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# Effects of Glyphosate and 2,4-D on Earthworms (*Eisenia foetida*) in Laboratory Tests

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**Abstract** Laboratory tests were conducted to compare the effects of various concentrations of glyphosate and 2,4-D on earthworms (*Eisenia foetida*) cultured in Argissol during 56 days of incubation. The effects on earthworm growth, survival, and reproduction rates were verified for different exposure times. Earthworms kept in glyphosate-treated soil were classified as alive in all evaluations, but showed gradual and significant reduction in mean weight (50%) at all test concentrations. For 2,4-D, 100% mortality was observed in soil treated with 500 and 1,000 mg/kg. At 14 days, 30%–40% mortality levels were observed in all other concentrations. No cocoons or juveniles were found in soil treated with either herbicide. Glyphosate and 2,4-D demonstrated severe effects on the development and reproduction of *Eisenia foetida* in laboratory tests in the range of test concentrations.

**Keywords** Terrestrial ecotoxicology · Earthworm · *Eisenia foetida* · Contaminated soils · Herbicides

Earthworms are important members of the soil fauna and possess a number of characteristics that make them appropriate organisms for use in assessing potential risks of contaminated soils. Earthworms are affected by a variety of organic and inorganic compounds, which may cause bioaccumulation. They are also important in the terrestrial trophic system, constituting a food source for a wide variety of organisms, including birds, mammals, reptiles, amphibians, fish, insects, and microorganisms.

*Eisenia foetida* has been chosen as a test species because: (1) it reproduces easily in the laboratory; (2) it is the most widely used species in laboratory experiments; (3) it was approved by the European Union and OECD for use in toxicity tests; and (4) it has been used by the U.S. Environmental Protection Agency (EPA) as a sweeping test for contaminant residues in several polluted sites.

Toxicity studies with earthworms are still scarce in Brazil. Preliminary results indicate that the behavioral test can serve as a rapid indicator of the toxicity of contaminated soils and can be used as a complementary test for risk assessment of polluted areas (Sisino et al. 2006).

Pesticides are important pollutants of natural habitats. Among the pesticides used in Brazil, glyphosate and 2,4-D are the most widely used herbicides. Glyphosate is a broad-spectrum, post-emergence, non-selective aminophosphonate-type herbicide. In the early twenty-first century, glyphosate became the largest-selling single crop-protection chemical product on the market (Woodburn 2000). Glyphosate is generally regarded as an environmentally friendly herbicide due to its biodegradation and strong adsorption to soil (Barja & dos Santos Alfonso, 2005; Vereecken 2005). However, evidence from some experiments suggests that glyphosate reduces the growth of *A.caliginosa* when applied repeatedly to laboratory cultures at 2-week intervals, at a rate lower than commercially recommended (Springett and Gray 1992), but had no effect on *A. caliginosa* in another pot experiment where the chemical was mixed with soil (Martin 1982). Another study suggests that glyphosate, even at the recommended field dose, can cause cell death and interfere with non-specific esterase activity in the intestinal epithelial lining of

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*P. elongate*, causing at least 50% mortality in this worm population (Morowati 2000).

2,4-D is a widely used herbicide in Southern Brazil due to its low cost and good selectivity. This herbicide has poor biodegradability and has been detected frequently in water courses (Chingombe et al. 2006). However, this pesticide causes several metabolic alterations and tissue necrosis in non-target organisms, including important members of the food chain such as fish (Gallagher and Di Giulio 1991). On the other hand, some authors have found that the acute risk to earthworms from use of 2,4-D is low (Dalby et al. 1995). Earthworms may be exposed from either single or multiple applications of 2,4-D to a wide variety of crops, but in particular from its use on pasture and turf. A laboratory toxicity study using the dimethylamine salt of 2,4-D on *Eisenia foetida* reported a 14-day LC50 of 350-mg/kg soil. This test was conducted by mixing a concentration of test substances in soil (WHO 1997).

The concentrations used in this experiment had been treated previously as recommended agricultural rates with the commercial product. However, depending on the specific weed control and management, the herbicide effect may be intensified due to the increased dose and the number of applications.

The current study aimed to test the effects of glyphosate and 2,4-D, two pesticides extensively used in Brazilian agriculture, on the growth and survival of *Eisenia foetida*.

## Materials and Methods

Glyphosate [N-(phosphonomethyl)glycine] purity 99.7% and 2,4-D [2,4-dichlorophenoxyacetic acid] purity 99% were obtained from SIGMA CHEMICAL and ACROS ORGANICS, respectively.

Soil samples used in the experiments were collected in the experimental area of Embrapa Agrobiologia, located in the municipal district of Seropédica (Rio de Janeiro) and classified as Argissol (EMBRAPA 1997). The source was selected as one of the most representative soils in Brazilian territory. The soil is used extensively in corn, soy, sugarcane, and other crops in tillage and non-tillage systems. The soil samples showed the following characteristics: 608 g/kg sand; 112 g/kg silt; 280 g/kg clay; 10.8 g/kg organic carbon; and pH 5.5.

The *Eisenia foetida* earthworms were obtained from the Minhocário Arborium (RJ). They were carefully transported to the laboratory and acclimatized for 4 weeks before any experiment, at 20°C ± 2, in bovine manure. All tests were performed using adult earthworms (age less than 2 months and clitella well developed) and with individual weight from 300 to 600 mg (ISO 11268-1 1993).

For the experiments, 10 earthworms were transferred to each container with 400 g of soil prepared by adding different concentrations of glyphosate and 2,4-D (dry weight basis). Four replicates were analyzed for each concentration and 4 control containers, prepared under identical conditions without the addition of the target pesticides. The concentrations used were 1; 10; 100; 500; 1,000 mg/kg. The containers were covered with paper-filter with holes to maintain aeration conditions during the 56 days of the test. Soil moisture was standardized at 60% of maximum water-holding capacity, and the samples were maintained at room temperature (20 ± 2°C) in the presence of light. During all the experiments, moisture content was checked and maintained at 60% by adjusting the weight of the container against the weight known from the previous week prior to sampling. After 14, 21, 28, 42, and 56 days of incubation, the containers were opened, surviving and dead earthworms were counted, and the survivors' average weight was verified. Earthworms were classified as dead when they did not respond to a gentile mechanical stimulus and morphological abnormalities were recorded. For the reproduction test, cocoon production and numbers of hatched juveniles were hand-sorted and returned and further incubated during the experiment (ISO 11268-2 1998).

All the data were analyzed by ANOVA using Tukey's test for comparing the treatments and duration of exposure for each herbicide and control groups. The results were expressed as means ± SD (standard deviation). Statistical significance was set at $p < 0.05$.

## Results and Discussion

The two herbicides showed different effects on the earthworms. In the growth test, earthworms from the soil treated with glyphosate showed a gradual reduction in mean weight during the experiment. The effect was observed at all test concentrations when compared to untreated soil (Fig. 1). The percentage of weight loss at the end of the experiment was approximately 50% of baseline weight. All earthworms were classified as alive at all moments of sampling. The growth rates of untreated worms were considered non-significant during the 56 days of the experiments. The average biomass of untreated worms was the same. In contrast, worms from glyphosate-treated soil samples, in terms of time dependency, were significantly different ($p < 0.05$) from the controls, whereas no concentration dependency was observed with this chemical.

For 2,4D, 100% mortality was observed a few hours after exposure of those organisms in soil treated with 500 and 1,000 mg/kg. After 14 days, mortality was around

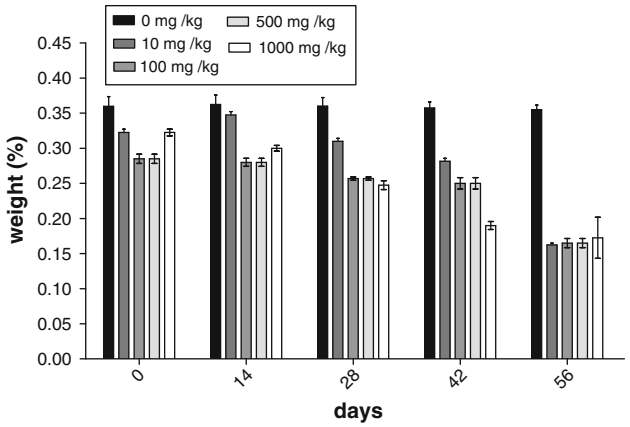


**Fig. 1** Effect of Glyphosate soil concentration and exposure time on *Eisenia foetida* biomass

30%–40% of specimens in all the other concentrations. A gradual reduction in the mean weight of exposed worms (<30%) was also observed after 56 days in soils containing 2,4-D concentrations of 10 and 100 mg/kg, when compared to the soil controls (Fig. 2). The general tendency of these results showed that the decrease in weight was time- and concentration-dependent over the 56 days of exposure, which indicated statistical significance for both parameters ($p < 0.05$).

Comparing the results of the growth tests in soils treated with the two pesticides (glyphosate and 2,4-D), although glyphosate did not kill the test organisms in the range of test concentrations, the decrease in mean weight may indicate a chronic effect of this herbicide. In soils treated with 2,4-D, the observed effects (death of specimens after a few hours of exposure and loss of weight) are consistent with acute toxicity symptoms.

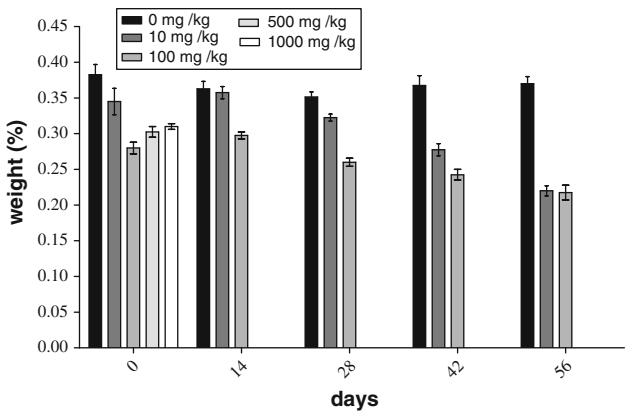


**Fig. 2** Effect of 2,4-D soil concentration and exposure time on *Eisenia foetida* biomass

The reduction in mean weight of worms exposed to soils treated with 2,4-D (≤30%) when compared to those from soil treated with glyphosate (50%) can be explained by the morphological changes caused by the herbicides. In the presence of 2,4D, the emergence of abnormal swelling in the clitellar region of live worms increases their body volume and consequently their mean weight.

Significant anatomical changes were observed at 30 days (Fig. 3). Morphological abnormalities like elevating the body, coiling, and curling were observed in all specimens exposed to the highest concentrations of glyphosate and 2,4D after 30 days of exposure. Similar abnormalities were also observed in the organisms exposed for 50 days at lower concentrations. Excessive mucous secretion was noticed in all exposed worms. The exposed worms were also much less active than the controls. Approximately 65% of exposed worms displayed surface lesions and extrusion of coelomic fluid resulting in bloody lesions on the posterior part of the body and resulting in death. In soil treated with glyphosate, morphological changes (ruptures in the body wall and bloody lesions) were also observed in the midsection of the earthworm bodies (Fig. 3a–c). Fragmentation of the body was also observed as a result of these changes. Fifty percent of all treated worms developed breakages and shedding of cuticle (dried), which resembles ecdysis (shedding of skin) in insects and snakes (Fig. 3c).

In soil treated with 2,4-D, about 80% of exposed worms developed constrictions and swelling in the clitellar region (Fig. 3e, f), and most of the worms displayed multiple ruptures in the rectal region, with collapsed annular segment (Fig. 3d).

Table 1 shows the results of the reproduction tests in the soils treated with glyphosate and 2,4-D. No cocoons or juveniles were found in any experiment using soil containing the target herbicides. For both herbicides, reproduction was always lower than 20% when compared to the control, so that according to Dechema (1995), both can be classified as toxic. Despite the low reproduction observed in the controls, the absence of cocoons or juveniles in soils treated with both glyphosate and 2,4-D may result from interference by these substances in the earthworms' reproductive mechanism. The significant reduction ($p < 0.05$) in cocoons and juveniles was observed in all treated worms for both herbicides, compared to untreated worms. Additional studies are required to fully understand this effect. In addition, loss of weight was observed in the earthworms at the end of the experiment as compared to the controls, suggesting a significant toxic effect.

Soil ingestion and dermal absorption are the most important intake routes of soil pollutants by earthworms. In the present study, when the earthworms were introduced

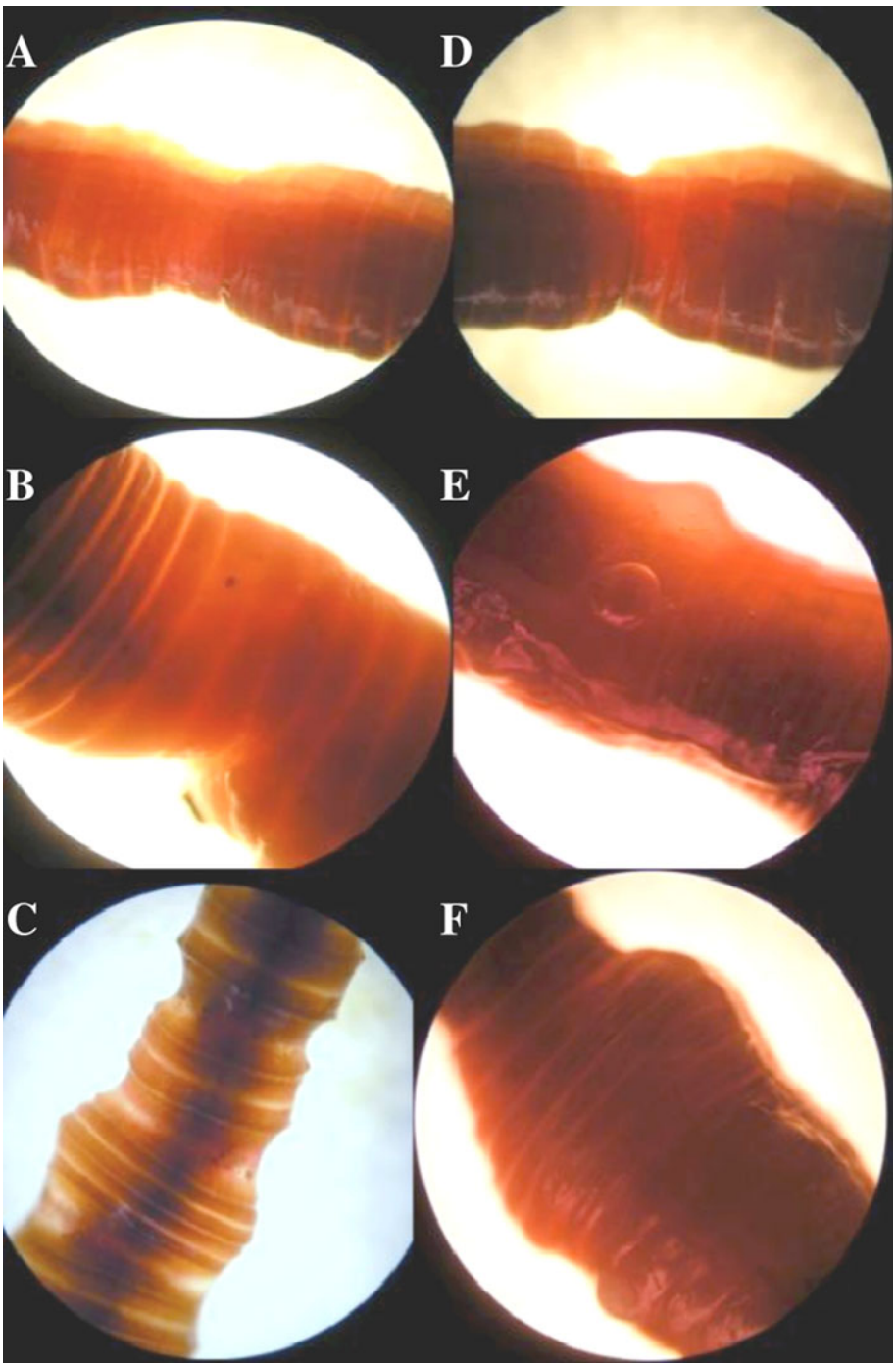


**Fig. 3** Effect of glyphosate and 2,4-D on morphology of the earthworm, *E. foetida*, during 60 days of exposure. **a** Self-protection mechanism of anterior fragmented portion; **b** Loss of mucous and shedding of cuticular membrane; **c** Body constrictions and degeneration on posterior end; **d** Fragmentation on posterior region, attached to tiny portion of denatured body; **e** Coiling of earthworm along with abundant mucous; **f** Abnormal swelling on clitellar region and ruptures on body surface

into soils contaminated with 2,4-D, they remained on the surface, with death following after a few days (avoidance behavior). This behavior was not observed in soils treated with glyphosate.

Glyphosate and 2,4-D demonstrated severe effects on the development and reproduction of *Eisenia foetida* in laboratory tests. The toxic effects observed after exposure to soils contaminated with 2,4-D were much more severe than those observed with exposure to glyphosate at the same concentration. Meanwhile, long-term exposure (56 days) to soil contaminated with glyphosate demonstrated a toxic effect on normal development and reproduction of *Eisenia foetida*, indicating that this substance may have significant toxic effects on soil biota. A field study is being conducted at this laboratory to confirm these results in earthworm populations under natural conditions.

**Table 1** Mortality (% of dead animals related to numbers of tested specimens) and reproduction test (total numbers of cocoons (28d) and hatched juveniles (56d))

These results were obtained from 4 replicates (n = 40 worms)

| Conc. mg/kg | Mortality (%) | Reproduction test | | Conc. mg/kg | Mortality (%) | Reproduction test | |
|---|---|---|---|---|---|---|---|
| | | Cocoons | Juveniles | | | Cocoons | Juveniles |
| Glyphosate | | | | 2,4-D | | | |
| 0 | 0 | 7 | 4 | 0 | 0 | 5 | 2 |
| 10 | 0 | 0 | 0 | 10 | 30 | 0 | 0 |
| 100 | 0 | 0 | 0 | 100 | 40 | 0 | 0 |
| 500 | 0 | 0 | 0 | 500 | 100 | 0 | 0 |
| 1,000 | 0 | 0 | 0 | 1,000 | 100 | 0 | 0 |

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