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Experimental study of the effect of a glyphosate-based herbicide on a species of earthworm *Aporrectodea caliginosa* (Savigny, 1826)

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Abstract: This work aims to demonstrate the effect of a glyphosate-based herbicide on a species of earthworm, *Aporrectodea caliginosa* (Savigny, 1826) to provide scientific justification for the species rarity in cultivated environments where this phytosanitary product is widely used. During the fifty-two days of contamination in the laboratory, the life traits monitored and evaluated were growth and mortality. Herbicide concentrations ranging from the lowest (C30) of around 240 l kg^{-1} to the highest (C210), equivalent to 1840 l kg^{-1} were tested. The study found that the herbicide is toxic to the A*porrectodea caliginosa* species, causing growth to slow and mortality to rise as concentrations rise. We report a delayed negative effect at low concentrations, which appears after several weeks of product exposure, depending on the exposure time. However, at the highest concentrations of the herbicide studied, the early negative effect is visible after the second week of exposure.

Key words: earthworm, *Aporrectodea caliginosa*, formulation, glyphosate, dose, toxicity

Raziskava učikov herbicidov na osnovi glifosata na vrsto deževnika *Aporrectodea caliginosa* (Savigny, 1826)

Izvleček: Namen raziskave je bil prikazati učinek herbicidov na osnovi glifosata na vrsto deževnika, *Aporrectodea caliginosa* za znanstveno potrditev ogroženosti te vrste v urbanem okolju, kjer se ta herbicid veliko uporablja. Med dvainpetdesetimi dnevi zastrupljanja v laboratoriju sta bili spremljani in ovrednoteni rast in mortaliteta. Preiskušene koncentracije herbicida so bile v območju od najmanjših, C30, okrog 240 l kg^{-1}, do največjih, C210, ki so ustrezale 1840 l kg^{-1}. Izsledki raziskave so pokazali, da je herbicid strupen za to vrsto in povzroča upočasnjevanje rasti in veča njeno mortaliteto s povečevanjem koncentracije. Opaženi so bili tudi negativni učinki manjših koncentracij, ki so se pojavili več tednov po izpostavitvi in so bili odvisni od časa izpostavitve. Pri večjih koncentracijah herbicida so bili negativni učinki vidni že po dveh tednih izpostavitve.

Ključne besede: deževnik, *Aporrectodea caliginosa,* odmerki herbicida, glifosat, doza, strupenost

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1 INTRODUCTION

Earthworms play an important role in many soils. They are commonly called "ecosystem engineers" due to their importance in pedogenesis and soil profile development. Their actions have an impact on the physical, chemical, and microbiological properties of the soil, as well as the activity of other soil organisms (Bartlett, 2010). Herbicides negatively impact them through the water that soaks into the soil (Mamy et al., 2011). Despite the efficacy of glyphosate-based herbicides (GBH) in controlling weeds in agricultural and non-agricultural soils, their residues in the soil system have long been a concern (Owagboriaye et al., 2020). After spraying, glyphosate is distributed between the plant, the soil, and the atmosphere (Beckert et al., 2011). It attacks specific rhizosphere functions, which can have environmental consequences due to its immobilization via cation chelation; it is thus highly stable and not easily degradable (Huber et al., 2005). Earthworms exposed to glyphosate show a decrease in growth rate and a tendency to avoid treated areas (Casabé et al., 2007; Gill et al., 2018).

Previous research has shown that certain earthworm species, such as *Octolasion tyrtaeum* (Savigny, 1826) and *Lumbricus terrestris* L., 1857, are sensitive to glyphosate. Other species, such as *Esisenia fetida* (Savigny, 1826) and *Aporrectodea caliginosa* (savigny, 1826), were less sensitive to glyphosate or not at all (Correia, Moreira, 2010; Domnguez et al., 2016; Garca-Torres et al., 2014; Gaupp-Berghausen et al., 2015). The species *Aporrectodea caliginosa* is declining in our country's cultivated land (vegetable and cereal crops), leading us to believe that it is sensitive to pesticides, particularly glyphosate-based pesticides, which are the most widely used in Algeria (Oultaf et al., 2022). To provide scientific answers to the species' scarcity in cultivated environments due to glyphosate-based pesticides, laboratory work was carried out in which the species' life history (mortality and growth) was studied. The current study focuses on two important points: demonstrating the sensitivity of *Aporrectodea caliginosa* to the herbicide glyphosate and determining the species' toxic concentration range.

2 METHODS

2.1 COLLECTION OF EARTHWORMS

The earthworms used in the experiment were *Aporrectodea caliginosa*, collected in sufficient quantity (to avoid the laboratory rearing stage) on a lawn far from any source of pollution (healthy soil) in Beni Douala, Kabylia. We extracted the earthworms using Bouché's (1972) physical method. The adult worms (clitellum clearly visible) were collected after the species *Aporrectodea caliginosa* was identified, primarily using morphological criteria that vary greatly between species. Two hundred and ten healthy earthworms were sent to the laboratory in preparation for herbicide exposure. At the laboratory, the individuals were introduced into a large plastic tray containing soil from the same sampling station, moistened and without any additions. For a two-day acclimatization period, the tray was kept in the dark at room temperature (22-23 °C)

2.2 SOIL SAMPLING AND PREPARATION

The soil used in the experiment is from the same worm sampling station, and we chose to use soil from the same station without any additions to get as close to natural conditions as possible. According to Chevillot (2017), using real soil in toxicological tests appears to be the most relevant choice for getting as close to a system representative of the environment as possible. After freeze-drying, the sampled soil was transported to the laboratory in plastic boxes and sieved to 2 mm to ensure an easy-to-handle texture and a suitable fraction for physicochemical analysis. According to the preliminary analysis, the soil was clayey in texture, with a slightly acidic pH (6.06) and 4.85 % organic matter.

2.3 PREPARATION OF TEST CONCENTRATIONS

Glyfozell 36SL®, a liquid formulation (soluble concentrate) containing glyphosate as the active ingredient, was used as the herbicide. The glyphosate content of one liter of commercial product (360 g l^{-1}) and the maximum authorized agronomic dose (6 l ha^{-1}) was used to calculate the amount of product to be mixed with the soil. Eight concentrations (C0, C30, C60, C90, C120, C150, C180, C210) were considered, each representing a quantity of product in l.

2.4 SOIL CONTAMINATION

The pre-prepared soil (40 kg) was distributed over forty-one-kilogram boxes of soil; into each box, a precise quantity of the product was added by spraying to ensure good distribution of the product in the soil, which was then well stirred by hand for better homogenization, and left to stabilize overnight.

2.5 EXPERIMENTAL DESIGN

The earthworms (200 individuals) are rinsed with distilled water, weighed, and distributed at a rate of five individuals per box and five replicates per concentration; to keep the soil moist, the soil surface is moistened each time, if necessary, with spring water to approximate natural conditions as closely as possible. The experiment was carried out in the dark and at room temperature. To avoid altering the worms' behavior or the concentrations tested, no additional food was added during the test (Fig. 1).

2.6 TOXICITY ASSESSMENT

Earthworms were removed from each box once a week for 52 days, counted, weighed, and returned to the box. Mortality and growth mass were also assessed.

2.7 GROWTH RATE (MASS)

The average mass was recorded at each exposure time interval allowed us to calculate the relative growth rate, which we calculated using the Martin (1986) equation:

$$Relative\ growth\ rate = ln\ \frac{P_m}{P_0} * 100$$

(1)

P_0 is the average mass of earthworms before exposure to a concentration, and P_m is the average mass of earthworms after exposure to glyphosate at each concentration.

2.8 MORTALITY

The number of dead earthworms in each box was used to calculate mortality. Earthworms were considered dead when an advanced body alteration was observed, and the individual did not respond to a stimulus. Throughout the experiment, abnormal behaviors such as restlessness and coiling were assessed.

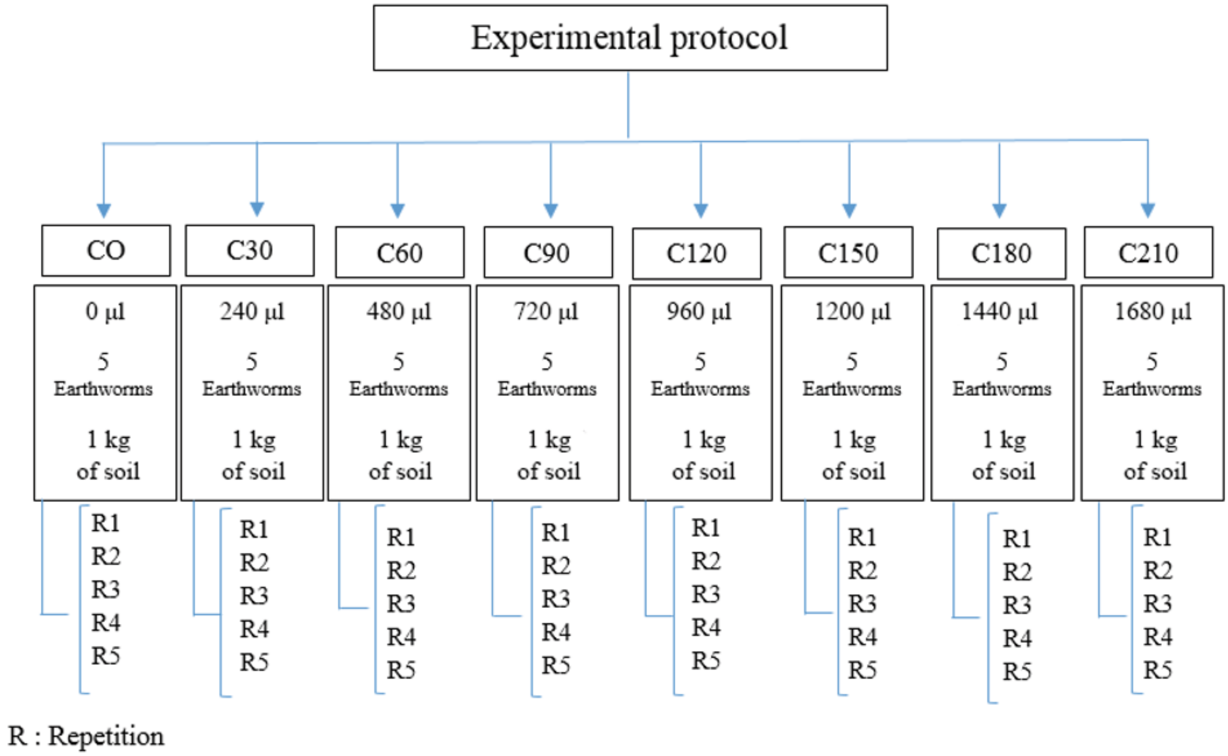

Figure 1: Summary diagram of the experimental protocol

2.9 DATA ANALYSIS

Multiple regressions were calculated using R software version 3.3.4 for both growth and mortality as functions of glyphosate concentration and exposure duration of earthworms.

3 RESULTS

3.1 RELATIVE GROWTH

The results of the multiple regression analysis conducted to explain the evolution of growth rate as a function of exposure duration and herbicide concentration are provided by the equation:

Growth = −0.0093 (Time) −0.00073 (Concentration) + 0.79

The low probability value (*p*-value: 1.351e-10) indicates that the regression model is significant. The coefficient of determination (0.5097) showed that the quality of the fit was good. Therefore, growth decreases as concentration increases and exposure is prolonged.

The results show that after the eighth day of pollutant exposure, there is difference in the growth of control worms. These differences were observed in comparison to the first day of the experiment and all other periods from day 16 to day 40. Indeed, these periods are distinguished by a mass loss from day eight to day forty-three. After 52 days of experimentation, a resumption of growth is observed for the control, as evidenced by an increase in mass (Fig. 2).

The results of earthworms exposed to various glyphosate concentrations show mass gain during the first week of exposure for all concentrations ranging from C30 to C210. These findings suggest that *Aporrectodea caliginosa* species can tolerate glyphosate concentrations equivalent to 1680 µl kg^{-1} soil for a short period (one week). Overall, a relative and gradual decrease in mass was observed beginning on the eighth day of exposure for all concentrations, except for concentrations C60, C150, and C210, where the decrease in mass began on the sixteenth day of exposure. Glyphosate has a negative effect on growth starting on the sixteenth day of exposure for concentrations C120 and C180 and on the twenty-fourth day of exposure for concentrations C150 and C210. After several weeks of exposure, the negative effect of contamination on growth is demonstrated for concentrations below C180, particularly the lowest C30 (240 µl kg^{-1}). However, high concentrations have a negative impact on earthworm growth after only a short period of exposure.

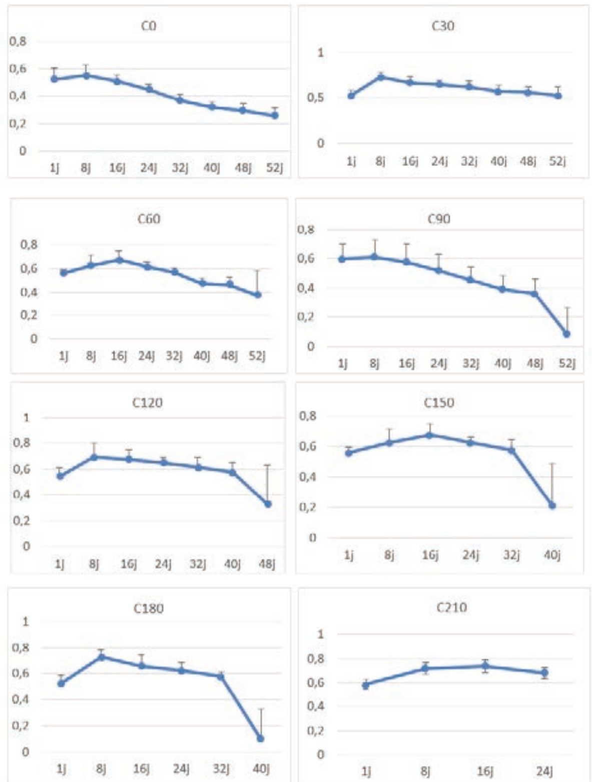

Figure 2: Variations in relative growth rate (%) as a function of time (j = day) and concentration

The multiple regression calculated to explain the evolution of mortality rate as a function of exposure duration and herbicide concentration is given by the equation:

Mortality = 0.078 (Time) + 0.0126 (Concentration) −2.17

The low probability value (7.321e-15) indicates that this regression model is significant. The coefficient of determination (0.6447) showed that the quality of the fit was good. Therefore, mortality increases as concentration increases and exposure is prolonged.

The results show that no mortality was observed for the control (C0 = 0 µl) for the entire duration of the experiment (52 days) (Fig. 3). For all concentrations tested, there was a very marked effect of time and degree of contamination on the mortality rate of *Aporrectodea caliginosa* earthworms. The lowest concentrations (240 µl kg^{-1}, 480 µl kg^{-1} and 720 µl kg^{-1}) had a delayed lethal effect, starting only on the forty-eighth day of exposure to the pesticide, with mortality rates of 1 ± 0.707, 2.2 ± 0.836 and 3 ± 0 respectively. This lag time decreases with increasing concentrations. In fact, at the highest concen-

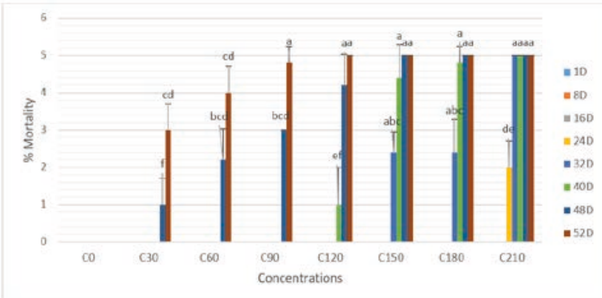

Figure 3: Mortality rate (number of individuals) of *Aporrectodea caliginosa* earthworms as a function of time and glyphosate concentration.

trations (1200 µl, 1440 µl, 1680 µl), the lag time is much reduced, resulting in 100 % mortality as early as day 32 of glyphosate exposure. According to the findings, mortality increases with time and concentration.

4 DISCUSSION

This ecotoxicity study aims to determine the toxicity of a glyphosate-based herbicide on *Aporrectodea caliginosa* earthworms. This species is uncommon in agricultural plots where the herbicide of choice is widely used. The biological parameters considered were mortality and growth. Throughout the experiment, no mortality was recorded for the control worms, indicating that the experimental conditions, aside from glyphosate contamination, did not contribute to earthworm mortality. Over the forty-eight days of experimentation, control earthworms showed a slight decrease in initial biomass. Conversely, the controls showed a significant mass recovery (0.266 ± 0.044 to 0.357 ± 0.181) at the end of the experiment.

The findings show that glyphosate is toxic to *Aporrectodea caliginosa* even at low soil concentrations. This toxicity is observed at low concentrations of around 240 µl kg^{-1} soil (C30). Toxicity levels vary according to exposure time and pollutant dose. Mass loss in contaminated individuals is significantly greater than in the control group. As a result, the sensitivity of *Aporrectodea caliginosa* to glyphosate was confirmed. Observing at least one death for low concentrations (C30) after forty-eight days of experimentation indicates that each concentration was potentially lethal for *Aporrectodea caliginosa* in this study. The worms' inability to resist the herbicide's lethal effects, particularly at high doses, may explain why mortality increased proportion to herbicide concentration. This finding is consistent with the findings of Casabé et al. (2007), who demonstrated that glyphosate is highly toxic to earthworms at recommended concentrations. Furthermore, for all doses above 960 mg kg^{-1}, this result could explain the total death of adults (100 % mortality) on the thirty-second day of contamination.

Thus, living in glyphosate-contaminated soil causes the species *Aporrectodea caliginosa* earthworms to lose mass, beginning as early as the first week of exposure to the pollutant. The researchers hypothesize that worms living in uncontaminated soil have more metabolic resources than worms living in contaminated soil; worms in uncontaminated soil are thus unaffected by contamination stress and can thus invest in mass gain (Pochron et al., 2017; Pochron et al., 2021). Body mass and survival time are affected by the glyphosate-based formula. Taken together, the data show that increasing glyphosate doses result in a significant decrease in survival time and a significant decrease in body mass when compared to the control. Toxicity studies on *Eisenia fetida* with the same contaminant show a steady decline in body mass in the organisms tested (Yasmin, D'Souza, 2007; Correia, Moreira, 2010). Tolerance to high concentrations of glyphosate was recently discovered in the same species, indicating its use in bioremediation processes (Lescano et al., 2020).

Growth, as measured by body mass, is a common assessment criterion in toxicological studies. However, body mass does not always decrease in response to contamination (Pochron et al., 2018; Pochron et al., 2019). When organisms are exposed to various contaminants, their body mass may increase. Recent research shows that earthworms living in nutrient-rich soil can gain mass even while metabolically processing contaminants (Pochron et al., 2020). However, our findings show that *Aporrectodea caliginosa* does not use this strategy in response to glyphosate. Herbicide concentrations of C30, C60, C90, C120, C150, C180, and C210 were tested on earthworms. Worms exposed to the lowest dose (240 µl kg^{-1}) for 48 days died at a low rate. At concentrations greater than 720 µl kg^{-1}, there was 100 % mortality at the end of the experiment. These findings are consistent with those obtained on the *Octolasion tyrtaeum* (Savigny, 1826): after 32 days of treatment, 100 % mortality was observed at the highest glyphosate concentration (50,000 mg kg^{-1}).

However, most studies on the acute effects of glyphosate at various concentrations on *Eisenia fetida* show that no product dose results in mortality. The effect of glyphosate-based herbicides on other earthworm species, *Lumbricus terrestris* and *Aporrectodea caliginosa* reveals that *L. terrestris* activity decreased three weeks after herbicide application. However, no change in activity was observed for *A. caliginosa* species (Gaupp-Berghausen et al., 2015), confirming that not all earthworm species respond similarly to soil contamination by glyphosate-based formulations and that the species *Aporrectodea caliginosa* exhibits a clear sensitivity to this herbicide. In addition to mass loss and mortality, individuals exposed to glyphosate showed behavioral abnormalities such as coiling and excitation, even at low concentrations.

Previous research on the toxicity of glyphosate indicates that while it does not directly cause earthworm mortality, it can have serious long-term consequences (Verrell, Van Buskirk, 2004). Sublethal effects on the population dynamics of the *Eisenia fetida* species exposed to glyphosate were observed, as evidenced by decreased cocoon fertility. This resulted in the extinction of the soil's earthworm population (Santadino et al., 2014). Anatomical changes were also observed after 30 days of experimentation. According to Correia, Moreira (2010), morphological abnormalities such as body elevation and curling were observed in all specimens exposed to high concentrations of glyphosate in soil. As a result, our findings support previous findings that different agrochemicals affect different earthworm body parameters (Van Gestel et al., 1992; Zaller et al., 2021).

Previous studies observed that earthworm biodiversity is reduced in intensively farmed fields (Smith et al., 2008), and laboratory studies on the species *Aporrectodea* and *Allolobophora* sp. conclude that pre-exposure to pesticides in the field enhances earthworm physiological responses (Givaudan, 2014).

Glyphosate is the most widely used herbicide in the world (Müller, 2021). Few experimental studies evaluating its impact on earthworms under field conditions have been carried out. Overall, these studies conclude that there is a high risk of chronic toxicity of these substances on earthworms if recommended doses are not respected (Pelosi et al., 2021). However, studies evaluating the impact of glyphosate-based pesticides under laboratory and controlled conditions are numerous, but their results remain controversial, due to the fact that these studies, do not reflect real field conditions and do not take into account potential variations in abiotic and biotic factors such as temperature and organic matter (Schmidt et al., 2024). In general, recent laboratory results still highlight the negative effect of glyphosate on earthworms, and they recommend further research on glyphosate in the laboratory and in the field, taking all variants into account, to prevent future threats to soil biodiversity from glyphosate (De Lima E Silva & Pélosi, 2024).

The present study will thus contribute to a broader understanding of the impact of glyphosate on soil fauna in general and earthworms in particular.

5 CONCLUSION

A study of the effect of the herbicide glyphosate on the earthworm *Aporrectodea caliginosa* revealed the species' sensitivity to this plant protection product. This sensitivity manifests itself in reduced growth and increased mortality in the species. Glyphosate has a negative effect that is dose and time-dependent. The effect appears after several weeks of exposure to glyphosate at the low concentration C30, equivalent to 240 µl kg^{-1} of product. At high concentrations (C210 equivalent to 1680 µl kg^{-1} product), however, the effect is visible after the second week of glyphosate exposure. According to our hypothesis, the use of glyphosate-based pesticides, in particular, significantly impacts the growth and mortality of the *Aporrectodea caliginosa* species, explaining its low abundance in cultivated land where the product is widely used. We believe this species could be a good candidate for bioindication of soil pollution caused by glyphosate-based pesticides. Its application in soil quality biomonitoring is also suggested.

6 CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

7 DATA AVAILABILITY STATEMENT

Data available on request from the corresponding author.

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