$ pose a minimal risk, while concentrations exceeding $1 \mu\text{g L}^{-1}$ represent a high risk to aquatic organisms.

The highest median value reported for 2,4-D was $10 \mu\text{g L}^{-1}$, and the median concentration in Brazilian surface freshwater was $0.05 \mu\text{g L}^{-1}$. Concentrations of 2,4-D lower than $1 \mu\text{g L}^{-1}$ pose a low risk, while concentrations higher than $10 \mu\text{g L}^{-1}$ could cause a high risk. Based on these findings, Brovini et al. (2021a) suggested that the permissible levels of glyphosate and 2,4-D by Brazilian legislation in freshwater ecosystems should be revised.

When we examined the pesticides separately, it was observed that the levels permitted in water by Brazilian laws showed cytotoxic and/or genotoxic effects on *A. cepa*. Our data are consistent with previous studies that assessed glyphosate and 2,4-D at concentrations allowed in water by Brazilian legislation using the *A. cepa* test. Bordin et al. (2023) reported that glyphosate at $65 \mu\text{g L}^{-1}$ induced chromosomal aberrations and nuclear abnormalities in *A. cepa* roots. In addition, Miranda et al. (2023) showed that glyphosate at concentrations of 50, 65, and $280 \mu\text{g L}^{-1}$ and 2,4-D at concentrations of 4, 10, and $30 \mu\text{g L}^{-1}$ were cytotoxic or genotoxic to

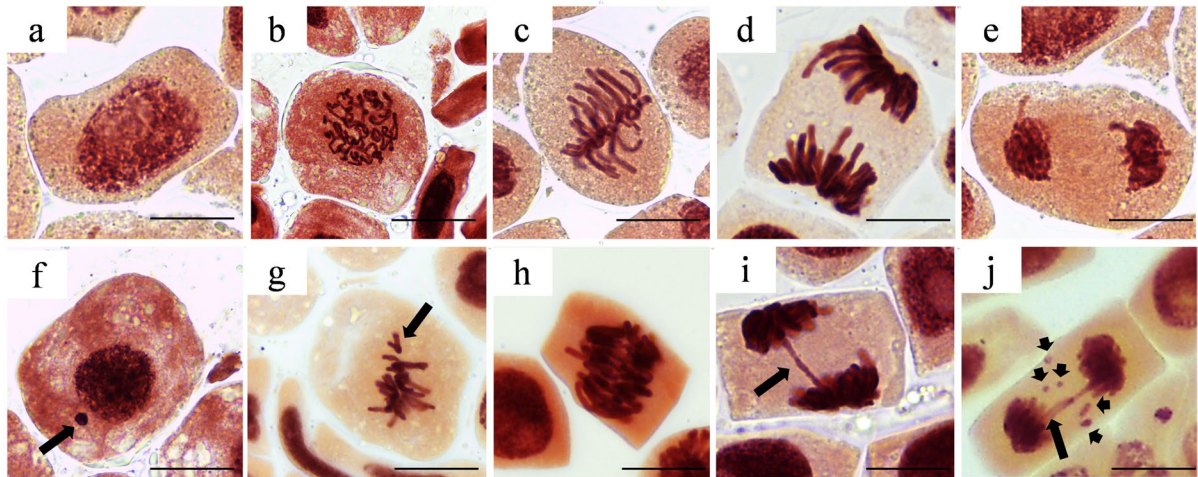


Fig. 1 Illustrative micrographs depicting the normal cell cycle of *A. cepa* (a-e) and main chromosomal aberrations and micronucleus identified following exposure to the isolated herbicides and the mixtures (f-j). (a) Interphase. (b) Prophase. (c) Metaphase. (d) Anaphase. (e) Telophase. (f) Micronucleus (arrow).

(g) Metaphase with chromosomal loss (arrow). (h) Anaphase with chromosome adhesion. (i) Anaphase with chromosome bridge (arrow). Telophase with bridge (bigger arrow) and chromosomal fragments (smaller arrows). Bar scale: 20 μm

onion. As *A. cepa* is a bioindicator of water quality, these studies support the previous findings of Brovini et al. (2021a) regarding the need to review the maximum allowable concentrations of glyphosate and 2,4-D in water.

Other studies have also reported the cytogenotoxic effects of the glyphosate on non-target organisms. However, it is important to note that the most studies conducted with plants assessed concentrations higher than those examined in this study, ranging from 0.36 to 7.2 mg/ml (Truta et al., 2011), 5 to 30 mg L^{-1} (Mercado & Caleño, 2020), 1.56 to 11.66 mg/ml (Finkler et al., 2022), and 1.34 to 13.40 mg L^{-1} (Vieira et al., 2024). Regarding aquatic organisms, Rodrigues et al. (2019) showed that glyphosate (1.7 to 100 mg L^{-1}), its primary degradation product aminoethyl phosphonic acid—AMPA (1.7 to 100 mg L^{-1}), the commercial formulation ATN (1.7 to 50 mg L^{-1}), and the surfactant polioxietil amina (0.4 to 4 mg L^{-1}) induced DNA damage in zebrafish larvae. In another study, Hong et al. (2018) found that glyphosate (0.35 – 5.60 mg L^{-1}) induced oxidative stress, DNA damage, and micronuclei in freshwater shrimp.

Similarly, 2,4-D at concentrations of 25.2 and 50.4 mg L^{-1} caused DNA strand breaks in fish (Arcaute et al., 2018), as did 2,4-D at concentrations of 10, 20 and 40 mg L^{-1} (Zafra-Lemos et al.,

2021). Tadpoles exposed at concentrations higher than 52.5 $\mu\text{g L}^{-1}$ died within 24 h of the start of the experiment. In addition, the occurrence of mouth and intestinal malformations (at 100 $\mu\text{g L}^{-1}$), erythrocyte nuclear abnormalities (at 30.0, 52.5, 75.0 and 100 $\mu\text{g L}^{-1}$) and micronuclei (at 52.5, 75.0, and 100 $\mu\text{g L}^{-1}$) were also recorded (Santos et al., 2024).

In studies on mammals, there is often conflicting evidence regarding the genotoxic effects of glyphosate. In the review study, Kier and Kirkland (2013) reported that the genotoxic properties of glyphosate are primarily attributed to the impact of "inert ingredients" present in commercial formulations rather than the active ingredient itself. In this sense, Chauhan et al. (2014) showed that the glyphosate formulation produced an increase in reactive oxygen species, nitrotyrosine formation, superoxide dismutase activity, and glutathione (GSH) levels in the HepG2 cell line. In addition, the commercial formulation induced apoptosis. On the other hand, no effects were found with acid glyphosate and AMPA treatments.

Mesnage et al. (2022) demonstrated that Roundup formulations induce more biological changes associated with carcinogenesis than glyphosate. In another study, glyphosate, Roundup 360 PLUS, and AMPA induced DNA damage and caused oxidation of purines and pyrimidines in human peripheral blood

mononuclear cells. Notably, the commercial formulation induced DNA damage at a concentration as low as 5 μM , while glyphosate and AMPA caused DNA lesions at concentrations of 250 μM and 500 μM , respectively (Woźniak et al., 2018). Additionally, Montero-Montoya et al. (2023) reported that glyphosate induce micronuclei, chromatin buds, nucleoplasmic bridges, and extranuclear fragments in peripheral lymphocytes.

Regarding 2,4-D, both the active ingredient at a concentration of 10 $\mu\text{g ml}^{-1}$ and the formulation Dedalo Elite at a concentration of 2 $\mu\text{g ml}^{-1}$ increased the frequency of micronuclei in Chinese hamster ovary cells. In addition, both compounds induced DNA damage and cytotoxicity (Laborde et al., 2020). Mesnage et al. (2022) evaluated the carcinogenic properties of 2,4-D. The researchers found that the herbicide is a strong inducer of oxidative stress and the unfolded protein response, while having relatively minimal effects on DNA damage.

As previously stated, glyphosate has been classified as a probable carcinogen (IARC, 2017), while 2,4-D is classified as a possible carcinogen to humans (IARC, 2018). Continuous exposure to cytogenotoxic and mutagenic agents can have an impact on human health, as noted by Fenech et al. (2020) and Marchetti et al. (2023). Therefore, the contamination of drinking water by mixtures containing proven, probable, or potential carcinogenic pesticides is a cause for concern, as highlighted by Panis et al. (2022).

Previous studies have reported the genotoxic activity of mixtures containing 2,4-D and glyphosate on various organisms (Bernardi et al., 2022; Carvalho et al., 2020; Finkler et al., 2022; Pavan et al., 2021). Our results are consistent with these findings. In this study, we prepared mixtures to replicate a range of contamination scenarios. Variations in cytogenotoxic responses may be due to differences in combined concentrations and analyzed parameters, such as mitotic index, chromosomal aberrations, and micronuclei. The results obtained for mixtures that exhibited additive/synergistic or potentiated effects are highlighted. The detection of these interactions warns of potential risks to exposed organisms. Therefore, the findings could assist pesticide regulators and environmental managers in improving pesticide legislation. However, there is currently no national discussion on the issue of pesticide mixtures in water.

In contrast, some countries have taken steps to decrease their use of pesticides. For example, the

European Union has implemented some of the strictest legislative limits globally, as demonstrated in Table 1. Additionally, Directive 2020/2184 of the European Union adopts the precautionary principle and establishes a maximum limit of 5 $\mu\text{g L}^{-1}$ for the sum of all pesticides in drinking water.

To the best of our knowledge, this is the first report with the large number of binary mixtures 2,4-D+glyphosate analyzed. The data obtained in this study help reveal the adverse effects of co-exposure to these herbicides when relevant concentrations to the aquatic environment are considered.

4 Conclusion

This study investigated the cytogenotoxicity of the herbicides 2,4-D and glyphosate, as well as their binary mixtures, using the *A. cepa* test. The results showed that even concentrations allowed by Brazilian legislation were cytotoxic and increased the frequency of chromosomal aberrations, indicating a potential risk to exposed organisms. Additionally, certain mixtures of 2,4-D and glyphosate were found to be cytotoxic and genotoxic, exhibiting additive, synergistic, or potentiated effects. The research emphasizes the importance of formulating pesticides that are less harmful to non-target organisms. Additionally, the findings may aid pesticide regulators and environmental managers in enhancing pesticide regulations and facilitating discussions on pesticide mixtures in water. Further research should prioritize investigating the toxic mechanisms of 2,4-D+glyphosate mixtures using different assays and biomarkers. Studies on both active ingredients and co-formulations are also necessary.

Acknowledgements The authors thank Scientific Initiation Program at the Federal University of Espírito Santo (PIIC UFES) and Viviane Tavares for technical support.

Authors Contributions Tatiana da Silva Souza: Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Supervision. Gabriele Bitencourt Tavares: Methodology, Forma, analysis, Data curation, Writing – review & editing. Victor Ventura de Souza: Conceptualization. Writing – review & editing.

Funding The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Data Availability The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Declarations

Ethical Approval This is an original article that did not use other information that requires ethical approval.

Consent to Participate All authors participated in this article.

Consent to Publish All authors have given consent to the publication of this article.

Competing Interests The authors have no relevant financial or non-financial interests to disclose.

References

- Aranha, A., & Rocha, L. (2019). “Coquetel” com 27 agrotóxicos foi achado na água de 1 em cada 4 municípios. Agência Pública/ Repórter Brasil. Retrieved August 7, 2022, from <https://portrasdoalimento.info/2019/04/15/coquetel-com-27-agrotoxicos-foi-achado-na-agua-de-1-em-cada-4-municipios/>
- Arcaute, C. R., Larramendy, M. L., & Soloneski, S. (2018). Genotoxicity by long-term exposure to the auxinic herbicides 2,4-dichlorophenoxyacetic acid and dicamba on *Cnesterodon decemmaculatus* (Pisces: Poeciliidae). *Environmental Pollution*, 243(Part A), 670–678. <https://doi.org/10.1016/j.envpol.2018.09.040>
- Argentina (2019). Comisión do Ambiente, da Saúde Pública e da Segurança Alimentar, Resolución Conjunta SRYGR y SAB N° 34 (2019). Retrieved June 7, 2023, from <https://www.argentina.gob.ar/normativa/nacional/resoluci%C3%B3n-34-2019-332261/texto>
- Bernardi, F., Lirola, J. R., Cestari, M. M., & Bombardelli, R. A. (2022). Effects on reproductive, biochemical and genotoxic parameters of herbicides 2,4-D and glyphosate in silver catfish (*Rhamdia quelen*). *Environmental Toxicology and Pharmacology*, 89, 103787. <https://doi.org/10.1016/j.etap.2021.103787>
- Bordin, E. R., Santos, F., Mannes, Y., Freitas, A. M., & Ramsdorf, W. A. (2023). Single and mixture cytogenetic effects evaluation of atrazine and glyphosate herbicides at environmentally relevant concentrations on *Allium cepa* root meristem cells. *Ecotoxicology and Environmental Contamination*, 18(1), 38–50. <https://doi.org/10.5132/eec.2023.01.06>
- Brasil (2005). Ministério do Meio Ambiente. Resolução n° 357 de 17 de março de 2005. Dispõe sobre a classificação dos corpos de água e diretrizes ambientais para o seu enquadramento, bem como estabelece as condições e padrões de lançamento de efluentes, e da outras providências. Diário Oficial da União, Brasília, DF, 18 mar. 2005. Retrieved June 10, 2022, from <http://www2.mma.gov.br/port/conama/legiabre.cfm?codlegi=459>
- Brasil (2011). Ministério da Saúde. Portaria n° 2.914 de 12 de dezembro de 2011. Dispõe sobre os procedimentos de controle e de vigilância da qualidade da água para consumo humano e seu padrão de potabilidade. Diário Oficial da União, Brasília, DF, 13 dez. 2011. Retrieved June 10, 2022, from https://bvsms.saude.gov.br/bvs/saudelegis/gm/2011/prt2914_12_12_2011.html
- Brovini, E. M., Deus, B. C. T., Vilas-Boas, J. A., Quadra, G. R., Carvalho, L., Mendonça, R. F., Pereira, R. O., & Cardoso, S. J. (2021a). Three-bestseller pesticides in Brazil: Freshwater concentrations and potential environmental risks. *Science of the Total Environment*, 771, 144754. <https://doi.org/10.1016/j.scitotenv.2020.144754>
- Brovini, E. M., Cardoso, S. J., Quadra, G. R., Vilas-Boas, J. A., Paranaíba, J. R., Pereira, R. O., & Mendonça, R. F. (2021b). Glyphosate concentrations in global freshwaters: Are aquatic organisms at risk? *Environmental Science and Pollution Research*, 28, 60635–60648. <https://doi.org/10.1007/s11356-021-14609-8>
- Brovini, E. M., Quadra, G. R., Paranaíba, J. R., Carvalho, L., Pereira, R. O., & de Aquino, S. F. (2023). Occurrence and environmental risk assessment of 22 pesticides in Brazilian freshwaters. *Aquatic Toxicology*, 260, 106566. <https://doi.org/10.1016/j.aquatox.2023.106566>
- Camilo-Cotrim, C. F., Bailão, E. F. L. C., Ondeí, L. S., Carneiro, F. M., & Almeida, L. M. (2022). What can the *Allium cepa* test say about pesticide safety? A review. *Environmental Science and Pollution Research International*, 29(32), 48088–48104. <https://doi.org/10.1007/s11356-022-20695-z>
- Carles, L., Martin-Laurent, F., Devers, M., Spor, A., Rouard, N., Beguet, J., Besse-Hoggan, P., & Batisson, I. (2021). Potential of preventive bioremediation to reduce environmental contamination by pesticides in an agricultural context: A case study with the herbicide 2,4-D. *Journal of Hazardous Materials*, 416, 125740. <https://doi.org/10.1016/j.jhazmat.2021.125740>
- Carvalho, W. F., Arcaute, C. R., Torres, L., de Melo e Silva, D., Soloneski, S., & Larramendy, M. L. (2020). Genotoxicity of mixtures of glyphosate with 2,4-dichlorophenoxyacetic acid chemical forms towards *Cnesterodon decemmaculatus* (Pisces, Poeciliidae). *Environmental Science and Pollution Research*, 27, 6515–6525. <https://doi.org/10.1007/s11356-019-07379-x>
- Champion, L., Linder, M. I., & Kutay, U. (2017). Cellular reorganization during mitotic entry. *Trends in Cell Biology*, 27(1), 26–41. <https://doi.org/10.1016/j.tcb.2016.07.004>
- Chaufan, G., Coalova, I., & Molina, M. C. R. (2014). Glyphosate commercial formulation causes cytotoxicity, oxidative effects, and apoptosis on human cells. *International Journal of Toxicology*, 33(1), 29–38. <https://doi.org/10.1177/1091581813517906>
- China (2006). Ministry of Health of China, National Standard of the People’s Republic of China GB 5749–2006. Retrieved June 10, 2023, from <http://tradechina.dairyaustralia.com.au/wp-content/uploads/2018/08/GB-5749-2006-Standards-for-Drinking-Water-Quality.pdf>
- Daam, M. A., Chelinho, S., Niemeyer, J. C., Owojori, O. J., De Silva, P. M. C. S., Sousa, J. P., van Gestel, C. A. M., & Römbke, J. (2019). Environmental risk assessment of pesticides in tropical terrestrial ecosystems: Test procedures, current status and future perspectives. *Ecotoxicology and*

- Environmental Safety*, 181, 534–547. <https://doi.org/10.1016/j.ecoenv.2019.06.038>
- Dhyèvre, A., Foltête, A. S., Aran, D., Muller, S., & Cotelte, S. (2014). Effects of soil pH on the *Vicia* micronucleus genotoxicity assay. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, 774, 17–21. <https://doi.org/10.1016/j.mrgentox.2014.09.004>
- Díaz-Martín, R. D., Valencia-Hernández, J. D., Betancourt-Lozano, M., & Yáñez-Rivera, B. (2021). Changes in microtubule stability in zebrafish (*Danio rerio*) embryos after glyphosate exposure. *Heliyon*, 7(1), e06027. <https://doi.org/10.1016/j.heliyon.2021.e06027>
- European Union. (2020). Directive (EU) 2020/2184 of the European Parliament and of the Council on the quality of water intended for human consumption. Official Journal of the European Union of 16 December 2020. Retrieved May 18, 2023, from Available at: <https://eur-lex.europa.eu/eli/dir/2020/2184/oj>
- Felisbino, K., Santos-Filho, R., Piancini, L. D. S., Cestari, M. M., & Leme, D. M. (2018). Mesotrione herbicide does not cause genotoxicity, but modulates the genotoxic effects of Atrazine when assessed in mixture using a plant test system (*Allium cepa*). *Pesticide Biochemistry and Physiology*, 150, 83–88. <https://doi.org/10.1016/j.pestbp.2018.07.009>
- Fenech, M., Knasmueller, S., Holland, N., Bonassi, S., & Kirsch-Volders, M. (2020). Micronuclei as biomarkers of DNA damage, aneuploidy, inducers of chromosomal hypermutation and as sources of pro-inflammatory DNA in humans. *Mutation Research - Reviews in Mutation Research*, 786, 108342. <https://doi.org/10.1016/j.mrrev.2020.108342>
- Finkler, M., Rodrigues, G. Z. P., Kayser, J. M., Ziulkoski, A. L., & Gehlen, G. (2022). Cytotoxic and genotoxic effects induced by associated commercial glyphosate and 2,4-D formulations using the *Allium cepa* bioassay. *Journal of Environmental Science and Health, Part B*, 57(2), 133–141. <https://doi.org/10.1080/03601234.2022.2034432>
- Fioresi, V. S., Vieira, B. C. R., de Campos, J. M. S., & Souza, T. S. (2020). Cytogenotoxic activity of the pesticides imidacloprid and iprodione on *Allium cepa* root meristem. *Environmental Science and Pollution Research*, 27, 28066–28076. <https://doi.org/10.1007/s11356-020-09201-5>
- Gaaied, S., Oliveira, M., Le Bihanic, F., Cachot, J., & Banni, M. (2019). Gene expression patterns and related enzymatic activities of detoxification and oxidative stress systems in zebrafish larvae exposed to the 2,4-dichlorophenoxyacetic acid herbicide. *Chemosphere*, 224, 289–297. <https://doi.org/10.1016/j.chemosphere.2019.02.125>
- Gaboardi, S. C., Candioto, L. Z. P., & Panis, C. (2023). Agribusiness in Brazil and its dependence on the use of pesticides. *Hygiene Environ Health Advances*, 8, 100080. <https://doi.org/10.1016/j.heha.2023.100080>
- Gomes, M. P., Brito, S. C., Vieira, F., Kitamura, R. S. A., & Juneau, P. (2022). Emerging contaminants in streams of Doce River watershed, Minas Gerais, Brazil. *Frontiers in Environmental Science*, 9, 801599. <https://doi.org/10.3389/fenvs.2021.801599>
- Han, M., Zhang, Z., Liu, S., Sheng, Y., Waigi, M. G., Hu, X., Qin, C., & Ling, W. (2023). Genotoxicity of organic contaminants in the soil: A review based on bibliometric analysis and methodological progress. *Chemosphere*, 313, 137318. <https://doi.org/10.1016/j.chemosphere.2022.137318>
- Health Canada. (2019). Guidelines for Canadian Drinking Water Quality—Summary Table. Water and Air Quality Bureau, Healthy Environments and Consumer Safety Branch, Health Canada, Ottawa, Ontario. Retrieved August 16, 2023, from <https://www.canada.ca/en/health-canada/services/environmental-workplace-health/reports-publications/water-quality/guidelines-canadian-drinking-water-quality-summary-table.html>
- Hong, Y., Yang, X., Huang, Y., Yan, G., & Cheng, Y. (2018). Assessment of the oxidative and genotoxic effects of the glyphosate-based herbicide roundup on the freshwater shrimp, *Macrobrachium nipponensis*. *Chemosphere*, 210, 896–906. <https://doi.org/10.1016/j.chemosphere.2018.07.069>
- IARC – International Agency for Research Cancer. (2017). World Health Organization. Monographs on the identification of carcinogenesis hazards to humans. 112. Retrieved June 10, 2022, from <https://publications.iarc.fr/549>
- IARC – International Agency for Research Cancer. (2018). World Health Organization. Monographs on the identification of carcinogenesis hazards to humans. DDT, Lindane, and 2,4-D. 113. Retrieved June 10, 2022, from <https://publications.iarc.fr/550>
- IBAMA. (2012). Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis. Relatórios de comercialização de agrotóxicos: 2012. Retrieved June 10, 2022, from <https://www.gov.br/ibama/pt-br/assuntos/quimicos-e-biologicos/agrotoxicos/relatorios-de-comercializacao-de-agrotoxicos>
- IBAMA. (2022). Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis. Relatórios de comercialização de agrotóxicos: 2022. Retrieved June 10, 2022, from <https://www.ibama.gov.br/agrotoxicos/relatorios-de-comercializacao-de-agrotoxicos>
- Ilyushina, N. A., Egorova, O. V., Masaltsev, G. V., Averianova, N. S., Revazova, Y. A., Rakitskii, V. N., Goumenou, M., Vardavas, A., Stivaktakis, P., & Tsatsakis, A. (2020). Genotoxicity of mixture of imidacloprid, imazalil and tebuconazole. *Toxicology Reports*, 307, 1090–1094. <https://doi.org/10.1016/j.toxrep.2020.08.021>
- India. (2012). Drinking Water Sectional Committee, Indian Standard Drinking Water — Specification, Second Revision. IS 10500:2012. Retrieved June 12, 2023, from <https://mpcb.mizoram.gov.in/uploads/attachments/d6c72ef548b2660da564372278a7c125/pages-160-indian-standards.pdf>
- Japan. (2006). Ministry of Health, Labor and Welfare, Outline of examination in reviewing water quality standards. GB 5749–2006. Retrieved June 12, 2023, from <https://www.mhlw.go.jp/topics/bukyoku/kenkou/suido/kijun/konkyo0303.html>
- Kier, L. D., & Kirkland, D. J. (2013). Review of genotoxicity studies of glyphosate and glyphosate-based formulations. *Critical Reviews in Toxicology*, 43(4), 283–315. <https://doi.org/10.3109/10408444.2013.770820>
- Laborde, M. R. R., Larramendy, M. L., & Soloneski, S. (2020). Cytotoxic and genotoxic assessments of 2,4-dichlorophenoxyacetic acid (2,4-D) in in vitro mammalian cells. *Toxicology in Vitro*, 65, 104783. <https://doi.org/10.1016/j.tiv.2020.104783>

- Leme, D., & Marin-Morales, M. A. (2009). *Allium cepa* test in environmental monitoring: A review on its application. *Mutation Research - Reviews in Mutation Research*, 682(1), 71–81. <https://doi.org/10.1016/j.mrrev.2009.06.002>
- Lima, I. B., Boëchat, I. G., Fernandes, M. D., Monteiro, J. A. F., Rivaroli, L., & Gücker, B. (2023). Glyphosate pollution of surface runoff, stream water, and drinking water resources in Southeast Brazil. *Environmental Science and Pollution Research*, 30, 27030–27040. <https://doi.org/10.1007/s11356-022-24167-2>
- Lima-Junior, D. P., Lima, L. B., Carnicer, C., Zanella, R., Prestes, O. D., Florianio, L., & Júnior, P. M. (2024). Exploring the relationship between land-use and pesticides in freshwater ecosystem: A case study of the Araguaia River Basin. *Brazil. Environmental Advances*, 15, 100497. <https://doi.org/10.1016/j.envadv.2024.100497>
- Liman, R., Ciğerci, İH., & Öztürk, N. S. (2015). Determination of genotoxic effects of imazethapyr herbicide in *Allium cepa* root cells by mitotic activity, chromosome aberration, and comet assay. *Pesticide Biochemistry and Physiology*, 118, 38–42. <https://doi.org/10.1016/j.pestbp.2014.11.007>
- Marc, J., Bellé, R., Morales, J., Cormier, P., & Mulner-Lorillon, O. (2004). Formulated glyphosate activates the DNA-response checkpoint of the cell cycle leading to the prevention of G2/M transition. *Toxicological Sciences*, 82(2), 436–442. <https://doi.org/10.1093/toxsci/kfh281>
- Marcato, A. C. C., de Souza, C. P., & Fontanetti, C. S. (2017). Herbicide 2,4-D: A review of toxicity on non-target organisms. *Water, Air & Soil Pollution*, 228, 120. <https://doi.org/10.1007/s11270-017-3301-0>
- Marchetti, F., Cardoso, R., Chen, C. L., Douglas, G. R., Ello-way, J., Escobar, P. A., Harper, T. J., Heflich, R. H., Kidd, D., Lynch, A. M., Myers, M. B., Parsons, B. L., Salk, J. J., Settivari, R. S., Smith-Roe, S. L., Witt, K. L., Yauk, C. L., Young, R., Zhang, S., & Minocherhomji, S. (2023). Error-corrected next generation sequencing - promises and challenges for genotoxicity and cancer risk assessment. *Mutation Research - Reviews in Mutation Research*, 792, 108466. <https://doi.org/10.1016/j.mrrev.2023.108466>
- Martins, R. X., Vieira, L., Souza, J. A. C. R., Silva, M. G. F., Muniz, M. S., Souza, T., Queiroga, F. R., Machado, M. R. F., Silva, P., & Farias, D. (2021). Exposure to 2,4-D herbicide induces hepatotoxicity in zebrafish larvae. *Comparative Biochemistry and Physiology Part c: Toxicology & Pharmacology*, 248, 109110. <https://doi.org/10.1016/j.cbpc.2021.109110>
- Mercado, S. A. S., & Caleño, J. D. Q. (2020). Cytotoxic evaluation of glyphosate, using *Allium cepa* L. as bioindicator. *Science and the Total Environment*, 700, 134452. <https://doi.org/10.1016/j.scitotenv.2019.134452>
- Mesnage, R., Brandsma, I., Moelijker, N., Zhang, G., & Antoniou, M. N. (2021). Genotoxicity evaluation of 2,4-D, dicamba and glyphosate alone or in combination with cell reporter assays for DNA damage, oxidative stress and unfolded protein response. *Food and Chemical Toxicology*, 157, 112601. <https://doi.org/10.1016/j.fct.2021.112601>
- Mesnage, R., Ibragim, M., Mandrioli, D., Falcioni, L., Tibaldi, E., Belpoggi, F., Brandsma, I., Bourne, E., Savage, E., Mein, C. A., & Antoniou, M. N. (2022). Comparative toxicogenomics of glyphosate and roundup herbicides by mammalian stem cell-based genotoxicity assays and molecular profiling in sprague-dawley rats. *Toxicological Sciences*, 186(1), 83–101. <https://doi.org/10.1093/toxsci/kfab143>
- Miranda, L. A., Souza, V. V., Campos, R. A., Campos, J. M. S., & Souza, T. S. S. (2023). Phytotoxicity and cytogenotoxicity of pesticide mixtures: Analysis of the effects of environmentally relevant concentrations on the aquatic environment. *Environmental Science and Pollution Research*, 30, 112117–112131. <https://doi.org/10.1007/s11356-023-30100-y>
- Montero-Montoya, R., Suárez-Larios, K., & Serrano-García, L. (2023). Paraoxon and glyphosate induce DNA double-strand breaks but are not type II topoisomerase poisons. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, 890, 503657. <https://doi.org/10.1016/j.mrgentox.2023.503657>
- Moreira, N. C. S., Lima, J. E. B. F., & Sakamoto-Hojo, E. T. (2021). Citometria de fluxo – fundamentos, aplicações e análise do ciclo celular e apoptose. In D. M. F. Salvadori, C. S. Takahashi, C. K. Grisolia, & R. A. Santos (Eds.), *Da Toxicogenética à Toxicogenômica* (1st ed., pp. 251–272). Rio de Janeiro: Atheneu.
- Moutinho, M. F., de Almeida, E. A., Espíndola, E. L. G., Daam, M. A., & Schiesari, L. (2020). Herbicides employed in sugarcane plantations have lethal and sublethal effects to larval *Boana pardalis* (Amphibia, Hylidae). *Ecotoxicology*, 29, 1043–1051. <https://doi.org/10.1007/s10646-020-02226-z>
- Nwani, C. D., Nagpure, N. S., Kumar, R., Kushwaha, B., & Lakra, W. S. (2013). DNA damage and oxidative stress modulatory effects of glyphosate-based herbicide in freshwater fish, *Channa punctatus*. *Environmental Toxicology and Pharmacology*, 36, 539–547. <https://doi.org/10.1016/j.etap.2013.06.001>
- Palma-Bautista, C., Cruz-Hipólito, H. E., Alcántara-de la Cruz, R., Vázquez-García, J. G., Yannicari, M., & De Prado, R. (2021). Comparison of premix glyphosate and 2,4-D formulation and direct tank mixture for control of *Conyza canadensis* and *Epilobium ciliatum*. *Environmental Pollution*, 281, 117013. <https://doi.org/10.1016/j.envpol.2021.117013>
- Panis, C., Candiotti, L. Z. P., Gaboardi, S. C., Gurzenda, S., Cruz, J., Castro, M., & Lemos, B. (2022). Widespread pesticide contamination of drinking water and impact on cancer risk in Brazil. *Environment International*, 165, 107321. <https://doi.org/10.1016/j.envint.2022.107321>
- Pavan, F. A., Samojuden, C. G., Rutkoski, C. F., Folador, A., da Fré, S. P., Müller, C., Hartmann, P. A., & Hartmann, M. T. (2021). Morphological, behavioral and genotoxic effects of glyphosate and 2,4-D mixture in tadpoles of two native species of South American amphibians. *Environmental Toxicology and Pharmacology*, 85, 103637. <https://doi.org/10.1016/j.etap.2021.103637>
- Pinheiro, A., Silva, M. R., & Kraisch, R. (2010). Presença de pesticidas em águas superficiais e subterrâneas na bacia do Itajaí, SC. *Revista de Gestão da Água da América Latina*, 7, 17–26. <https://doi.org/10.21168/rega.v7n2.p17-26>
- Pires, N. L., de Araújo, E. P., Oliveira-Filho, E. C., & Caldas, E. D. (2023). An ultrasensitive LC-MS/MS method for the

- determination of glyphosate, AMPA and glufosinate in water - analysis of surface and groundwater from a hydrographic basin in the Midwestern region of Brazil. *Science of the Total Environment*, 875, 162499. <https://doi.org/10.1016/j.scitotenv.2023.162499>
- Rank, J., & Nielsen, M. H. (1994). Evaluation of the Allium anaphase-telo phase test in relation to genotoxicity screening of industrial wastewater. *Mutation Research - Environmental Mutagenesis and Related Subjects*, 312(1), 17–24. [https://doi.org/10.1016/0165-1161\(94\)90004-3](https://doi.org/10.1016/0165-1161(94)90004-3)
- Rocha, G. M., & Grisolia, C. K. (2019). Why pesticides with mutagenic, carcinogenic and reproductive risks are registered in Brazil. *Developing World Bioethics*, 19, 148–154. <https://doi.org/10.1111/dewb.12211>
- Rodrigues, L. B., Costa, G. G., Thá, E. L., Silva, L. R., Oliveira, R., Leme, D. M., Cestari, M. M., Grisolia, C. K., Valadares, M. C., & Oliveira, G. A. R. (2019). Impact of the glyphosate-based commercial herbicide, its components and its metabolite AMPA on non-target aquatic organisms. *Mutation Research - Genetic Toxicology and Environmental Mutagenesis*, 842, 94–101. <https://doi.org/10.1016/j.mrgentox.2019.05.002>
- Santos, G., Rutkoski, C. F., Folador, A., Skovronski, V. J., Müller, C., Pompermaier, A., Hartmann, P. A., & Hartmann, M. (2024). 2,4-D-based herbicide underdoses cause mortality, malformations, and nuclear abnormalities in *Physalaemus cuvieri* tadpoles. *Comparative Biochemistry and Physiology Part c: Toxicology & Pharmacology*, 277, 109840. <https://doi.org/10.1016/j.cbpc.2024.109840>
- Schatten, H. (2013). Mitosis. In S. Maloy & K. Hughes (Eds.), *Brenner's encyclopedia of genetics* (2nd ed., pp. 448–451). Academic Press. <https://doi.org/10.1016/B978-0-12-374984-0.00962-1>
- Seong, Y. S., Kamijo, K., Lee, J. S., Fernandez, E., Kuriyama, R., Miki, T., & Lee, K. S. (2002). A spindle checkpoint arrest and a cytokinesis failure by the dominant-negative polo-box domain of Plk1 in U-2 OS cells. *Journal of Biological Chemistry*, 277(35), 32282–32293. <https://doi.org/10.1074/jbc.M202602200>
- Souza, C. P., Guedes, T., & Fontanetti, C. S. (2016). Evaluation of herbicides action on plant bioindicators by genetic biomarkers: A review. *Environmental Monitoring and Assessment*, 188, 694. <https://doi.org/10.1007/s10661-016-5702-8>
- Souza, R. B., Souza, C. P., & Guimarães, J. R. (2022). Environmentally realistic concentrations of eprinomectin induce phytotoxic and genotoxic effects in *Allium cepa*. *Environmental Science and Pollution Research*, 29(53), 80983–80993. <https://doi.org/10.1007/s11356-022-21403-7>
- Souza, M. C. O., Cruz, J. C., Cesila, C. A., Gonzalez, N., Rocha, B. A., Adeyemi, J. A., Nadal, M., Domingo, J. L., & Barbosa, F. (2023a). Recent trends in pesticides in crops: A critical review of the duality of risks-benefits and the Brazilian legislation issue. *Environmental Research*, 228, 115811. <https://doi.org/10.1016/j.envres.2023.115811>
- Souza, V. V., Souza, T. S., Campos, J. M. S., Oliveira, L. A., Ribeiro, Y. M., Hoyos, D. C. M., Xavier, R. M. P., Charlie-Silva, I., & Lacerda, S. M. S. N. (2023b). Ecogenotoxicity of environmentally relevant atrazine concentrations: A threat to aquatic bioindicators. *Pesticide Biochemistry and Physiology*, 189, 105297. <https://doi.org/10.1016/j.pestbp.2022.105297>
- Takano, H. K., Oliveira Junior, R. S., Constantin, J., Biffe, D. F., Franchini, L. H. M., Braz, G. B. P., Rios, F. A., Gheno, E. A., & Gemelli, A. (2013). Efeito da adição do 2,4-D ao glyphosate para o controle de espécies de plantas daninhas de difícil controle. *Revista Brasileira De Herbicidas*, 12, 1. <https://doi.org/10.7824/rbh.v12i1.207>
- Truta, E., Vochita, G., Rosu, C., Zamfirache, M.-M., & Olteanu, Z. (2011). Evaluation of roundup-induced toxicity on genetic material and on length growth of barley seedlings. *Acta Biologica Hungarica*, 62, 290–301. <https://doi.org/10.1556/ABiol.62.2011.3.8>
- Türkoglu, Ş. (2012). Determination of genotoxic effects of chlorfenvinphos and fenbuconazole in *Allium cepa* root cells by mitotic activity, chromosome aberration, DNA content, and comet assay. *Pesticide Biochemistry and Physiology*, 103(3), 224–230. <https://doi.org/10.1016/j.pestbp.2012.06.001>
- US EPA. (2024). National Primary Drinking Water Regulations. Retrieved July 3, 2024, from <https://www.epa.gov/ground-water-and-drinking-water/national-primary-drinking-water-regulations#Organics>
- Verma, S., & Srivastava, A. (2018). Morphotoxicity and cytogenotoxicity of pendimethalin in the test plant *Allium cepa* L. - A biomarker based study. *Chemosphere*, 206, 248–254. <https://doi.org/10.1016/j.chemosphere.2018.04.177>
- Vieira, C., Marcon, C., & Droste, A. (2024). Phytotoxic and cytogenotoxic assessment of glyphosate on *Lactuca sativa* L. *Brazilian Journal of Biology*, 84, e257039. <https://doi.org/10.1590/1519-6984.257039>
- WHO - World Health Organization. (2018). A global overview of national regulations and standards for drinking-water quality. Retrieved June 13, 2023, from <https://www.who.int/publications/i/item/9789241513760>
- Woźniak, E., Sicińska, P., Michałowicz, J., Woźniak, K., Reszka, E., Huras, B., Zakrzewski, J., & Bukowska, B. (2018). The mechanism of DNA damage induced by Roundup 360 PLUS, glyphosate and AMPA in human peripheral blood mononuclear cells - genotoxic risk assessment. *Food and Chemical Toxicology*, 120, 510–522. <https://doi.org/10.1016/j.fct.2018.07.035>
- Zafra-Lemos, L., Cusioli, L. F., Bergamasco, R., Borin-Carvalho, L. A., & Portela-Castro, A. L. B. (2021). Evaluation of the genotoxic and cytotoxic effects of exposure to the herbicide 2,4-dichlorophenoxyacetic acid in *Astyanax lacustris* (Pisces, Characidae) and the potential for its removal from contaminated water using a biosorbent. *Mutation Research*, 865, 503335. <https://doi.org/10.1016/j.mrgentox.2021.503335>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.