Transgenerational Effects of Environmentally Relevant Concentrations of Atrazine and Glyphosate Herbicides, Isolated and in Mixture, to Freshwater Microcrustacean *Daphnia Magna*

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Abstract

Herbicide mixture is used as an alternative to obtain different mechanisms of action acting on weeds, resulting in the frequent presence of pesticides in environmental compartments. As they are products used worldwide, this study evaluated effects of environmentally relevant concentrations of the analytical standards and commercial formulations of the herbicides atrazine ($2 \mu\text{g L}^{-1}$) and glyphosate ($65 \mu\text{g L}^{-1}$), in isolation and also in mixture ($2 + 65 \mu\text{g L}^{-1}$) on the microcrustacean *Daphnia magna*. Through chronic exposure (21 days) of two generations of organisms, effects on survival and reproductive capacity were observed, as well as responses regarding oxidative stress, determined through the analysis of biochemical biomarkers such as catalase and glutathione S-transferase. In the evaluation of the first generation of test organisms, no significant results related to biochemical biomarkers were observed, only effects over sexual maturation of organisms. However, in the second generation of exposed organisms, changes were observed in all parameters evaluated, with the mixture of herbicide active principles being the treatment responsible for more significant responses ($p < 0.05$). A statistical difference ($p < 0.05$) was also observed between analytical standards and commercial formulations, indicating that other components present in the formulations can change the toxicity of the products. Given the difficulty of estimating the effects of mixtures and considering that various stressors are found in the environment, our results support the need to carry out studies that address long-term effects and, above all, that verify what the impacts are across generations, so that the toxicity of products is not underestimated.

1. Introduction

Weeds are among the greatest restrictions for agricultural production, as they cause agronomic and economic damage (Alonso et al., 2018, Montiel-León et al., 2019, Bordin et al., 2020). For its control, herbicide mixtures are widely used in order to obtain different action mechanisms, thus, they are also considered more effective in combating resistance (Moss et al., 2019).

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is an herbicide that acts over the photosynthetic system, and has been recognized as an endocrine disruptor (García-Espiñeira et al., 2018, Kar et al., 2020). Glyphosate (N - (phosphonomethyl) glycine) is one of the most used herbicides in the world and acts in the chimitate pathway, inhibiting the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthetase (Brilisauer et al., 2019, Carles et al., 2019). Due to overuse and continuous use, and their chemical and physical characteristics such as persistence in soil and water, the permanent presence of these pesticides in surface and underground water may cause damage to several non-target organisms (Alonso et al., 2018, Cleary et al., 2019).

The ecotoxicological properties of pesticide mixtures, when compared to the same individual compounds, are poorly investigated. However, in environmental compartments, a wide variety of these compounds is often detected, in low concentrations which are considered environmentally relevant (Srivastava, Mishra, 2009, Bianchi et al., 2015). Moreover, effects caused by the active ingredients of pesticides usually differ

from the effects from commercial products, due to the presence of adjuvants in their formulations (Bridi et al., 2017, Zocchi, Sommaruga, 2019, Nagy et al., 2020).

Considering the use of combinations of atrazine and glyphosate, and that both can coexist in different environmental matrices (Bonfleur et al., 2015, Mahler et al., 2017), toxicity of these herbicides alone and also their mixture has already been evaluated over some bioindicator organisms, which expressed reproductive, biochemical and genotoxic effects and, even reported additive effects when studying the mixture of herbicides (Mer et al., 2013, Santos, Martinez, 2014, García-Espiñeira et al., 2018).

Aquatic invertebrate organisms are subject to exposure to numerous pollutants, in concentrations that vary according to multiple factors, including climatic conditions, losses through aerial drift and applicator handling (Alonso et al., 2018). Although, these organisms can accumulate various chemical products in concentrations higher than those detected in waters in which they live, through bioaccumulation or adhere to suspended particulate matter, which can be ingested by aquatic organisms (Blahova et al., 2020).

Acute toxicity is primarily observed to assess damage of certain products to the environment, however, the effects of chronic exposure are still poorly investigated (Pérez, Hoang, 2018). The transmission, across generations, of characteristics induced by environmental disturbances has been the subject of some studies (Zhang et al., 2013, Bhandari et al., 2015, Cleary et al., 2019, Religia et al., 2019). Considering that healthy offspring are generated from a healthy matrix, which lives in an adequate environment, nutritional, hormonal and immunological components are determining factors for the development and maintenance of offspring in a variety of organisms (Cleary et al., 2019, Hedayatirad et al., 2020).

Biomarkers are sensitive indicators of rapid biological responses, which reflect toxic effects and disturbances due to the presence of compounds dispersed in the environment (Peakall, 1994, Xiong et al., 2018). By determining the activity of enzymes such as catalase and glutathione S-transferase, it is possible to obtain information on parameters such as oxidative stress, since these enzymes act in the oxidant defense systems of organisms (Yang et al., 2019).

The presence of xenobiotic compounds can interfere with the ecological balance, and the exposure of different organisms to chemicals can be manifested both in adults and in their descendants (Hedayatirad et al., 2020, Kar et al., 2020). Knowing that pesticides can act indirectly over non-target aquatic species, especially when leached into rivers or through spray drift, it is necessary to know the effects and anticipate remediation, seeking to reduce the possible consequences that these organisms may suffer.

In this sense, the objective of this study was to investigate the chronic transgenerational effects caused by active principles and commercial formulations of the herbicides atrazine and glyphosate, isolated and in mixture, in environmentally relevant concentrations, to freshwater microcrustacean *Daphnia magna*.

2. Materials And Methods

2.1 Chemicals

Atrazine analytical standards stock solutions (20 mg L^{-1}) (CAS nº 1912-24-9, Sigma Aldrich, pureza $\geq 98\%$) and glyphosate (500 mg L^{-1}) (CAS nº 1071-83-6, Sigma Aldrich, pureza $\geq 98\%$) were prepared in purified water, with a reverse osmosis system. Stock solutions of commercial herbicide formulations atrazine (40% active ingredient atrazine m/v, 66% other ingredients m/v) and glyphosate (48% active ingredient glyphosate m/v, 35.56% acid equivalent m/v, 68.39% other ingredients m/v) were prepared at a concentration of 100 mg L^{-1} in purified water, with a reverse osmosis system, based on the active ingredient amount present in the formulations.

2.2 Environmental concentrations

Concentrations of $2 \text{ } \mu\text{g L}^{-1}$ for atrazine and $65 \text{ } \mu\text{g L}^{-1}$ for glyphosate were evaluated, in addition to the mixture of $2 + 65 \text{ } \mu\text{g L}^{-1}$ of atrazine and glyphosate, respectively, resulting in 7 experimental groups: standard analytical atrazine (ATSA), glyphosate analytical standard (GPSA) and mixture of analytical standards (MIXSA), atrazine commercial formulation (ATCOM), glyphosate commercial formulation (GPCOM) and mixture of commercial formulations (MIXCOM) and the negative control group (NC), containing only the culture medium, prepared according to NBR 12.713 (ABNT, 2016).

These concentrations were defined based on the analysis of studies that determined the presence of these herbicides in environmental samples (Alonso et al., 2018, Carles et al., 2019, Chen et al., 2019, Mahler et al., 2017, Montiel Leon et al., 2019, Vieira et al., 2017), with concentrations being more realistic and close to those frequently reported in the environment. In addition, these concentrations are allowed in waters that can be used for public supply after simplified treatment (Class 1), according to Brazilian legislation (Brasil, 2005).

2.3 Test organisms

Daphnia magna organisms used in the parental generation in this study were cultivated at the Laboratory of Ecotoxicology at the Federal Technological University of Paraná, in Curitiba (Brazil). The methodology related to the cultivation of the microcrustacean *Daphnia magna* followed the requirements of NBR 12.713 (ABNT, 2016). Approximately 40 *D. magna* organisms were kept in glass beakers containing 1.5 L of reconstituted water prepared according to NBR 12.713 (ABNT, 2016). Culture medium renewal was carried out five times a week and simultaneously the feeding of the organisms was carried out with a cell suspension of *Desmodesmus subspicatus* ($10^6 \text{ cells mL}^{-1}$), also cultivated in the same laboratory according to the methodology presented in NBR 12.648 (ABNT, 2018).

2.4 Chronic test of *Daphnia magna* as a bioindicator

Chronic ecotoxicity tests were performed according to standard protocols (OECD, 2012), which lasted 21 days and consisted of 12 replicates per treatment ($n = 12$). Figure 1 presents a summary of the experimental design used. The tests were conducted in an aquatic ecotoxicology test room, where the test

organisms were subjected to samples of analytical standards and commercial formulations, at environmental concentrations of $2 \mu\text{g L}^{-1}$ and $65 \mu\text{g L}^{-1}$ of isolated atrazine and glyphosate, respectively, and also the mixture of herbicides at the same concentrations, with controlled temperature of $20 \pm 2 \text{ }^{\circ}\text{C}$ and photoperiod of 16 hours of light and 8 hours of dark.

The parental generation (F0) was composed of newborn *D. magna* individuals (2 to 24 hours of life), which were individually added in containers containing 40 mL of test solution. The organisms remained in a BOD incubator, with the total renewal of the medium being carried out five times a week with solutions of the same concentration, which were prepared daily from stock solutions. Feeding took place on the same days of solution renewal, with approximately 500 μL of a cell suspension of *Desmodesmus subspicatus* (10^6 cels mL^{-1}).

Throughout the experiment, the organisms were checked daily to assess survival and reproductive status, with newborn individuals being counted and discarded.

Transgenerational effects were observed when a new generation of organisms was exposed for the same period and under the same test conditions. The second generation (F1) was composed of young individuals (2 to 24 hours of life) obtained from the parental generation (F0). After the period corresponding to the third litter birth from the control group of F0 generation, F1 generation began. The F0 generation neonates obtained from all replicates of the same group were transferred to the same container, so that the organisms were randomly arranged at the beginning of the new generation. These neonates were then individually arranged in 12 replicates ($n = 12$), according to the corresponding groups, representing the F1 generation.

The parameters analyzed in the chronic test were survival, sexual maturation and reproduction. Survival was determined by counting the surviving adult individuals after the 21 days exposure period. Sexual maturation was determined by observing the beginning of the offspring production per parent (primiparous), and reproduction was evaluated by counting the number of offspring produced per alive parent during the 21 days of the test.

2.5 Biochemicals biomarkers

Test organisms surviving after 21 days of exposure were distributed into three pools composed of four organisms. For samples where matrix deaths occurred, the pools were composed of four or three organisms. These individuals were transferred to microtubes (2 mL) where they were homogenized in 250 μL of potassium phosphate buffer (0.1 M, pH 7.2). The homogenized samples were kept refrigerated throughout the process ($4 \text{ }^{\circ}\text{C}$), and centrifuged at 8,000 G for 10 minutes at $4 \text{ }^{\circ}\text{C}$. Biochemical biomarkers analyzes were carried out immediately, using the supernatant from samples obtained after centrifugation, and the buffer used in the homogenization of the samples was used as a blank. For all activity calculations, blank values were previously discounted. All biomarkers determinations were performed using a microplate reader (FLUOstar Omega, BMG Labtech).

2.5.1 Total protein concentration

To determine total protein concentration, Bradford method was performed (Bradford, 1976) adapted to 96 well-plate using bovine serum albumin (BSA, Sigma-Aldrich) as protein standard, and measurements were made at 595 nm, in order to express the enzymatic activities as a function of the protein amount.

2.5.2 CAT activities analysis

Catalase (CAT) activity was based on the method described by Aebi (1984), using a quartz microplate where the decomposition of substrate H_2O_2 was measured at 240 nm for 2 min (20 reading cycles).

2.5.3 GST activities analysis

Glutathione S-transferase (GST) activity was determined by the conjugation of glutathione (GSH) with chloro-2,4- dinitrobenzene (CDNB) monitored at 340 nm for 6 min (20 reading cycles) following the methodology described by Keen et al. (1976).

2.6 Statistical analysis

The data obtained were checked for normality using the Kolmogorov Smirnov test, followed by ANOVA - one way and Dunnett and Tukey post-test for parametric data, or the Kruskal-Wallis test followed by Dunn test for non-parametric data, with 95% confidence interval ($p < 0.05$) using GraphPad Prism software.

3. Results And Discussion

After chronic exposure (21 days) of *Daphnia magna* organisms it was possible to verify that the survival parameter was not affected in the first generation of exposed organisms (F0). However, in the next generation (F1) effects were observed, mainly when related to the mixture of atrazine and glyphosate herbicides, with the group corresponding to the mixture of commercial formulations being responsible for the highest number of deaths (Table 1).

Table 1: Percentage of survival of test organisms *Daphnia magna*, obtained after chronic transgenerational exposure to analytical standards and commercial formulations of atrazine and glyphosate herbicides, isolated and in mixture.

Group	Survival (%)	
	F0	F1
NC	100	100
ATSA	100	92
GPSA	100	100
MIXSA	100	92
ATCOM	100	100
GPCOM	92	92
MIXCOM	100	83

NC = negative control, ATSA = standard analytical atrazine, GPSA = standard analytical glyphosate, MIXSA = mixture of analytical standards (AT + GP), ATCOM = commercial formulation atrazine, GPCOM = commercial formulation glyphosate, MIXCOM = commercial formulation mix (AT + GP).

We observed that the groups corresponding to the mixture of herbicides were responsible for the greatest effects, when compared to the groups of isolated herbicides, since they had the highest organisms lethality. This lethality occurred increasingly over generations, indicating that chronic exposure, even at low concentrations, considerably affects non-target organisms. Furthermore, it was possible to notice that the mixture of commercial products, probably due to the presence of adjuvants, was more toxic to the organisms, when compared to its active ingredients.

This fact has already been reported by other authors, where the commercial formulation containing glyphosate as the active ingredient was more toxic to dragonfly larvae *Coenagrion pulchellum*, when compared to the active ingredient alone, with negative effects on survival, behavior and physiological characteristics, when evaluating concentrations of 1000 and 2000 $\mu\text{g L}^{-1}$ (Janssens, Stoks, 2017). Loughlin et al. (2016) did not observe significant lethality effects, when compared to the control, in crayfish *Cherax quadricarinatus* exposed to commercial formulation of atrazine, even the evaluated concentrations (100, 500 and 2500 $\mu\text{g L}^{-1}$) being higher than the environmental concentrations tested in our study.

The effects of the herbicides atrazine (0.006, 0.06, 0.6, 6, 60 and 600 μM) and glyphosate (0.01, 0.1, 1, 10, 100 and 1000 μM), isolated and in mixture, were also evaluated by García-Espiñeira et al. (2018), on the organism *Caenorhabditis elegans*. The authors found an increasing lethality as the concentrations of the isolated herbicides increased, and when the mixture of atrazine (600 μM) and glyphosate (1000 μM) was evaluated, 80% lethality was found. These results were similar to those observed in our study, considering that the treatment consisting of a mixture of commercial herbicide formulations was the group responsible for the highest organisms lethality (83%).

Although many studies demonstrate the toxicity of active ingredients and commercial herbicide formulations in terms of non-target species mortality, much information is still required on sublethal effects involving realistic environmental concentrations (Janssens, Stoks, 2017, Séguin et al., 2017). It is important to study the sublethal effects since it is estimated what happens in the real ecosystem, mainly related to herbicides applied seasonally in agricultural activities (Religia et al., 2019).

The microcrustacean *Daphnia magna* is one of the most used species in ecotoxicological tests due to its sensitivity, and the ecology of these aquatic invertebrates is adversely affected by relevant environmental concentrations (García-Espiñeira et al., 2018, Moreira et al., 2020). Thus, the study of sublethal effects in this biological model reflects much important information, which allows the estimation of the effects aimed at preventing damage to non-target species, and thus avoiding the biomagnification of damage (Cuhra et al., 2013, Zocchi, Sommaruga, 2019).

3.2 Effects on reproduction on *Daphnia magna*

Results obtained for the parameter related to the mean time (days) for the beginning of the production of offspring per female (primiparous) are shown in Figure 2. It is possible to observe that in the first generation (F0), the statistical difference ($p < 0.05$), when compared to the control group, it was verified for the ATSA, MIXSA, GPCOM and MIXCOM groups, and in the second generation (F1), for the MIXSA and GPCOM groups. Furthermore, in the first generation (F0) a statistical difference ($p < 0.05$) was also observed between the treatments composed by the analytical standard herbicide glyphosate and the commercial formulation.

In this analysis, it can be seen that all groups in which there was a significant difference ($p < 0.05$) presented a delay in sexual maturation, that is, in the beginning of the production of offspring per female. Furthermore, the MIXSA and GPCOM groups showed statistical difference ($p < 0.05$) in both generations (F0 and F1) when compared to the control, demonstrating that this effect remained in the offspring of the first generation.

For the analysis of the reproduction parameter, only offspring from females that remained alive until the end of the 21 days exposure period were considered. Figure 3 shows the results corresponding to the average number of offspring produced by alive female, obtained in the two generations evaluated. In the first generation of exposed offspring (F0), there was no statistical difference ($p > 0.05$) between the samples. However, for the second generation (F1), there was a statistical difference ($p < 0.05$) when compared to the results obtained in the control group for the GPSA and ATCOM samples, resulting in the inhibition of reproductive capacity.

In addition, the statistical response ($p < 0.05$) obtained between treatments is highlighted, as in the second generation (F1) there was a difference in all groups between active ingredients and commercial formulations, that is, between ATSA and ATCOM, GPSA and GPCOM, and MIXSA and MISCOM. Also, some groups presented a high number of offspring produced by females, which may be associated with the stress caused by exposure to herbicides, where organisms direct their energy to reproduction,

increasing the number of offspring (Moreira et al., 2020). Or it may be related to the hormesis effect, which corresponds to a stimulating or beneficial response at low concentrations and inhibitory or toxic at high concentrations, when organisms are exposed to toxic molecules (Drzymała, Kalka, 2020). This effect can be directly induced or occur due to homeostasis imbalance as a result of compensatory actions (Moreira et al., 2020).

The sublethal effects presented by the organisms are usually due to biochemical or molecular interferences. Parameters that involve reproduction, as well as behavior and interactions with the environment at the beginning of the life cycle, are fundamental to establish the survival and permanence of aquatic organisms in their ecosystem (Folle et al., 2020). Therefore, when evaluating the second generation of organisms (F1), there is a need to obtain greater answers about continuous exposure to different environmental contaminants (Pérez, Hoang, 2018).

García-Espiñeira et al. (2018), verified effects on the reproduction of *C. elegans*, reporting a reduction in the organisms population exposed to a mixture of atrazine and glyphosate, with a reduction in litter size by 93% for atrazine (6 µM) and glyphosate (10 µM) isolated. The results obtained in our study regarding treatment with commercial formulation atrazine in the second generation (F1) are similar to those observed by García-Espiñeira et al. (2018), however, the results presented by the treatment composed by the commercial glyphosate formulation are divergent. Still, regarding the mixture of herbicides, a significant reduction ($p < 0.05$) was observed, similarly to authors, however, only when compared to the group composed by the mixture of analytical standards of herbicides.

When evaluating environmentally relevant concentrations, as performed in our study, Moreira et al. (2020) observed that the reproduction of *Daphnia magna* was not affected when exposed to sublethal concentrations (0.002, 0.004 and 0.006 µg L⁻¹) of the pesticides Kraft® 36 EC and Score® 250 EC, which have abamectin and difenoconazole as active principles, respectively, isolated and also in mixture.

Considering that concentrations of atrazine that induce toxicity can also delay development and cause abnormalities in some species (Yoon et al., 2019), Religia et al. (2019) also evaluated generations of *D. magna*, which were fed *Raphidocelis subcapitata* exposed to the herbicide atrazine (150 µg L⁻¹). The authors verified that the matrices fed with this phytoplankton did not show abnormalities, however, they produced non-viable offspring. Initially, the number of unviable offspring was high, but it decreased in later stages, which indicated that *R. subcapitata* exposed to the sublethal concentration of this herbicide affected the population dynamics of *D. magna*. Marcus and Fiumera (2016) also suggest that the herbicide atrazine, at ecologically relevant doses (2 and 20 µg L⁻¹ and 2 and 20 mg L⁻¹), presents effects on the physical characteristics of *Drosophila melanogaster*, and that the reduction in life span of the evaluated organisms may be due to oxidative stress caused by the herbicide.

The importance of long-term exposure was also exposed by Xu et al. (2017), when reporting that after prolonged exposure to sublethal concentrations of the herbicide glyphosate, effects such as the inhibition of food intake were observed, which limits the growth and changes the metabolic profile of the mollusc

Pomacea canaliculata. Similarly to what was reported in our study, the transgenerational or multigenerational evaluation of effects proved to be necessary, when Cleary et al. (2019) evaluated *Oryzias latipes* organisms exposed to atrazine (5 and 50 $\mu\text{g L}^{-1}$), and their results suggested that even though early exposure to this herbicide did not cause significant phenotypes in the first directly exposed generation, subsequent generations of fish were susceptible to increased reproductive dysfunction risks.

Through the studies presented, which used the same herbicides or the same biological model used in ours, the responses of different sensitivities between the organisms can be seen, and that even indirectly, as in the case presented by Religia et al. (2019), effects caused by pesticides can be observed. This fact encourages the search for information about contamination processes at different levels, because when low concentrations are not tested on non-target organisms, many products are considered harmless to the environment, considering that toxicity is underestimated when inadequately evaluated.

3.3 Biochemical biomarkers

Considering that *Daphnia* species are widely used indicators in ecotoxicological tests, there is still a scarcity of studies that address a transgenerational analysis and assess multiple biomarkers in these organisms. Therefore, this work addressed such issues, and also, through the use of pesticides, which are a global concern, enabled satisfactory results to encourage the realization and deepening of studies on these themes.

Among the responses obtained through exposure to sublethal concentrations, biochemical biomarkers allow us to assess in an early and more detailed way the effects of different compounds (Moreira et al., 2020). The use of these methodologies aids monitoring and environmental management activities, allowing for the revision, when pertinent, of legislation that stipulates the maximum acceptable limits for chemical products in the environment.

The production of free radicals and reactive oxygen species (ROS) causes oxidative damage, affecting the DNA molecule, proteins and lipids, interrupting cellular physiological processes in several living organisms (Yoon et al., 2019). Responsible for the antioxidant mechanisms, by reducing the hydrogen peroxide content, catalase (CAT) acts as a molecular biomarker helping to assess the effects of herbicides, due to its role in the detoxification of hydrogen peroxide (H_2O_2) generated under stress conditions (Mona et al., 2013).

In this study, catalase activity was not statistically different ($p > 0.05$) when compared to the control in the first generation (F0) evaluated (Figure 4). However, for the second generation (F1), the statistical difference ($p < 0.05$) when compared to the control, occurred for the ATSA and MIXSA groups, and also between the groups corresponding to the active ingredient (ATSA) and the commercial formulation (ATCOM) of atrazine. It can be inferred that the exposed organisms presented a response mechanism to oxidative stress in the groups in which statistical differences were verified ($p < 0.05$) due to the increased activity of this enzyme.

Glutathione S-transferases (GST) are widely studied when referring to herbicides, due to their role in detoxification processes against xenobiotics (Peragón, Amores-Escobar, 2018). These enzymes play an important role in removing excess reactive oxygen species (ROS), which cause oxidative stress in living organisms, and the significant increase in their activity has already been reported as a response to environmental stressors in aquatic organisms (Yoon et al., 2019).

In this work, the glutathione S-transferase activity did not show statistical difference ($p > 0.05$) for the first generation (F0) evaluated (Figure 5). For the second generation (F1), a statistical difference ($p < 0.05$) when compared to the control group was observed for the ATSA, MIXSA and GPCOM groups. There was also a significant difference ($p < 0.05$) between treatments, highlighting the difference between the GPSA and GPCOM, and MIXSA and MIXCOM groups. This indicates that organisms present different responses when isolated molecules or commercial products of herbicides, which contain other compounds in their formulations, are evaluated.

According to the results obtained from the analysis of the two generations, an increase in the activity of this enzyme is observed in most treatments from the second generation (F1). According to Agathokleous et al. (2020), chemical-induced hormesis also presents a temporal variation as a result, with an initial increase, followed by a decline. Moreover, authors state that the response of antioxidant enzymes to chemicals has a tendency to increase initially, due to activation of enzyme synthesis, followed by reduction or inhibition of activity (Viarengo et al., 2007, Séguin et al., 2017). Transgenerational assessment allows us to observe these responses, however, if more generations were studied, the information would be even more accurate.

In our study, the ATSA and MIXSA groups presented statistical difference ($p < 0.05$) when compared to the control, for the two biochemical biomarkers evaluated, CAT and GST, when they were determined in the second generation studied (F1).

Contardo-Jara et al. (2009) did not find changes in CAT activity after exposure of *Lumbriculus variegatus* to glyphosate and its commercial formulation, however, when they evaluated the biotransforming enzyme GST, a significant increase in activities was observed for the two treatments, at a concentration of $50 \mu\text{g L}^{-1}$. The authors state that, as glyphosate's biochemical pathway of action is unique to plants and some microorganisms, it is expected that the toxicity to non-target organisms will be reduced.

Séguin et al. (2017) observed a significant increase in CAT activity evaluated in the digestive gland of *Crassostrea gigas* in the groups exposed to glyphosate, and a significant difference in GST activity only when examining temporal variations. The authors suggest that herbicides containing glyphosate as an active ingredient have no effects at concentrations of 0.1, 1 and $100 \mu\text{g L}^{-1}$, requiring the association with other biomarkers to understand the effects. These results are similar to those obtained in our study when the analytical glyphosate standard was evaluated, however, the information is divergent regarding the commercial formulation, because in the GST evaluation the group corresponding to the commercial

product glyphosate, besides presenting statistical difference ($p < 0.05$) compared to the control, it also showed a difference between the treatment with the active ingredient glyphosate alone.

Moreira et al. (2020) evaluated environmental concentrations of the pesticides Kraft® 36 EC (abamectin) and Score® 250 EC (difenoconazole), isolated and in mixture, in *D. magna* organisms, and verified that these compounds, when isolated, did not cause effects on the catalase activity (CAT), but the mixtures promoted an increase in this enzyme. The same was observed in our study, since the mixture of herbicides was significantly different ($p < 0.05$) from the control in the second generation of *D. magna* evaluated.

Santos and Martinez (2014) also evaluated biochemical biomarkers after exposure to atrazine and glyphosate herbicides, isolated and in mixture in the snail *Corbicula fluminea*, and found no statistical difference for GST activity. On the other hand, CAT enzyme activity presented statistical difference for the group exposed to glyphosate (10 mg L^{-1}). The data presented by the authors do not corroborate those reported in our study, but the concentrations we evaluated are lower and closer to the environmental reality.

According to Message et al. (2015) the effects caused by exposure to the herbicide glyphosate may be due to endocrine disruption and oxidative stress, resulting in metabolic changes, which depend on the concentration and time of exposure. In addition, the toxic effects of commercial products can be explained by the presence of adjuvants, which have their own toxicity, but can also increase the toxicity of active principles, indicating that the formulations may be of greater ecotoxicological relevance (Contardo-Jara et al., 2009, Cavas, 2011).

Some studies report that the activities of antioxidant enzymes increase when organisms are exposed to low concentrations of chemicals or when exposures occur in a short period of time however, they can decrease or be inhibited when organisms are exposed to high concentrations or after a prolonged exposure, depending on the concentration tested (Wang et al., 2011, Moreira et al., 2020). Effects induced by mixing pesticides and other environmental contaminants have already been reported for different systems, which indicated that the components of a mixture are responsible for antagonistic or synergistic effects on the stimulatory response (Chamsi et al., 2019, Agathokleous et al., 2020).

The influence on the enzymatic activities of CAT and GST in *D. magna* has been related to the presence of contaminants from the pesticide class, with the values determined for these enzymes slightly varying when compared to data reported in several studies (Rivetti et al., 2015). This fact may be linked to several factors that can cause variability in these parameters, such as the algae species used in feeding, the age and litter of organisms, and also experimental conditions such as temperature and photoperiod (Moreira et al., 2020).

Our results showed that the biochemical biomarkers evaluated CAT and GST started to present statistical significance ($p < 0.05$) from the second generation of exposed organisms (F1). This fact denotes the importance of evaluating concentrations close to those detected in ecosystems, and how this affects

non-target organisms over generations, so that the effects are closer to reality. Furthermore, these results highlight the need for an assessment of multiple biomarkers after different exposure times, and above all, that even at environmental concentrations, lower than the normally assessed concentrations, it is possible to obtain an answer about the oxidative stress indirectly caused to non-target species.

4. Conclusion

This study presents primordial results regarding studies that evaluate the mixture of compounds, in environmentally relevant concentrations, and the comparison between active ingredients and commercial products. Changes in reproductive parameters and biochemical biomarkers were observed after chronic exposure of *D. magna* organisms to sublethal concentrations of the herbicides atrazine and glyphosate, isolated and in mixture. The importance of evaluating the effects across generations is highlighted, since significant responses ($p < 0.05$) were mostly observed in the second generation of organisms evaluated.

Important differences were also observed between the active principles and commercial products, which demonstrates the need to seek alternatives for the synthesis of pesticides. Seeking to use less toxic adjuvants, or other ways to obtain the same desired effects, since the active principles can be less toxic when isolated, compared to a formulation, which contains other molecules.

Considering that concentrations higher than those evaluated in this work have already been found in environmental samples in different regions of the world, the potential risk to which non-target aquatic organisms are exposed is perceived. This fact encourages the search for damage prevention measures for the preservation of aquatic ecosystems.

Declarations

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Conflicts of interest

No potential conflict of interest was reported by the authors.

Availability of data and material

Not applicable.

Code availability

Not applicable.

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Figures

Figure 1

Experimental design for the ecotoxicological testing.

Figure 2

Start of offspring production per female (mean $\pm$ SD), for the first (F0) and second (F1) generation of *D. magna* evaluated after chronic exposure to analytical standards and commercial formulations of the herbicides atrazine and glyphosate, isolated and in mixture. Asterisk indicates the statistical difference ($p < 0.05$) in relation to the negative control, and different lowercase letters indicate the statistical difference ($p < 0.05$) between treatments with active principles and commercial formulations for the same herbicide. NC = negative control, ATSA = standard analytical atrazine, GPSA = standard analytical glyphosate, MIXSA = mixture of analytical standards (AT + GP), ATCOM = commercial formulation atrazine, GPCOM = commercial formulation glyphosate, MIXCOM = commercial formulation mixture (AT + GP).

Figure 3

Newborns produced by alive female (mean $\pm$ SD), for the first (F0) and second (F1) generation of *D. magna* evaluated after chronic exposure to analytical standards and commercial formulations of the herbicides atrazine and glyphosate, isolated and in mixture. Asterisk indicates the statistical difference ($p < 0.05$) in relation to the negative control, and different lowercase letters indicate the statistical difference ($p < 0.05$) between treatments with active principles and commercial formulations for the same herbicide. NC = negative control, ATSA = standard analytical atrazine, GPSA = standard analytical glyphosate, MIXSA = mixture of analytical standards (AT + GP), ATCOM = commercial formulation atrazine, GPCOM = commercial formulation glyphosate, MIXCOM = commercial formulation mixture (AT + GP).

Figure 4

Enzymatic activities of catalase (mean $\pm$ SD) obtained after chronic exposure of the first (F0) and second (F1) generation of *D. magna* to analytical standards and commercial formulations of the herbicides atrazine and glyphosate, isolated and in mixture. Asterisk indicates the statistical difference ($p < 0.05$) in relation to the control, and different lowercase letters indicate the statistical difference ($p < 0.05$) between treatments with active principles and commercial formulations for the same herbicide. NC = negative control, ATSA = standard analytical atrazine, GPSA = standard analytical glyphosate, MIXSA = mixture of analytical standards (AT + GP), ATCOM = commercial formulation atrazine, GPCOM = commercial formulation glyphosate, MIXCOM = commercial formulation mixture (AT + GP).

Figure 5

Glutathione S-transferase enzymatic activities (mean $\pm$ SD) obtained after chronic exposure of the first (F0) and second (F1) generation of *D. magna* to analytical standards and commercial formulations of the herbicides atrazine and glyphosate, isolated and in mixture. Asterisk indicates the statistical difference ($p < 0.05$) in relation to the control, and different lowercase letters indicate the statistical difference ($p < 0.05$) between treatments with active principles and commercial formulations for the same herbicide. NC = negative control, ATSA = standard analytical atrazine, GPSA = standard analytical glyphosate, MIXSA = mixture of analytical standards (AT + GP), ATCOM = commercial formulation atrazine, GPCOM = commercial formulation glyphosate, MIXCOM = commercial formulation mixture (AT + GP).