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Toxicity of atrazine- and glyphosate-based formulations on *Caenorhabditis elegans*

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ABSTRACT

Atrazine and Glyphosate are herbicides massively used in agriculture for crop protection. Upon application, they are available to the biota in different ecosystems. The aim of this research was to evaluate the toxicity of Glyphosate and Atrazine based formulations (GBF and ABF, respectively). *Caenorhabditis elegans* was exposed to different concentrations of each single formulation, and to the mixture. Lethality, locomotion, growth, and fertility were measured as endpoints. Effects on gene expression were monitored utilizing green fluorescence protein transgenic strains. ABF caused lethality of 12%, 15%, and 18% for 6, 60, and 600 µM, respectively, displaying a dose dependence trend. GBF produced lethality of 20%, 50%, and 100% at 0.01, 10, and 100 µM, respectively. Locomotion inhibition ranged from 21% to 89% at the lowest and maximum tested concentrations for Atrazine; whereas for Glyphosate, exposure to 10 µM inhibited 87%. Brood size was decreased by 67% and 93% after treatment to 0.06 and 6 µM Atrazine, respectively; and by 23% and 93% after exposure to 0.01 and 10 µM Glyphosate, respectively. There were no significant differences in growth. Changes in gene expression occurred in all genes, highlighting the expression of *sod-1, sod-4*, and *gpx-4* that increased more than two-fold after exposure to 600 µM ABF and 10 µM GBF. The effects observed for the mixture of these formulations were additive for lethality, locomotion and fertility. In short, GBF, ABF, and their mixture induced several toxic responses related to oxidative stress on *C. elegans*.

1. Introduction

Atrazine and Glyphosate are herbicides widely used in agriculture for crop protection and illegal crop eradication; however, there is little information regarding the toxicity of the mixture of their commercial formulations in soil animals. The toxicity of Atrazine has been studied in several biological models such as microalgae (Baxter et al., 2016; Esperanza et al., 2016), worms (Chen et al., 2014), rodents (Mansour et al., 2014; Samardzija et al., 2016), fish (De Paiva et al., 2017; Liu et al., 2016; Wirbisky et al., 2016), crustaceans (Schmidt et al., 2017) and amphibians (Hoskins and Boone, 2017; Ji et al., 2016; Rutkoski et al., 2018; Saka et al., 2018), among others.

Atrazine has also been recognized as an endocrine disrupter (Kucka et al., 2012; Qin et al., 2015). The effect of Atrazine-based formulations (ABF) on red claw crayfish *Cherax quiadricarinatus* strongly suggests it could be causing endocrine disruption on the hormonal system responsible for sexual differentiation, increasing the female proportion (Mac Loughlin et al., 2016). Atrazine shortened the maturation process of *Xenopus laevis* oocytes and caused a higher death rate during early embryogenesis (Ji et al., 2016). In mammals, studies with immature gonadotropin-treated rats reported that Atrazine blocks ovulation via suppression of luteinizing hormone receptor and estradiol secretion (Samardzija et al., 2016). Mouse pups were more sensitive to Atrazine than their mothers, and exposure during gestation was more harmful than during lactation (Mansour et al., 2014).

On the other hand, several studies have been carried out regarding the toxicity of Glyphosate or its formulations, also declared as an endocrine disrupter. For instance, decreased fecundity has been reported in *Caenorhabditis elegans* hatched from eggs exposed to Glyphosate-based formulations when compared to untreated worms (McVey et al., 2016). In rats, it decreased the weight of the seminal vesicle and coagulating glands, as well as the total sperm count (Dai et al., 2016). Neonatal exposure to Glyphosate-based formulations (GBF) leads to endometrial hyperplasia and increases proliferation and disruption of proteins involved in uterine organogenetic differentiation (Guerrero Schimpf et al., 2017). Early exposure to GBF had neurobehavioral effects in the offspring of rats (Gallegos et al., 2016).

Glyphosate has also been identified as a neurotoxicant; in zebrafish

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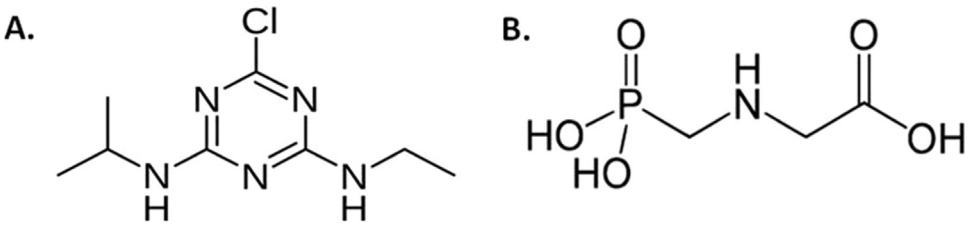

Fig. 1. Structural formula of pesticides. A. Atrazine B. Glyphosate.

embryos it induces loss of delineated brain ventricles and cephalic regions (Roy et al., 2016). Dopaminergic neurons on *C. elegans* hatched from eggs exposed to GBF show early modified neurodevelopment (McVey et al., 2016). Glyphosate has been further reported as nephrotoxic (Mohamed et al., 2016; Wunnapuk et al., 2014). Studies on amphibians found that Glyphosate and its formulations cause distinct skin alterations and altered the respiratory function (Rissoli et al., 2016).

It is quite important to mention that Glyphosate and Atrazine may co-exist as pollutants in several environmental matrices (Bonfleur et al., 2015; Muturi et al., 2017). The toxicity of the mixture has been reported in several studies, including cell genotoxicity (Roustan et al., 2014), cytotoxicity on snails (Mona et al., 2013), reproductive effects on fish (Le Mer et al., 2013), changes in microbial communities of aquatic habitats (Muturi et al., 2017), biochemical and genotoxic effects on *Corbicula fluminea* (Dos Santos and Martinez, 2014) and alterations of the physiology of *Crassostrea gigas* (Geret et al., 2013), among others.

Several biological models have been used to evaluate the toxic effect of different pesticides. In this study, we employed the nematode *C. elegans* because of its convenient features. For instance, its transparency allows for transgenic proteins fused to fluorescent markers to be visible in living animals on *in vivo* experiments (Giles and Rankin, 2009; Tejeda-Benitez and Olivero-Verbel, 2016); its generation time, four days, is short and occurs by self-fertilization, ensuring rapid reproduction in the laboratory (Zhuang et al., 2014); each adult hermaphrodite produces 200–300 progeny (Tejeda-Benitez et al., 2016; Yu et al., 2013). In addition, the genomic response to endocrine disrupters could be translated to development functions such as growth and reproduction (Höss and Weltje, 2007; Tejeda-Benítez et al., 2018).

C. elegans has been used in toxicological research, from the whole animal level to the level of individual cells (Zhuang et al., 2014). Bioassays to assess the effects of a toxicant on *C. elegans* can be carried out through different biological endpoints, for instance, lethality, growth, locomotion, and reproduction. It is also possible to use molecular markers to determine oxidative stress or changes in the expression of stress response genes through the use of reporter genes such as the gene of the Green Fluorescence Protein (GFP). The detoxification battery of *C. elegans* includes the group of heat shock proteins (HSP) that work as molecular chaperones, supporting refolding and repairing of denatured proteins and assisting protein synthesis (Anbalagan et al., 2013; Helmcke and Aschner, 2010); the glutathione peroxidases (GPx), the first line of defense against peroxides, superoxide anion and hydrogen peroxide (Doyen et al., 2012); and superoxide dismutase (SODs), protecting cells from oxidative damage (Back et al., 2010).

The aim of this research was to evaluate the toxicity of GBF, ABF, and their mixture, using *Caenorhabditis elegans* as a biological model, through the assessment of growth, locomotion, reproduction, and changes in the expression of *hsp-3, hsp-70, sod-1, sod-4, gpx-4* and *gpx-6,* using *gfp* reporter genes.

2. Materials and methods

2.1. Nematodes and bacteria

The *C. elegans* wild-type strain Bristol N2 was used in the bioassays of lethality, locomotion, growth, and fertility. *Escherichia coli* OP50 was used as food in K agar prepared with KCl, NaCl, agar, peptone, cholesterol, $CaCl_2$ and $MgSO_4$ (Williams and Dusenbery, 1990). Strains were kept at 20 °C in Petri dishes with K agar, and age-synchronized via bleach solution prepared with NaOH and HClO, which destroys the worms, but eggs are protected by their shell. Approximately 14 h after synchronization eggs have hatched, and larvae in L1 stage are placed in fresh dishes until used in the bioassays. Worms were fed *ad libitum*.

2.2. Solutions

A commercial formulation of Glyphosate (Roundup®) and Atrazine (Atrazine 50 SC) were used in this study. The structures of both herbicides are displayed in Fig. 1.

The concentration of Atrazine in the formulation was analyzed by gas chromatography (GC). The preparation of samples was made by dilution with dichloromethane and direct injection into an Agilent AT 6890 N gas chromatograph (Agilent Technologies, Palo alto, CA, US) with flame ionization detector. The column DB-5 (J&W Scientific, Folsom, CA, US) [5% Phenyl 95% dimethylpolysiloxane, 60 m, 0.25 mm, 0.25 μm] was used in the analysis. Injection (1 μL) was performed in split mode, and quantification was carried out by the external standard method using Atrazine G3C027 as reference material. The concentration of Glyphosate was determined by high performance liquid chromatography (HPLC). Preparation of samples was performed by dilution with HPLC grade water. The sample was analyzed in a Prominence-Shimadzu HPLC, with autosampler, diode array detector, and LC Solution Software. The Whatman Partisil 10 SAX HPLC column, 4.6 mm × 250 mm, 10 μm, was used in the analysis. The mobile phase was acetonitrile: buffer (1:24), flow rate of 1.5 mL/min under isocratic elution, 20 μL injection volume, 220 nm UV detection and 12.5 min total running time. Quantification was carried out by the external standard method employing Glyphosate DRE-C14050000 (Laboratory Labor Dr. Ehrenstorfer-Schafers) as reference material.

2.3. Exposure

Age-synchronized nematodes were exposed to 0.01, 0.1, 1, 10, 100 and 1000 μM of GBF; and 0.006, 0.06, 0.6, 6, 60 and 600 μM of Atrazine and mixtures of both, based on the concentration previously detected. K medium (52 mM NaCl and 32 mM KCl in ultra-filtered water) was utilized as a control.

The toxicity of mixtures of both pesticides was tested following two approaches: The first included the 1:1 combination of the herbicides at corresponding tested concentrations (Table S1A in Supplementary Data). The second involved the development of an additive model keeping constant the No Observed Adverse Effect Level (NOAEL) and the Lowest Observed Adverse Effect Level (LOAEL) values for a given herbicide, and varying the concentrations of the second one, starting from its NOAEL (Table S1B). All experiments detailed below were carried out three times, with four replicates per treatment.

2.4. Mortality

Nematodes in L4-larval stage were exposed 24 h to herbicide solutions. Approximately 10 ± 1 worms were used for treatment. Then, the