Soils, Section 4: Ecotoxicology

Research Article

Ecotoxicological Assessment of the Effects of Glyphosate and Chlorpyrifos in an Argentine Soya Field*

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Abstract

Background, Aim and Scope. Continuous application of pesticides may pollute soils and affect non-target organisms. Soil is a complex ecosystem; its components can modulate the effects of pesticides. Therefore, it is recommended to evaluate the potential environmental risk of these compounds in local conditions. We performed an integrated field-laboratory study on an Argentine soya field sprayed with glyphosate and chlorpyrifos under controlled conditions. Our aim was to compare the sensitivity of a series of endpoints for the assessment of adverse effects of the extensive use of these agrochemicals.

Materials and Methods. A RR soya field in a traditional farming area of Argentina was sprayed with glyphosate (GLY) or chlorpyrifos (CPF) formulations at the commercially recommended rates, according to a randomized complete block design with 3 replicates. In laboratory assays, *Eisenia fetida andrei* were exposed to soil samples (0–10 cm depth) collected between the rows of soya. Endpoints linked to behavior and biological activity (reproduction, avoidance behavior and bait-lamina tests) and cellular/subcellular assays (Neutral Red Retention Time – NRRT; DNA damage – Comet assay) were tested. Field assays included litterbag and bait-lamina tests. Physico/chemical analyses were performed on soil samples.

Results. GLY reduced cocoon viability, decreasing the number of juveniles. Moreover, earthworms avoided soils treated with GLY. No effects on either reproduction or on avoidance were observed at the very low CPF concentration measured in the soils sampled 10 days after treatment. Both pesticides caused a reduction in the feeding activity under laboratory and field conditions. NRRT was responsive to formulations of CPF and GLY. Comet assay showed significantly increased DNA damage in earthworms exposed to CPF treated soils. No significant differences in DNA migration were observed with GLY treated soils. Litterbag field assay showed no differences between treated and control plots.

Discussion. The ecotoxicological effects of pesticides can be assessed by monitoring the status of communities in real ecosystems or through the use of laboratory toxicity tests. Litterbag field test showed no influence of the treatments on the organic matter breakdown, suggesting a scarce contribution of soil macrofauna. The bait-lamina test, however, seemed to be useful for detecting the effects of GLY and CPF treatments on the activity of the soil fauna. CPF failed to give significant differences with the controls in the reproduction test and the results were not conclusive in the avoidance test. Although the field population density of earthworms could be affected by multiple factors, the effects observed on the reproduction and avoidance tests caused by GLY could contribute to its decrease, with the subsequent loss of their beneficial functions. Biomarkers measuring effects on suborganism level could be useful to predict adverse effects on soil organisms and populations. Among them, NRRT, a lysosomal destabilization biomarker, resulted in demonstrating more sensitivity than the reproduction and avoidance tests. The Comet assay was responsive only to CPF. Since DNA damage can have severe consequences on populations, it could be regarded as an important indicator to be used in the assessment of soil health.

Conclusions. Reproduction and avoidance tests were sensitive indicators of GLY exposure, with the former being more labor intensive. Bait-lamina test was sensitive to both CPF and GLY. NRRT and Comet assays revealed alterations at a subcellular level, and could be considered complementary to the biological activity tests. Because of their simplicity, some of these bioassays seemed to be appropriate pre-screening tests, prior to more extensive and invasive testing.

Recommendations and Perspectives. This study showed deleterious effects of GLY and CPF formulations when applied at the nominal concentrations recommended for soya crops. Further validation is needed before these endpoints could be used as field monitoring tools in Argentine soya soils (ecotoxicological risk assessment – ERA tools).

Keywords: Agrochemicals; avoidance behavior; bait-lamina test; chlorpyrifos; Comet assay; *Eisenia fetida andrei*; glyphosate; litterbag test; neutral red retention time (NRRT); pesticide application; reproduction test; soya field

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Introduction

Increasing world use of synthetic herbicides and insecticides to sustain high-yielding crop varieties may affect non-target organisms and, in the long run, lead to the development of non-reversible damage to the structure and function of soil ecosystems (Muthukaruppan et al. 2004, Römbke et al. 2005, Reinecke and Reinecke 2007). Soil is a complex dynamic ecosystem; its components can modulate the effects of pesticides (Kördel and Römbke 2001). Therefore, it is recommended to assess these effects under local conditions. Experiments made with soil-dwelling fauna showed that acute tests were generally too insensitive and suggested that the protection of the habitat function could only be achieved if more sensitive parameters were taken into consideration (Kördel and Römbke 2001, Booth et al. 2005). Links between field trials and laboratory experiments, and between responses at different levels of biological organization (cell, organism, population), should be established (Vasseur and Cossu-Leguille 2003, Van Gestel and Weeks 2004, Jänsch et al. 2006). Earthworms are considered highly appropriate terrestrial model organisms for ecotoxicity tests. Organisms of the species *Eisenia fetida* sensu lato (*E. fetida* and *E. andrei*) are, because of their low cost, easy handling and the standardization of acute and subchronic ecotoxicological tests, suitable biomonitors used in developed countries for assessing pesticide risks to soil invertebrates (OECD 1984, Jänsch et al. 2006). In our country, the evaluation of the habitat function of soils has just started. In order to improve this situation, we performed an integrated field-laboratory study on a soya field of a traditional agricultural area of Argentina, sprayed with the herbicide glyphosate and the organophosphorus insecticide chlorpyrifos at the recommended doses. Both pesticides are frequently used not only in Argentine soya fields but also worldwide (Jergentz et al. 2005, Marc et al. 2005, Sandahl et al. 2005, Micucci and Taboada 2006). Our aim was to evaluate the usefulness of a series of endpoints for assessing adverse effects of the extensive use of these agrochemicals. Two field bioassays related to organic matter breakdown (OMB) (litterbag and bait-lamina assays) were included. Laboratory tests were performed on *Eisenia fetida andrei* (neutral red retention time, Comet assay, avoidance behavior, bait lamina laboratory test, and reproduction test). Some of the performed tests are at present not standardized, but might be complementary to the existing standardized tests and could be useful for the ecotoxicological risk assessment (ERA) of agricultural soils. The ultimate end of these studies is to encourage a more responsible agriculture, where the ecological cost is taken into account together with other production costs.

1 Materials and Methods

1.1 Study area and experimental field design

RR (Roundup Ready®, glyphosate resistant) soya was machine-planted in mid-November 2004 near the experimental station of INTA (National Institute for Agricultural Technology) in Oliveros, Santa Fe Province, Argentina (32°48' S, 62° W) on a clay silty soil (typic Argiudoll Maciel, INTA 1985). Results of physicochemical analyses of soil samples (0–10 cm layer) performed by the Geochronology and Isotopic Geology Institute (INGEIS), Buenos Aires, Argentina, were: pH 5.64–5.79, electrical conductivity 53.7–69.7 ∝s, organic C 1.56–1.60%, organic N 0.134–0.136%, C/N 11.6–11.9, Ca 1,250–1,317 ppm, K 292–317 ppm, Mg 183–184 ppm, and P 14.7–26.3 ppm. This soil presented a compacted structure and the loss of the superficial layer due both to the parent material and to too many years of continuous agriculture. The mean annual precipitation and temperature are 1,006 mm and 17.7°C respectively. The experimental design consisted of randomized complete blocks with 3 replications, each block comprising 3 plots of 13 x 5 m. The distance between the blocks was at least 500 m. At each block, randomly chosen plots were sprayed using a calibrated hand pressure sprayer with commercial chlorpyrifos (CPF) or glyphosate (GLY) at the manufacturers' recommended rate, or with water (control). Applied doses were: CPF (ATANOR 48) 620 g ai/ha once 30 days after seeding (t_0); GLY (Roundup FG) 1,440 g ai/ha 24 h after seeding and at t_0; water (control plots) at t_0. The study was conducted in summer (December 2004-March 2005).

For laboratory bioassays, soil samples (10–15 Kg) of the different plots (0–10 cm depth) were collected between the rows of soya one day (t_1) and 10 days (t_2) after t_0. For pesticide determinations, samples (0–3 cm and 0–10 cm depth) were taken and kept refrigerated until analysis. CPF determinations were performed by the **National Agrifood and Quality Service** (SENASA), Buenos Aires, Argentina, using GC techniques. Glyphosate determinations were performed by HPLC-fluorescence detector, by the Institute of Technology for Chemical Industry (INTEC), Santa Fe, Argentina.

1.2 Laboratory assays

1.2.1 Earthworms and test soil preparation

Soil samples were 2-mm sieved and adjusted to 50–60% of maximum water holding capacity before running the bioassays. *Eisenia fetida andrei* earthworms 0.30–0.60 g fresh weight, maintained in our laboratory, were exposed to soils prepared as described above. Before starting the bioassays, earthworms were washed with dechlorinated tap water and placed on moist filter paper for a minimum of 3 h, in order to let them empty their guts. As far as possible OECD/ISO guidelines were used for the tests.

1.2.2 Reproduction test

The test was performed according to ISO 11268-2 (1998), with slight modifications. Briefly, to each of three replicate containers per treatment, 300 g soil and six adult earthworms were added. Food (2 g dry baby cereal mixture) was added at the beginning of the experiment and then once weekly. Humidity of soils was maintained during the entire experiment. After 28 days of exposure, earthworms were removed from soil and survival rates recorded. The remaining soil (containing cocoons) was returned to its respective containers and incubated for another 28 days to continue the exposure of cocoons and juveniles to the test soil. At the end of the test (56 days), the number of hatched and non-hatched cocoons and the number of juveniles were recorded.

1.2.3 Avoidance behavior

ISO N 281 (2004) was followed. Experiments were conducted using a plastic, rectangular, two-chamber container (20 x 10 x 10 cm). Containers were divided in half with a plastic split, 520 g of control soil were placed in one half of the container, and 520 g of treated soil were placed on the other side. After the separator was removed, ten adult earthworms were placed on the centerline of the soil surface, and the containers were covered, while allowing sufficient aeration. After an incubation time of 3 days, the split was reintroduced and the number of individuals in each compartment was counted. Four replicates were run for each test. For each block, a control dual test (each test box contained the same control soil in both chambers) was performed with four replicates.

1.2.4 Bait-lamina test

Bait-lamina consist of plastic strips about 12 cm long, 1 cm broad, and 1 mm thick. The lamina are perforated in 5 mm-distances by 16 small (1 mm) holes, filled with a bait substance (a mixture of cellulose, wheat bran and activated carbon). In the laboratory, an adaptation of Helling et al. (1998) was performed. Containers with 350–400 g soil, 4 bait-lamina and 6 earthworms each were used (4 replicates/treatment/block). After exposure of 3 days, the number of empty or perforated holes in each lamina was counted and the percentage of feeding activity was calculated.

1.2.5 Neutral red retention time (NRRT) assay

After 7 days or 28 days exposure to soils as in section 1.2.2, worms were removed, rinsed in dechlorinated tap water and blotted dry. Coelomic fluid containing coelomocyte cells from individual earthworms was extruded through dorsal pores after stimulation with an electric current (Kobayashi et al. 2001). Viability of coelomocytes was measured using the trypan blue exclusion technique. The NRRT on the coelomocyte cells of each worm was measured according to Weeks and Svendsen (1996) with slight modifications. Coelomic fluid (20 µL) was placed on a microscope slide and the cells allowed adhering for 60 sec prior to the application of 20 ∝L neutral red working solution (80 ∝g/mL) and a coverslip. Each slide was scanned for 2 min at 5-min intervals under a light microscope (400 x). Observation was stopped when the ratio of cells with fully stained cytoplasm reached >50% of the total number of cells counted. This time was recorded as the NRRT.

1.2.6 Comet assay

Earthworms were exposed to soil samples during 7 days as in section 1.2.2. Comet assay was performed by adaptation of Singh et al. (1988), on coelomic fluid obtained as described in section 1.2.5. Immediately after extrusion, 10 ∝L coelomocyte suspension were mixed with 75 ∝L 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) and a starting current of 250 mA. Cell nuclei were rated visually and classified into five categories according to the tail intensity. An arbitrary damage index (DI) was defined as: $DI = \sum n_i \times i$, where n*i* is number of cells with damage class *i* (0, 1, 2, 3 or 4).

1.3 Field assays

1.3.1 Litterbag test

OMB was tested by an adaptation of Ingelsfield (1989), using bags filled with dried lucerne (*Medicago sativa* L.), with holes of either 0.2 mm (M_1) or 3.6 mm (M_2). Litterbags were buried in the soil before treatments with the pesticides. After 10 (t_2), 50 (t_3) or 90 (t_4) days, they were removed from the soil, their contents were dried and weighed. OMB was calculated as the percentage of organic material mass loss at the sampling time t (start weight – end weight) x 100/start weight). Macrofauna index (MI) was calculated as: MI= $OMB(M_1)/OMB(M_2)$.

1.3.2 Bait-lamina test

The feeding activity of soil organisms was assayed according to von Törne (1990). 16 bait-lamina strips (as a 4 x 4 matrix) were inserted vertically into the top soil layer (3 replicates/treatment/block). After 50 days exposure, lamina were removed from the soil, carefully washed, and examined. The number of pierced holes in each lamina was counted. The vertical distribution of the feeding activity was also recorded.

1.4 Statistical analysis

Statistical analyses were performed with GraphPad InStat 3 (GraphPad Software, San Diego, USA). Data were first tested for normality (Kolmogorov-Smirnov's test) and for homogeneity of variances (Bartlett's test). Depending on these results, means were compared by one-way ANOVA (parametric) or non-parametric Kruskal-Wallis tests. When significance was demonstrated ($p<0.05$), Tukey-Kramer or the non-parametric Dunn's tests were applied for post-hoc comparison of means. For avoidance experiments, Student t-test was used (one-tailed test for control-treated experiments; two-tailed test for the dual control tests) (ISO N 281 2004, Natal Da Luz et al. 2004).

2 Results

2.1 Chemical analysis

Soils of CPF treated plots presented an average concentration of 0.14 ± 0.02 ppm in the 0–3 cm layer and of 0.04 ± 0.01 ppm in the 0–10 cm layer at t_1. CPF was detected neither in the GLY treated nor in the control plots. 10 days after spraying, CPF decayed to 0.02 ppm ± 0.01 ppm in the

upper layer and was not detectable in the 0–10 cm layer. 50 days after treatment, CPF was not detected (detection limit: 0.01 ppm). GLY was below the detection limit (0.05 ppm) in all samples. Two differential characteristics of GLY treated plots, compared to the other plots, provided evidence of the application and efficacy of this pesticide: the absence of weeds between the soya rows, and the significantly higher soybean yield (in the same order as its regional average) at the end of the soya growing cycle. Yields were 3146 ± 93 kg/ha in the GLY treated plots, 1453 ± 13 kg/ha in the CPF treated plots, and 1248 ± 17 kg/ha in the control plots.

2.2 Bioassays

2.2.1 Reproduction test

After 28 days of exposure to soils sampled at t_2, the survival rate of earthworms in all treatments was 100%. No significant differences in cocoon production between earthworms exposed to treated soils and controls were observed (**Table 1**). However, the number of hatched cocoons was significantly reduced ($p<0.01$) in earthworms exposed to GLY treated soils (hatchability: 41% of control). The number of juveniles was also significantly lower than in controls, without modification in the ratio juveniles/hatched cocoons, indicating a deleterious effect on the viability of the cocoons. No significant effects on reproduction were observed in earthworms exposed to CPF treated soils.

2.2.2 Avoidance behavior

Fig. 1 shows the results of the avoidance behavior test after earthworm exposure to soil samples of each block collected at t_2. In dual control tests, no significant differences ($p>0.05$) were found in the distribution of the worms between both chambers of the containers. Earthworms exposed to GLY treated soils, however, exhibited significant avoidance responses ($p<0.05$). With respect to CPF treated soils, the avoidance response was controversial. When exposed to soils from blocks 1 and 2, earthworms preferred the treated substrates instead of the control ones. This preference was statistically significant with block 1. In contrast, worms tended to avoid CPF treated soils from block 3.

2.2.3 Feeding activity (Bait-lamina test)

Laboratory assays. The response of bait-lamina consumption after 3 days of earthworm exposure to soils collected at t_1 is shown in **Fig. 2**. Substrate consumption rates, measured as the percentage of open holes in the bait-lamina sticks, showed a significant reduction in GLY and CPF treated soils ($p<0.001$). For control soils the average consumption was 68.2 ± 2.7%, whereas for GLY and CPF treated soils it was of 25.1 ± 11.3% and 19.0 ± 8.9% respectively.

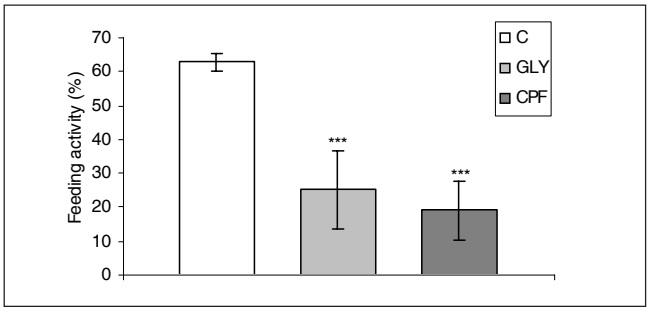

Fig. 2: Bait-lamina laboratory assay. Percentage of feeding activity (3 days) of *Eisenia fetida andrei* in controls, GLY and CPF treated soils. Mean ± SD (4 replicates/treatment/block). *** Significantly different from control (Kruskal-Wallis and Dunn's post test, $p<0.001$)

Table 1: Effects of exposure to glyphosate (GLY) and chlorpyrifos (CPF) treated soils on reproductive parameters of *Eisenia fetida andrei*

Parameter	C	GLY	CPF
Mortality % (28 days)	0	0	0
Nº of cocoons (56 days)	11.2 ± 4.3	8.2 ± 3.0	11.3 ± 5.1
Nº of unhatched cocoons (56 days)	3.0 ± 1.0	5.6 ± 2.1	4.3 ± 2.1
Hatchability % (hatched x 100 / total Nº of cocoons)	71 ± 12	29 ± 23 *	55 ± 28
Nº of juveniles (56 days)	7.6 ± 3.6	1.1 ± 1.7 *	3.4 ± 3.7
Nº of juveniles/hatched cocoons	1.1 ± 0.3	0.4 ± 0.7	0.5 ± 0.4

Mean ± SD (3 replicates/ treatment/ block). *Significantly different from control (ANOVA and Tukey's post test, $p<0.05$)

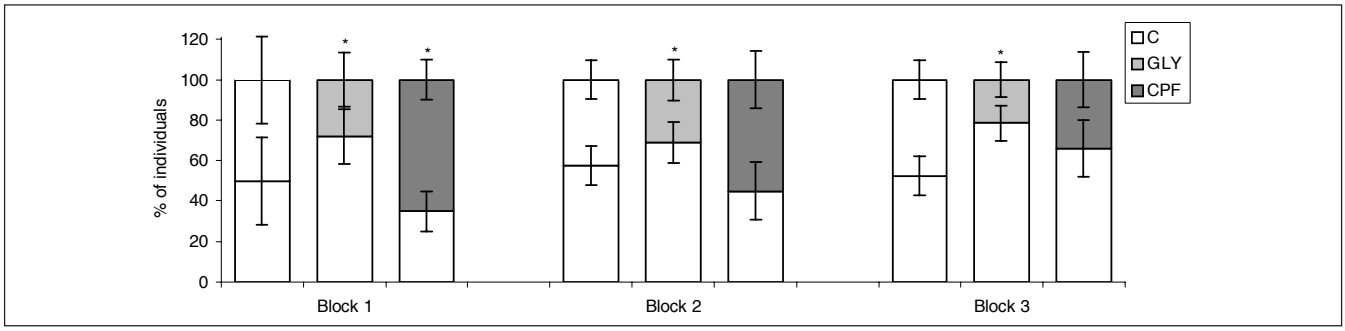

Fig. 1: Avoidance behavior assay. Distribution of *Eisenia fetida andrei* on each section of the dual chamber test container. Mean ± SD (4 replicates/ assay). * Significantly different from control (Student t-test, $p<0.05$)

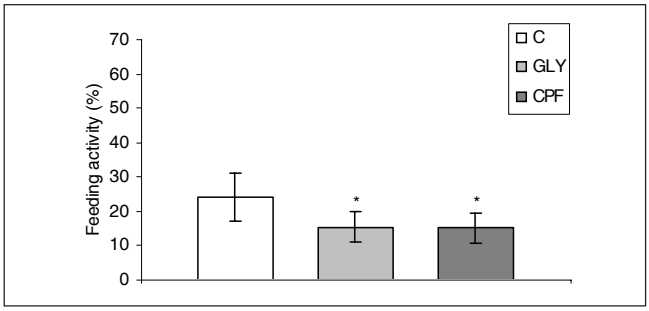

Fig. 3: Bait-lamina field assay. Percentage of feeding activity (50 days) of soil fauna in controls, GLY and CPF treated soils. Mean ± SD (3 replicates/treatment/block). * Significantly different from control (ANOVA and Tukey's post test, p<0.05)

Field assays. After 50 days exposure of bait-lamina into field soils, the consumption of bait by soil macrofauna was low (**Fig. 3**); however, the feeding activity was significantly reduced (p<0.05) in treated plots when compared to the control (C: 24.0 ± 7.0%; GLY: 15.5 ± 4.4%; CPF: 15.3 ± 4.4%). **Fig. 4** shows the vertical distribution of bait perforation; in general a decrease of the piercing activity with depth was observed.

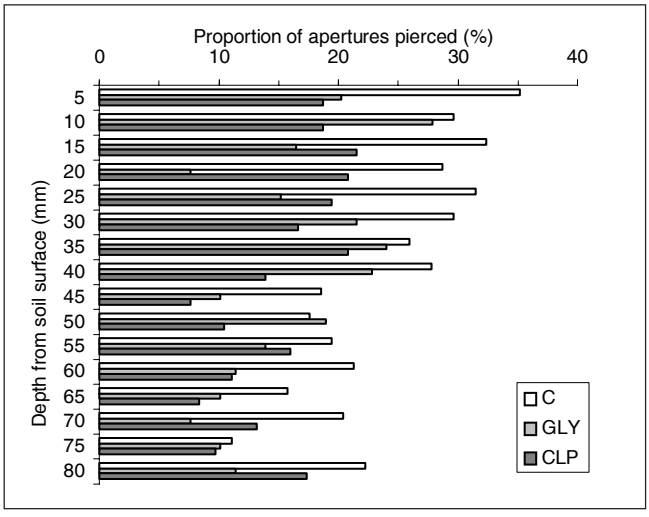

Fig. 4: Bait-lamina field assay. Distribution of pierced apertures with the depth in controls, GLY and CPF treated soils (n=144)

2.2.4 Lysosomal destabilization (NRRT)

Fig. 5 shows the results of NRRT bioassay in earthworms exposed for 7 days (**A**) or 28 days (**B**) to soils sampled at t_1 and t_2, respectively. In control soils, NRRT were longer than 60 min after 7 days exposure, whereas they fell to 45 min after 28 days exposure, showing some stress probably due to the longer earthworm exposure time. After a period of 7 days, lysosomal NRRT was significantly reduced in earthworms exposed to GLY (47.0 ± 6.3 min, p<0.01) and CPF (18.3 ± 8.8 min, p<0.001) treated soils collected at t_1, when compared to controls (63.7 ± 2.5 min). When exposed to soils sampled at t_2, the reduction in NRRT was only observed with CPF treated soils (30.0 ± 3.2 min, p<0.001).

After 28 days exposure, NRRT was responsive to GLY and CPF treated soils, both with soils collected at t_1 (C: 45.0 ± 4.1 min, GLY: 23.3 ± 2.6 min, CPF: 13.3 ± 2.6 min) or at t_2 (C: 42.5 ± 2.9min, GLY: 22.5 ± 2.7 min, CPF: 15.0 ± 3.2 min).

2.2.5 DNA damage (Comet assay)

DNA damaging effects on coelomocytes of earthworms exposed to controls and GLY and CPF treated soils collected at t_1 are shown in **Fig. 6**. Two parameters, percentage of damage (% D, **Fig. 6 A**) and Damage Index (DI, **Fig. 6 B**) were used for comparison. In all experiments, viability of cells was around 95%. CPF produced a significant increase in% D (97.4%, p<0.001) and DI (346.5, p<0.001) when compared to controls (% D: 35.3, DI: 82.9). On the other hand, no significant differences in DNA migration (% D= 42.0, DI=104.2) were observed when earthworms were exposed to GLY treated soils.

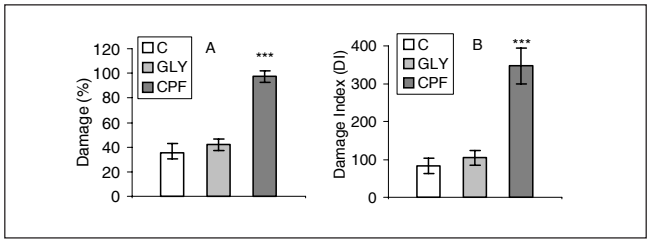

Fig. 6: Comet assay. Effects on coelomocytes DNA migration of *E. fetida andrei* exposed to controls, GLY and CPF treated soils. A. Percentage of damage (% D). B. Damage Index (DI). Mean ± SD (3 earthworms/treatment/block). *** Significantly different from control (Kruskal-Wallis and Dunn's post test, p<0.001)

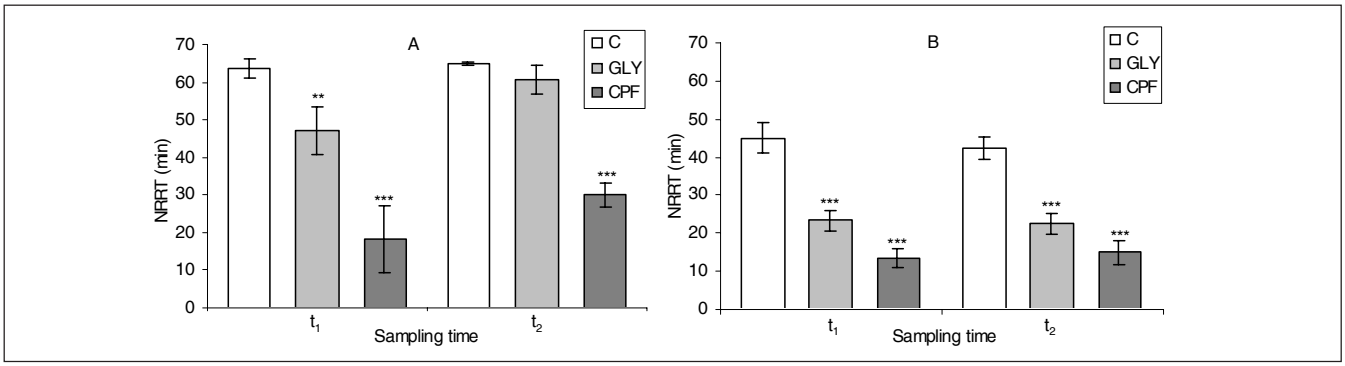

Fig. 5: Neutral red retention time (NRRT) in coelomocytes of *E. fetida andrei* exposed for 7 days (A) or 28 days (B) to GLY and CPF treated soils sampled at t_1 and t_2. Mean ± SD (3 earthworms/treatment/block). **p<0.01; ***p<0.001: Significant differences with control, ANOVA and Tukey's post test

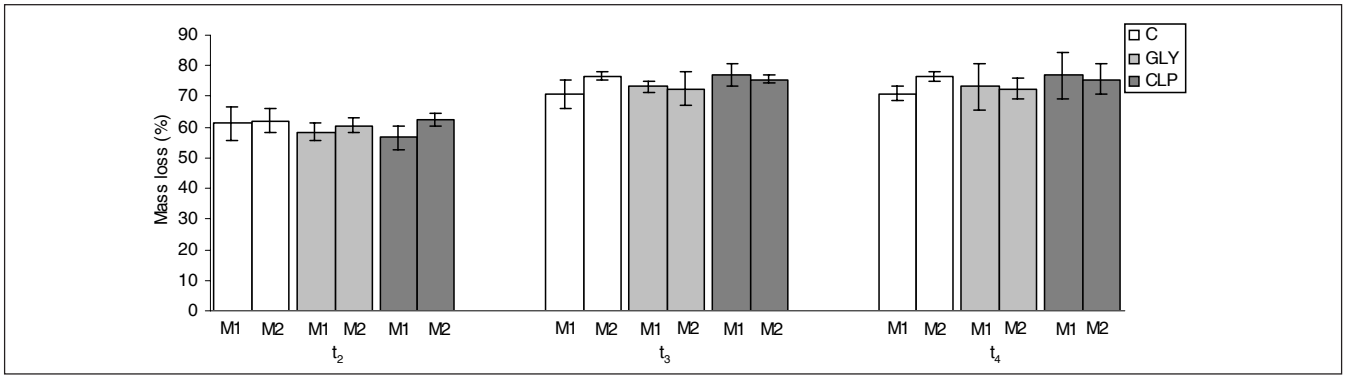

Fig. 7: Litterbag field assay. Percentage of organic mass loss in fine (M_1) and coarse (M_2) meshed bags. Mean ± SD (2 replicates/treatment/block). (ANOVA and Tukey's post test, p>0.05)

2.2.6 Organic matter breakdown (litterbag)

Fig. 7 shows the decomposition rate of litter after 10, 50 and 90 days of field exposure of bags to treated soils. Litterbag loss was not significantly different between treatments and controls at any assayed time. No mesh effect was observed (MI close to 1 in all cases).

3 Discussion

3.1 Effects on biological activity and behavior

3.1.1 Reproduction

Contaminants may reduce the reproductive success, or offspring survival of earthworms. The effects on reproductive output can be interpreted in two ways: as a direct effect due to an interaction with key mechanisms for reproduction, or as an indirect effect, via assimilation of nutrients, growth, and maintenance of the energetic balance (Booth and O'Halloran 2001). In the present study, a significant effect of GLY on hatching of cocoons, the process by which juveniles are liberated from their protective envelopes, was observed, whereas cocoon production was not reduced (see Table 1). Although physiological processes between terrestrial and aquatic organisms differ, an interference of GLY with the expression of the hatching enzyme, as found by Marc et al. (2005) in sea urchin *Sphaerechinus granularis* embryos, might have occurred. Differing with GLY, CPF reproduction test failed to give significant differences with the controls. Recently, Jager et al. (2007) suggested some deleterious effects of CPF on reproduction of the springtail *Folsomia candida*. In laboratory-simulated field experiments, Booth and O'Halloran (2001) found a significant decrease in cocoon production and in viability in *Aporrectodea caliginosa* exposed to soils containing 28 ppm of CPF. In our case, the mean values of the number of juveniles and hatchability are between those obtained for the controls and for GLY treated soils, but these resulting values were not statistically different. The low concentration in the field (0.02 ppm) could have been one of the causes of the lack of CPF effects on reproduction.

3.1.2 Avoidance behavior

Earthworm reproduction tests are long and labor intensive (56 days tests); the avoidance test, on the other hand, is a faster technique. Earthworms, by having chemoreceptors and sensory tubercles, present a high sensitivity to chemicals in soils (Reinecke et al. 2002). However, some chemicals cannot be detected by the earthworms, and they may die in the test soil without trying to escape (Garcia et al. 2004). In our laboratory studies, earthworms avoided soils treated with GLY sampled at t_2, while the CPF effects are unclear (see Fig. 1). The number of replicates should be adjusted to ensure reliable results. Many authors (Hund-Rinke et al. 2005, Loureiro et al. 2005) concluded that the avoidance assay is useful as a first screening test. Avoidance behavior may be of crucial importance for the species populations and can significantly contribute to their exposure and survival in field conditions (Lukkari and Haimi 2005). Although the field population density of earthworms could be affected by multiple factors, the effects observed on the reproduction and avoidance tests caused by GLY could contribute to its decrease, with the subsequent loss of their beneficial functions.

3.1.3 Organic matter breakdown: Litterbag and bait-lamina tests

Organic matter breakdown is one of the most integrating processes in the soil ecosystem. According to Knacker et al. (2003), litterbag and bait-lamina tests may reveal a specific pattern of responses related to OMB, which might not be correlated to each other, and this could be attributed to the differences in the substrate and in the way of exposure to the organic matter used. Therefore, it is worth studying OMB by both techniques. In the present study, the litterbag field test (see Fig. 7) showed neither influence of the treatments on the OMB nor mesh effect, suggesting a scarce contribution of soil macrofauna. The bait-lamina test, which provides an artificial organic substrate, in spite of the low consumption of bait, seemed to be useful for detecting the effects of GLY and CPF treatments on the feeding activity of the soil fauna (see Fig. 3). Förster et al. (2004) established correlations between bait-lamina feeding rate and soil earthworm density. Our results, in accordance with the low density of earthworms, were revealed by occasional examinations during the course of the experiment, thereby providing a simple and practical tool to complement studies of the effects of chemicals on functional aspects of soil ecosystems. When we evaluated the feeding profiles under field conditions, the biological activity decreased with the depth of soil layers (see Fig. 4). Soil compaction of the agricultural soil,

may have had an influence on the stratification of this response (Taboada et al. 1998, Förster et al. 2004). Few studies applied the bait-lamina test in laboratory assays (Helling et al. 1998, Van Gestel et al. 2001). In our laboratory tests, a similar pattern of effects of GLY and CPF treatments as in bait-lamina field test was observed (see Fig. 2). The evaluation of the 'feeding activity' under laboratory conditions resulted simply and quickly (3 days) and could be useful as a screening test.

3.2 Cellular/subcellular biomarkers.

The assessment of responses on sub-organismal level (biomarkers) may be useful to predict adverse effects on soil organisms and populations (Scott Fordsmand and Weeks 2000, Maboeta et al. 2002, Vasseur and Cossu Leguille 2003, Van Gestel and Weeks 2004).

3.2.1 Lysosomal destabilization

In order to evaluate the biological effects of the field soils, relatively broad responses and changes that could provide information about the general health status at the cellular level were tested by the NRRT, a lysosomal destabilization biomarker, as a general indicator of physiological stress, and by the Comet assay, as a measure of the general DNA integrity damage (Singh et al. 1988, Weeks and Svendsen 1996). NRRT was responsive to formulations of GLY and CPF even at the low dose sprayed (see Fig. 5). Moreover, when *Eisenia fetida andrei* earthworms were exposed to CPF treated soils in samples taken 10 days after treatments, it was significantly decreased, providing more sensitive results than the reproduction and the avoidance tests (which were also sensitive endpoints). The NRRT has been tested for both organic and inorganic compounds in terrestrial systems, and, in many cases, it has been advocated as a sensitive biomarker (Maboeta et al. 2002, Xiao et al. 2006). Booth et al. (2005) studying exposure of earthworms of the species *Aporrectodea caliginosa* to petroleum hydrocarbons, also found that NRRT was the most sensitive biomarker, compared with cocoon production, and juvenile maturation parameters. Using earthworms of the same species, exposed to soils contaminated with CPF, other authors also concluded that NRRT is a sensitive biomarker for this pesticide (Booth et al. 2001, Reinecke and Reinecke 2007). As in the case of other biomarkers, little information is available on the linkage of this biomarker response to effects at soil population or community levels (Scott-Fordsmand and Weeks 2000). Owing to its sensitivity, this biomarker could provide an early warning of impending ecological damage.

3.2.2 DNA damage (Comet assay)

The Comet assay (single cell gel electrophoresis), is a rapid and sensitive method for detection of primary DNA damage on the individual cell level, which is increasingly used in biomedical research, human and environmental bio-monitoring studies and genotoxicity testing (Zang et al. 2000, Reinecke and Reinecke 2004). Results document that it is capable of detecting xenobiotic interaction with DNA in consequence of exposure to complex environmental samples (Kosmehl et al. 2004). In the present study, the Comet assay was responsive to exposure to CPF treated soils collected one day after treatment. The percentage of damaged cells and the degree of DNA damage on coelomocytes of organisms were significantly different from controls, being that the score obtained for DNA damage was rather high (346.5 compared to a maximum of 400) (see Fig. 6). No effects were observed on the earthworms exposed to GLY-treated soils. Earthworm coelomic fluid harboring cells, the coelomocytes, have properties similar to mammalian leucocytes (Burch et al. 1999). Leucocytes of albino rats exposed in vivo to CPF also showed DNA damage with the Comet assay 24 h after exposure, although a reparation apparently took place with time (Rahman et al. 2002). Since DNA damage can have severe consequences on populations, it could be regarded as an important indicator to be used in the assessment of soil health. More studies on the time course of CPF effects on the coelomocytes DNA will be necessary.

4 Conclusions

Soil screening could be a process of identifying and defining areas, contaminants, and conditions at sites that need further attention. In this work, a battery of bioassays and biomarkers have been assessed in agricultural soils treated with GLY and CPF. Reproduction and avoidance tests were sensitive indicators of GLY exposure; although a reproduction test is more labor intensive. The bait-lamina test was sensitive to both CPF and GLY. NRRT and Comet assays revealed alterations at a subcellular level, before damage became visible at higher organization levels, and could be considered complementary to the biological activity related tests. Because of their simplicity, these bioassays seemed to be appropriate pre-screening tests to evaluate effects of both pesticides in Argentine agricultural soils, prior to more extensive and invasive testing.

5 Recommendations and Perspectives

Transgenic soya is the most extensively cultivated crop in Argentine agricultural lands. Intensive use of GLY and CPF in this crop could represent a risk for soil biota. This study showed deleterious effects of GLY and CPF formulations when applied at the nominal concentrations recommended for soya crops. Complementary laboratory and field studies may facilitate the selection of the appropriate biomarkers for the ecotoxicological assessment of the risk associated with the extensive use of pesticides. Further validation is needed before these endpoints could be used as field monitoring tools in Argentine soya soils (ecotoxicological risk assessment – ERA tools).

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