Biomarkers for the assessment of chlorpyrifos effects on earthworms and on soil functional parameters

Lucas Piola(1), Julio Fuchs(2), María Luisa Oneto(1), Silvana Basack(1), Rosana Giménez(3), Rubén Massaro(4), Juan Carlos Papa(4), Eva Kesten(1) and Norma Casabé(2)

(1) Universidad de Buenos Aires (UBA), Facultad de Ciencias Exactas y Naturales (FCEN), Departamento de Química Biológica (DQB), Toxicología y Química Legal, Ciudad Universitaria, Pabellón 2, 4º piso, 1428 Buenos Aires, Argentina. E-mail: lpiola@qb.fcen.uba.ar, mloneto@qb.fcen.uba.ar, basack@qb.fcen.uba.ar, ekesten@qb.fcen.uba.ar
(2) Consejo Nacional de Investigaciones Científicas y Técnicas, Toxicología y Química Legal, UBA, FCEN, DQB, Ciudad Universitaria, Pabellón 2, 4º piso, 1428 Buenos Aires, Argentina. E-mail: juliof@qb.fcen.uba.ar, nbcm@qb.fcen.uba.ar
(3) UBA, Facultad de Agronomía, Área de Protección Vegetal, Avenida San Martín 4453, 1417 Buenos Aires, Argentina. E-mail: rgimenez@agro.uba.ar
(4) Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria Oliveros, Ruta 11, Km 353, 2206 Oliveros, Santa Fe, Argentina. E-mail: rmassaro@correo.inta.gov.ar, jcpapa@correo.inta.gov.ar

Abstract – The objective of this work was to evaluate the effects of chlorpyrifos on earthworms and on soil functional parameters. An integrated laboratory-field study was performed in a wheat field in Argentina, sprayed with chlorpyrifos at two recommended application rates (240 or 960 g ha\(^{-1}\) a.i.). Laboratory tests included neutral red retention time, comet assay (single cell gel electrophoresis), and avoidance behavior, each using the earthworm Eisenia andrei exposed in soil collected 1 or 14 days after pesticide application, and the bait-lamina test. Field tests assessed organic matter breakdown using the litterbag and bait-lamina assays. Earthworm populations in the field were assessed using formalin application and hand-sorting. The neutral red retention time and comet assays were sensitive biomarkers to the effects of chlorpyrifos on the earthworm E. andrei; however, the earthworm avoidance test was not sufficiently robust to assess these effects. Feeding activity of soil biota, assessed by the bait lamina test, was significantly inhibited by chlorpyrifos after 97 days, but recovered by the 118th day of the test. Litterbag test showed no significant differences in comparison to controls. Earthworm abundance in the field was too low to adequately test the sensitivity of this assessment endpoint.

Index terms: Eisenia andrei, agricultural soil, field assay, laboratory bioassay.

Introduction

It is increasingly recognized that the protection of soils and their inherent communities must become a primary goal of environmental policy worldwide (Römbke et al., 2005; Filser et al., 2008). Chlorpyrifos (O,O-diethyl-O-3,5,6-trichloro-2-pyridyl phosphorothioate), a broad spectrum systemic organophosphorus anticholinesterase insecticide, is widely used in Argentina in direct soil application for the control of agricultural pests.
The toxicity of pesticides to soil organisms depends on the compound bioavailability, which is affected by the physiochemical properties of the compound and the soil, and by the uptake routes of exposed organisms. Therefore, ecotoxicity studies can benefit from using experimental designs that improve data relevance for local exposure conditions in the field (Yu et al., 2006; Filser et al., 2008). However, due to this complexity, assessment of the risks for soil organisms cannot rely exclusively on chemical analysis. In-vivo assays using sentinel species provide a more reliable assessment of toxicity because they are more representative of the natural soil conditions.

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maintained in laboratory culture, by exposure to soil samples sieved (≤2 mm) and adjusted to 50–60% of the soil water holding capacity (WHC = 48.6±2.4%). Unless other specifications, OECD/ISO guidelines were followed for the tests. To perform cellular/subcellular assays, six adult earthworms were added to 300 g soil (three replicate containers per treatment). After a 7-day exposure, each earthworm was washed with water, dry blotted using a filter paper, and placed individually in a glass conical tube containing 1.5 mL of freshly prepared alkaline electrophoresis solution (0.4 mol L\(^{-1}\) Tris-HCl, pH 7.5) and immersed in 1 mmol L\(^{-1}\) NaOH and 1 mmol L\(^{-1}\) Na\(_2\)EDTA) at 4ºC 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. One hundred cell nuclei per slide were rated visually and classified into four categories, according to the tail intensity (size and shape). The extent of DNA damage was measured as damage index (DI), a weighted value of damage according to cells distribution, defined as DI = \(\sum(n_i x_i)\), in which \(n_i\) is the number of cells with damage class \(i\) (0, 1, 2 or 3).

The avoidance behaviour was studied according to Weeks & Svendsen (1996) with slight modifications. Twenty microliters of coelomic fluid were placed on a microscope slide, and the cells were allowed to adhere for 60 s, prior to the application of 20 μL of neutral red working solution (80 µg mL\(^{-1}\)) and a coverslip. Each slide was scanned for 2 min, at 5-min intervals under a light microscope (400 x). Observation went on until the ratio of cells with fully stained cytoplasm exceeded 50% of the total number of cells counted. This time was recorded as the neutral red retention time (NRRT).

The comet assay was performed on the same coelomic fluid (Casabé et al., 2007). We mixed 10 μL of the coelomocyte suspension with 75 μL of 0.75% low-melting-point agarose at 37ºC, and spread it over a microscope slide precoated with 100 μL of 0.4% trypan blue in PBS. The percentage viability was calculated based on the percentage of unstained cells.

The neutral red retention assay on the coelomocyte cells of each worm was measured according to Weeks & Svendsen (1996) with slight modifications. Twenty microliters of coelomic fluid were placed on a microscope slide, and the cells were allowed to adhere for 60 s, prior to the application of 20 μL of neutral red working solution (80 µg mL\(^{-1}\)) and a coverslip. Each slide was scanned for 2 min, at 5-min intervals under a light microscope (400 x). Observation went on until the ratio of cells with fully stained cytoplasm exceeded 50% of the total number of cells counted. This time was recorded as the neutral red retention time (NRRT).

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The laboratory bait-lamina test was performed as an adaptation of Helling et al. (1998). Containers were used with 350–400 g of soil, four bait-laminas and six earthworms, in each of the four replicates per treatment per block. The bait-lamina is a plastic strip perforated at 5-mm distances with 16 small (1-mm diameter) holes, filled with cellulose, wheat bran and activated carbon. After exposure for three days, the number of pierced holes in each lamina was counted.

Field assays to determine the effects of chlorpyrifos on organic matter decomposition included the litterbag test and the bait-lamina test. The litterbag test was performed according to Ingelsfield (1989) using bags filled with lucerne (*Medicago sativa* L.), with mesh size of either 0.2 mm (microflora, microfauna and mesofauna allowed), or 3.6 mm (microflora, meso- and macrofauna allowed). Litterbags were buried at 10 cm depth in the soil, before treatments with the pesticides (two replicates per treatment per block). Litterbags were sampled after 97 and 118 days. The contents were dried (50ºC, 48 hours) and weighed. Organic matter breakdown was calculated as the percentage of organic material mass loss at the sampling
time \( t \) [(start weight \( - \) end weight) \( \times \) 100/start weight]. Bait-lamina test was performed according to Von Törne (1990). Sixteen bait-lamina strips (as a 4x4 matrix) were inserted vertically into the top soil layer (three replicates per treatment per block). After 14, 97, and 118 days of exposure, laminas were removed from the soil and examined. The number of pierced holes in each lamina was counted.

To study natural earthworm populations, formalin (0.2\%) was used to extract them from the soil. Aclitellate and clitellate earthworms were counted and taxonomically identified.

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). Means were compared by one-way ANOVA (parametric) or nonparametric Kruskal-Wallis tests. Tukey-Kramer or the nonparametric Dunn's test was applied for post-hoc comparison of means. Linear correlations were performed using the Pearson linear correlation test. For avoidance experiments, pair-wise Student's \( t \) test was used (one-tailed test for control-treated experiments; two-tailed test for dual control tests) (da Luz et al., 2004; International Organization for Standardization, 2008; Marques et al., 2009).

Results and Discussion

Soils treated with 240 g ha\(^{-1}\) a.i. chlorpyrifos showed the same average concentration (0.02±0.01 mg kg\(^{-1}\)) after 1 or 14 days of treatment application. In plots sprayed with 960 g ha\(^{-1}\) a.i., concentration was 0.10±0.02 mg kg\(^{-1}\) after 1 day, and 0.03±0.01 mg kg\(^{-1}\) after 14 days from treatment application.

Chlorpyrifos was neither detected in soils sampled 97 days after treatment, nor in the control plots (detection limit: 0.01 mg kg\(^{-1}\)). As chlorpyrifos soil concentration was similar for both treatments after 14 days, and due to the large number of soil samples to be processed, laboratory bioassays were only performed on samples of soils sprayed with the high rate of pesticide. Lysosomal neutral red retention time was significantly reduced in earthworms exposed for seven days to chlorpyrifos low-rate and high-rate treated soils, collected one day after spraying, when compared to controls (Figure 1). A significant concentration-response relationship was observed (\( r = 0.888, p<0.001 \)). The reduction was also observed in earthworms exposed to soils sampled 14 days after treatment.

Neutral red retention assay has been used to assess concentration-response relationships for a variety of metals and organic compounds (Svendsen et al., 2004; Gastaldi et al., 2007). Few studies, however, showed effects on neutral red retention time in earthworms exposed to chlorpyrifos contaminated soils (Hodge et al., 2000; Casabé et al., 2007; Reinecke & Reinecke, 2007). Our results showed that the neutral red retention time was sensitive to the low chlorpyrifos concentrations present in soil, and may constitute an early indication of impending physiological damage in the earthworms.

Chlorpyrifos produced a significant increase in damage index with respect to controls in coelomocytes of earthworms exposed to soils sampled one day after treatments (Figure 2). A significant increase was also observed on high-rate treated soils collected 14 days after spraying, although DNA migration was lower. In all experiments, the viability of cells was approximately 95\%. Damage to DNA, as measured with comet assay, may lead to mutations, strand breaks, altered bases and, finally, health disorders, resulting in severe disturbances in ecosystems which may lead, in some cases, to an elevated extinction risk of sensitive species.

Although comet assay is considered an important biomarker of DNA damage in earthworm ecotoxicology (Fourie et al., 2007), few studies have been reported on terrestrial environments (Martin et al., 2005; Xiao

![Figure 1. Neutral red retention time, in coelomocytes of *Eisenia andrei* exposed for seven days to control and chlorpyrifos-treated soils, sampled at different times after treatments. Mean±SD (three earthworms per treatment per block). Different letters denote significant differences between groups.](image-url)
The comet assay applied on earthworm coelomocytes showed a high level of DNA damage, by exposure to chlorpyrifos treated soils. Neutral red retention and comet assays revealed alterations at subcellular level, and can be regarded as indicators to be used in the assessment of soil earthworms health. Previous results on a soybean field of the Santa Fe Province, sprayed with chlorpyrifos, showed a similar pattern in both cellular biomarkers (Casabé et al., 2007). In terms of the avoidance, earthworms exposed to soils sampled 14 days after treatment had varied results: when exposed to soils from block 1, earthworms preferred the treated substrate; in contrast, worms tended to avoid treated soils from blocks 2 and 3 (Figure 3). The responses were statistically significant. Soil physicochemical analysis showed no differences between blocks. No significant difference was found in the distribution of the worms between both chambers of the containers in dual control tests, which indicates an even distribution of individuals among the two sections of the test container.

Avoidance behaviour is an ecologically relevant endpoint, directly related to the energy budget of the worms, and indirectly to the soil structure. Exposure to pesticides that alters earthworm behaviour can induce migratory behaviour, which can lead to modifications in population abundance or biomass, and to changes in species diversity. Although avoidance tests with earthworms have been considered as suitable screening tools for the assessment of potentially contaminated soils (da Luz et al., 2004; Sousa et al., 2008), few studies were done with chlorpyrifos treated soils (Hodge et al., 2000; Zhou et al., 2007). The results of this study showed that the avoidance behaviour was not a sufficiently sensitive endpoint for assessment of the effects of chlorpyrifos on the earthworms.

Bait-lamina laboratory test showed a nonsignificant decrease in the bait consumption of earthworms exposed to treated soils collected 1 and 14 days after spraying, compared to controls (Figure 4). These results contrast with those reported previously for a soybean field on a clay silty soil sprayed with chlorpyrifos (Casabé et al., 2007). Physical structure and chemistry of the soils have a strong influence on this functional endpoint, and could account for the observed differences. In field experiments, feeding activity of soil macrofauna assessed with the field bait-lamina test increased with increasing exposure time (Figure 5). Fourteen days after introduction of the bait-laminas into soils, the substrate consumption rates, measured as the percentage of open holes in the bait-laminas, were very low, probably as a consequence of the low density of earthworms and of the short exposure time. No significant differences between treated and control soils were observed. Feeding activity was significantly decreased in both treatments, compared to control soils, after 97 days of exposure; however, no concentration-dependent relationship was observed. After 118 days exposure, a nonsignificant decrease was observed.

Chlorpyrifos treatment of the soil did not affect litter decomposition, after 97 or 118 days of the litterbag exposure in the field (Figure 6). The overall litter
decomposition rate was very slow. No difference was detected between the decomposition rates of the leaves enclosed in the fine and coarse mesh litterbags. Decomposition of organic matter is one of the most integrating processes in the soil ecosystems. Pesticides affecting this function might adversely influence nutrient cycling and soil fertility (Förster et al., 2004). The two assayed field methods showed a different pattern of response that could be attributed to the different quality and way of exposure of the organic matter used (Knacker et al., 2003). Besides, both functional endpoints are closely related to soil moisture content and abundance of earthworms in soil (Förster et al., 2004).

The number of earthworms was very low in the first 14 days of the experiment – Octolasion cyaneum (Lumbricidae) and Microscolex dubius (Acanthodrilidae) occurrences –, and no significant differences between control and treated soils were observed. No earthworms were found at the subsequent sampling dates.

**Conclusions**

1. The neutral red retention time and comet assays are sensitive biomarkers of chlorpyrifos effects, on the exposed earthworm Eisenia andrei.

2. The earthworm avoidance test was not sufficiently robust for assessing the effects of chlorpyrifos on Eisenia andrei.

3. Feeding activity of soil biota, assessed by the bait-lamina test, is significantly inhibited by chlorpyrifos after 97 days, but recovers by the 118th day of the test.

4. Chlorpyrifos treatment of the soil does not affect litter decomposition after the 118-day exposure of the litterbag in the field.

5. The earthworm abundance was too low to adequately test the sensitivity of this assessment endpoint.

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**References**


