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Glyphosate Sublethal Effects on the Population Dynamics of the Earthworm *Eisenia fetida* (Savigny, 1826)

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Abstract Pesticides' sublethal effects are not regularly taken into account when assessing agrochemical's toxicity. With the objective of detecting chronic, sublethal effects of the widely used herbicide glyphosate, an experiment was performed using the earthworm *Eisenia fetida* as model organism. Earthworm adults were randomly assigned to three glyphosate treatments: control (no glyphosate), regular dose for perennial weeds, and double dose. Six *E. fetida* individuals were placed in each pot. Two random pots were taken weekly from each treatment and the number of adults, individual weight, number of cocoons, and presence and number of young earthworms were recorded. A matrix analysis was performed with the data. The matrix population model built showed that while the control population had a positive growth rate, both glyphosate treatments showed negative growth rates. The results suggest that under these sublethal effects, non-target populations are at risk of local extinction, underscoring the importance of this type of studies in agrochemical environmental risk assessment.

Keywords Ecotoxicology · Chronic effects · Earthworms · Pesticides · Agrochemicals

Capsule Non-target organisms can be at risk of local extinction due to agrochemicals chronic sublethal effects, which are not consistently taken into account in toxicity and risk assessment studies.

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1 Introduction

Since the mid 1990s, the use of genetically modified crops has been rapidly adopted worldwide (Qaim 2005). Argentina is the third producer of transgenic crops in the world, with about 15 % of the global surface dedicated to transgenic crops, only surpassed by the USA and Brazil (James 2011). Out of all the crops, soybean is the one that presents the greatest growth in Argentina. Since the 1970s, the surface has grown steadily. While in 1971, only 37,700 ha were occupied with soybean by 2012, the surface dedicated to this crop was 19.7 million hectares (FAO 2012), reaching almost 66 % of the overall cropping surface in Argentina. The rapid adoption of new technologies (i.e., the use of transgenic soybeans resistant to glyphosate and no tillage) contributed to make the soybean exports and its derivatives one of the main sources of the country's foreign exchange with a contribution of approximately US$**2500** million for 2013. By the 2006 cropping season, almost 100 % of the surface sown with soybean in Argentina was already transgenic (Trigo and Cap 2003). The increase in the use of these

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soybeans resistant to glyphosate-based herbicides, or RR (Roundup Ready®), produced a steady increase in the consumption of this herbicide (Pengue 2000).

The mechanism of action of glyphosate is the inhibition of the 5-enolpyruvilshikimate-3-phosphate synthase enzyme (EPSPs). The inhibition of the EPSPs prevents the formation of enolpyruvil shikimato-phosphate and the biosynthesis of aromatic amino acids (phenylalanine, tyrosine, and tryptophan), which are the precursors of important secondary metabolites in plants, such as lignin, flavonoids, and alkaloids (Pérez-Jones et al. 2005; Wakelin et al. 2004).

Glyphosate deactivates due to its adsorption to soil particles (Sprankle et al. 1975), and its degradation is related to the bacterial activity in the soil, particularly bacteria from the genus *Pseudomonas* (Gimsing et al. 2004). The USA Environmental Protection Agency has reported that its average lifespan is 60 days (USEPA 1999). Cox (1995) presented data collected from different authors which indicate that depending on the soil's characteristics and climatic conditions, the persistence of this compound varies from a few days to 1 year. Glyphosate can also be adsorbed by clays and organic matter; Piccolo and Celano (1994) determined that this herbicide can be transported deep into the soil by humic substances. These soil particles can be toxic for organisms that ingest significant amounts of soil (Welten 2000) during their normal feeding, such as earthworms.

Considering the wide rural extensions that are treated annually with this herbicide worldwide, it is important for ecological risk assessment to study not only the acute but also the sublethal, chronic effects that it has on non-target species (Casabé et al. 2007). This is of special interest for those ecological groups that have a relevant importance for soil conservation and fertility, being earthworms one of the most important of such groups.

Earthworm activity modifies both physical and chemical soil properties, and their abundance and distribution are strongly influenced by the environmental conditions and the ecological state of the system (Edwards and Bohlen 1996; Lavelle and Spain 2001; Lee 1985). They also play a key role for soil functioning, and are therefore used extensively in terrestrial ecotoxicity studies.

Eisenia fetida (Savigny, 1826) is an exotic species in Argentina, native to the Nearctic region and it is found in most Argentinean soils (Momo and Falco 2009). In its adult state, it weighs between 400 and 600 mg, and it is capable of ingesting large quantities of cellulosic matter, being able to ingest up to its own weight per day. Based on its feeding and other habits, it is ecologically classified as an epigean species, a mulch eater, which includes all types of decomposing organic matter. This species has a high reproduction rate as long as the environmental conditions are good, and it reproduces year around, placing a cocoon per individual approximately every 10 days. The incubation time varies between 14 and 44 days, producing more than one descendent per cocoon (from 2 to 20 individuals) (Reynolds 1996; Venter and Reinecke 1988).

E. fetida is also an internationally validated species for ecotoxicological tests (ISO 1993; ISO 1998; OECD 2004) due to its cosmopolitan distribution and its easiness to raise and handle. This species is among the most commonly used one in environmental risk assessment and testing with chemicals such as Imidacloprid and RH-5849 (Zang et al. 2000). In a test with ten organic substances, Neuhauser and Callahan (1990), found that sublethal concentrations (lower than the LC50) of Carbaryl and Dieldrin produce a decrease in the growth rate and reproduction of this earthworm. Tests in microcosmos with Cypermethrin showed that *E. fetida* bioconcentrates this pesticide inside its body by a factor of 2 to 6 and biotransforms 92 % of the absorbed product without presenting adverse effects at the studied concentrations (Viswanathan 1992).

Zoran et al. (1986) also found teratogenic effects that depend on the dosage of the Benomyl fungicide during the regeneration of the posterior segments of these earthworms after amputation.

Toxicology tests are usually carried out as acute (48 or 72 h) or subchronic (7 to 17 days) tests. However, there is an increasing recognition for the need of longer-term tests to determine the chronic and sublethal pesticide effects on non-target populations (Antón et al. 1993; Spurgeon et al. 2004; Venkateswara Roa et al. 2003).

Therefore, the objective of this work was to determine the chronic, sublethal toxic effects of glyphosate in its commercial presentation as Roundup® (Monsanto, SL at 48 %) on populations of *E. fetida* and to evaluate the ecological importance of those effects on earthworms' demographic dynamics.

2 Materials and Methods

2.1 Experimental Design

The test was carried out in pots located inside 1 m×1 m×0.60 m containers, under controlled temperature (25±3 °C), humidity (80 %), and light 12 L:12D conditions. Twenty-four pots were arranged in three treatments. Each pot had a surface of 0.0392 m^2 (28×14 cm), which were filled halfway up with sifted soil from the upper 10 cm of an Argiudol soil (USDA 2010). On the surface of each pot, a layer of finely chopped plant material was added to add fresh organic food for the earthworms. In each pot, six weighed adult specimens of *E. fetida* were placed.

After 6 days of acclimation, glyphosate herbicide was added in its commercial presentation Roundup® (Monsanto, SL, 48 %) with the equivalent dose for perennial weeds (6 L of formulated/ha) and also a double dose (12 L of formulated/ha) as follows: (a) Control (treatment 0): 50 cm^3 of distilled water; (b) dosage 1 (treatment 1): 0.024 cm^3+distilled water to complete 50 cm^3; (c) dosage 2 (treatment 2): 0.048 cm^3+distilled water to complete 50 cm^3. For each treatment, eight replicates were made. Every 10 days, fresh food was added to each pot.

Soil humidity was kept constant (80 %), near the optimal humidity for these earthworms (Reinecke and Venter 1987).

At days 12, 21, 28, and 40 after acclimation, two pots were randomly chosen from each treatment. The samples were manually examined, and the number of adult earthworms, their individual weights, the number of cocoons, and presence and number of young earthworms were recorded. Before discarding the samples, the cocoons were separated and incubated in Petri dishes over humid filter paper, and egg fertility was registered as well.

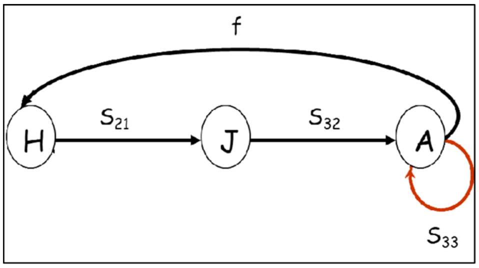
With these data, the following demographic parameters were calculated: survival of adults, fecundity, and cocoon's fertility. Young individuals were found in several pots probably due to cocoons which hatched before each sampling. However, the initial number of youngsters was unknown, and as a consequence to build the matrices, young survival was assumed to be equal to that of the adults (S_{23}=S_{33}).

2.2 Statistical Analyses

The number of earthworms per pot, the total weight per pot, the mean individual weight per pot, the fertility per pot and the number of cocoons per pot, and cocoon fertility were analyzed with ANOVA (α=0.05).

2.3 Population Dynamic Matrix Model

The collected data were used to build a matrix population model (MPM) of the earthworms' dynamic. Matrix models (Caswell 2001) are an adequate tool for studying the effects agrochemicals on non-target populations. A MPM is a population dynamics model which has two main characteristics: first, it is a discrete model (that is, the time is considered as a discrete variable and a fix time step is defined); second, the population is modeled having an age or stage structure, in consequence, the population is represented by a vector containing the abundance of each considered stage, and the time dependent transition is given by a transition matrix. For this species in particular, the collected data correspond to several stadia on the life cycle of the earthworms thus allowing testing the long-term sublethal effects on its population dynamics.



Matrix Population Model (H: Eggs; J: Juveniles; A: Adults)

The MPM is useful to test for disturbance effects on the population dynamics, taking into account different survival, fecundity (*f*), and fertility (S_{21}) parameters.

In this study, a Leslie matrix was used with a structure for a population of three stages, as follows:

$$A = \begin{bmatrix} 0 & 0 & f \\ S_{21} & 0 & 0 \\ 0 & S_{32} & S_{33} \end{bmatrix}$$

where *f* represents the fecundity of the last stage, assumed to be the only one capable of reproducing, S_{ij} represent the survival from the *j*- stage to the *i*- stage. The Leslie matrix assumes that the chosen time lapse is such that the organisms that do not go on to the next stage die and that all of the adults die as well. In this work, we cannot ignore the survival of adults within the period and, therefore, there is a diagonal term S_{33} that is not null. However, we can ignore the other terms of the diagonal, and we know that only the adult individuals reproduce.

In these matrixes, the eigenvalue of greater module (called λ_1) gives an indication of the asymptotic dynamic of the population and the logarithm of its module is the intrinsic growth rate of the population (r). This means that the population grows if the module of λ_1 is greater than 1 (or $r>0$). The data obtained allows the estimation of different elements of the B matrix for *E. fetida*, under different glyphosate doses.

A sensitivity analysis was performed in the matrix built. In a population matrix, the eigenvalue of higher module determines the asymptotic behavior of the population, and it is calculated solving the equation:

$$\det[A - \lambda I] = 0,$$

where I is the identity matrix.

Then eigenvectors are calculated as follows:

$$Aw_1 = \lambda_1 w_1$$

which represents the asymptotic age distribution, and

$$v_1 A = \lambda_1 v_1$$

which represents the reproductive value of each age or stage, that is the relative contribution of each age or stage.

Then the sensitivity of the eigenvalue of each parameter of the matrix can be calculated solving the following equation:

$$S_{ij} = \frac{d\lambda_1}{da_{ij}} = \frac{v_{1i} w_{j1}}{\langle v_1 w_1 \rangle}$$

where the denominator is the scalar product of both eigenvectors, which allows for the calculation of the sensitivity of the population parameters for each treatment.

3 Results

3.1 Glyphosate Effects on *E. fetida* Number and Biomass

No significant differences were registered for total weight per treatment or for the number of individuals when considering time as co-variable.

The number of cocoons accumulated per treatment increased significantly ($p<0.05$) with the glyphosate dose. The fertility of these cocoons was significantly lower in the treatment with the higher dose than in the other treatments.

3.2 Effects of Glyphosate on the Population Dynamics of *E. fetida*

The demographic matrixes obtained using the population parameters obtained are the following:

$$\text{Dosage 0: } A_1 = \begin{bmatrix} 0 & 0 & 0.04 \\ 1 & 0 & 0 \\ 0 & 1 & 1 \end{bmatrix}$$

$$\text{Dosage 1: } A_2 = \begin{bmatrix} 0 & 0 & 1.7 \\ 0.76 & 0 & 0 \\ 0 & 0.07 & 0.07 \end{bmatrix}$$

$$\text{Dosage 2: } A_3 = \begin{bmatrix} 0 & 0 & 0.81 \\ 0.84 & 0 & 0 \\ 0 & 0.04 & 0.04 \end{bmatrix}$$

For the three matrices, the first eigenvalues were real numbers, which indicates absence of long-term fluctuations and have the following modules: λ_1=1.037; λ_2= 0.473; λ_3=0.315.

The larger than 1 eigenvalue in the case of the controls indicates that the population is growing, while the lower than 1 eigenvalues for the other treatments show that the population size diminishes in the long run (Fig. 1). The long-term projection indicates that populations under the chronic effect of glyphosate present a negative growth rate, which could lead to local extinction. Interestingly, the lowest dose showed an initial increase in growth rate after the application of the pesticide. This result is likely caused by an increase in the fecundity as an

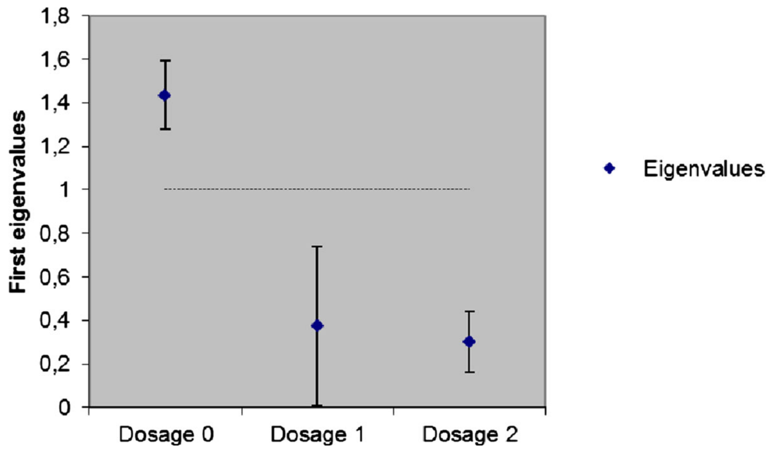


Fig. 1 Matrix population model eigenvalues for the three treatments. Mean±SE shown. Vales below the survivorship threshold (dotted line) indicate population diminishing in the long run, that could lead to local extinction

initial response to stress. However, the final result shows both treatment populations having a negative growth rate (Fig. 2).

In the treatments with glyphosate, the number of cocoons per adult is higher than those of the controls but the survival of juveniles and adults and the fertility are lower than in the control treatment. A sensitivity analysis of the matrixes built (Table 1) shows that in the controls, the most sensitive parameter (the one that modifies the eigenvalue of the matrix the most) is the survival of the adults, while in the treatments with glyphosate, the most sensitive one is the survival from juveniles to adults.

4 Discussion

One of the reasons why earthworms are considered to be good indicators of the local soil conditions is because of their low colonization capacity (Lavelle et al. 1989), as a result of their scarce mobility at a meso-geographical scale (Judas 1988; Lavelle et al. 1989) and their relatively low fertility.

The results presented here show that the biomass, the number of earthworms, and the dynamics of the earthworm populations can be affected by the regular use of herbicides in agriculture.

The number of eggs increased with the glyphosate doses. Morse (1998) points out that hormesis or hormoligosis takes place when small quantities of a stressful agent, among them pesticides, can be useful for an organism in suboptimal environments. This occurs in numerous invertebrates (Abdullah et al. 2006) and *E. fetida*, much like other earthworms, usually responds to different stress factors, increasing their reproductive rate.

The matrix population model shows that glyphosate negatively affects the fertility of the *E. fetida* eggs and,

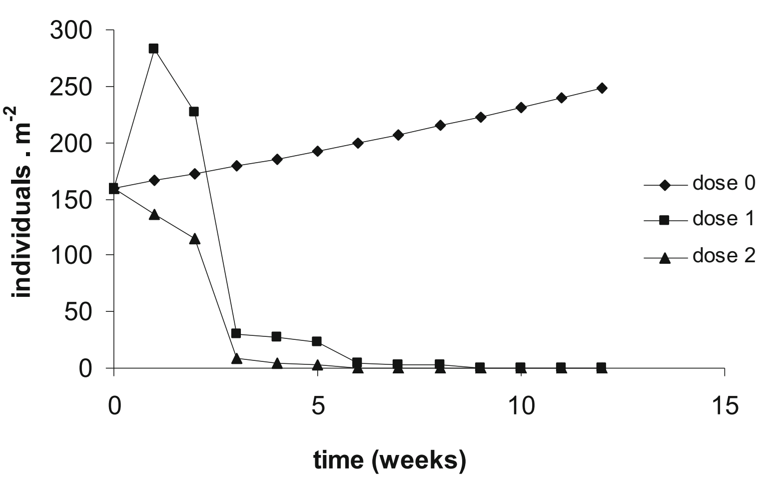


Fig. 2 Population dynamics projections according to the results of the matrix population model. Results show population having a positive growth rate for the controls and an overall negative growth rate for both glyphosate treatments. Both treatment populations are predicted to go locally extinct after 6 weeks

Table 1 Sensitivities of the population parameters for each treatment. Adult survival is the most sensitive parameter for the controls, while survival of young turning into adults is the most sensitive parameter explaining the dynamics for both glyphosate treatments

Parameter	Dosage 0	Dosage 1	Dosage 2
f (individual fecundity)	0.87	0.09	0.12
S_{21} *(egg fertility)*	0.03	0.20	0.12
S_{32} *(survival of young turning into adults)*	0.03	2.13	2.50
S_{33} *(adult survival)*	0.93	0.37	0.36

consequentially, impacts the long-term dynamics of its populations.

The isolated analysis of pesticide effects on the different demographic parameters as they are often studied do not usually show the real effects of different factors on the dynamics of the populations. The environmental characteristics that affect the performance of the organisms have different impacts on their demography. Determining the relative importance of these characteristics on the population dynamics has both a theoretical and practical importance. On one hand, it helps in the understanding of events such as spatial distribution, habitat selection, and dispersion. On the other hand, it also helps in clarifying the relative importance of the characteristics that affect the dynamics of a population allowing to improve the conservation state of a species or to plan for its management.

The viability population analysis presented in this work indicates that in the absence of the disturbance, earthworm populations would increase in size. However, a scenario of absence of disturbance of soil organisms in agroecosystems is not realistic.

Matrix population models following Leslie (Leslie 1945, 1948) have been used now for close to 30 years, becoming one of the most used tools for investigating the dynamics of age-structured populations (Caswell 2001). These models have been successful in improving population analyses by explicitly including population structure and especially in describing toxic effects on population dynamics (Charles et al. 2009). The complex relationships between toxic compounds and population dynamics and life history traits, requires the use of modeling in order to predict the ecotoxicological effects, especially when sublethal effects are expected (Billoir et al. 2007). Matrix population models are also particularly relevant, when the ecotoxicological effects have different effects on the different age or stages of the population being analyzed (Emlen and Springman 2007).

Population matrix analysis is therefore a very useful tool to clarify these situations. The demographic matrix of this species showed how the long-term effects of glyphosate can put a population in danger of local extinction if exposure to this type of factors is sustained over time (Harper et al. 2008).

In this work, the matrix population model was used to calculate the asymptotic rate of population increase, showing it to be negative for high glyphosate levels. A sensitivity analysis on the parameters of the matrix indicates the results on the growth rate of the population if a parameter is changed in a small amount. If the sensitivity value for a given parameter is close to zero, that parameter is said to have a small effect on the population dynamics. On the other hand, if the sensitivity value of a parameter is high, it indicates that changing it has a strong effect on the population dynamics (Farji-Brenner et al. 2003).

The sensitivity analysis shows that the most important parameters in the population dynamics were adult survival and fecundity for the control. When exposed to glyphosate, the most sensitive parameter in the population dynamics is the survival of the young turning into adults. These results strongly suggest sublethal effects that can lead to the local extinction of the population.

5 Conclusions

Glyphosate is a herbicide and, therefore, its target organisms are not animals. However, the results presented show that at least in the case of epigean earthworms such as *E. fetida*, it has long-term effects that cannot be ignored.

Considering the important role that earthworms have in the maintenance of the structure and fertility of soils, the effect that glyphosate has on its populations can cause medium- to long-term edaphic deterioration and can even drastically deplete the local earthworm populations.

These results also underscore the importance of sublethal effects in ecological risk assessment studies. Protocols for acute toxicity studies usually used in pesticide assessment may not show significant mortalities in the short term. However, long-term sublethal effects such as the ones identified in this study must be taken into account, in particular when these effects are identified in an internationally validated species for ecotoxicological tests, such as *E. fetida*.

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