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Effects of environmentally relevant concentrations of atrazine and glyphosate herbicides, isolated and in mixture, on two generation of the freshwater microcrustacean *Daphnia magna*

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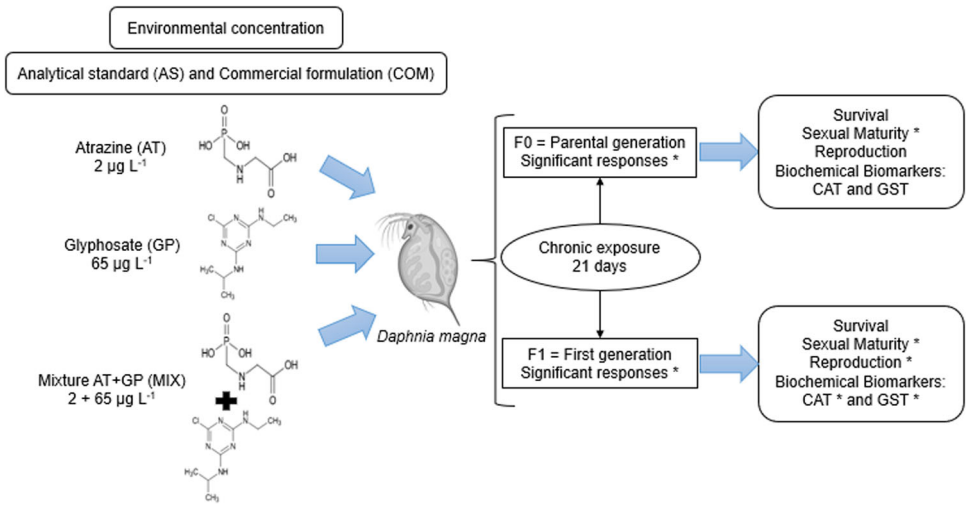
Abstract

The herbicides atrazine and glyphosate are used worldwide and their excessive usage results in the frequent presence of these pesticides in environmental compartments. We evaluated the effects of environmentally relevant concentrations of analytical standards and commercial formulations of atrazine (2 µg L^{-1}) and glyphosate (65 µg L^{-1}), isolated and in mixture (2 + 65 µg L^{-1}) on the microcrustacean *Daphnia magna*. Through chronic exposure (21 days) of two generations, we observed effects on survival, reproductive capacity and responses of the antioxidant defense system (catalase) and biotransformation system (glutathione S-transferase). The survival of organisms was affected in the second generation (F1) with a mortality of 17% in the mixture of commercial formulations treatments. In the evaluation of the first generation (F0) we observed only effects on sexual maturation of organisms, while in the F1, changes were observed in all parameters evaluated. A statistical difference ($p < 0.05$) was also observed between the analytical standards and the commercial formulations for all parameters evaluated, indicating that other components present in the formulations can change the toxicity of products. We suggest that atrazine can modulate toxicity when mixed with glyphosate, as the standard analytical atrazine and mixture of analytical standards results were similar in most parameters. Given the difficulty in estimating effects of mixtures and considering that various stressors are found in the environment, our results support the need to carry out long-term studies and, above all, to verify what are the impacts across generations, so that the toxicity of products is not underestimated.

Graphical abstract



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Keywords Active ingredient · Biochemical biomarkers · Chronic toxicity · Commercial formulations · Pesticides

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 their control, herbicide mixtures are widely used in order to obtain different action mechanisms, thus; they are also considered more effective in combating weed resistance (Moss et al., 2019).

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5 -triazine) is an herbicide that acts on 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). In addition to overuse and continuous use, the chemical and physical properties of these pesticides, such as persistence in soil and water, has caused the permanent presence of these pesticides in surface and ground water which may cause damage to several non-target organisms (Alonso et al., 2018; Cleary et al., 2019).

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 are 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). Adjuvants are considered inert substances as they do not have pesticidal activity (Cossi et al., 2020). Although, in some cases, an increase in the toxicity of commercial formulations versus their active principles is reported (Nagy et al., 2020). Besides that, toxicity from products may also differ according to the commercial formulation (Reno et al., 2018).

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 in mixture has already been evaluated in multiple bioindicator organisms. These studies 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 exposured to numerous pollutants, in concentrations that vary according to multiple factors, including climatic conditions, losses through aerial drift and application handling (Alonso et al., 2018). However, these organisms can accumulate various chemical products in higher concentrations than those detected in waters in which they live, through bioaccumulation or adherence to suspended particulate matter, which can be ingested by aquatic organisms (Blahova et al., 2020).

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 reveals important information, which allows the estimation of the effects aimed at preventing damage to non-target species, and thus avoiding biomagnification damage (Cuhra et al., 2013; Zocchi; Sommaruga, 2019).

Acute toxicity is observed primarily to assess the environmental damage caused by certain products; however, generational effects are 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 and biotransformation, respectively (Yang et al., 2019; Santana et al., 2021).

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 indirectly act on non-target aquatic species, especially when leached into rivers or through spray drift, it is necessary to know the effects and anticipate remediation, seeking reduction of possible consequences that these organisms may suffer.

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

Materials and methods

Chemicals

Atrazine analytical standards stock solutions (20 mg L^{-1}) (CAS no. 1912-24-9, Sigma Aldrich, pureza ≥98%) and glyphosate (500 mg L^{-1}) (CAS no. 1071-83-6, Sigma Aldrich, pureza ≥98%) were prepared with purified water, from 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} with purified water, from a reverse osmosis system, based on the active ingredient amount present in the formulations.

Environmental concentrations

Concentrations of 2 µg L^{-1} for atrazine and 65 µg L^{-1} for glyphosate were evaluated, in addition to the mixture of 2 + 65 µg 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), mixture of commercial formulations (MIXCOM) and the negative control group (NC), containing only 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 closer 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).

Test organisms

Daphnia magna organisms used in the first 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 *D. 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 cells mL^{-1}), also cultivated in the same laboratory according to the methodology presented in NBR 12.648 (ABNT, 2018).

Chronic test of *Daphnia magna* as a bioindicator

Chronic ecotoxicity tests were performed according to standard protocols (OECD, 2012), which lasted for 21 days and consisted of 12 replicates per treatment ($n = 12$). Fig. 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 µg L^{-1} and 65 µg L^{-1} of

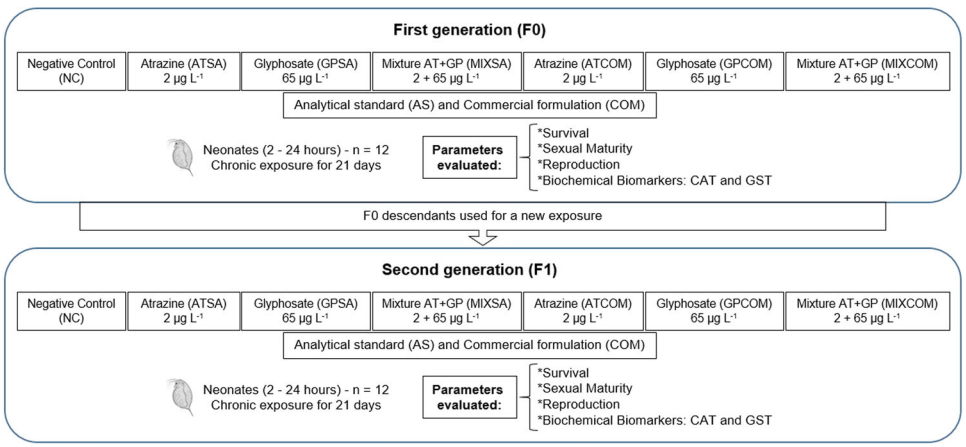

Fig. 1 Experimental design for the ecotoxicological testing

isolated atrazine and glyphosate, respectively, and also the mixture of herbicides at the same concentrations, with controlled temperature of 20 ± 2 °C and photoperiod of 16 h of light and 8 h of dark.

The first 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.

Effects on two generations 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 h of life) obtained from the first generation (F0). After the period corresponding to the third litter birth from the control group from F0 generation, F1 generation began. For treatments where the number of neonates was not enough to start the second generation (F1), we waited until the next day when the number of neonates was enough to start a new generation.

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 remaining adult individuals after 21 days of exposure. 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 live adult during the 21 days of the test.

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). Homogenized samples were kept refrigerated throughout the process (4 °C), and centrifuged at 8,000 G for 10 min at 4 °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).

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.

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).

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).

Statistical analysis

The data obtained were checked for normality using the Kolmogorov Smirnov test, followed by ANOVA - two way, using generation and treatment as factors, 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.

Results and discussion

Survival rate

After chronic exposure (21 days), 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).

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. The lethality increased in the second generation, indicating that chronic exposure, even at low concentrations, considerably affects non-target organisms. Furthermore, it was noticed that the mixture of commercial products, probably due to the presence of adjuvants, was more toxic to the organisms than its individual active ingredients.

This fact has already been reported by other authors, in which the commercial formulation containing glyphosate as the active ingredient was more toxic to dragonfly larvae *Coenagrion pulchellum*, than to the active ingredient alone, with negative effects on survival, behavior, and physiological characteristics, when evaluating concentrations of 1000 and 2000 μg 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 with evaluated concentrations (100, 500, and 2500 μg L^{-1}) being higher than the environmental concentrations tested in our study.

The effects of the herbicides atrazine (1.29, 12.94, 129.40, 1,294.08, 12,940.8, and 129,408 μg L^{-1}) and glyphosate (1.69, 16.90, 169.07, 1,690.7, 16,907, 169,070 μg L^{-1}), 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 (129,408 μg L^{-1}) and glyphosate (169,070 μg L^{-1}) was evaluated, 80% lethality was detected. 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 organism 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 of herbicides applied seasonally in agricultural activities, since that is a better estimate of what happens in the real ecosystem (Religia et al., 2019).

Table 1 Survival percentage of test organisms *D. magna*, obtained after chronic exposure of two generations 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)

Effects on reproduction on *Daphnia magna*

Results obtained for the parameter related to the mean time (days) for the beginning of offspring production per female (primiparous) are shown in Fig. 2. It is possible to observe that in the first generation (F0), the statistical difference ($p < 0.05$), when compared to the control group, was verified for the ATSA, MIXSA, GPCOM and MIXCOM groups, and in the second generation (F1), for the MIXSA group. 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. We also verified statistical difference ($p < 0.05$) between generations (F0xF1) for all treatments with herbicides ($ATSA_{F0}xATSA_{F1}$; $GPSA_{F0}xGPSA_{F1}$; $MIXSA_{F0}xMIXSA_{F1;}$ $ATCOM_{F0}xATCOM_{F1}$; $GFCOM_{F0}xGFCOM_{F1}$; $MIXCOM_{F0}xMIXCOM_{F1}$).

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 offspring production per female. Furthermore, the MIXSA group presented 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 second generation. The toxicity assessed through the reproductive cycle of *D. magna* is associated with hormones, which are targets of endocrine disruptors (Jeong; Simpson, 2020). We observed that some of the effects of atrazine and glyphosate on *D. magna* fecundity can be explained by the reduced age of first reproduction when compared to unexposed organisms, which can cause disturbance on *Daphnia* populations in aquatic ecosystems. However, it is suggested that these effects do not affect the population's intrinsic growth rate, but it may take from 1 to 5 generations to recover from this disturbance considering a stress scenario (Ginjupalli; Baldwin, 2013).

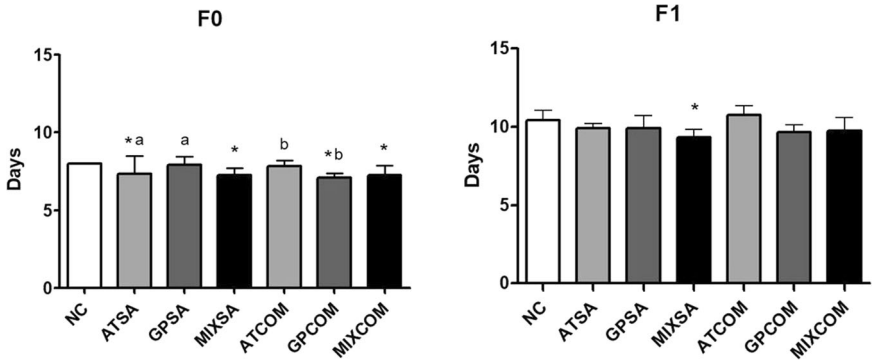

Fig. 2 Start of offspring production per female (mean ± SD), for the first (F0) and second (F1) generation of *D. magna* evaluated after chronic exposure to analytical standards and commercial formulations of the herbicides atrazine and glyphosate, isolated and in mixture. Asterisk indicates the statistical 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)

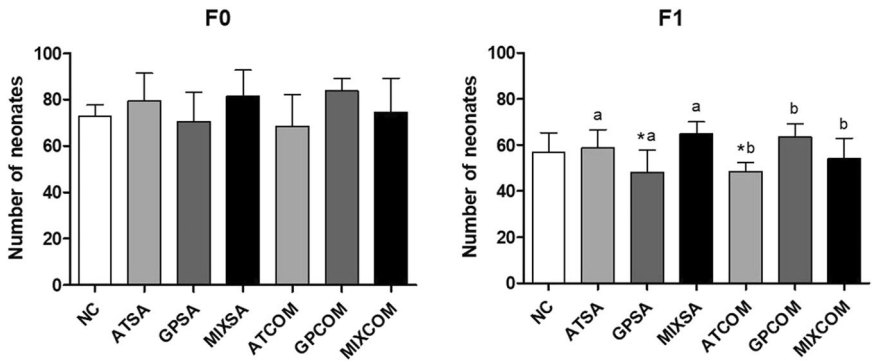

Fig. 3 Newborns produced by alive female (mean ± SD), for the first (F0) and second (F1) generation of *D. magna* evaluated after chronic exposure to analytical standards and commercial formulations of the herbicides atrazine and glyphosate, isolated and in mixture. Asterisk indicates the statistical 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)

For the reproduction parameter analysis, only offspring from females that remained alive until the end of the 21 day exposure period were considered. Figure 3 shows the results corresponding to the average number of offspring produced by live females, obtained in the two generations evaluated. In the first generation of exposed offspring (F0), there was no statistical difference ($p > 0.05$) between 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. Furthermore, as verified for the primipara parameter, we also identified statistical difference ($p < 0.05$) between generations (F0xF1) for all treatments with herbicides.

Observing the graphs in Fig. 3, we can see that in the second generation (F1) all treatments followed the same pattern presented in the first generation (F0); however, with a reduced number of neonates produced by alive female. Furthermore, we can observe that MIXSA and ATSA presented a very similar response, and both diverged from GPSA, which may suggest a synergism between the herbicides when in the mixture, with atrazine being the modulator of this effect. For commercial formulations, on the other hand, the same effect was not verified, since the average of neonates produced by females exposed to the herbicide mixture was between the average of neonates produced by females exposed to isolated herbicides. Regarding the mixture, the underlying toxicity mechanisms could be clarified through studies at the molecular level, as

each commercial formulation has molecules that can change the toxicity of the product (Magdaleno et al., 2015; Sanches et al., 2017). Therefore, our results confirm the difficulty in predicting the effects obtained by mixtures when based only on information from individual compounds. This fact is probably due to biological complexity and redundancy in the response pathways from organisms (Moreira et al., 2020).

In addition, the statistical response ($p < 0.05$) obtained between treatments is highlighted. Once, 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. Besides that, 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 offspring number (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). However, we recognize that to confirm a hermetic effect, it would be necessary to evaluate a dose-response curve and a stimulus on lower concentrations would have to be detected.

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 population exposed to a mixture of atrazine and glyphosate, with a reduction in litter size by 93% for atrazine (1,294.08 $\mu g\,L^{-1}$) and glyphosate (1,690.7 $\mu g\,L^{-1}$) 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 group composed of the commercial glyphosate formulation are divergent. Still, regarding the mixture of herbicides, a significant reduction ($p < 0.05$) was observed similar to previous authors; however, only when compared to the group composed of 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 *D. magna* was not affected when exposed to sublethal concentrations (0.002; 0.004 and 0.006 $\mu g\,L^{-1}$) of the pesticides Kraft® 36 EC and Score® 250 EC, which have abamectin and difenoconazole as active principles, respectively, isolated and also in mixture.

Religia et al. (2019) also evaluated generations of *D. magna*, which were fed *Raphidocelis subcapitata* exposed to the herbicide atrazine (150 $\mu g\,L^{-1}$). 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*.

The importance of long-term exposure was also demonstrated by Xu et al. (2017), when reporting that after prolonged exposure of the mollusc *Pomacea canaliculata* 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. Similar to what was reported in our study, the multigenerational evaluation proved to be necessary, when Cleary et al. (2019) studied *Oryzias latipes* organisms exposed to atrazine (5 and 50 $\mu g\,L^{-1}$). 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 as 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. Furthermore, it is noteworthy that in our study, the effects were obtained through chronic exposure of *D. magna* to concentrations below the $LC_{50\text{-}48h}$ determined for atrazine (35.5– 50.41 $mg\,L^{-1}$) (Palma et al., 2008; Moreira et al., 2014) and glyphosate (11.68–21.34 $mg\,L^{-1}$) (Reno et al., 2018; Gustinasari et al., 2020). And the concentrations that we evaluated are also lower than the other concentrations already detected in the environment for these herbicides (Alonso et al., 2018; Berman et al., 2018; Carles et al., 2019; Loro et al., 2015, Sousa et al., 2015).

These facts encourage 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. When the toxicity of herbicides is based only on the LC50, its release to the market and, consequently, its continuous

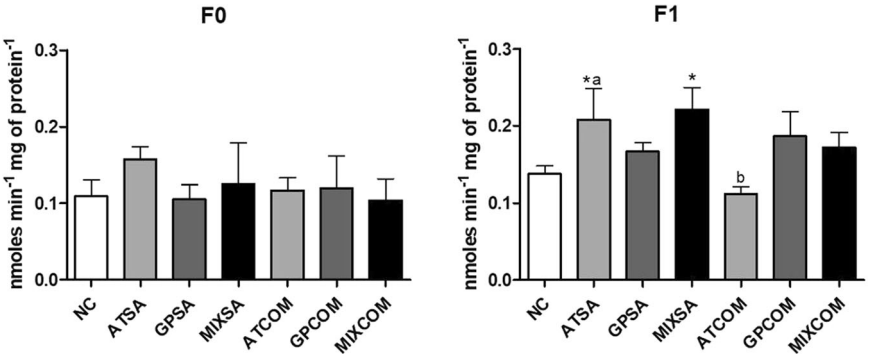

Fig. 4 Enzymatic activities of catalase (mean ± SD) obtained after chronic exposure of the first (F0) and second (F1) generation of *D. magna* to analytical standards and commercial formulations of the herbicides atrazine and glyphosate, isolated and in mixture. Asterisk indicates the statistical difference ($p < 0.05$) 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)

and excessive use can cause severe environmental damage, since the toxicity is underestimated when inadequately evaluated. Therefore, we found impacts on the reproduction of this organism even at concentrations considerably lower than the LC50 for these products. This alerts us to the real estimation of pesticide effects, based on ecotoxicological tests in different organisms, and with prolonged exposure to contaminants.

Biochemical biomarkers

Considering that *Daphnia* species are widely used as indicators in ecotoxicological tests, there is a surprising scarcity of studies that analyze multiple biomarkers over several generations in these organisms. Therefore, this study 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 reactive oxygen species (ROS) occurs naturally; however, when there is an imbalance between the generation of oxidant compounds and the antioxidant system action, there is initially oxidative stress, and more severely oxidative damage, which affects the DNA molecule, proteins and lipids, interrupting cellular physiological processes in several living organisms (Yoon et al., 2019; Pastorino et al., 2021). Due to its role in the detoxification of hydrogen peroxide (H_2O_2) generated under stress conditions, catalase (CAT), acts as a molecular biomarker, helping to assess the effects of herbicides (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 (Fig. 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. Furthermore, we also verified statistical difference ($p < 0.05$) between generations (F0xF1) for MIXSA.

As observed for the reproductive effect, CAT activity for the second generation (F1) was similar for ATSA and MIXSA, and both different from GPSA, indicating that the herbicide atrazine could be modulating the interaction between the herbicides and generating that response in MIXSA. As for commercial formulations, it can be inferred that GPCOM can cause a protective effect when compared to ATCOM, since the CAT activity was similar between GPCOM and MIXCOM.

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).

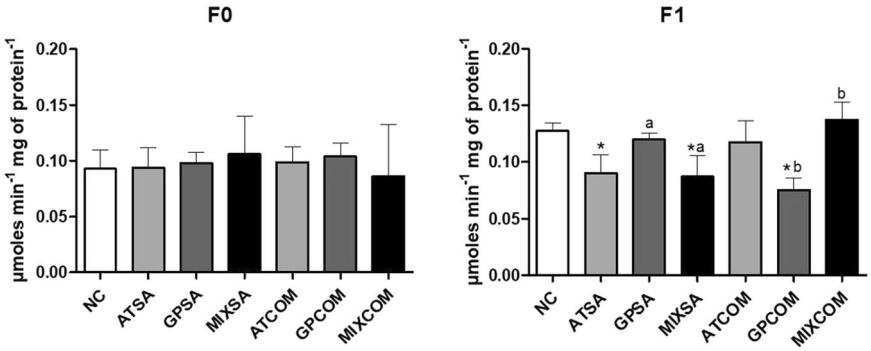

Fig. 5 Glutathione S-transferase enzymatic activities (mean ± SD) obtained after chronic exposure of the first (F0) and second (F1) generation of *D. magna* to analytical standards and commercial formulations of the herbicides atrazine and glyphosate, isolated and in mixture. Asterisk indicates the statistical 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)

In this study, the glutathione S-transferase activity was not statistically elevated ($p > 0.05$) for the first generation (F0) (Fig. 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. Furthermore, for the GST biomarker we did not verify statistical difference ($p > 0.05$) between generations (F0xF1).

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 µg 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. Even though, in our study, the higher GST activity obtained in MIXCOM compared to MIXPA, and higher activity obtained in GPSA compared to GPCOM, indicates a greater demand for antioxidant or conjugation activities (Osório et al., 2014). Biotransformation of these herbicides may have generated an excess of ROS, stimulating an antioxidant defense system response (Liu, 2020; Santana et al., 2021). The decrease in GST activity, which occurred in the same groups where there was an increase in CAT activity, is possibly due to the action of this antioxidant enzyme (CAT), as it is the first line of defense against the action of ROS produced by exposure to herbicides (Destro et al., 2021). This would explain the decrease in GST we observed in treatments exposed to ATSA, MIXSA, and GPCOM (Fig. 5).

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 µg 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, not only presented statistical difference ($p < 0.05$) compared to the control, but 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 Mesnage 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 exposure time. Moreover, 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. 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 CAT and GST presented statistical significance ($p < 0.05$) only in the second generation of exposed organisms (F1). This fact denotes the importance of evaluating the effects of contaminants over generations. Since, through the assessment of concentrations detected in environmental matrices, the observed effects can be closer to the real effects that occur in ecosystems. When comparing the commercial formulations with their respective active ingredients, we found a statistical difference ($p < 0.05$) for CAT where the highest activity occurred in the treatment with the active ingredient atrazine (Fig. 4), and for GST where the highest activity occurred in the treatments with the active ingredient glyphosate and the commercial formulation of the mixture (Fig. 5). We found reduced toxicity for treatments with atrazine (CAT) and glyphosate (GST) isolated, and greater toxicity for the mixture (GST), when compared with their respective active ingredients. Therefore, we found that both active ingredients and commercial formulations may be toxic to *Daphnia*. This toxicity may vary according to different commercial formulations due to the presence of different ingredients (Bridi et al., 2017). Besides that, the toxicity presented by the mixture of herbicides, both the active ingredients and the commercial formulations, may be the result of the interaction between the herbicides and adjuvants present in the formulations. This information helps to understand the effects that commercial products can cause to non-target organisms, and shows that the adjuvants that make up the formulations change the toxicity of the products.

Conclusion

This study presents primary results in the evaluation of the effects of herbicide mixtures at environmentally relevant concentrations and in the comparison between active ingredients and commercial products. The MIXCOM resulted in the highest mortality of organisms (17%) in the second generation (F1). We emphasize the importance of evaluating the chronic and over generations effects, since significant responses ($p < 0.05$) in reproductive parameters and biochemical biomarkers were observed mainly in the second generation (F1) of the evaluated organisms when compared to the first generation (F0).

We verified that, the herbicide atrazine may be modulating the toxicity presented by the mixture of active ingredients, since ATSA and MIXSA presented similar responses, and both differ from GPSA. We also observed important differences between the active ingredients and commercial products, which demonstrates the need to seek alternatives for the synthesis of pesticides. When the toxicity of formulations is increased by the presence of adjuvants, as in the case of mixture of herbicides (MIXCOM), a search for less toxic adjuvants or other ways to obtain the same desired effects on weeds should occur.

We emphasize the importance of evaluating multiple biomarkers and, above all, the evaluation of environmental concentrations, lower than those normally evaluated, so that it is possible to obtain an early response on the effects caused to non-target species. Considering that concentrations higher than those evaluated in this study have already been found in environmental samples from different regions of the world,

the potential risk to which non-target aquatic organisms are exposed can be seen. This fact encourages the search for measures to prevent damage to aquatic ecosystems.

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Compliance with ethical standards

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