

Full Length Research Paper

Effects of contaminated natural soil by Glyphosan® SL on biochemical responses of the earthworm *Eisenia* sp.Leida Marcano^{1,2*}, Juanny Hernández¹, Edgar Zapata-Vívenes² and Analía León²¹Postgrado Biología Aplicada, Departamento de Biología, Universidad de Oriente, Cumaná-Venezuela.²Departamento de Biología, Universidad de Oriente, Escuela de Ciencias, Cumaná-Venezuela.

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Biochemical biomarkers are widely used for the monitoring environmental quality since they can act as early warning signals, for potential ecosystem degradation caused by contaminants. In order to investigate the acute and chronic effects of a commercial formulation of glyphosate on antioxidant defenses (glutathione peroxidase, GPx; glutathione-S-transferase, GST; and reduced glutathione, GSH) and oxidative damage (malondialdehyde levels, MDA), *Eisenia* sp was exposed in plastic containers to natural soils contaminated with Glyphosan® SL (100 g kg⁻¹ of soil) during 7 and 21 days. Following exposure for 21 days, another group of worms were placed on herbicide-free soils to recovery for 30 days. Treatment with the herbicide significantly affected to all the biochemical markers evaluated. Antioxidant defenses (GSH, GPx and GST) and MDA, in general, increased at both exposure periods. These results showed that Glyphosan® SL; the active ingredient (glyphosate) and/or its chemical additives exerts its toxic effects in *Eisenia* sp by altering cellular antioxidant defenses, inducing a condition of oxidative stress. During the recuperation phase of the earthworms previously exposed for 21 days to the herbicide, GPx decreased to values similar to those of the control group and MDA although decreased, control values were not reached. These results indicate that in 30 days *Eisenia* sp exhibits a partial recovery of the oxidative stress induced by the herbicide exposure, probably the activation of mechanisms clearance of the Glyphosan® SL metabolites justify the high levels of GSH and GST at this stage. In conclusion, herbicide exposure induced changes in the biochemical responses of *Eisenia* sp highlight the importance of these responses as useful tools in the evaluation of impacts by pesticides in terrestrial organisms.

Key words: Biomarker, *Eisenia*, glyphosate, antioxidant enzymes, glutathione, lipid peroxidation.

INTRODUCTION

In ecotoxicological risks assessment research there is a great interest in the monitoring of terrestrial pollution induced by the widespread use of agrochemicals worldwide, which endangers the ecosystems and the

natural resources of the planet (Tiwari et al., 2016). Glyphosate (N-(phosphonomethyl) glycine) is a herbicide widely used for its effectiveness in destroying plants and microorganisms by inhibiting shikimate pathway, and thus

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it was considered as completely non-toxic to animals and human (Aparicio et al., 2013; Kwiatkowska et al., 2016). This herbicide is considered environmentally friendly because it degrades rapidly, having a short half-life in the soil ranging from 5 to 23 days and its strong adsorption to soil particles that decrease its availability for terrestrial biota (Domínguez et al., 2016). However, adverse effects of glyphosate, particularly as commercial formulations, have been evidenced in non-target organisms (Piola et al., 2013; Balbuena et al., 2015; Mattos et al., 2016).

Earthworms play an essential role in providing soil fertility and may represent an important soil contamination biomonitor, principally due to their ability to ingest soil particles, absorb substances throughout the intestinal epithelium into the coelomic cavity, where chemical can come in direct contact with coelomic fluid (Mincarelli et al., 2016). When the well-being of earthworms is impaired, for example due to soil contamination with pesticides or heavy metals, the physiology of the organisms that inhabit the soil can be compromised (Calisi et al., 2012). Some studies have shown that glyphosate has no direct effects on earthworms (Casabé et al., 2007; García-Pérez et al., 2014) or has stimulating effects on earthworm reproduction (Santos and Ferreira, 2012). Conversely, other studies showed harmful effects of glyphosate on reproductive parameters in earthworms (Santadino et al., 2014; Gaupp-Berghausen et al., 2015).

The metabolism of xenobiotics, including pesticides, can often induce oxidative stress (Contardo-Jara et al., 2009; Sinhörin et al., 2014) as resulting from an overproduction of reactive oxygen species (ROS) such as the superoxide radical ($\cdot\text{O}_2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\cdot\text{OH}$), which affect mainly lipids, proteins and nucleic acids (Feng et al., 2015). Molecular mechanisms of protection against ROS include enzymes as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione-S-transferase (GST) and small molecules (that is, reduced glutathione, GSH), which play a crucial role in scavenging ROS generated during aerobic metabolism (Tiwari et al., 2016). GSH plays a key role in the cellular antioxidant defense mechanisms acting directly as oxyradical scavenger and as substrate for GPX and GST (Zhou et al., 2012). Since GSH-status is essential to cellular detoxification of many toxic xenobiotics, monitoring this endogenous thiol and GSH-dependent enzymes during pesticide exposure is very important.

The measurement of biomarkers in living organisms have recently become an integral component of environmental monitoring programme in several countries as a supplement to the commonly used contaminant monitoring (Buch et al., 2013; Salvio et al., 2016). In addition, biochemical biomarkers can act as early warning signals to prevent environmental damage (Tiwari et al., 2016). Some studies in *Eisenia* sp have revealed that malathion and chlorpyrifos produce immunotoxicity

(Cortésia et al., 2015) and inhibit growth and reproduction (Granadillo and Marcano, 2013). However, in this earthworm the effect of glyphosate-based herbicides is unknown. In this study were evaluated the levels of GSH, MDA and GSH-dependent enzyme activities in *Eisenia* sp, during acute and chronic exposure to Glyphosan® SL and after a 30-day recovery period.

MATERIALS AND METHODS

Earthworm maintenance

Earthworms were obtained from the Vermiculture Department of National Institute of Socialist Educative Cooperation (INCES, the acronym in Spanish) from Cumaná, Sucre state, Venezuela. Before experimental bioassays were performed, these worms were acclimatized for 14 days under laboratory conditions at $25 \pm 1^\circ\text{C}$ in plastic containers with a natural organic substrate prepared with 60% of horse manure and 40% of pruning wastes, humidity (80%), and 12:12 h light dark cycle (Polo et al., 2012).

Exposure to herbicide

The exposure test to Glyphosan® SL was conducted in pre-cleaned 500-mL plastic containers. Each container was filled with 300 g of natural organic substrate (horse manure: 60 % and vegetable waste: 40 %) and was homogeneously mixed with 100 g of herbicide kg^{-1} soil; sublethal dose that guaranteed a 100% of survival during the treatment with the herbicide. The control group was mixed with the same volume of water. Before placing the earthworms, the pH (7.6 ± 0.2) and moisture of the substrate (35%) were checked. Healthy earthworms with an average biomass of 200-350 mg and sexually mature (well-developed clitellum) were used; two earthworms in each container were placed and five replicates were set for each herbicide-treatment and control group. Weekly replacement of the exposure system was performed to maintain herbicide concentrations, substrate moisture and food supply to organisms. Earthworms ($n=8$) were collected randomly from each replicate on the 7th and 21st day. Knowing that organism responses to pollutants vary with exposure time and generally, acute assays are less sensitive than chronic assays, we select a short exposure time (7 days) and a longer exposure time (21 days) to contrast the pattern of response of *Eisenia* sp in both periods. Following exposure for 21 days, only this group of earthworms ($n=8$) were transferred to plastic containers with herbicide-free natural substrates and were kept in recovery for 30 days. The containers were checked daily during herbicide exposure and recovery and no mortality was observed during the assay.

Enzyme activities

The carcass tissue of each earthworm was individually homogenized in Tris-buffer (20 mM, pH 7.6, 1 mM DTT, 1 mM EDTA, 500 mM sucrose, 150 mM KCl and 10 mM PMSF) in a 1:9 w v^{-1} ratio using Polytron homogenizer at 12,000 rpm and at 4°C . After centrifugation at 4°C at 10,000 g for 30 min, supernatant was collected for biochemical measurements.

Glutathione peroxidase (GPX, EC 1.11.1.9) activity was measured following the rate of NADPH oxidation at 340 nm in 1 mL of reaction mixture: 100 mM phosphate buffer 7.5, 2 mM NaN_3 , 1.8 mU glutathione reductase (Sigma Chemical Co), 0.1 mM NADPH, 0.03% H_2O_2 , 0.67 mM GSH and 100 μL of earthworm supernatant

(Nuseti et al., 2001).

Glutathione-S-transferase (GST, EC 2.5.1.18) activity was determined following the procedure of Habig et al. (1974), using 1-chloro-2,4-dinitro-benzene (CDNB) as substrate for spectrophotometrical measurement at 340 nm and at 37°C. The reaction mixture (1.0 mL) consisted of 100 mM phosphate buffer, pH 7.5, 20 mM CDNB, 3.4 mM GSH and 100 μ L of earthworm supernatant. The optical density variation was followed for 1 min.

Glutathione

For the glutathione assay, the GSH-400 method from OxisResearch® based on a chemical reaction that occurs in two steps was used. The first step leads to the formation of substitution products (thioethers) between a patented reagent, R1 (4-chloro-1-methyl-7-trifluoromethyl-quinolinium methylsulphate), and all mercaptans (RSH) present in the sample. The second step is a β -elimination reaction which takes place under alkaline conditions in the presence of 30% NaOH (R2) that transforms the thioether obtained with GSH in a chromophoric thione having a maximal absorbance at 400 nm. Briefly, carcass tissues were homogenized in 20% phosphoric acid in a 1:4 w/v ratio using Polytron homogenizer at 12,000 rpm and at 4°C. Homogenate was centrifugated at 3,000 g for 10 min at 4°C and supernatant was conserved for GSH assay. The GSH concentration was estimated using a standard curve prepared with GSH (Sigma Co) whose concentration range was of 20-100 μ mol L⁻¹ in the reaction medium (Blume et al., 1975).

Lipid peroxidation

Lipid peroxidation was determined using the LPO-586 method from OxisResearch® based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole (R₁), with malondialdehyde (MDA), a product of polyunsaturated fatty acid peroxidation, at 45°C to yield a stable chromophore with maximal absorbance at 585 nm. For the tissue homogenate preparation, One gram of carcass tissue was homogenized in 9 mL of cold phosphate buffer (20 mM, pH 7.4). Prior to homogenization, 10 μ L 0.5 M butylated hydroxytoluene (BTH) was added. The homogenate was centrifugated at 3,000 g at 4°C for 10 min and clear supernatant was used for the MDA assay. Aliquots of 100 μ L of supernatant were mixed with R₁ and HCl, after incubation for 60 min at 45°C, the absorbance at 586 nm was measured. A standard curve was prepared with an acetal 1, 1, 3-tetrametoxyp propane (TMOP) because the aldehyde itself is not stable. The TMPO is hydrolyzed during the acid incubation step at 45°C, which generates MDA (Esterbauer et al., 1991).

Statistical analysis

The experimental values of GSH concentrations and antioxidant enzyme activities in the earthworms were tabulated as the mean \pm standard deviation (SD) of eight determinations. Test of significance was performed by two-way analysis of variance (ANOVA) followed by Duncan's multiple range test considering significant at $P < 0.05$ (Sokal and Rohlf, 2012).

RESULTS

The Table 1 shows the results of the biochemical markers evaluated in *Eisenia* sp exposed Glyphosan® SL and

after the recovery by 30 days. After both periods of exposure, the biochemical parameters of control and herbicide-exposed earthworms showed statistically significant differences. A significant increase was observed in GPx (Fs=13.12; $P < 0.01$) and GST activity (Fs=12.62; $P < 0.05$), as well as in the GSH content (Fs=6.34; $P < 0.05$) and MDA levels (Fs=518.14; $P < 0.001$). These effects were similar during acute (7 days) and chronic (21 days) herbicide exposure.

During the recovery phase of the earthworms previously exposed for 21 days to the herbicide, GPx declined (Fs=11.22; $P < 0.05$) to values similar to those of the control group and, MDA although decreased significantly (Fs=39.12; $P < 0.001$), control values were not reached. In comparison with the exposure period, GST and GSH remained unchanged in the recovery phase.

DISCUSSION

Measurements of biomarkers responses in earthworms induced by chemical contaminants are widely used in pollution monitoring in soil environments and, alterations in antioxidants such as GSH and related-enzymes has been used as an index of unbalanced ROS generation and oxidative stress in physiological systems (Feng et al., 2015). The present investigation clearly demonstrates that the dose of Glyphosan® SL used significantly affected GST, GPx, and GSH in *Eisenia* sp. Increased levels of MDA are associated with an enhancement of GSH level and enzymatic activities of GST and GPx. These results showed that generation of oxyradicals has occurred in earthworms following herbicide exposure and, stimulation of the GSH-dependent antioxidant system was not sufficient to counteract oxidative stress.

Lipid peroxidation is a well-established mechanism of cellular injury, and is used as an indicator to oxidative stress in cells and tissues. Lipid peroxides are unstable and decompose to form a complex series of compounds including reactive carbonyl compounds. Since polyunsaturated fatty acid peroxides generate MDA, measurement of MDA has been used as an indicator of lipid peroxide (Esterbauer et al., 1991). GPx provides a mechanism for cellular detoxification of peroxides, so this enzymatic reaction plays a crucial role in protecting cell from damage against free radicals, which can be generated by peroxide decomposition (Tiwari et al., 2016). Increased GPx activity in *Eisenia* sp could be associated with the generation of H₂O₂; a pro-oxidant agent that diffuses easily through the membranes and can react with transition metals, generating the hydroxyl radical (\cdot OH). Elevated concentrations of this oxyradical induce lipid peroxidation of membranes and oxidation of proteins that damage cellular integrity (Da Rosa et al., 2008).

According to the results of this study, Xue et al. (2009) showed that exposure to tetrabromobisphenol A (TBBPA)

Table 1. Activities of GPx and GST, and concentrations of GSH and MDA from *Eisenia* sp exposed to Glyphosan® SL (7 and 21 days) and during the recovery phase (30 days). Values are presented as means ± SD (n=8). C= control organisms; E= Exposed organisms to Glyphosan® SL. (gww⁻¹= grams wet weight).

Response		Exposure period		Recovery
		7 days	21 days	30 days
GPx (U gww ⁻¹)	C	1.78 ± 0.76	1.75 ± 0.39	0.96 ± 0.26
	E	2.26 ± 0.94	3.62 ± 0.46	1.46 ± 0.32
GST (U gww ⁻¹)	C	418.93 ± 149.17	571.55 ± 143.26	455.63 ± 71.59
	E	603.18 ± 86.54	634.29 ± 147.75	313.13 ± 101.98
GSH (mmol gww ⁻¹)	C	4.37 ± 0.58	3.60 ± 0.22	4.02 ± 0.28
	E	5.44 ± 0.08	5.16 ± 0.39	5.04 ± 0.45
MDA (nmol gww ⁻¹)	C	4.32 ± 2.59	4.62 ± 2.61	5.17 ± 3.00
	E	26.67 ± 4.54	50.51 ± 5.13	25.50 ± 4.61

produced significant alterations in antioxidant enzymes, GSH and MDA levels. The authors indicate that TBBPA exerts its toxic effects on *E. fetida* by inducing the generation of [•]OH radical, which lead to oxidative stress in the tissues of the earthworm. In the fish *Carassius auratus*, Fan et al. (2013) showed that the exposure to Roundup formulation was more harmful than glyphosate and, the hydroxyl radical generation increased with the concentration of Roundup®.

GST activity also exhibited a significant increase during acute and chronic exposure of *Eisenia* sp to Glyphosan® SL. Organic hydroperoxides, alkenals and epoxides resulting from oxidative metabolism may be regarded as natural substrates for GST (Canesi et al., 1999). GST is a detoxifying enzyme that catalyze the conjugation of GSH with a group of compounds having electrophilic centers e.g., nitrocompounds, organophosphates and organochlorides (Otitoju and Onwurah, 2007). Thus, increased GST may reflects an increase in the use of GSH in conjugation reactions involved in the metabolism of lipid hydroperoxydes and carbonyl compounds formed by herbicide-induced peroxidation of cellular membranes, such as has been proposed for earthworms exposed to others xenobiotics (Folarin et al., 2016).

GSH (γ-glutamylcysteinylglycine) is a naturally occurring tripeptide whose nucleophilic and reducing properties play a central role in metabolic pathways, as well as in the antioxidant system of most aerobic cells. This, GSH is crucial to a variety of process, including the detoxification of xenobiotics, removal of hydroperoxides and free radicals (Hermes-Lima, 2004). The GSH concentrations in *Eisenia* sp appear to be highly regulated since in spite of their use as cofactor in GST and GPx reactions, the GSH level was elevated during acute and chronic exposure to the herbicide. Possibly, a high activity of glutathione reductase (GR) in the

presence of NADPH reduce the product of the GPx and GST reactions; oxidized glutathione (GSSG) and by this way, GSH levels remain high (Tiwari et al., 2016).

Cells and tissues protect themselves from the oxidative damage using antioxidant enzymes and glutathione as they act as scavengers of ROS. Nevertheless, individual and inter specific differences in the induction of this response have been reported (Contardo-Jara and Wiegand, 2008). Our results are in agreement with the GSH and MDA contents increased in *E. fetida* after exposure to di-*n*-butyl phthalates during 7 and 28 days (Du et al., 2015). On the other hand, Zhang et al. (2014) showed that low doses of fomesafen (≤ 500 μg kg⁻¹) may result in oxidative damage and lipid peroxidation in *E. fetida* by inducing generation of ROS at short exposure periods (14 days). However, the adverse effects of fomesafen gradually disappear as the cooperation of antioxidant enzymes and exposure time are prolonged. Significant depletion in cellular GSH content was noted after 2 days exposure to lead. However, after a period of 21 days of exposure, significant enhancement of GSH level was observed in the earthworms treated with the metal (Maity et al., 2008).

GST stimulation in *Pheretima posthuma* exposed to the pesticides lindane, aldrin, and endosulfan was reported (Booth et al., 2000). Conversely, the activity of GST did not change in *Aporrectodea caliginosa* exposed to diazinon and chlorpyrifos (Booth and O'Halloran, 2001). Generally commercial preparations containing glyphosate cause stronger changes than glyphosate itself. These findings may be due to significant toxicity of additives (adjuvants or co-formulates) present in the pesticide preparations such as alcohol ethoxylates and alkylamine ethoxylates that enhance glyphosate stability and penetration into cell (Ibrahim, 2016). A higher increase in GST was recorded in *Lumbricus variegatus* treated

with Roundup Ultra compared to pure glyphosate, suggesting this response to a possible synergistic effect of the chemical mixtures in the formulation, as well as confirming the fact that toxic effects of adjuvants and surfactants in formulations may be underestimated (Contardo-Jara et al., 2009).

The research conducted by Martínez et al. (2007) on human blood cells has shown that cytotoxic effects caused by Roundup were stronger than induced by glyphosate. IC₅₀ values determined for glyphosate formulation and pure glyphosate were estimated to be 1.64 mg L⁻¹ and 56.4 mg L⁻¹, respectively. Herbicide formulations containing ethoxylaed surfactants were found more toxic than glyphosate itself in different mammalian cell lines (Larsen et al., 2014).

In the present study, GSH levels in the recovery phase remained as high as in the 21-day herbicide exposure, possibly indicating the participation of the tripeptide in the clearance of the metabolites resulting from the biotransformation of the herbicide such as the aminomethylphosphonic acid (AMPA) and methylphosphonic acid (Kwiatkowska et al., 2016). In this phase, MDA levels decreased with respect to the values shown by the earthworms exposed to the herbicide at 21 days, indicating that *Eisenia* sp can stimulate mechanisms of herbicide depuration GSH-mediated to reduce oxidative damage. However, possibly a longer time (>30 days) is required for the complete depuration of the organism. In agreement with the results of the present study, Menezes et al. (2011) reported alterations in the antioxidant system and elevation of reactive species to thiobarbituric acid (TBARS) in the fish *Rhamdia quelen* treated with a glyphosate-based formulation. The antioxidant defenses and TBARS levels decreased to values similar to those of the control during the recovery phase. After exposure of *E. fetida* to pesticide thiacloprid, inhibition of GST and catalase activities and then increasing during the recovery course compared with the control was observed (Feng et al., 2015).

Conclusion

This study demonstrated that the acute and chronic exposure of *Eisenia* sp to Glyphosan® SL activated GSH-dependent mechanisms as a compensatory biochemical response possibly to control an overproduction of pro-oxidant agents. However, this molecular strategy was not sufficient to prevent oxidative damage in the tissue earthworms with lipid peroxidation of cellular membranes. The significant changes in measured biomarkers after application of a dose of Glyphosan® SL highest that doses recommended for use in the agriculture indicate that the investigated herbicide could have not a harmful effect on earthworms in the context of the realistic environment. The biomarkers

evaluated in this study should not be used as a replacement for conventional soil monitoring techniques, but should be applied as supplementary approaches for demonstrating links between sublethal biochemical alterations and adverse effects in natural populations in field studies.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This research adheres to the ASAB/ABS Guidelines for the Use of Animals in Research (2012). All treatments of the experimental animals were in compliance with Venezuelan regulations on the protection of animals used for experimental and scientific purposes by National Fund on Science and Technology, Commission on Bioethics and Biosecurity (FONACIT, 2008 in Spanish).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests

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