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Comparative toxicity of two glyphosate-based formulations to *Eisenia andrei* under laboratory conditions

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HIGHLIGHTS

- Compared toxicity of two glyphosate formulates on *Eisenia andrei* was determined.
- Based on LC_{50}, Roundup FG (72% ea) was 4.5-fold more toxic than MON 8750 (85.4% ea).
- Roundup FG showed sublethal effects on weight change, DNA and lysosomal damage.
- MON 8750 affected NRRT and growth at high doses, close to its LC_{50}.
- Differences found would be due to some components of the formulations.

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ABSTRACT

Glyphosate-based products are the leading post-emergent agricultural herbicides in the world, particularly in association with glyphosate tolerant crops. However, studies on the effects of glyphosate-based formulations on terrestrial receptors are scarce. This study was conducted to evaluate the comparative toxicity of two glyphosate-based products: Roundup FG (monoammonium salt, 72% acid equivalent, glyphosate-A) and Mon 8750 (monoammonium salt, 85.4% acid equivalent, glyphosate-B), towards the earthworm *Eisenia andrei*. Median lethal concentration (LC_{50}) showed that glyphosate-A was 4.5-fold more toxic than glyphosate-B. Sublethal concentrations caused a concentration-dependent weight loss, consistent with the reported effect of glyphosate as uncoupler of oxidative phosphorylation. Glyphosate-A showed deleterious effects on DNA and lysosomal damage at concentrations close to the applied environmental concentrations (14.4 μg ae cm^{-2}). With glyphosate-B toxic effects were observed at higher doses, close to its LC_{50}, suggesting that the higher toxicity of formulate A could be attributed to the effects of some of the so-called "inert ingredients", either due to a direct intrinsic toxicity, or to an enhancement in the bioavailability and/or bioaccumulation of the active ingredient. Our results highlight the importance of ecotoxicological assessment not only of the active ingredients, but also of the different formulations usually employed in agricultural practices.

1. Introduction

Glyphosate (GLY) (N-[phosphonomethyl]glycine) based products are the leading post-emergent herbicides for the control of annual and perennial weeds in the world. It is a broad-spectrum, non-selective, systemic herbicide, which is directly applied to foliage. In plants, GLY disrupts the shikimic acid pathway through inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, leading to a reduction in aromatic amino acids vital for protein synthesis and plant growth. It has also been shown to modulate plant cytochrome P_{450} (Lamb et al., 1998).

The mechanism by which this herbicide exerts toxic effects in humans or experimental mammals is not clear. Two specific biochemical mechanisms have been identified or proposed: uncoupling of oxidative phosphorilation and inhibition of hepatic mixed function oxidases (USDA/FS, 2011).

In Argentina, due to the use of GLY-based herbicides for weed control in GLY-resistant crops and in chemical fallow in direct sowing, increased use of this herbicide was encouraged. According to CASAFE (2011), about 200 million kg of GLY-based herbicides were applied in 2010. GLY is believed to be less toxic to the ecosystem than other herbicides; transgenic plants tolerant to this compound

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have been developed following this argument. However, non-target organisms may be exposed to herbicide residues.

The Ecological Risk Assessment (ERA) of pesticides requires data regarding their toxicity to aquatic and terrestrial non-target species. Studies usually focus on the deleterious effects of the active ingredient (a.i.), generally not considering the potential toxicity of commercial formulations. Toxicity testing of formulates, however, is likely to provide more realistic information. In commercial-GLY formulations, herbicide is generally present in the salt form. Products typically also include surfactants to facilitate the movement of the polar compound GLY through the waxy cuticle of plant foliage and other minor ingredients (WHO, 1994). While these ingredients are typically classified as "inert", some may be more toxic than the herbicides with which they are used.

Laboratory bioassays under controlled conditions using relevant bioindicator organisms constitute a first approach for the evaluation of pesticides risk in soil compartment (Scott-Fordsmand and Weeks, 2000; Sánchez-Hernández, 2006; Environment Agency, 2009). In a search for non-mammalian species for evaluating the impact due to anthropogenic compounds released to the terrestrial environment, earthworm is the species of choice (Scott-Fordsmand and Weeks, 2000). Standard methods with *Eisenia fetida/andrei* have been used in ecotoxicological research for decades (OECD, 1984). Increasing efforts have been focused on the study of biomarkers as early warning indicators of ecosystem deleterious effects (Scott-Fordsmand and Weeks, 2000; Sánchez-Hernández, 2006; Domingues et al., 2010). In particular, the assessment of responses at the sub-organismal level may be useful to predict adverse effects on soil organisms and populations. Non-specific suborganism biomarkers, such as lysosomal membrane stability measured as neutral red retention time (NRRT) and DNA damage estimated by the comet assay, are indicative of effects of more than one detrimental factor, and they may be relevant to assess the risk of multiple chemicals (Reinecke and Reinecke, 2004; Xiao et al., 2006).

The presence of GLY may cause deleterious changes to earthworm populations and soil functioning (Römbke et al., 2003; Casabé et al., 2007). However, studies on the effects of GLY-based formulations on terrestrial receptors are scarce (Casabé et al., 2007; Yasmin and D'Souza, 2007).

In this work, the filter paper contact test is proposed as a method to assess the effects of different GLY-based formulations on *E. andrei*. Our aims were to evaluate and compare lethal toxicity and two unspecific sublethal responses, lysosomal destabilization and DNA damage, in *E. andrei* exposed to GLY formulations.

2. Materials and methods

2.1. Chemicals

Roundup FG (monoammonium salt, soluble granules, 72% p/p acid equivalent (ae); GLY-A) and Mon 8750 (monoammonium salt, soluble granules, 85.4% p/p ae; GLY-B) were provided by Monsanto Argentina SAIC. All other reagents were from Sigma (St. Louis, MO, USA).

2.2. Bioassays

2.2.1. Lethal toxicity

Mature adults of *E. andrei* with well-developed clitella were selected from the colony maintained in our laboratory. Filter paper contact exposure was performed according to OECD (1984) with slight modifications. Filter papers (60 cm^2) were impregnated with a range of GLY solutions in distilled water (1 mL). Earthworms were exposed to the following concentrations, selected after a preliminary range-finding: GLY-A: 30, 45, 67.5, 101.25 μg ae cm^{-2}; GLY-B: 240, 360, 540 μg ae cm^{-2}.

Five earthworms per concentration were placed individually into test tubes with their inner surface lined with the treated filter papers. Tubes were covered with nylon gauze, sealed with a tight fitting rubber and incubated for 72 h at 22 ± 2 °C in continuous darkness. Controls were exposed to water impregnated papers. After 72 h, the number of live and dead worms was recorded. Two independent experiments were performed. Median lethal concentration (LC_{50}) and the highest concentration at which no mortality was observed were calculated. As a sublethal endpoint, growth of surviving earthworms was determined. The weight was measured at the beginning (Wi) and at the end of the experiment (Wf), and its change was calculated as percentage relative to initial weight: $100^{*}(Wf - Wi)/Wi$.

2.2.2. Sublethal toxicity

Adult earthworms were exposed to sublethal concentrations of the two formulates, as determined in the LC_{50} studies: GLY-A (7.5, 15.0, 30.0 μg ae cm^{-2}) and GLY-B (60, 120, 240 μg ae cm^{-2}). Sets of five worms were exposed to each sublethal concentration; two independent experiments were performed. Controls were run simultaneously.

2.2.2.1. Coelomocytes collection. After 72 h exposure, organisms were removed, rinsed and blotted dry. Coelomic fluid from individual earthworms, containing coelomocytes, was extruded through dorsal pores after stimulation with an electric current (Fuchs et al., 2011) and transferred to an eppendorf tube. Viability of coelomocytes was measured using the trypan blue exclusion method.

2.2.2.2. Comet assay. Comet assay was performed according to Casabé et al. (2007), on coelomic fluid obtained as described above. Immediately after extrusion, 10 μL coelomocyte suspension were mixed with 75 μL 0.75% low melting point (LMP) agarose at 37 °C, and spread over a microscope slide precoated with 100 μL 1% normal melting point agarose. After 5 min of solidification at 4 °C, a layer of LMP agarose was placed on top and left to harden for 5 min at 4 °C. Slides were immersed in alkaline lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% N-lauryl sarcosinate, 10% DMSO and 1% Triton X-100, pH 10) and stored overnight at 4 °C. After lysis, slides were rinsed with neutralization buffer (0.4 M Tris–HCl, pH 7.5) and immersed in freshly prepared alkaline electrophoresis solution (300 mM NaOH and 1 mM Na_2EDTA) at 4 °C for 12 min, to allow DNA unwinding. Electrophoresis was conducted for 20 min at 25 V (1 V cm^{-1}) and a starting current of 250 mA. After electrophoresis, microgels were drained and washed with three changes, 5 min each, of neutralization buffer and washed two times with 1 mL distilled water during 5 min. Moisture excess was removed in an oven at 37 °C and slides were stored in a dust-free box until stained with ethidium bromide (20 μg mL^{-1}), just prior to microscopic observation. The whole procedure was performed under dim light, to minimize artifactual DNA damage. The slides were independently coded and scored by a single observer. 100 randomly selected cells per organism (50 cells from each of two replicate slides) were analyzed using an Axioplan (Zeiss, West Germany) fluorescent microscope. Cell nuclei were rated visually and classified into five categories according to the tail intensity (size and shape), and given a value of 0, 1, 2, 3 or 4 from undamaged, 0, to maximally damaged, 4. Damage index (DI), a weighted value of damage according to scoring of cells, was calculated as $DI = \Sigma n_i \times i$, where n_i is number of cells with damage class i. The percentage of damaged cells (D%) was also calculated.

2.2.2.3. NRRT assay. Coelomocytes of the same groups of earthworms utilized for the comet assay were used, but only three

earthworms for each group. The NRRT on the coelomocytes of each worm was measured according to Weeks and Svendsen (1996) with minor modifications. Coelomic fluid (20 μL) was placed on a microscope slide; the cells allowed adhering for 60 s prior to the application of 20 μL neutral red (NR) working solution (80 μg mL^{-1}) and a coverslip. The NR solution was renewed each hour to avoid crystallization of the non-polar NR in the PBS solution. Each slide was scanned for 2 min at 5 min intervals under a light microscope (400×). In healthy cells, NR is retained in the lysosomes and the cytosol is colorless. For damaged cells, efflux of dye into the cytosol results in its redness. Observation was stopped when the number of cells with fully stained cytoplasm reached >50% of the total number of cells counted. This time was recorded as the NRRT. As in comet assay, evaluations of NRRT were performed by a single operator and they were blind.

2.2.3. Statistical analysis

Statistical analysis was performed with GraphPad InStat 3 (GraphPad Software, San Diego, USA). Data were tested for normality (Kolmogorov–Smirnov's test) and for homogeneity of variances (Bartlett's test). Means were compared by one-way ANOVA (parametric) or non-parametric Kruskal–Wallis tests. Tukey–Kramer or the non-parametric Dunn's test was applied for post-hoc comparison of means. Linear correlations were performed using the Pearson linear correlation test. The probability level of significance was considered $p < 0.05$.

3. Results

3.1. Survival and growth

Lethal toxicity tests showed a concentration-dependent response for both formulations, the percentage of survival decreasing with increasing pesticide concentration. No mortality was observed in control organisms. LC_{50} and respective 95% confidence intervals were: 66.0 (54.6–82.1) μg ae cm^{-2} for GLY-A and 293.9 (254.0–339.8) μg ae cm^{-2} for GLY-B.

According to classification of Roberts and Dorough (1984), based on the resulting LC_{50} values for *Eisenia* sp. exposed to impregnated papers, GLY-A was classified as very toxic (LC_{50}: 10–100 μg ae cm^{-2}), whereas GLY-B was moderately toxic (LC_{50}: 100–1000 μg ae cm^{-2}). The exposure of earthworms to both formulates resulted in an increase in the percentage of weight loss relative to the initial weight (Fig. 1). Only the worms exposed to the highest concentrations showing no earthworms mortality (67.5 and 240.0 μg ae cm^{-2} for GLY-A and GLY-B respectively) exhibit significant differences compared to controls ($p < 0.01$). In control worms the decrease was less than 20% (13.4 ± 6.3%), showing that the earthworm culture and exposure conditions were suitable.

3.2. Comet assay

DNA damaging effects on coelomocytes of earthworms exposed to GLY-A are shown in Fig. 2. Two parameters, D% (Fig. 2a) and DI (Fig. 2b) were used for comparison. In all experiments, viability of cells was above 85%. Background level of DNA damage (control) was low, with 32.5 ± 6.7% of damaged cells and a DI of 75.5 ± 16.7. Comparing to controls, a significant increase in D% (15 μg ae cm^{-2}: 78.0 ± 5.3%; 30 μg ae cm^{-2}: 85.0 ± 7.1%, $p < 0.001$) and DI (264.7 ± 38.1 and 287.0 ± 28.3, for 15 and 30 μg ae cm^{-2} respectively, $p < 0.001$) was observed.

Fig. 3 shows the results of comet assay obtained with GLY-B. For 120 and 240 μg ae cm^{-2}, DNA damage, measured as D% (39.0 ± 7.0%; 43.3 ± 3.2%; Fig. 3a) and DI (105.5 ± 21.4; 87.0 ± 10.4; Fig. 3b), did not differ significantly from control.

The distribution of DNA damage in the different damage classes are shown in Fig. 4. The control group presented the highest proportion of undamaged cells (67.5%, level 0). GLY-A treatments (Fig. 4a) resulted in a significant decrease in the percentage of cells in class 0 for both concentrations ($p < 0.001$), with a significant increase in the percentage of comets at the high class ($p < 0.01$), which is reflected in an increase in D% and DI. As it can be seen in Fig. 4b, no significant differences in cell distribution were observed at any damage level with GLY-B.

3.3. NRRT

As shown in Fig. 5a, NRRT of coelomocytes of worms exposed to GLY-A showed a concentration-dependent decrease ($r = 0.8374$), significantly different ($p < 0.001$) for all the assayed concentrations

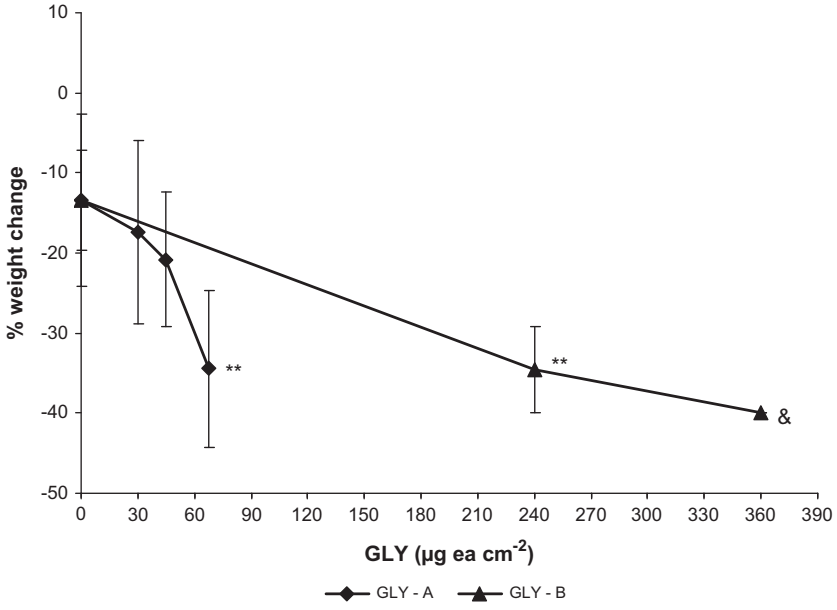

Fig. 1. Percentage of weight change in *Eisenia andrei* exposed to sublethal concentrations of two formulations of GLY. Data are expressed as mean ± SD. $^{**}p < 0.01$; $^{***}p < 0.001$: significant differences when compared to controls. &: only one earthworm survived.

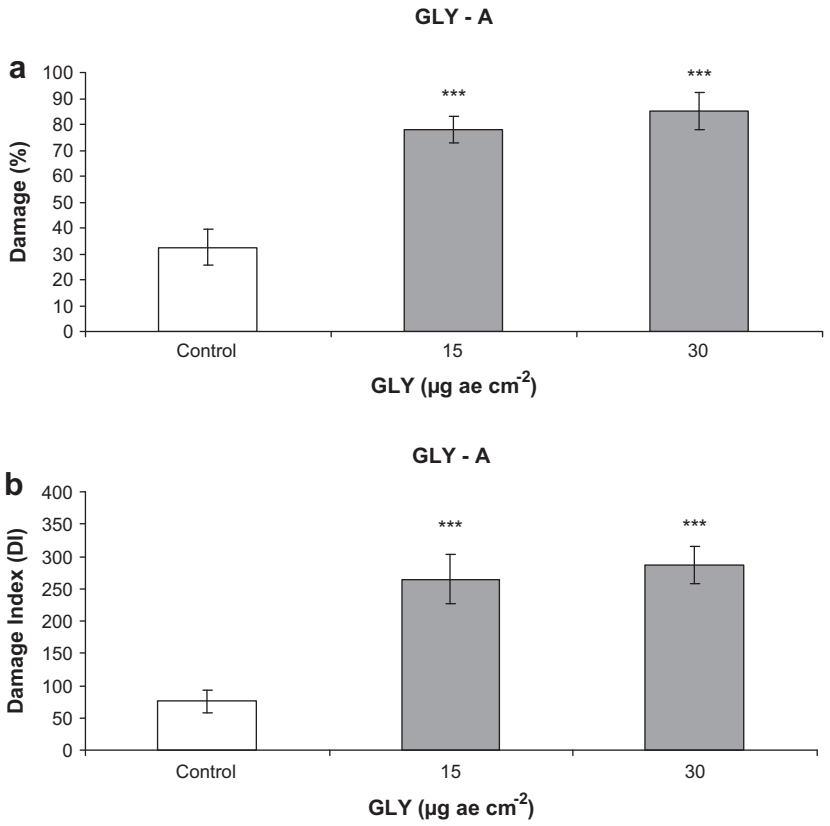

Fig. 2. Damage % (a) and damage index (b) in coelomocytes of *Eisenia andrei* exposed to GLY-A. Data are expressed as mean ± SD. $^{***}p < 0.001$: significant differences when compared to controls.

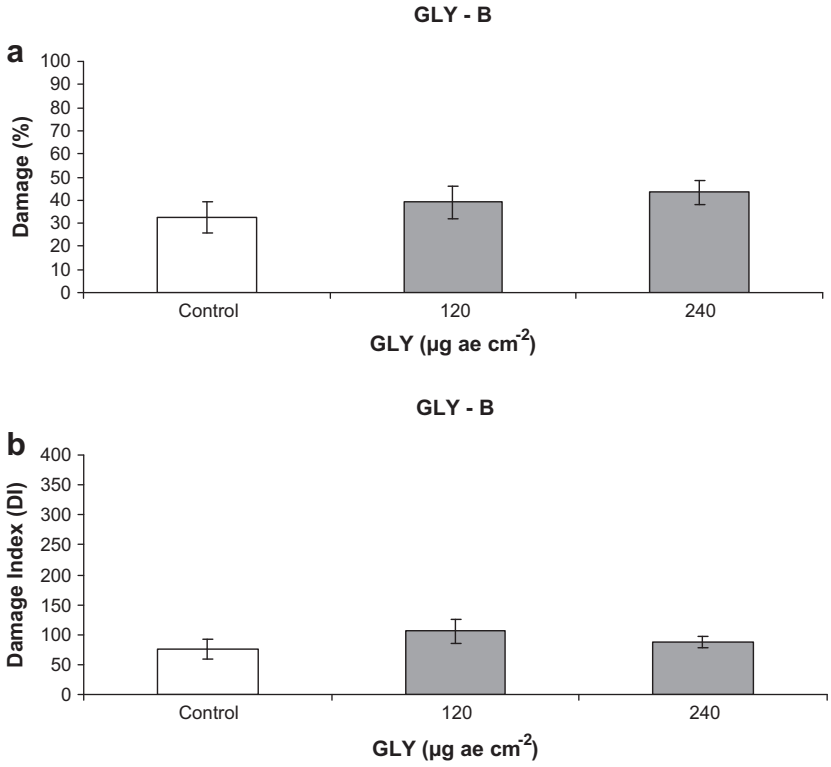

Fig. 3. Damage % (a) and damage index (b) in coelomocytes of *Eisenia andrei* exposed to GLY-B. Data are expressed as mean ± SD.

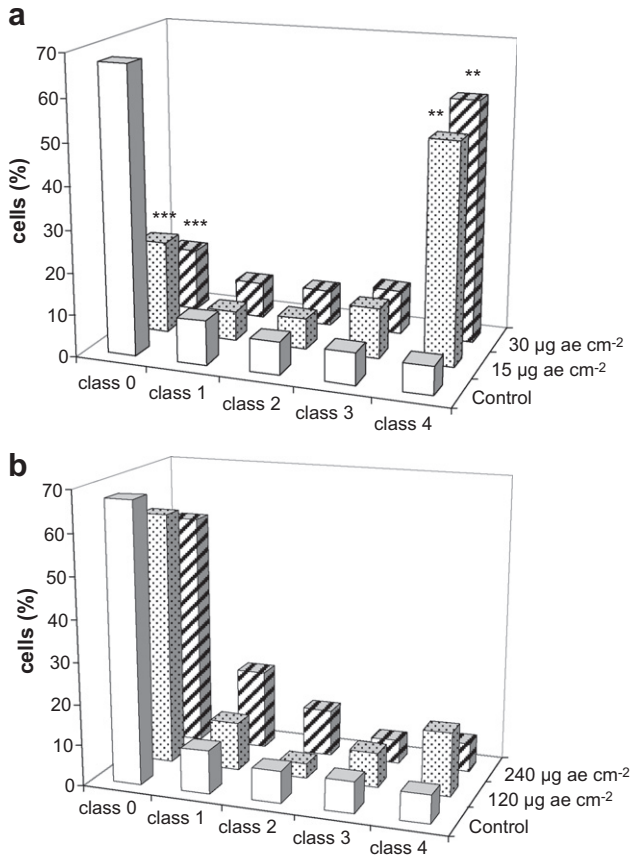

Fig. 4. Percentage of cells in each damage class in coelomocytes of *Eisenia andrei* exposed to GLY-A (a) and GLY-B (b). Data are expressed as mean ± SD. $^{**}p < 0.01$; $^{***}p < 0.001$: significant differences when compared to controls.

(28.3 ± 5.8, 15.0 ± 7.1; 10.7 ± 7.3 min for 7.5, 15.0 and 30.0 µg ae cm^{-2} respectively), comparing to controls (64.0 ± 2.2 min). The NRRT of the lowest concentration differed significantly ($p < 0.05$) from the others; the two highest concentrations did not differed significantly from each other ($p > 0.05$).

Only the coelomocytes of earthworms exposed to the highest concentration of GLY-B (240 µg ae cm^{-2}) showed a significant decrease (NRRT = 35.0 ± 3.8 min) compared to controls, as well as to 60 and 120 µg ae cm^{-2} (Fig. 5b; $p < 0.001$).

4. Discussion

GLY and its formulations could be classified as important environmental hazards due to their frequent use nowadays. Since the patent for GLY expired in most of the world, new GLY-based herbicides have entered the market.

Toxicity testing has been the main instrument for legal requirements and environmental management decisions, which has led to the development of multiple standardized protocols (Sánchez-Hernández, 2006). When the toxicity of herbicides is discussed, the focus is mostly on the active compound. However, herbicides are formulated to increase their efficacy against target plants. Several studies have found that commercial GLY-based formulations are more toxic to the organisms than GLY itself because of the adjuvants present in the formulations (Bonnet et al., 2007; Lajmanovich et al., 2011). However, few studies were reported on the toxicity of GLY formulates to earthworms (Yasmin and D'Souza, 2007; Pereira et al., 2009; Santos et al., 2011).

In this study we compared the toxicity of two different GLY formulates in *E. andrei*, using the filter paper contact test. We hypothesized that their toxic (lethal and sublethal) effects might differ because of the differences in the components of the formulations.

Median lethal concentration (LC_{50}), calculated from concentration-mortality data, showed a significant variation in lethal toxicity between GLY-A and GLY-B. Both formulates contain the same a.i. (monoammonium salt); however GLY-A (72% ea) is 4.5-fold more toxic than GLY-B (85.4% ea). Preliminary studies of our laboratory (unpublished results) with a formulated product of GLY containing a different a.i. (isopropylamine salt, 36% ae) showed similar lethal toxicity (LC_{50}: 276.4 µg ae cm^{-2}) between this formulate and GLY-B. Wang et al. (2012), exposing *E. fetida* on filter paper, reported a moderately toxic lethality for technical GLY (LC_{50}: 566.1 (437.4–905.4) µg cm^{-2}). Our results reinforce the inadequacy on the use of toxicity values based in assessment that consider solely the a.i. of a pesticide.

Sublethal endpoints seem to be more sensitive indicators of effects than lethality. Harmful chemicals trigger molecular, biochemical, physiological or morphological alterations in living organisms that can potentially be used as biomarkers. Its use as surrogate measures of biological impact within laboratory and field studies has been prevalent for years (Scott-Fordsmand and Weeks, 2000; Booth and O'Halloran, 2001).

Growth is one of the physiological indicators which best reflects the well-being of an organism in its medium. It is the net result of many essential life processes, such as consumption, excretion and energy expenditure (Booth and O'Halloran, 2001). Contaminants can reduce the energy available for growth, since detoxification processes are energy demanding. Santos et al. (2011), exposing *E. andrei* to recommended field doses of GLY, found a decrease in earthworm's weight, although no statistical differences were observed. Correia and Moreira (2010) found that GLY (99.7%) applied to soil had a substantial adverse effect on the growth rate of the earthworm *E. fetida*. According to Yasmin and D'Souza (2007) exposure of the earthworm *E. fetida* to the GLY formulation 'Glycel 41% SL' at commercial application rates, caused a 50% reduction in weight but no significant reduction in the number of organisms. In the current research, a concentration-dependent weight loss in *E. andrei* exposed to sublethal concentrations of both assayed formulates was observed, indicating a potential growth inhibitory effect. Our observations could be related to the reported effect of GLY as uncoupler of oxidative phosphorylation, which could result in energy loss and lead to death.

Bearing in mind the relevance of DNA in the maintenance of homeostasis and in the transfer of information to offspring, assessment of DNA damage is highly relevant in ERA. Comet assay is able to detect the interaction between xenobiotics and DNA as a consequence of exposure to complex environmental samples. This assay applied to earthworms (mostly *E. fetida*) has been used in previous studies to investigate the genotoxicity of artificial or natural soils spiked with various chemicals (Reinecke and Reinecke, 2004; Xiao et al., 2006; Klobučar et al., 2011). Muangphra et al. (2012) detected no differences in tail DNA%, tail length, and tail moment of coelomocytes from earthworms (*Pheretima peguana*) exposed to concentrations <LC_{50} of GLY-based herbicides on filter paper.

In our hands, comet assay showed significant DNA damage in earthworms exposed to GLY-A. However, no dose-related response was evident. A high percentage of highly damage cell nuclei was observed, resulting in high values of DI. This bimodal distribution of DNA damage, which is characteristic of apoptosis, could be due to early apoptotic cells. Coelomocytes of earthworms exposed to GLY-B showed no significant increase in DNA damage, measured as % of damage cells or DI. There was a high percentage of undamaged cells; the majority of comets fell in class 0.

Lysosomal compartment is sensitive to different stressors; one of the characteristic changes is the increased fragility of the lysosomal membrane. The neutral red retention assay, used to

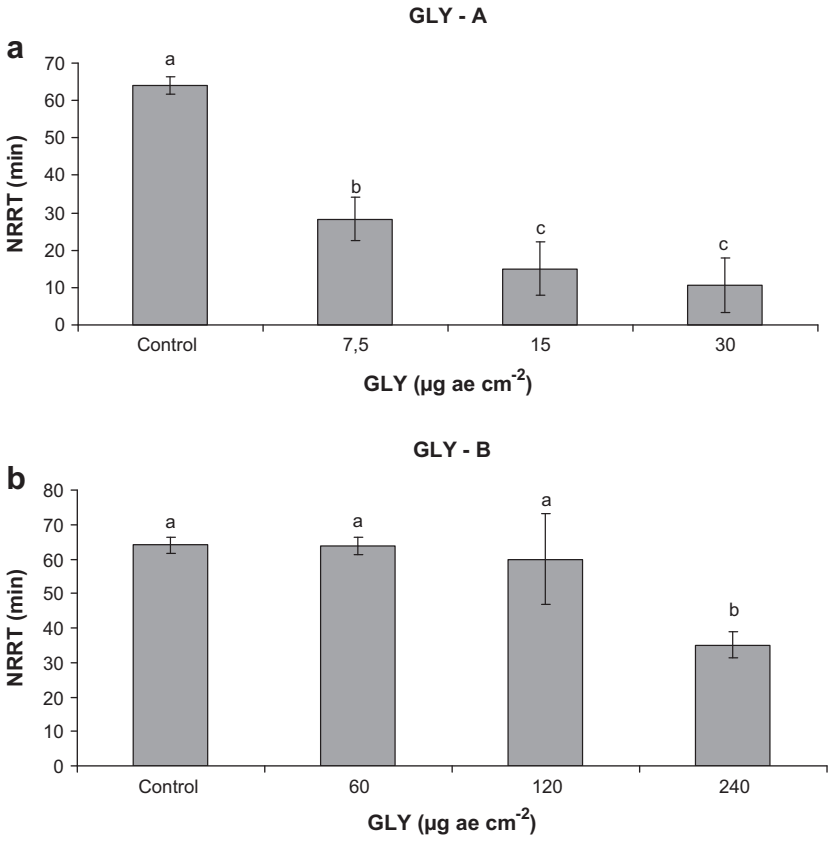

Fig. 5. NRRT in coelomocytes of *Eisenia andrei* exposed to GLY-A (a) and GLY-B (b). Data are expressed as mean ± SD. Different lowercase letters denote significant differences between groups ($p < 0.05$).

determine lysosomal damage, is based on the ability of viable cells to incorporate NR into the lysosomal compartment. If lysosomal membrane is damaged, NR leaks out into the cytosol and the time taken for redness the cytosol is related to the degree of membrane damage. The mechanisms underlying this alteration in membrane stability are not well understood, but it is suggested that these effects can be mediated by direct binding to the lysosomal membrane, by activating calcium- and tyrosine kinase-dependent cell signaling pathways and indirectly by the enhanced formation of oxyradicals. The magnitude of lysosomal rupture may induce reparable sublethal damage, apoptosis, or necrosis (Hwang et al., 2004). NRRT has already been established as responsive in earthworms exposed to metals, such as copper, cadmium, zinc, lead, organic contaminants like polycyclic aromatic hydrocarbons, energetic compounds, and pesticides (Xiao et al., 2006; Casabé et al., 2007; Klobučar et al., 2011). It was also previously established as a useful biomarker of TNT exposure in *E. andrei* (Robidoux et al., 2002; Fuchs et al., 2011).

A significant lysosomal membrane destabilization, slightly dose-related, was evidenced in earthworms exposed to GLY-A, with NRRT severely decreased at 7.5 µg ae cm^{-2}. Exposure to GLY-B caused a significant NRRT diminution only at the higher assayed concentration. It should be noted that at this concentration, close to the LC_{50}, earthworms showed a high weight loss (greater than 30%), indicating a great impairment in its general health status, anticipating a lethal consequence in a short time. In fact, earthworms exposed to this concentration showed clear behavioral alterations, such as agitation and rolling. A good correlation has been postulated between lysosomal damage and changes in DNA integrity, although both assays evaluate two different types of responses. According to Monteiro et al. (2011) the membrane destabilization promotes the rupture of lysosomes, releasing a large amount of endonucleases that might act on the DNA molecule causing a disruption.

In our research with GLY-A, the sensitivities of the NRRT and of the comet assays have shown to be very similar, whereas both biomarkers were more sensitive than body weight. With GLY-B, instead, NRRT and growth were adversely affected at the same concentration (240 µg ae cm^{-2}), whereas no DNA damage was detected at any assayed concentration.

GLY-A showed deleterious effects on growth, DNA and lysosomal damage at concentrations close to the applied environmental concentrations (14.4 µg ae cm^{-2}). With GLY-B, however, toxic effects were observed with higher doses, close to its LC_{50}. Having in mind that GLY-B is a concentrated product for use in manufacturing and formulating, containing a higher content of a.i. (93.96%) than GLY-A (79.2%), we suggest that the higher toxicity of GLY-A could be attributed to the effects of some of the so-called "inert ingredients", either due to a direct intrinsic toxicity, or to an enhancement in the bioavailability and/or bioaccumulation of the a.i.

5. Conclusions

The present study provides relevant information on the toxicity of two GLY commercial formulates on *E. andrei*. According to our results, differing effects of the different formulates were seen in our experiments.

The obtained results suggests some components of the formulation and not GLY, could be responsible for the differences found, highlighting the importance of ecotoxicological assessment not

only of the a.i., but also of the different formulations usually employed in agricultural practices.

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