

Study of the effects of glyphosate application on Collembola populations under controlled conditions

Estudio de los efectos de la aplicación de glifosato sobre poblaciones de Colémbolos en condiciones controladas

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ABSTRACT

Glyphosate is the most widely used herbicide worldwide. However, the effects of this molecule on non-target populations are still a subject of study. The objective of this research was to determine the effect of the application of different glyphosate doses on variation in collembolan (springtail) populations. To accomplish this goal, samples of organic substrate that contained different collembolan populations were collected. Samples were taken to the laboratory and acclimatized for 48 h. Glyphosate C¹⁴ was then applied to the samples in doses equivalent to 0 L ha⁻¹, 2 L ha⁻¹, and 4 L ha⁻¹ under a completely randomized experimental design with three treatments and five replicates. Population counts were performed by implementing the flotation method at 0, 4, 7 and 11 d after application (DAA). We found that individuals were distributed in the families Isotomidae and Entomobryidae and divided into species of the genus *Proisotoma* (Börner), *Lepidocyrtus* (Bourlet) and *Seira* (Lubbock). A decrease in the number of arthropods between 40% and 60% was reported for the treatments with herbicide application at 4 and 7 DAA, showing a drop in the size of the community in those treatments in which the herbicide was applied compared to the control. However, no differences were observed between herbicide doses. Additionally, the presence of glyphosate C¹⁴ was demonstrated in dead individuals. This confirms a possible effect of the herbicide on some biological systems that led to a decrease in the size of the population.

Key words: bioindicator, ecotoxicology, herbicide, springtails.

RESUMEN

El glifosato es el herbicida más utilizado a nivel mundial. Sin embargo, los efectos de esta molécula sobre poblaciones no objetivo aún es tema de estudio. La presente investigación tuvo como objetivo determinar el efecto de la aplicación de diferentes dosis de glifosato sobre la variación de las poblaciones de colémbolos. Para esto, se recolectaron muestras de un sustrato orgánico que contenía diferentes poblaciones de colémbolos. Las muestras se llevaron al laboratorio y se aclimataron durante 48 h. Después se les aplicó glifosato C¹⁴ en dosis equivalentes a 0 L ha⁻¹, 2 L ha⁻¹, y 4 L ha⁻¹ bajo un diseño experimental completamente al azar, con tres tratamientos y cinco repeticiones. Se realizaron conteos poblacionales implementando el método de flotación a los 0, 4, 7 y 11 d después de aplicación (DDA). Se encontró una distribución de individuos en las familias Isotomidae y Entomobryidae, divididos en especies de los géneros *Proisotoma* (Börner), *Lepidocyrtus* (Bourlet) y *Seira* (Lubbock). Se reportó una disminución entre el 40% y 60% en el número de artrópodos para los tratamientos donde se utilizó el herbicida a los 4 y 7 DDA, mostrando una disminución del tamaño de la comunidad en aquellos tratamientos en los cuales se aplicó el herbicida en relación con el testigo. Sin embargo, no se observaron diferencias entre las dosis de herbicida. Adicionalmente se demostró la presencia de glifosato C¹⁴ en los individuos muertos; esto confirma un posible efecto del herbicida sobre algunos sistemas biológicos que llevo a una disminución del tamaño de la población.

Palabras clave: bioindicador, ecotoxicología, herbicida, colémbolos.

Introduction

The use of herbicides in agricultural systems can have effects on non-target organisms (Santos *et al.*, 2010), affecting certain edaphic populations. This probably implies an alteration of the diversity, structure, and function of ecosystems (Casabé *et al.*, 2007). Lins *et al.* (2007) suggest that undisturbed ecosystems have greater macro- and mesofaunal biodiversity compared to agricultural systems

that have been subjected to monocultural practices. This can be observed in that portion of the mesofauna that is in greater abundance, where mites and collembolans are found (Rusek, 1998; Johnston, 2000; Gómez-Anaya *et al.*, 2010). The Collembola (Hexapoda:Collembola), commonly known as springtails, are hexapods of sizes between 250 µm and 10 mm. They are wingless, have ametabolous development and entognathous mouthparts. Springtails are considered a very important functional unit among the soil's

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mesofauna since they contribute to the decomposition of the soil organic phase through the mechanical disaggregation of plant materials (Lins *et al.*, 2007). They also promote nutrient mineralization (Al-Assiuty & Khalil, 1996) and the formation and maintenance of the soil structure (Lins *et al.*, 2007), having a direct influence on soil formation and fertility and on the levels of energy production of an ecosystem (Gómez-Anaya *et al.*, 2010).

Palacios-Vargas and Castaño (2014) state that this group is of interest as an ecological indicator of the conservation or alteration of a given environment.

Several methods have been proposed for determining the reasons behind the presence or absence of this species in specific habitats. One of these methods seeks to explain the effect of changes in land use and diverse agricultural practices on collembolan populations (Palacios-Vargas & Castaño, 2014). This method compares different species in altered agricultural habitats to those in one or more unaffected environments in order to analyze the effect of agricultural practices (Ponge *et al.*, 2003). In addition, monitoring springtail populations on site before and after an alteration (such as the application of fertilizers, amendments, pesticides, or crop planting, etc.) can also serve this purpose (Palacios-Vargas & Castaño, 2014). All these practices can be performed to detect changes in the structure of communities and predict the effects of a habitat's alterations. In agriculture, it is possible to use edaphic entomofauna as a bioindicator (Pereira *et al.*, 2018). In this context, springtails stand out since they have the capacity to colonize practically any agricultural environment, are easy to breed in the laboratory, have a short life cycle and, therefore, can respond quickly to environmental changes. All these characteristics make them a group of great importance as a bioindicator of environmental quality in edaphic systems (Palacios-Vargas & Castaño, 2014).

Ecotoxicological studies in springtails usually use one or several species onto which the toxic substance is sprayed on the substrate or the base diet. The model species *Folsomia candida* (Willem, 1902) is frequently used to perform standardized tests of acute toxicity by lethality and chronic toxicity of reproduction (Hopkin, 2002; Santos *et al.*, 2012). These tests can be complemented with an avoidance test, in which the arthropod's migration into a contaminated medium is analyzed. All these tests offer advantages such as greater speed, economy, sensitivity, and environmental reliability since springtails can flee from the sources of pollution in real-life scenarios (Palacios-Vargas & Castaño, 2014).

Glyphosate is the most popular herbicide used around the world. It is an organic compound formed by a fraction of glycine and an amino phosphate radical that can be found in acid form or be used commercially as an isopropylamine salt of N-(phosphonomethyl) glycine. It behaves as a non-selective, systemic herbicide of foliar action (Duke & Powles, 2008). In plants, it acts on the shikimic acid pathway by competitive inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase (Niemeyer *et al.*, 2018). The lack of control in catalysis due to the enzyme's inhibition reduces the biosynthesis of amino acids and compounds such as tetrahydrofolate, ubiquinone, and vitamin K (Salazar-López & Madrid, 2011). Although glyphosate is applied foliarly, a great amount of this herbicide reaches the soil where it is strongly adsorbed by clay minerals and the organic fraction. There, it can be degraded by edaphic microorganisms into aminomethylphosphonic acid (AMPA) and sarcosine, depending on the soil type, nutrient concentration, pH, temperature and humidity. Nevertheless, this herbicide can be a residue for beneficial soil invertebrates (Niemeyer *et al.*, 2018). Although the metabolic pathway in which glyphosate acts has not been reported in animal cells, there are multiple reports of harmful effects on non-target organisms such as insects (Schneider *et al.*, 2009; Benamú *et al.*, 2010; Santos *et al.*, 2010; Santos *et al.*, 2012; Al-Daikh *et al.*, 2016). As glyphosate can affect different arthropods, we formulate the hypothesis that this herbicide may have a negative impact on the population structure of springtails.

The objective of this research was to determine the effect of two different glyphosate doses on the changes in collembolan populations in an organic substrate under laboratory conditions. Changes in the abundance and diversity of these beneficial arthropods have been identified as a contribution to an understanding of the agricultural system as a whole.

Materials and methods

The study was performed in two phases: a field phase related to the procurement of the living material and a laboratory phase in which the test was performed.

Field collected and samples preparation

In order to obtain a considerable number of springtails for the experiment, organic substrate was collected from the composter at Universidad Nacional de Colombia, Bogota campus (4°38'10.5" N, 74°05'22.5" W). The soil contained enough hexapods for taking samples to expose them to different treatments. Samples were collected in pitfall traps

and supplemented by taking the organic phase located between 0 and the first 10 cm of the pit's depth.

A plastic container with dimensions 27.2 cm x 21 cm x 14.5 cm was filled to 60% of its capacity with Pindstrup® plus Orange substrate (blonde peat with low nutrient content): (23 g Mg Om⁻³, 240 g K₂Om⁻³, 50 g NH₄⁺m⁻³, 70 g NO₃⁻m⁻³, pH 6, and electrical conductivity 0.8 dS m⁻¹). The remaining 30% was filled with the different collected samples, and an empty space of 10% was left for air circulation. Subsequently, the peat and organic matter with springtails were mixed and homogenized to obtain a similar number of samples in each treatment. Collembolans were bred until there was a population greater than 1000 individuals m⁻². These were observed under a VanGuard stereo microscope (1122SP, VEE GEE Scientific, Vernon Hills, IL, USA) taking samples of the substrate of a known area. The peat was moistened with water and sprayed with an atomizer to increase the relative humidity. The sample was left in a drying chamber at 22°C ± 2 to allow springtails to acclimatize to the new conditions before applying the treatments.

Experimental procedure

The sample was divided into 15 Fido jars of 125 ml, guaranteeing homogeneous experimental units (EU) with 80 g of the substrate added with the help of a digital scale. Different treatments were applied in these jars. Population counts were made 1 d before application (DBA), the day of application (day 0), and at 4, 7, and 11 d after application (DAA), by taking 2.5 g of substrate sample with a closed base plastic cylinder with 1.5 cm radius × 1.5 cm height for each count. Subsequently, population densities were projected in m². Counting was carried out by placing the samples from the plastic cylinder into another plastic container of greater volume. Subsequently, 10 ml of water was added to bring all the arthropods to the surface, using the flotation technique for sampling and collection (Álvarez *et al.*, 2001). Live individuals were collected for counting with a fine tip brush. These individuals were returned to the substrate to avoid a population decrease in the following sample. Dead individuals were collected in Eppendorf tubes to subsequently quantify the possible amount of herbicide present.

The amount of herbicide was determined in order to establish the interaction between glyphosate and the arthropods, based on the collection of dead individuals. Dead collembolans were processed in biological oxidizer OX600 and taken for quantification performed in a Tri-carb liquid scintillation counter (2910TR, PerkinElmer, Waltham, MA, USA), adding aliquots of the analyte to 5 ml vials containing a liquid scintillation cocktail.

Data analysis

To run the experiment two treatments with radio labeled herbicide + one control treatment were established using a completely randomized statistical design (CRSD). Each treatment had five replicates for a total of 15 EU. The treatments were as follows: T0 - absolute control without glyphosate application, adding 10 ml of water in the same amount as in the other treatments; T1 - treatment with glyphosate application (commercial formulation Roundup®) at a rate of 2 L ha⁻¹ (1.76 µl); T2 - treatment with glyphosate application (Roundup®) at a rate of 4 L ha⁻¹ (3.52 µl). For the treatment with glyphosate, a solution of the commercial glyphosate formulation Roundup® and 0.2671 µl Ci of C¹⁴ glyphosate was used. This solution was applied with a micropipette dividing the total area of the container into five equal parts. The data obtained were analyzed in R® 3.5, with tests of analysis of variance and Tukey's comparison of means (α = 0.05). The assumption of normality was confirmed with the Shapiro-Wilk test (α = 0.05).

Mounting process and taxonomic identification

For taxonomic identification, individuals were randomly collected from three samples taken from the original container with peat and organic matter using the previously described flotation method. Samples were processed using a stereomicroscope, and then transferred to plastic test tubes containing 70% alcohol. A total of 30 slides were examined to identify each specimen to the genus level. The methodology proposed by Palacios-Vargas and Mejía (2007) was followed for slides that consisted of transferring the arthropods to 10% KOH on a concave slide for two minutes. Subsequently, the specimens were placed in lactophenol that was heated with a lighter until steam was released. Hoyer's solution was used as a mounting medium, and each slide was covered and placed on a griddle plate for drying at 70°C for a week. The mounts were covered and marked with a label with the geographical coordinates, collecting method, and collector's name.

Taxonomic keys were used to identify individuals to genus level (Bellinger *et al.*, 1996-2019) using a Nikon E600 phase contrast optical microscope (Nikon, Japan). A photographic record was performed to support the results, using Image Pro Insight® (Media Cybernetics, Inc. Rockville, USA).

Results

After counting the population, there was a greater abundance of collembolans of the family Isotomidae with 283 individuals corresponding to 86% of the total number

where the genus *Proisotoma* was found. The family Entomobryidae contributed to the remaining 14% with 47 individuals divided into the genus *Seira* and *Lepidocyrtus*, in a 6:1 ratio (Fig. 1). So, considering a reference measurement such as m^2 , there were up to 10,500 individuals m^{-2} .

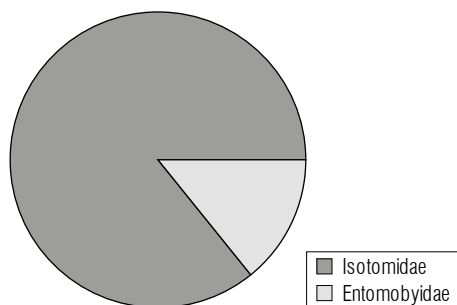


FIGURE 1. Percentage of individuals per family with percentage distribution. Values correspond to the total individuals found in the four samples.

Exposure to the herbicide

Population counts were performed at 0, 4, 7 and 11 DAA to measure the effect of the herbicidal application on the collembolan populations. In the first sample, populations of all treatments were kept at similar levels without showing significant differences and with values between 8000 and 9000 individuals m^{-2} . A significant population decrease was observed in the treatments with glyphosate at 4 and 7 DAA, showing values between 5000 and 6000 individuals m^{-2} , whereas the control exceeded 10,000 individuals m^{-2} . These values were reflected in mortality rates of 40% and 57.4% for treatments with 2 L ha^{-1} and 4 L ha^{-1} at 4 DAA, and 60% and 44.6% for treatments with 2 L ha^{-1} and 4 L ha^{-1} at 7 DAA (Fig. 2). No statistically significant differences were found between treatments with herbicide application.

According to Figure 2, a representative change on the population structure was observed compared to these values of the control treatment, especially at 4 and 7 DAA. A negative effect of the application of the herbicide on the mortality percentage at 4 DAA was observed for the treatment with 4 L ha^{-1} , decreasing with time. The treatment with 2 L ha^{-1} showed a slower effect and increased progressively with time until 7 DAA. At 11 DAA there were no significant differences between treatments with a low mortality percentage that did not exceed 23%.

With the treatment of glyphosate application at an equivalent dose of 2 L ha^{-1} , we observed a reduction in the number of individuals throughout the different samples (Figs. 2-3). A considerable decrease in the number of living springtails and an increase in the number of dead individuals was evident at 4 and 7 DAA compared to the base population

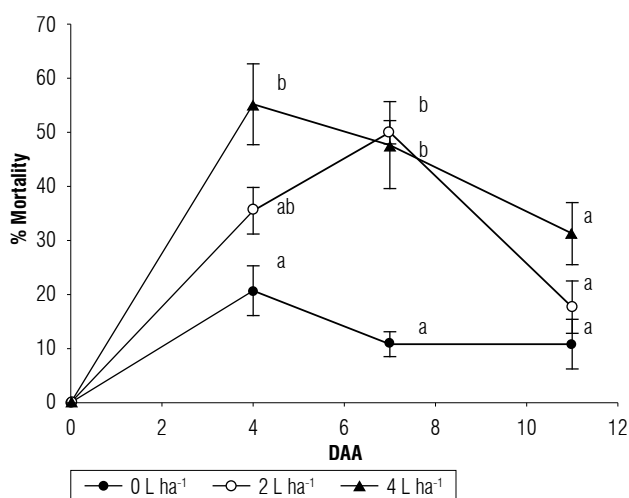


FIGURE 2. Mortality percentages of springtails with the different treatments. Each value on the X axis represents the sample number starting from the first sample (0 days after application (DAA)) in which the treatments were applied. Letters indicate significant differences between treatments over time. Means denoted by the same letter do not significantly differ at $\alpha = 0.05$ according to the Tukey's test.

at 0 DAA (Fig. 3). However, the last sample date (11 DAA) showed a different behavior compared to 7 and 4 DAA, since more live arthropods than dead ones were found. In addition, an increase in the number of live individuals was highlighted compared to 7 and 4 DAA, considering that the mortality percentage was lower at this sampling date (Fig. 2).

Figures 2 and 3 allow the variation in the number of individuals in the treatment with glyphosate to be apparent at a rate of 4 L ha^{-1} . There was a reduction in the number of arthropods throughout the different samples. Although this treatment showed the highest initial average of living individuals, it displayed a significant decrease compared to the control at 4 DAA. In addition, this sampling date was the only one with a greater number of dead individuals than live ones. The numbers of live springtails at 7 and 11 DAA were higher, whereas the number of dead arthropods decreased on those days, showing a possible trend towards a population recovery.

Figure 4 shows the relative values accumulated throughout the four samples. The herbicide application caused a decrease in the population size and indicated that exposure to the herbicide generated a moderate population detriment that is not exclusively quantifiable with dead arthropods.

With the result of the quantification of radiolabeled glyphosate present in the dead springtails collected in the different treatments, we showed that an exposure to the herbicide

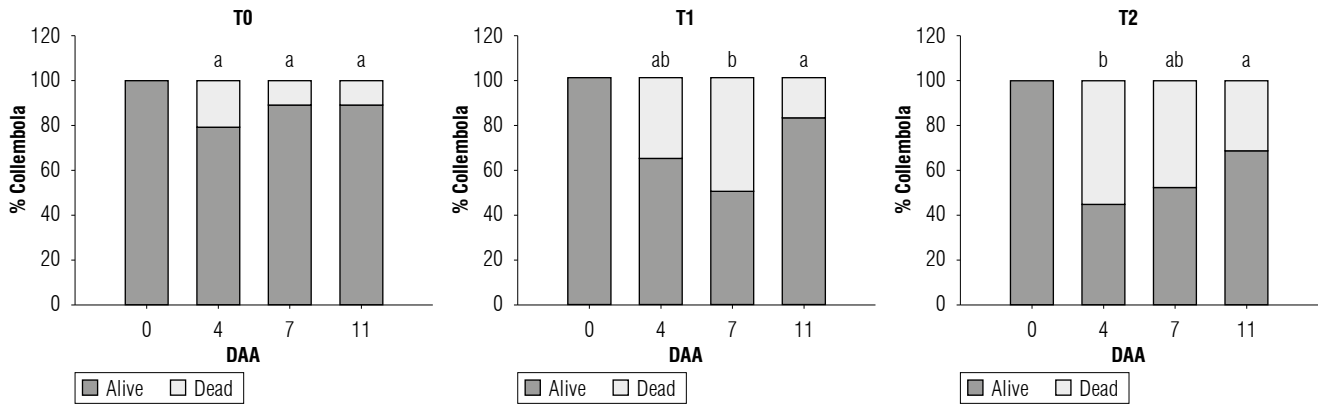


FIGURE 3. Relative values of the collembolan population changes with the different treatments T0: 0 L ha⁻¹, T1: 2 L ha⁻¹; T2: 4 L ha⁻¹. Each series of bars on the X-axis represents the sample number starting from the first sampling (0 days after application (DAA)) in which the treatments were applied. Letters indicate significant differences between treatments over time. The means denoted by the same letter do not significantly differ at $\alpha = 0.05$ according to the Tukey's test.

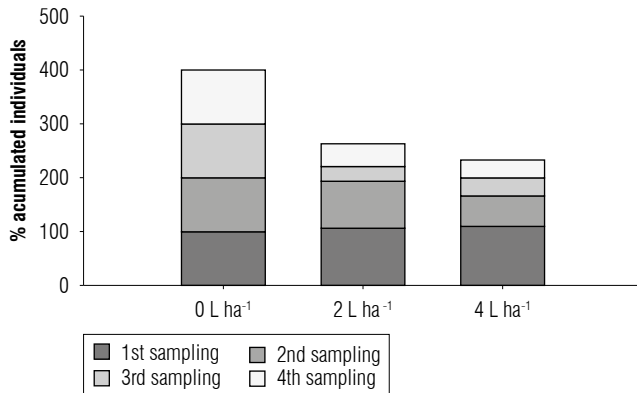


FIGURE 4. Cumulative relative values of the springtail population size in the different treatments over time.

close to 0.0059% and 0.0071% for 2 L ha⁻¹ and 4 L ha⁻¹ of the total applied dose (Tab. 1) has a possible direct effect on the decrease of the springtail population.

TABLE 1. C¹⁴ glyphosate (Roundup®) quantified in dead arthropods. The reported concentration indicates the total herbicide quantified in dead springtails collected at the end of the experiment.

	Herbicide dose	
	4 L ha ¹	2 L ha ¹
C ¹⁴ glyphosate (μ Ci)	0.2671	0.2671
Applied glyphosate (μ l)	3.52	1.76
Total glyphosate quantified in dead springtails (μ l)	2.0776E-06	1.2465E-06
% of glyphosate quantified in dead springtails	0.00590219	0.00708263

Discussion

Springtails along with mites are common organisms found in compost and usually represent more than 90% of the arthropod cluster (Palacios-Vargas & Mejía, 2007). Their

abundance is related to the composting and decomposition processes of the organic phase through mechanical-enzymatic fractionation and disintegration (Rusek, 1998), nutrient mineralization (Al-Assiuty & Khalil, 1996), the formation of humic substances (Rusek, 1998; Arbea & Blasco-Zumeta, 2001), and the stimulation of fungal and bacterial activity (Robles *et al.*, 2012). For this reason, it is not uncommon to find thousands of springtails in the relatively small area of m². This behavior can be seen in the data about springtails per m² that was around 10,000 individuals on day 0 before any application. Similar values were found in diversity samplings in composters by Palacios-Vargas and Mejía (2007) and Robles *et al.* (2012).

The great success of springtails as colonizers of this environment is due to the compost being a medium rich in resources that allow their reproductive success. It is a medium that allows the establishment of bacteria and fungus from which springtails can feed (Castaño-Meneses *et al.*, 2004). Also, it is a favorable habitat for the development of the biological and physiological processes that maintain the average temperatures above 20°C and the high humidity. These variables were reflected in high populations before the application of treatments on 0 DAA.

Figure 1 shows that only specimens of the families Isotomidae and Entomobryidae were found in percentages of 86% and 14%, respectively. However, this result does not represent the totality of possible families and genera that may exist at the sampling site. These results agree with Cutz-Pool (2008) and Palacios-Vargas and Castaño (2014), who showed the dominance of three families in composters in Mexico. Two of those families were those found in this study. In Colombia, Arango and Macías (2004) identified only the family Entomobryidae in composters.

Regarding population changes in Collembola, the results agree with field and laboratory tests in which this herbicide was used on springtail populations or species (Santos *et al.*, 2010; Reimche, 2014; Al-Daikh *et al.*, 2016; Mohammed *et al.*, 2017; Vinod & Sanal-Kumar, 2017; Pereira *et al.*, 2018) and other arthropods such as isopods, Neoptera, Coleoptera and spiders (Niemeyer *et al.*, 2006; Schneider *et al.*, 2009; Benamú *et al.*, 2010; Santos *et al.*, 2010; Al-Daikh *et al.*, 2016). The results indicate an effect on the decrease of the population size with treatments of glyphosate (Figs. 2-4). This effect is confirmed by the presence of the herbicide in dead arthropods demonstrated by radiolabeled quantification (Tab. 1). It is important to clarify that currently it is not clear how the herbicide could directly affect these populations, since the herbicidal site of action does not exist in these arthropods, and it is exclusive to autotrophic organisms (Niemeyer *et al.*, 2018; Pereira *et al.*, 2018). However, both the metabolism of the herbicide in animals and the compounds accompanying the molecule's formulation must be taken into account, since there are often unknown inert substances that act as surfactants and can interact with arthropods (De Aguiar *et al.*, 2016; Niemeyer *et al.*, 2018).

In the case of herbicides, it is common to find that methyl, ethyl, and thiocyanate groups can negatively influence the functioning of some biological systems (Vinod & Sanal-Kumar, 2017). Therefore, the herbicide or the accompanying molecules may have suffered this type of degradation, and their derivatives may have become toxic to the springtails, with a possible effect on the reproductive or respiratory systems. Regarding respiration in springtails, we know that the process is carried out through the cuticle. The cuticle in springtails is very thin, and it may allow the accumulation of the chemical groups already mentioned in the integumentary system of these animals. This might interfere in the gas exchange with the possibility of a subsequent diffusion towards the hemolymph of the arthropods (Al-Assiuty & Khalil, 1996; Mohammed *et al.*, 2017; Vinod & Sanal-Kumar, 2017). There may be an indirect effect on reproduction through abnormalities of the reproductive system, especially in the ovaries. Additionally, an alteration of reproductive hormones, a decrease in the number of ovipositions, and an increase in the development time of the instars can be seen (Schneider *et al.*, 2009; Benamú *et al.*, 2010; Mohammed *et al.*, 2017; Vinod & Sanal-Kumar, 2017; Abdullahi & Gbarakoro, 2019). Moreover, *in vitro* studies in animals classified glyphosate as an endocrine disruptor that increases the mortality of placental cells.

Hypothetically a similar pathway could be occurring in these arthropods (Schneider *et al.*, 2009) which may be related to a reduction in fecundity and fertility. It is also possible that there is an effect of the herbicide on other food source organisms or endosymbiont bacteria, since some of these microorganisms have different degrees of sensitivity to the herbicide, depending on their population density (Bórtoli *et al.*, 2012).

As shown in the different figures that illustrate the population variations, it is important to highlight that there were no significant changes between the doses used. This result could be explained by the possible effect of the treatments even at lower doses (Santos *et al.*, 2010). These authors observed a significant decrease in the number of adults and juveniles from 900 individuals in the control to values of 100 individuals under 1, 1.5 and 2 mg glyphosate kg⁻¹ of soil. Although the herbicide could affect springtail populations at a moderate level, these arthropods were never entirely eliminated. Of the total population on the last sampling date, herbicide treatments obtained 42% (for T1) and 46% (for T2) fewer individuals than the absolute control treatment and a percentage of mortality of less than 23%. Under field conditions, populations might increase their density and recover from the negative impacts of a pesticide as observed by Frampton (2000) and Vinod and Sanal-Kumar (2017). Additionally, we underline that the number of individuals increased from 7 d to 11 d for treatments where glyphosate was used. This fact may be related to the degradation of the pesticide in the substrate and the reduction of harmful radicals for springtails.

Although the role of springtails on soil health has been amply documented and recognized (Hopkin, 2002), the controversy over the effects of glyphosate on the development, physiology, behavior, biochemical and immunological alterations of non-target organisms is still ongoing; and practices to preserve beneficial arthropods in the agriculture are receiving little attention.

Aspects such as the dosage, form, and frequency of application must be considered in order to allow the recovery of the population. Thus, marginal effects on fitness and a considerable reduction in the population growth for the next generations (Schneider *et al.*, 2009) would be avoided and, thus, a negative effect on the physicochemical properties of soils. In conclusion, the results found in this study provide evidence of the negative effects of the herbicide glyphosate on these arthropods.

Conclusions

The predominant families in the trial were Isotomidae and Entomobryidae, in which the genera *Proisotoma*, *Seira* and *Lepidocyrtus* were found. This study is the first report on Collembola present in a composter in Bogota. The sensitivity of Collembola to external stress factors, such as the application of this herbicide at both doses, led to a reduction of the population size that was measurable on the first sampling date, and that tended to recover at the end of the experiment. However, there were no significant differences between treatments. The presence of radiolabeled herbicide in dead individuals is highlighted, confirming that the application of Roundup® had a detrimental effect on the springtail population. This study contributes to understanding the impact of the most widely used herbicide on non-target organisms that perform a vital function in the soil. It is necessary to carry out other trials, such as migration and field tests, to analyze the behavior of this phenomenon in an agroecosystem.

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Author's contributions

MDR and FTM designed the experiments, FTM carried out the field and laboratory experiments, FTM contributed to the data analysis, MDR and FTM wrote the article. All authors reviewed the manuscript.

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