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Genotoxic Evidences of Glyphosate and Chlorpyriphos on *Eisenia fetida* Coelomocytes

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Abstract

The organophosphorus herbicide glyphosate (GLY) and the organophosphate insecticide chlorpyriphos (CPF) are key pesticides in modern management cultures worldwide. Sublethal toxicity of the commercial herbicide formulation Roundup® and the insecticide formulation Terfos® were evaluated on *Eisenia fetida* coelomocytes exposed under *in vivo* and *ex vivo* laboratory conditions. Induction of DNA single-strand breaks evaluated by the single cell gel electrophoresis assay and coelomocyte viability as well as alterations in coelomocyte trophic indexes were employed as endpoints for genotoxicity and cytotoxicity, respectively. Specimens were exposed at concentrations corresponding to recommended pesticide field application rate, and endpoints were evaluated after 7 and 14 days of treatment (*in vivo* exposure). In addition, coelomocytes were exposed to aqueous leachate of pesticide-contaminated soils during 1 h (*ex vivo* exposure). Earthworms exposed to Roundup® and Terfos® showed an increased frequency of DNA damage. Also, a decrease of coelomocyte viability and decrease of trophic indexes were observed in all treatments. The results demonstrate that either GLY- and CPF-based formulations exerted genotoxic as well as cytotoxic effects in coelomocytes of *E. fetida* exposed *in vivo* and *ex vivo*.

Keywords

Commercial formulations, DNA damage, Herbicides, Insecticides, Single cell gel electrophoresis assay, Sublethal effects

Introduction

The widely use of pesticides in modern agricultural enables increased crop yields. However, pesticides residues can contaminate agricultural and adjacent lands and become an ecotoxicological threat to non-target organisms, included humans. Nowadays, results almost impossible for many countries to decrease the use of pesticides without altering crop yields as agriculture gradually transformed into a high-tech system for satisfying the world's growing demands for food, feed, fiber and fuel [1,2]. It is known that pesticides can also be hazardous whether not appropriately employed since many of them may represent potential hazards to the environment due to the contamination of soil, water, air and food [3]. In addition, anthropogenic activities are continuously introducing large amounts of these compounds into the environment regardless of their persistence, bioaccumulation and toxicity and thus increasing their jeopardizing effects (www.epa.gov/pesticides).

Glyphosate is a non-selective herbicide widely used worldwide for post-emergent control of annual and perennial plants including weeds on a great variety of crops. It is a polar, highly water soluble substance that makes complexes easily. It binds tightly to the soil particles, reaching a usual half-life of 45-60 days in soil and persistence from 222 to 835 days [4]. By the other hand, Chlorpyrifos is a broad spectrum organophosphate in-

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secticide that is widely used to control insect pest in agricultural fields. Likewise, Chlorpyrifos is a moderately persistent environmental contaminant, with a half-life ranging from several days to months [5]. High volumes of agrochemicals applied to great variety of crops, together with agricultural expansion and its persistence in the ecosystem generate great concerns due to the impact for the environment and large risk implicated for wildlife.

The ecotoxicological effects of pesticides can be assessed by monitoring the use of laboratory toxicity test. Earthworms, among soil organisms, are considered highly appropriate terrestrial model organisms for ecotoxicity test. They are powerful regulators of soil processes participating in the maintenance of its structure and regulation of organic matter dynamic [6-8]. Not only due to their natural contact with the soil but also for the ingestion of soil, earthworms can be easily influenced by pollutants. Thus, earthworms are sensitive indicators of anthropogenic stress factors and then are used as model organisms in the environmental risk assessments of chemicals [6,9]. In particular, the species *Eisenia fetida* has been widely used for soil toxicity assessments because standardized tests are available [10-12] Survival, growth, and reproduction are the usual endpoints for such tests, which provide eco-toxicologically relevant information. However, ecotoxicological approach by using biomarkers at low levels of biological organization is useful for damage detection before the community is affected [13]. The need to detect and assess the effects of contamination at sublethal levels has led to the development of molecular and cellular indicators of exposure to and effects of contaminants, referred as biomarkers [14,15].

The use cellular and molecular biomarkers can be complementary approach providing information about organism-stress response to toxicants before higher levels are affected [14]. In this sense, genotoxic evaluation is very important due to genotoxic influence can lead changes in one or more generations [16,17]. It can result in reduced fertility of soil populations, and thus causing biodiversity depletion [18].

It is well known that the single cell elelectrophoresis (SCGE) assay, also called comet assay, has been shown to be a sensitive and recommended method for the evaluation of DNA damage in individual cells induced by different xenobiotics [16,18-21]. In particular, the SCGE when applied on earthworms has resulted as a high sensitivity bioassay for evaluating the genotoxic damage induced for wide group of pollutants. Among them, metals [22-25] polycyclic aromatic hydrocarbons [23] and other organic compounds [26], ionic [27] and, overall, several pesticides [28-32] can be included.

Earthworm coelomocytes are the group of circulating cells present in the coelomic cavity [23,33,34,35]. Based on cytomorphometric, ultrastructural and cytochemical properties, coelomocytes can be classified into three major cell groups, namely eleocytes, hyaline amoebocytes and granular amoebocytes [36]. It has been reported that the proportion of the different cellular types of coelomocytes may be related to the health and the immune earthworm responses [23,28,35]. Thus, changes in cell proportions in the coelomic fluid of organisms exposed to different pollutants can be evaluated by means of this parameter as a reliable cytotoxic biomarker, as suggested elsewhere [15,37,38]. In agreement, it has been demonstrated that eleocyte proportions of another oligochaeta *Dendrobaena veneta* decreased after exposure to cadmium and copper [36].

The purpose of this study was to evaluate the toxicity of two commercial formulations of GLY and CPFon *E. fetida* exposed *in vivo* under laboratory conditions to treated soils and to its aqueous leachates as *ex vivo* exposure of extruded coelomocytes. DNA single-strand breaks, viability and coelomocyte counts of exposed organisms were employed as endpoints for genotoxicity and cytotoxicity, respectively. Pesticides were selected because they are commonly used as agricultural chemicals with intensive and overlapping applications in agricultural fields, not only in Argentinean soybean crops, but also around the world in many applications for the treatment of transgenic and non-transgenic agronomic crops [39].

Materials and Methods

Chemicals

Agrochemicals used included the 48% isopropylamine salt of glyphosate-based [N-(phosphonomethyl] glycine; CAS1071-83-6) commercial grade trade formulation Roundup® (Monsanto S.A.I.C., Buenos Aires, Argentina) and the 48% chlorpyriphos-based (*O*,*O*-diethyl *O*-3,5,6-trichloropyridin-2-ylphosphorothioate; CAS 2921-88-2) commercial grade trade formulation Terfos® (Chemotecnica S.A., Buenos Aires, Argentina). Hydrogen peroxide (H_2O_2, CAS 7722-84-1) was obtained from Sigma-Aldrich Co. (St. Louis, MO) whereas copper (II) chloride 2-hydrate ($CuCl_2.2H_2O$, CAS 10125-13-0) was purchased from Biopack Co. (Buenos Aires, Argentina). All other chemicals and solvents were of analytical grade. Nominal concentrations of GLY and CPF were controlled by HPLC-UV and GC-MS methods respectively according APHA [40].

Test organism

Specimens of *E. fetida* adults, average wet weight 300 mg, were purchased from local source (Luján, Buenos Aires, Argentina). Earthworms were maintained in

moistened control soil (pH 6.6 ± 0.26, 25% sand, 48% slime, 27% clay, moisture 40-60% of water holding capacity, WHC 60 ± 5 mL/100 g), at room temperature (RT) under natural photoperiod and fed with 10% of alfalfa forage. The worms were allowed to acclimate to laboratory conditions for several weeks before testing. Specimens were maintained in plastic containers in a control soil corresponding to a natural soil of the experimental surrounding field of National University of Lujan, previously characterized elsewhere [24].

Pesticide exposure

The genotoxicity, cytotoxicity and trophic indexes in coelomocytes of *E. fetida* exposed *in vivo* and *ex vivo* were determinate. The control soil was artificially treated with both pesticides chosen. The applications rate corresponded to the manufacturers recommended application dose. Applied dose were CPF 1 and 2 L/ha (480 and 960 g CPF/ha) and GLY 2.5, 4 and 6 L/ha (1200, 1920 and 2880 g GLY/ha). The artificial treatment of soils was performed simulating the conditions of application followed by producers in the field. A soil surface of minimum depth and 0.04 m^2 was used. Thus, soils sieved to 1000 µm were distributed in a homogeneous layer of 1 cm of depth in a glass vessel. Soil was treated with the corresponding amount of pesticide as application recommended rate of manufacturers. Commercial formulations were dispersed in Milli-Q water and applied into the soil by using a commercial sprayer. Once treated with pesticides, soils were mechanically homogenized and used immediately to avoid volatilization losses. To evaluate endpoints of *ex vivo* manner, coelomocytes were extruded of untreated organisms and incubated 1 h (RT) in the soil leachates. Soil leachates were prepared from pesticides-treated soils to evaluate the mobility of self inter environment compartments. Soil leachates were prepared according to US EPA [41] recommendations. For SCGE assay, solutions of PBS and H_2O_2 (100 µM in PBS) were used as negative and positive controls, respectively [19,23]. Solutions of PBS and $CuCl_2$ were used as negative and positive controls, in cytotoxicity evaluation, respectively [15,38]. To evaluate endpoints of *in vivo* manner, coelomocytes were extruded of organism exposed during 7 and 14 days in pesticides-treated soils. For each test, 750 g fresh weight of the test medium (control soil and pesticides-treated soils) was placed into each plastic container and then adult earthworms with clitellum observable were added. The containers were covered with perforated plastic film to prevent the test medium from drying and kept under the test conditions for 7 and 14 days. Two replicates for each treatment were performed.

Coelomocyte extrusion

At the end of the exposure period either for *in vivo* and *ex vivo* treatments, a non-invasive extrusion method was used for collecting earthworm coelomocytes according to Di Marzio, et al. [23]. Earthworms were rinsed in tap water at RT and placed on a damp paper towel overnight to void gut contents during the extrusion procedure. Afterwards, organisms were immersed in an extrusion medium consisted in 5% v/v ethanol in saline solution (0.85% NaCl, 2.5 mg/mL EDTA, pH 7.5). A pooled castings of five organisms was obtained and then placed into centrifuge tubes containing 2 mL of extrusion medium/individual and incubated for 1 min at RT. Coelomic fluid containing the extruded cells was diluted in calcium and magnesium free phosphate-buffered saline (PBS), washed twice, and centrifuged at 2000 rpm (4 °C, 10 min), and then coelomocyte pellets resuspended in 2 mL of PBS.

Coelomocyte counts and *in vivo* and *ex vivo* cytotoxicity

Extruded cells were counted using a counting chamber improved Neubauer hemocytometer. The extruded cells were characterized according to their morphology as eleocytes, amoebocytes or granulocytes according to Adamowicz and Wojtaszek and Adamowicz [34,36]. After cell counting, the following trophic indexes were calculated as follows:

Absolute trophic index earthworm (ATIE): *En/Cn,* where *En* is total eleocytes number average per individual/mL of celomic fluid and *Cn* is total coelomocytes number average per individual/mL of celomic fluid. Relative trophic index earthworm (RTIE): ATIE/*wwf* where *wwf* is wet weight without feces.

The cell viability was expressed as the percentage of viable cells measured with 0.4% of Trypan blue. One hundred cells were counted on each slide and three replicate slides were analyzed per specimen. Data were expressed as average of total viable coelomocytes per individual/mL of coelomic fluid. Copper, as $CuCl_2$, was used as positive control according Irizar, et al. [38] and Svendsen and Week [15].

Single cell electrophoresis (SCGE) assay

The SCGE assay protocol proposed by Di Marzio, et al. [23] was used. Assays were performed under indirect incandescent light at 4 °C. Gels were composed of three layers of agarose. The suspensions of earthworm's cells were diluted (1:2) with 1% low-melting-point agarose (LMPA) giving a final agarose solution of 0.66% and then 80 µL of the cell suspension were transferred to a slide having a thin layer of solidified 0.5% normal-melting agarose. The slides were covered with a coverslip and left on ice for 10 min to allow the second layer of agarose to solidify. The coverslip was gently removed, and 80 µL of 0.5% LMPA were spread over the second layer. A coverslip was placed

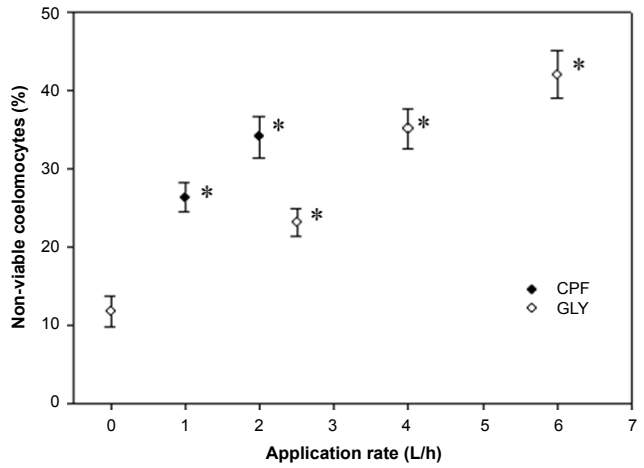

Figure 1: Cytotoxic effects of soil leachates in coelomocytes *E. fetida* exposed *ex vivo* for 1 h. Error bars represent standard deviation of the mean. *Statistically significant differences with control group ($p < 0.05$).

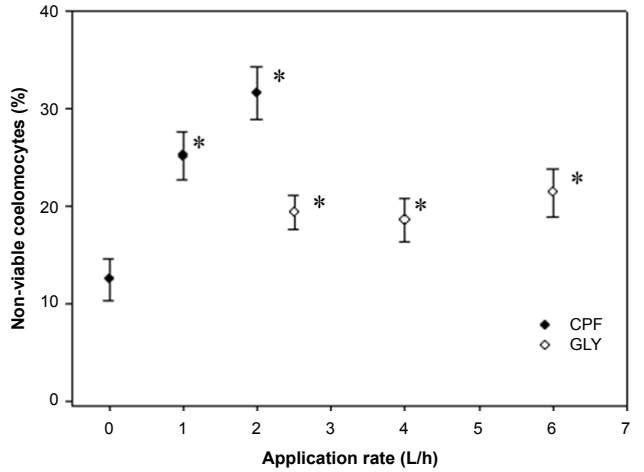

Figure 2: Cytotoxic effects of chlorpyriphos (CPF) and glyphosate (GLY) contaminated-soils on coelomocytes of *E. fetida* exposed *in vivo* for 14 days. Error bars represent standard deviation of the mean. *Statistically significant differences with control group ($p < 0.05$).

on top of third layer and the agarose solidified. This last coverslip was removed and each slide was immersed in freshly prepared cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris (pH 10), 1% N-laurylsarcosinate, 1% Triton X-100 and 10% dimethyl sulfoxide (DMSO) during 10 min. Slides were then placed in an electrophoresis tank and covered with electrophoresis buffer (300 mM NaOH, 1 mM Na_2EDTA, pH 13.5) for 25 min at RT to allow unwinding. Electrophoresis (300 mA, 30 min, 1 V/cm) was then performed in the some buffer. The slides were washed in the neutralization buffer (0.4 M Tris, pH 7.5, 10 min). Afterwards, slides were stained with 30 µL of 20 µ/mL ethidium bromide solution. The images of nucleoids were analyzed with Nikon Eclipse 600, microscope provided with epifluorescence (541-560 nm excitation filter and 590 nm emission filter) linked to an image analysis system (Image Pro Plus, V4.0, Media Cybernetics, Maryland, USA). The images obtained were analyzed with the CASP software [42]. Tail DNA% was used as final genotoxicity endpoint. The % Tail DNA comet assay parameter was chosen as it is not measured in arbitrary units, being more meaningful and advisable for regulatory purposes and for inter-laboratory comparisons [43].

Statistical analysis

Cytotoxicity and genotoxicity data were analyzed by non-parametric Kruskal-Wallis, and median and Dunn tests using Statistica software version 8.0 [44,45]. The Student t-test was used for pair comparisons in *in vivo* experiments between 7 and 14 days. The chosen level of significance was 0.05 unless indicated otherwise.

Results

Cell viability

All assayed concentrations of $CuCl_2$, ranged between 1 to 100 µg/mL, were statistically different with respect to negative controls (PBS). Coelomocyte cytotoxicity fell within the expected values showing confidence limits at 95% between 13.68 - 80.07 µg/mL, and LC50-1 hour mean of 33.10 µg/mL. The values for coelomocyte viability for *ex vivo* and *in vivo* exposure are indicated in Figure 1 and Figure 2, respectively. Coelomocytes exposed to aqueous leachate of pesticides-treated soils showed a significant increase ($p < 0.05$) in the percentage of non-viable cells (Figure 1). Both pesticides produced the increase of coelomocytes cytotoxicity in a positive concentration-response relationship. Earthworms exposed *in vivo* of pesticide-treated soils during 14 days showed a significant increase ($p < 0.05$) in the percentage of non-viable coelomocytes (Figure 2). CPF-based formulation showed an increase of non-viable cells concomitant with increase of application rates. On the contrary, GLY-based formulation showed a similar and significant increase ($p < 0.05$) of non-viable cells at all application rates evaluated. For the same application rate coelomocytes exposed of *ex vivo* manner showed higher toxicity than coelomocytes exposed of *in vivo* manner.

Trophic indexes (ATIE and RTIE)

Values for calculated trophic indexes are showed in Figure 3A and Figure 3B. The number of eleocytes in control earthworms during *in vivo* and *ex vivo* exposure remained without significant differences among all of them during the experiment. After *in vivo* exposure to pesticides-treated soils, the total number of eleocytes decrease significantly ($p < 0.05$) after 7 and 14 days of treatment. ATIE and RTIE indexes, that have into account the total number of eleocytes, decreased for both pesticides and exposure times ($p < 0.05$).

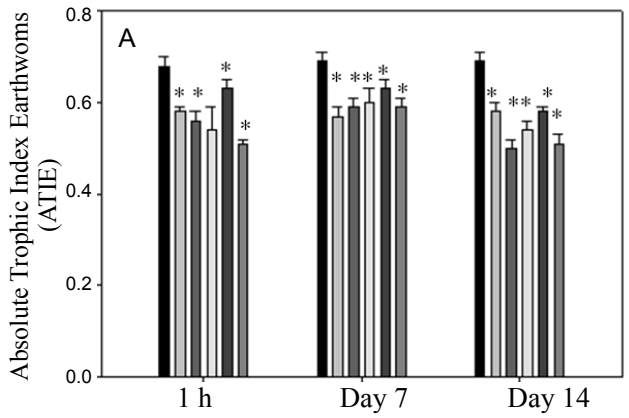

Figure 3A: Absolute trophic index earthworm (ATIE). after 1 h (*ex vivo*), 7 and 14 days (*in vivo*) exposure to CPF and GLY-contaminated soils and unpolluted soils (control). Values are represented as mean values of six cell counts and standard deviations. *Statistically significant differences with respect to the control group ($p < 0.05$).

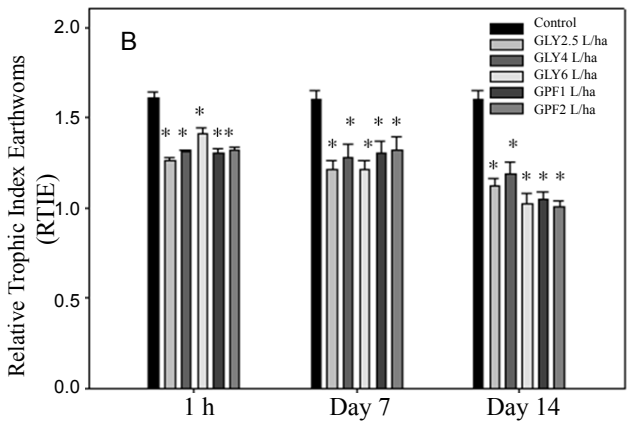

Figure 3B: Relative Trophic Index Earthworm (RTIE) after 1 h (*ex vivo*), 7 and 14 days (*in vivo*) exposure to CPF and GLY-contaminated soils and unpolluted soils (control). Values are represented as mean values of six cell counts and standard deviations. *Statistically significant differences with respect to the control group ($p < 0.05$).

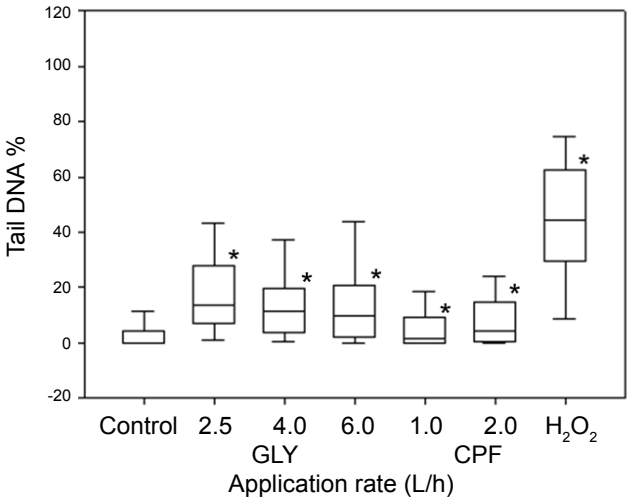

Figure 4: Genotoxicity of soils leachates and hydrogen peroxide on DNA damage in coelomocytes exposed *ex vivo* during 1 hour. (Line: median, box limits: 25% to 75%, bars: min-max values). *Statistically significant differences with negative control ($p < 0.001$).

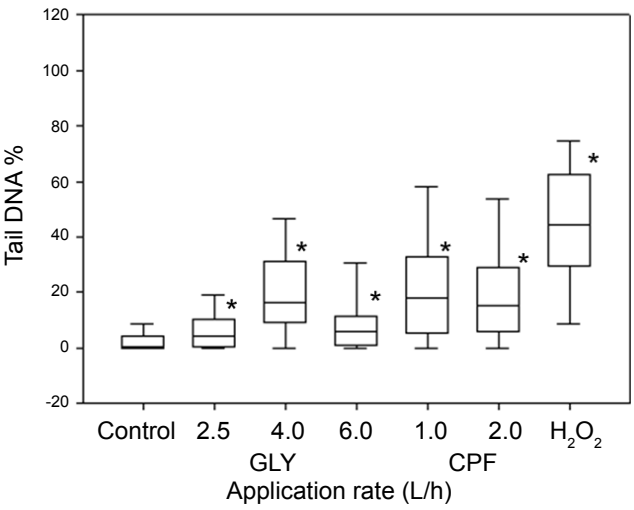

Figure 5: Genotoxic effects of chlorpyriphos (CPF) and glyphosate (GLY) contaminated-soils and hydrogen peroxide in coelomocytes exposed *in vivo* during 7 days. (Line: median, box limits: 25% to 75%, bars: min-max values). *Statistically significant differences with negative control ($p < 0.001$).

SCGE assay

DNA damage, measured as % of tail DNA, in coelomocytes exposed to 100 µM H_2O_2 was statistically different with respect to PBS solutions (negative controls). All pesticides-treated soils leachates exerted genotoxic effects in coelomocytes after 1 h of exposure (Figure 4). In the case of DNA damage after 7 and 14 days of exposure, the entire evaluated application rates were statistically different with respect to the control soil for both pesticides (Figure 5 and Figure 6). With the respect to the exposure time, different results were observed between both pesticides. In the case of GLY-based formulation a significant increase ($p < 0.05$) was observed in DNA damage for the application rates of 2.5 and 6 L/ha between 7 and 14 days of exposure. By the other hand the application rate of 4 L/ha did not show difference between 7 and 14 days of exposure. In the case of CPF-based formulation, the application rate of 1 L/ha showed a significant decrease ($p < 0.05$) in the Tail DNA% between both exposure time. On the contrary, the application rate of 2 L/ha showed a significant increment in the Tail DNA% between 7 and 14 days of exposure.

Discussion

Pesticides usually enter the soil as sprays applied to crop plants from above ground, and in a lesser extend when applied directly on soils. In the environment, terrestrial organisms are most commonly exposed to appli-

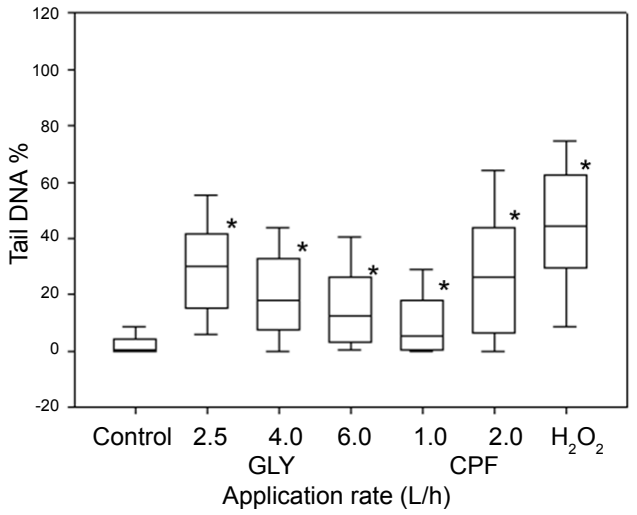

Figure 6: Genotoxic effects of chlorpyriphos (CPF) and glyphosate (GLY) contaminated-soils and hydrogen peroxide in coelomocytes exposed *in vivo* during 14 days. (Line: median, box limits: 25% to 75%, bars: min-max values). *Statistically significant differences with negative control ($p < 0.001$).

cation rate of pesticides recommended by manufacturers. The current study examined the toxic effects of two pesticides widely used at application rate recommended and over a non-target organism such as *E. fetida.* The results considering the viability response, DNA damage and trophic indexes showed that both pesticides exerted deleterious response in coelomocytes of *E. fetida* exposed *in vivo* and *ex vivo*.

Beside, results further demonstrated that it was possible to evaluate the DNA damage by using the SCGE assay, cytotoxicity and cellular proportions as non-invasive biomarkers starting from the coelomic cells or coelomocytes from exposed earthworms.

Ecological hazard assessment of chemicals has traditionally relied on the use of Standard Toxicity Tests (OECD, ISO), which in soils are based on short and long-term experiments using several terrestrial organisms [46]. It was recommended that an acute toxicity test with *E. fetida* was being employed as a valid test standard for soil evaluation [46]. Because of the low sensitivity of the acute endpoint mortality, alternative methods with more sensitive endpoints have to be checked as the chronic earthworm reproduction test [10]. However, the application of the latter is complicated because it requires 56 days of exposure against 14 days of acute exposure. Thus, it is necessary the development of sensitive biomarkers at short exposure times. In this aspect, the analysis of toxicity response taking into account cell types and trophic indexes was used as effective biomarker. Coelomocytes are free-circulating immune cells in the coelomic fluid, and have a central function in the earthworm's immunity against environmental pathogens and toxicants [28,35,36,38]. Coelomocytes are the first line of active defense. Due to this functional characteristic could led us to compare earthworm coelomocytes with human leukocytes as they share similar immunobiology [28]. Thus, the use of coelomocytes of *E. fetida* is an accurate technique for the toxicity assessment of pesticides in earthworms. The absolute and relative trophic index earthworm (ATIE and RTIE) were sensitive for to evaluate short and longer exposure times. These indexes take in account the density of eleocyte cells, which are mainly related with nutritive and immune functions [36]. The change in the relative proportions of coelomocytes seems indicate a physiological stress in earthworms provoked for the pesticides exposure [35].

Studies on the biological effects of currently used pesticides have increased in recent years. Evaluation of genotoxic effects of treated-soil pesticides and its leachate have acquired particular importance, especially in the case of a constant exposure as result of repeated applications. Thus, due to genotoxic influence can lead to changes in one or more generations [16,17], is very important to determinate in a sensitive manner any plausible interactions between pesticides and DNA. Pesticides tend to be very reactive compounds that can form covalent bonds with various nucleophilic centers of cellular biomolecules, including DNA [47]. Besides, it was also showed that several pesticides induce reactive oxygen species (ROS) formation which may be involved in the production of DNA-single strand breaks [30,32,48].

Two methods can be used to evaluate DNA damage: the micronucleus test and Comet assay, the latter being much more sensitive than the former [23,29]. In the present study, the SCGE or comet assay was used as a rapid and sensitive method for determine genotoxicity by measuring DNA damage such as single- and double-stranded DNA breaks as well as alkali-labile sites [16,20,21]. In the case of GLY, the results observed in the present study showed an increase of DNA migration in coelomocytes of *E. fetida* exposed both *in vivo* and *ex vivo*. These results are in accordance with previous reports of genotoxic effects provoked by GLY-based formulations by using several endpoints. Bolognesi, et al. [48] observed that GLY increase sister chromatid exchanges in human peripherical blood and adducts formation in kidney and liver cells in bone marrow cells. Grisolia [49] observed an increase in MN frequency in *Tilapia rendalli* fish. Vera-Candioti, et al. [50] observed the induction of primary DNA damage in peripheral blood cells of the ten spotted live-bearer fish *Cnesterodon decemmaculatus.* Also, different authors have obtained positive genotoxic results for GLY-based formulations through comet assay using a variety of cells and organisms. Clements, et al. [51] observed DNA damage in circulation blood cells of *Rana catesbeiana* tadpoles. Cavas and Konen [52] observed an

increase of DNA damage on peripheral erythrocytes in *Carassius auratus* fish. Mañas, et al. [53] observed an increase in DNA migration in Hep-2 cells exposed *in vitro* manner. Schaumburg, et al. [54] observed an increase in DNA damage in *Salvator merianae* neonates after embryonic exposed to GLY-based formulations. In the case of earthworm, Casabé, et al. [29] observed no difference in DNA migration when earthworms were exposed to GLY-treated soils during 7 days. However, it has been reported that for GLY formulations, toxicity depends greatly on the surfactant employed in the formulation [55].

In our results, GLY-based formulations showed an increase in DNA damage in coelomocytes exposed *in vivo* manner between 7 and 14 days at application rates of 2.5 and 6 L/ha. Accumulation of DNA damage may occur either through and increase in the number of DNA-damaging events or due to a decrease in DNA repair capacity and/or antioxidant system [56]. Also this results shows that GLY-based formulations is persistent in the soil for 14 days in our assays conditions, according to its usual half-life of 45-60 days in soil and persistence from 222 to 835 days data [4].

In the case of CPF, we observed an increase of DNA migration due to the induction of DNA damage in coelomocytes of *E. fetida* exposed both *in vivo* and *ex vivo*. Few reports have determined the relationship between CPF and DNA damage. Exposure to CPF-based formulations, acutely or chronically, caused a dose-dependent increase in DNA damage in the liver and brains of rats [57] and also the production of reactive oxygen species that caused considerable cytotoxicity in PC12 cells [58]. By the other hand, Li, et al. [59] observed an increase in DNA migration on HELA and HEK293 cells exposed to CPF. In terrestrial organisms, Casabé, et al. [29] and Piola, et al. [31] observed an increase in DNA damage in coelomocytes of earthworm *E. Andrei* exposed to CPF.

With the respect to time of exposure, coelomocytes exposed to CPF-based formulations showed two responses. In organisms exposed at lowest application rate, DNA migration showed an decrease ($p < 0.05$) between 7 and 14 day of exposure. This result could be explained for the stimulation of DNA repair machinery. On the contrary, at highest application rate, DNA migration showed an increase ($p < 0.05$).

Both pesticides increased the extent of DNA migration in *E. fetida* coelomocytes exposed *in vivo* and *ex vivo* manner. This increase it is connected with the induction of cytotoxicity, since the Tripan Blue method showed that the tested concentration were not toxic to these cells.

When leachate effects are analyzed, we observed an increase of DNA damage in *E. fetida* coelomocytes exposed *ex vivo* to both pesticides. Previous reports have demonstrated that runoff is a one of the major source of non-point pesticide contamination of steams [60]. Thus, pesticides are capable to migrate into the liquid phase and representing, then, a high ecological risk for aquatic biota [39,61,62]. The behavior of pesticides on the environment will depend not only on their intrinsic properties but also on environmental conditions and agricultural practices. Thus, pesticides can exert deleterious effects in aquatic organisms as previously reported. Biomarkers are an important element in the ecological risk assessment of pesticide pollution. Also, pesticides genotoxicity is a matter of interest, and its environmental detection is an important topic. The different ways in which pesticides are being applied are continuously increasing, as well as the resultant risks. The use of pesticides is recommended, in order to obtain the beneficial effects in crops, but without to produce effects in the biota and in human health.

Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

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