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Research Paper

Chronic effects of clothianidin to non-target soil invertebrates: Ecological risk assessment using the species sensitivity distribution (SSD) approach

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ABSTRACT

This study aimed to assess the chronic toxicity and risk of clothianidin in a seed dressing formulation to non-target soil invertebrates. The toxicity assays were performed with two oligochaetes (earthworms *Eisenia andrei* and enchytraeids *Enchytraeus crypticus*) and three collembolans (*Folsomia candida*, *Proisotoma minuta* and *Sinella curviseta*) species following ISO protocols. Risk assessment (via Hazard Quotient approach – HQ) was based on the hazardous concentrations for 95% of the species (HC_5), derived from chronic Species Sensitivity Distributions (SSD) for clothianidin, and on its predicted environmental concentrations (PEC). Four SSD scenarios were generated with literature and/or this study data, following different data selection criteria (i.e., general, only data from tests using similar formulations, similar soils, or identical soil/formulation). In our experiments, a higher clothianidin toxicity (EC_{50}-based) was found for collembolans (varying from 0.11 to 0.28 mg kg^{-1} between species) followed by the earthworms (4.35 mg kg^{-1}), while the enchytraeids were the least sensitive (33.5 mg kg^{-1}). HQ indicated a significant risk of clothianidin to soil invertebrates because the estimated PEC were at least 16.6 times higher than HC_5 and are expected to affect the whole group of collembolans. Despite the criteria for data inclusion have influenced the HC_5 values, no substantial changes were observed for the risk outcomes. To our knowledge, this is the first study assessing the chronic ecological risk of clothianidin to beneficial soil fauna based on a probabilistic SSD approach. Data from this study can help to derive more reliable protection thresholds for clothianidin in soils.

1. Introduction

The treatment of seeds with pesticides is a technique applied worldwide in commercial crops, such as soybean, corn and wheat (Labrie et al., 2020). Neonicotinoids are the most widely used insecticide class for seed treatment and are registered in more than 120 countries (Borsuah et al., 2020). Clothianidin is an N-nitroguanidine neonicotinoid insecticide used through foliar spraying, soil treatment and especially for seed dressing (Grout et al., 2020). This molecule acts competing with acetylcholine by the nicotinic receptors, thus promoting the paralysis and/or nervous hyperstimulation of exposed organisms (Hilton et al., 2018; Ihara and Matsuda, 2018). In this way, clothianidin protects the crop by providing a systemic, broad-spectrum control of soil-inhabiting insect pests (Atwood et al., 2018).

Although its use has been restricted and/or prohibited in some European Union since 2013, mainly due to its adverse effects on honey bee colonies (EFSA (European Food Safety Authority), 2018c), some EU member states are still using clothianidin through emergency authorizations to prevent severe pest damage to crops (Carrasco-Navarro and Skaldina, 2019; EFSA (European Food Safety Authority), 2018a, 2018b). Also, clothianidin sales outside Europe have continued to grow in the last years, and this active ingredient (a.i.) remains intensively used in agricultural areas of the American and Asian continents (Chevillot et al., 2017; Bass and Field, 2018; Grout et al., 2020; Ramasubramanian, 2021). In 2012, clothianidin was among the three most used neonicotinoids and accounted for 14.7% of the total neonicotinoid sales (Bass

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et al., 2015).

Despite its importance for food production, it is estimated that the major amount (normally > 90%) of the active ingredient involving the seed stays in soil (Alford and Krupke, 2017). Clothianidin has been found in agricultural soils at concentrations ranging from <0.002–2.06 mg kg^{-1} (De Perre et al., 2015; Limay-Rios et al., 2015; Jones et al., 2014; Schaafsma et al., 2016; Chowdhury et al., 2012; Ramasubramanian, 2013; Zhang et al., 2016). There is evidence of high persistence of clothianidin in soil, with the half-life dissipation time estimated to range from 148 to 6931 days (Goulson, 2013; Wood and Goulson, 2017). Residues of clothianidin may also accumulate after successive pesticide applications throughout the year (Van Gestel et al., 2017). Therefore, soil organisms can be chronically exposed to clothianidin, which may cause unpredictable long-term effects on different species of non-target soil invertebrates (De Lima e Silva et al., 2017, 2018; Ritchie et al., 2019).

Sublethal effects of clothianidin have been observed in soil organisms, such as decreases in earthworm's weight, cocoon and juveniles production (Ge et al., 2018; Wang et al., 2015a; De Lima e Silva et al., 2019) and decreases in collembolans and mites reproduction (Ritchie et al., 2019; De Lima e Silva et al., 2019). However, the toxic effects of this compound were mainly studied in a few standard soil invertebrate species separately, while alternative species were less included in ecotoxicological assessments of this insecticide so far (Ritchie et al., 2019; De Lima e Silva et al., 2019). Despite the functional redundancy of some species/taxonomic groups in supporting ecosystem services (Ertiban, 2019), understanding the ecotoxicological effects of clothianidin on a greater number of species/taxonomic groups, with different sensitivities and exposures, is fundamental to make feasible the generation of a species sensitivity distribution (SSD) for this compound in the soil compartment.

The SSD is a cumulative (probabilistic) distribution fitted to toxicity data sets obtained from laboratory toxicity tests with multiple species (Posthuma et al., 2002; Gredelj et al., 2018), which allows quantifying the ecological risk of pollutants from an ecosystem perspective, and thus reducing the uncertainty associated with the species-specific differences in toxicity responses and on the risk estimative (Gao et al., 2014). The advantages of this approach are widely recognized to assess the ecological risk of pollutants (Fox et al., 2021), which have been recommended by regulatory agencies in various countries (RIVM (National Institute of Public Health and the Environment), 2007; EC, 2003; USEPA (United States Environmental Protection Agency), 2005; NEPC (National Environment Protection Council), 2011). SDDs have been used in the risk assessment of endocrine disruptors (Kim et al., 2018, 2020), metals (Cândido et al., 2020; Wang et al., 2015b, 2018) and pesticides (Claus and Spanoghe, 2020; Wu et al., 2020) to non-target soil organisms. Recently, Humann-Guilleminot et al. (2019) failed to elaborate a SSD for clothianidin in the soil compartment due to the lack of toxicity data for soil organisms, and Wu et al. (2020) constructed an acute SSD using toxic values of clothianidin derived mostly from tests with insects. Then, a chronic SSD approach could substantially improve the ecological risk assessment (ERA) of clothianidin for in-soil organisms.

Currently, there are still no well-established criteria defining how SSDs should be generated (Renaud et al., 2019). However, when literature data are used, attention should be paid to the type of soil, the formulation, and the methodology used in the toxicity test to obtain the endpoint, as these parameters can significantly affect the toxicity value obtained and, therefore, impact the SSD outcomes (Silva et al., 2014; Li et al., 2018).

This study aimed: 1) to assess the chronic toxicity of clothianidin (via the seed dressing formulation Inside FS®) to two oligochaete species (earthworms *Eisenia andrei* and enchytraeids *Enchytraeus crypticus*) and three species of collembolans (*Folsomia candida*, *Proisotoma minuta* and *Sinella curviseta*) and; 2) to generate a general chronic species sensitivity distribution (SSD) for clothianidin with data from this study and literature to estimate the hazardous concentrations (HCs) in a way to assess its ecological risk for non-target soil invertebrates in a prospective worst-case scenario of sowing corn treated seeds. Additionally, the implications of using data from the literature to generate the SSD in the risk assessment of clothianidin in the soil will be discussed.

To our knowledge, this is the first study assessing the chronic ecological risk of clothianidin to beneficial soil fauna based on a probabilistic SSD approach. The results of this study allow anticipating the hazardous clothianidin concentrations to non-target soil invertebrates, which may provide a basis to establish regulatory threshold criteria for this insecticide in soils. Our work also can contribute to the development of ERA methodologies in Brazil, a segment that has recently been fomented by the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA) (IBAMA, 2020).

2. Materials and methods

In this study, chronic toxicity assays with five species of soil invertebrates were performed in a tropical artificial soil (TAS), which were grouped with literature data on the toxic effects of clothianidin on non-target soil invertebrates (Ritchie et al., 2019; Wang et al., 2015a; Ge et al., 2018; Chèvre et al., 2017) to generate a species sensitivity distribution (SSD) and to assess the ecological risk for this compound. As recommended for SSD generations (NEPC (National Environment Protection Council), 2011; Kim et al., 2020), the species selected for our assays belong to three different taxonomic groups. Three standard species (*E. andrei*, *E. crypticus* and *F. candida*), globally recognized as ecotoxicological models and with known sensitivity to several contaminants, as well as two alternative species representants of sexually reproducing collembola community (*P. minuta* and *S. curviseta*) were selected for the chronic toxicity assays (Buch et al., 2016; Zhang et al., 2019; De Lima e Silva et al., 2021).

2.1. Test soil and substance

A Tropical Artificial Soil (TAS) was used as a soil test on the chronic toxicity assays. The TAS was proposed by Garcia (2004) as an adaptation of OECD soil (OECD, 1984), and has been recommended for terrestrial ecotoxicological assays in tropical and subtropical regions (De Silva and van Gestel, 2009). This soil was composed of 75% fine sand, 20% kaolin clay and 5% coconut fiber. The pH of TAS was adjusted to 6.0 ± 0.5 with $CaCO_3$. The water holding capacity (WHC) of TAS was determined according to the Annex C of ISO 11268-2 (ISO, 2012).

The seed treatment formulation Inside FS (600 g clothianidin L^{-1}) was used to spike the TAS, according to ISO (2012). A stock solution containing 6000 mg L^{-1} was firstly prepared. Soil samples were spiked with contaminated aqueous solutions containing aliquots of the stock solution. Soil moisture was adjusted to approximately 60% of the maximum water holding capacity (WHC) of TAS. Nominal clothianidin concentrations tested ranged from 0.08 to 0.61 mg of a.i. per kg of dry soil (mg kg^{-1}) for collembolans and 1.5–80 mg kg^{-1} for oligochaetes. These concentrations were based on preliminary tests previously performed (data not shown) and on literature data (De Lima e Silva et al., 2019; Ritchie et al., 2019).

2.2. Chronic toxicity assays

The chronic toxicity assays were performed following ISO protocols (ISO, 2004, 2012, 2014), in a laboratory room with controlled temperature (20 ± 2 °C) and luminosity (12:12 h light:dark). The assays with each species were run independently. Soil moisture and pH were measured at the beginning and the end of each test (Table S1). Detailed descriptions of the procedures adopted in the assays can be found in Alves et al. (2015).

2.2.1. Earthworm toxicity assay

Test with the earthworm *E. andrei* was performed following ISO

(2012). Concentrations of 1.5, 2.4, 3.8, 6.1, 9.8 and 15.7 mg kg^{-1}, plus a negative control (only distilled water) were tested. Ten clitellate earthworms with an initial weight of 433 ± 78 mg (randomly selected from a pool of organisms acclimatized in TAS 24 h before the test, as recommended by the protocol) were placed in plastic containers with approximately 650 g of moist soil (contaminated or control). Recipients remained closed with perforated lids to allow gas exchange during the assay. Six replicates for the control and four replicates for each clothianidin treatment were prepared. The weekly maintenance of replicas, as well as the assessment of adult survival and growth (day 28) and the number of juveniles generated (day 56), were performed with minor adaptations of ISO (2012), as described in Bandeira et al. (2020b).

2.2.2. Enchytraeid toxicity assay

Enchytraeids *E. crypticus* were used in the chronic assays according to ISO 16387 (ISO, 2004). Six clothianidin concentrations (2.5, 5, 10, 20, 40 and 80 mg kg^{-1}) were tested. Ten individuals with similar length and visible clitellum were placed in 200 mL glass containers with 30 g of moist soil treated with clothianidin or only distilled water (control soil). Oat flakes were supplied as food. Eight replicates for the control and four replicates for treatments containing clothianidin were performed. Food and soil moisture was replenished weekly. After 21 days of exposure, enchytraeids were fixed in 70% ethanol, stained with five drops of Bengal rose solution (1% in ethanol), and the juveniles produced were counted as described in Li et al. (2018).

2.2.3. Collembola toxicity assays

Tests with collembolans were performed with the standard species *F. candida* and with two alternative species, *P. minuta* and *S. curviseta*. Collembola assays followed the recommendations of the ISO 11267 (ISO, 2014). Tests with the three species were performed in the same way. Six clothianidin concentrations (0.08, 0.12, 0.18, 0.27, 0.40 and 0.61 mg kg^{-1}) were tested. A control treatment with distilled water was also carried out. Ten 10 – 12 days old collembolans (twenty individuals for *S. curviseta*) randomly selected were inserted in 200 mL glass jars containing 30 g of moist soil (contaminated or control). In each test, ten replicates were prepared for the control and five replicates were prepared for each clothianidin treatment. Collembolans were fed with granulated dry yeast (*Saccharomyces cerevisiae*) at the beginning of the test and on the 14th day, and soil moisture was replenished weekly. After 28 days of exposure, the number of juveniles produced was counted as described in Bandeira et al. (2020b).

2.3. Data analysis

2.3.1. Estimative of effect concentrations

Data from each assay were checked for normality and homoscedasticity through the Kolmogorov-Smirnov and Bartlett's tests, respectively. Once the ANOVA's assumptions were fulfilled, the mean number of juveniles produced in treated soils were compared with the control treatment through Dunnett's post-hoc test to determine the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC). The concentrations that decreased reproduction by 10% and 50% (EC_{10} and EC_{50}, respectively) were estimated by fitting a logistic regression model to the data (Environmental Canada, 2007).

2.3.2. Species sensitivity distribution (SSD)

To compare the sensitivity of non-target soil invertebrates to clothianidin, a species sensitivity distribution (SSD) was generated by using literature toxicity data together with the toxicity values gained from our experiments. The following criteria were established for the selection of literature data: 1) toxicity assays must be performed with non-target soil-inhabiting invertebrate species (so, studies with agricultural pest species were not included in our review scope) and following standard methods (e.g. ISO, OECD, or Environment Canada protocols); and 2) toxicity values must be derived from chronic exposure, based on reproduction endpoints (e.g. number of juveniles, cocoon production and/or cocoon hatching). Based on these criteria, four studies were selected (Table 1), which vary mainly in the soil type and formulation used in the assays.

The EC_{10}s values were preferably adopted in the construction of SSD. In the studies in which EC_{10} was not available, the LOEC was used. The only exception was the data for *Oppia nitens* (Ritchie et al., 2019), where the NOEC was used because no EC_{10} or LOEC values were presented. Our main SSD (called $SSD_{general}$) was generated aiming to include as many species as possible, but restricting the selection criteria to only toxicity data from similar commercial formulations whenever possible. Only when no data was available for a certain species, the toxic values obtained from assays with the pure active ingredient were included (namely, for *E. fetida* and *F. fimetaria*). When more than one assessment endpoint was available for a single species, a mean endpoint value was calculated.

Three alternative approaches were developed to check the suitability of our $SSD_{general}$ and to elucidate the potential implications of the chosen selection criteria on SSD generation and risk assessment: 1) $SSD_{formulations}$ – only toxicity data from assays with similar commercial formulations (different soil types were considered); 2) $SSD_{soil\ types}$ – only toxicity data from tests in similar (artificial) soil types (assays based on pure a.i. were considered); and 3) – $SSD_{standard}$ – only data with the same commercial formulation and soil type (i.e., data from our experiments). For all approaches, the assessment endpoints were summarized to create a ranking of species' sensitivity. The SSDs and the hazardous concentration for 5% and 50% of the species (HC_5 and HC_{50}, respectively) were estimated by fitting a log-normal distribution to the dataset through the USEPA Species Sensitivity Distribution generator program (USEPA, 2005).

2.3.3. Predicted environmental concentration (PEC)

The predicted environmental concentration of clothianidin 28 days after the sowing of treated seeds (PEC) was calculated through the software ESCAPE®, following the recommendations of EPPO (2003). It was assumed a sowing density of 30 kg of corn seeds per hectare (ha), and a manufacturer recommended dosage of 4 mL of the commercial formulation Inside FS per kg of seed, resulting in 72 g a.i. ha^{-1} fully incorporated in the 5 cm of the topsoil layer. A soil density of 1.0 g cm^{-3} was adopted to calculate the mass of soil in contact with the amount of clothianidin applied. Insecticide interception by plants (1.5%; Alford and Krupke, 2017) and the dissipation over time following a single-first order kinetics (DT50 = 155 days; De Lima e Silva et al., 2019) were also considered in the calculations (EPPO, 2003). The PEC was estimated at 0.133 mg kg^{-1}.

2.3.4. Ecological risk estimation

The ecological risk of clothianidin to soil organisms was calculated following the guideline for the risk assessment of new and existing substances from the European Commission (EC, 2003). The Predicted No Effect Concentration (PNEC) was defined as the concentration that protects 95% of soil species ($PNEC = HC_5$). The ecological risk was calculated through the Hazard Quotient (HQ) approach ($HQ = PEC/PNEC$; EC, 2003), which was considered significant when $HQ > 1$.

3. Results

3.1. Chronic toxicity assays

The performance of control treatments is presented in Table S2. All chronic toxicity assays with the standard species met the validity criteria proposed by the guidelines. Alternative species of collembolans performed well in the assays, with adult survival rate > 80% and mean number of juveniles ≥ 100 in the controls. Despite the CV value for *P. minuta* (33.8%, Table S2) slightly above the limit established by the ISO guideline for *F. candida* (CV < 30%), a clear dose-response could be

Table 1
Chronic toxicity data used to generate the general Species Sensitivity Distributions (SSDs). For more information on the restriction criteria used for alternative SSD approaches, see the material and methods section.

Species	Assessment endpoint	Measured endpoint (days of exposure)	Test methodology	Soil	OM[a] (%)	Clothianidin compound	Temperature (°C)	Original endpoint value (mg kg^{-1})	Mean endpoint value (mg kg^{-1})	References
E. andrei	EC_{10}	Rep. juv.[b] (56d)	Environment Canada (2004)	Natural sandy loam	2.5%	Titan (600 g a.i. L^{-1})	20 ± 3	0.0031 (0.00058–0.017)[f]	0.407	Ritchie et al. (2019)
E. andrei	EC_{10}	Rep. juv.[b] (56d)	ISO (2012)	Tropical artificial soil (5% coconut fiber)	1.4%	Inside FS (600 g a.i. L^{-1})	20 ± 2	0.81 (0.39–1.24)[f]		This study
E. fetida	LOEC	Rep. cocoon[c] (56d)	OECD (2004)	Artificial soil (10% sphagnum peat)	6.17%[d]	Pure a.i.	20 ± 1	0.8	0.95	Wang et al. (2015a)
E. fetida	LOEC	Rep. cocoon[c] (56d)	OECD (2004)	Artificial soil (10% sphagnum peat)	6.17%[d]	Pure a.i.	20 ± 1	1.1		Ge et al. (2018)
E. crypticus	EC_{10}	Rep. juv.[b] (21d)	ISO (2004)	Tropical artificial soil (5% coconut fiber)	1.4%	Titan (600 g a.i. L^{-1})	20 ± 2	21.3 (3.4–39.2)[f]		This study
F. candida	EC_{10}	Rep. juv.[b] (28d)	Environment Canada (2014)	Natural sandy loam	2.5%	Titan (600 g a.i. L^{-1})	20 ± 3	0.045 (0.027–0.074)[f]	0.062	Ritchie et al. (2019)
F. candida	EC_{10}	Rep. juv.[b] (28d)	ISO (2014)	Tropical artificial soil (5% coconut fiber)	1.4%	Inside FS (600 g a.i. L^{-1})	20 ± 2	0.08 (0.05–0.12)[f]		This study
F. fimetaria	EC_{10}	Rep. juv.[b] (21d)	OECD (2009)	LUFA 2.2	4.6%[d]	n.a.	20 ± 2	0.11 (n.a.)[f]		Chèvre et al. (2017)
O. nitens	NOEC	Rep. juv.[b] (28d)	Princz et al. (2010)[e]	Natural sandy loam	2.5%	Titan (600 g a.i. L^{-1})	20 ± 3	92		Ritchie et al. (2019)
P. minuta	EC_{10}	Rep. juv.[b] (28d)	ISO (2014)	Tropical artificial soil (5% coconut fiber)	1.4%	Inside FS (600 g a.i. L^{-1})	20 ± 2	0.04 (0.01–0.07)[f]		This study
S. curviseta	EC_{10}	Rep. juv.[b] (28d)	ISO (2014)	Tropical artificial soil (5% coconut fiber)	1.4%	Inside FS (600 g a.i. L^{-1})	20 ± 2	0.12 (0.08–0.16)[f]		This study

[a] OM – organic matter; [b] rep. juv – mean number of juveniles produced; [c] rep. cocoon – mean hatchlings per cocoon; [d] value taken from Garcia (2004) because the OM value was not presented in the study; n.a. - not available; [e] the methods described in Princz et al. (2010) were adopted by Ritchie et al. (2019) because at that moment the standard test method for assessing contaminant effects on oribatid mite reproduction was still in development (draft form), but the methods and procedures used were equivalent of those in the standard protocol recently published (Environment and Climate Change Canada, 2020); [f] 95% confidence intervals.

observed for both species (*S. curviseta* and *P. minuta*), with results showing low variability.

The growth of *E. andrei* (measured as weight change) was inhibited when earthworms were exposed to clothianidin, with a LOEC of 6.1 mg kg^{-1}. The greatest weight loss was observed at 15.7 mg kg^{-1}, where earthworms have lost almost 40% of their initial weight. The reproduction of the earthworms was even more affected by clothianidin, since the number of juveniles significantly started to decline at the lowest concentration tested (LOEC = 1.5 mg kg^{-1}) and a reduction up to 92% of the generated population was observed at 15.7 mg kg^{-1} (Fig. 1).

Enchytraeids *E. crypticus* was the least sensitive species from our experiments (NOEC = 20 mg kg^{-1}; Table 2). The greatest inhibition on reproduction (almost 90%) was observed at 80 mg kg^{-1} (Fig. 1). Depending on the endpoint considered, enchytraeids were a factor of 7–26 less sensitive than earthworms (Table 2).

All collembolan species showed high sensitivity to clothianidin (Table 2). The reproduction of *F. candida* and *S. curviseta* was significantly inhibited starting at 0.18 mg kg^{-1}, while for *P. minuta* this effect began at the lowest concentration tested (0.08 mg kg^{-1}). The number of juveniles of the three species decreased in a dose-dependent manner, and at the highest clothianidin exposure level (0.61 mg kg^{-1}) the inhibitory effect was greater than 90% (Fig. 1). Based on EC50 values (and their 95% confidence intervals), the toxicity was statistically greater (likelihood ratio test, $p < 0.05$) for *F. candida* and *P. minuta* than for *S. curviseta* (Table 2).

3.2. Species sensitivity distribution and ecological risk estimation

The assessment endpoints (EC_{10}/LOEC/NOEC) values used to elaborate the SSDs are shown in Table 1. The toxicity data of eight species (*E. andrei*, *E. fetida*, *E. crypticus*, *F. candida*, *F. fimetaria*, *O. nitens*, *P. minuta* and *S. curviseta*) from four taxonomic groups (earthworms, collembolans, enchytraeids and mites) were included in the construction of $SSD_{general}$ (Fig. 2). Collembola was the most sensitive group (from the most to the least sensitive: *P. minuta*, *F. candida*, *F. fimetaria* and *S. curviseta*). Earthworms *E. andrei* and *E. fetida* showed intermediate sensitivity, while *E. crypticus* was less affected by clothianidin compared to the other oligochaete species. Finally, the oribatid mite *O. nitens* was the least sensitive species to clothianidin (Fig. 2; Table 1). Alternative SSDs had a lower number of species compared to the $SSD_{general}$. $SSD_{formulations}$ and $SSD_{soil\ types}$ encompassed six species from four and three taxonomic groups (respectively), while the $SSD_{standard}$ was

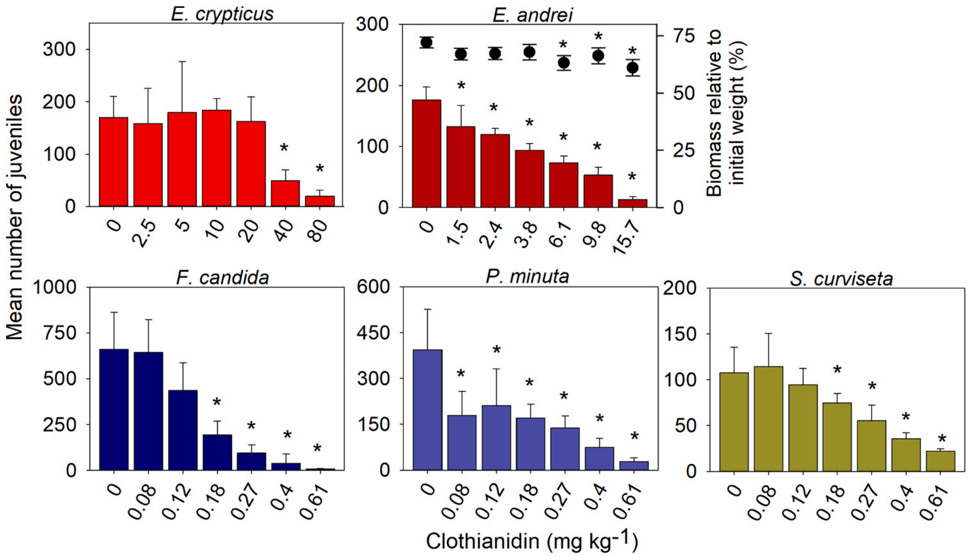

Fig. 1. Mean number of *Enchytraeus crypticus*, *Eisenia andrei*, *Folsomia candida*, *Proisotoma minuta* and *Sinella curviseta* juveniles (+ standard deviation; bars based on the left Y-axis) and *E. andrei* biomass (± standard deviation; points based on the right Y-axis) found after the exposure to increasing clothianidin concentrations in tropical artificial soil (TAS). Asterisk (*) indicates a significant difference compared to the respective control treatment (Dunnett's post-hoc test, $p \leq 0.05$).

Table 2
Assessment endpoints from chronic toxicity assays performed with five species of non-target soil invertebrates in TAS spiked with clothianidin concentrations. The 95% confidence intervals are presented in parenthesis.

Test species	Measured endpoint	NOEC (mg kg^{-1})	LOEC (mg kg^{-1})	EC$_{10}$ (mg kg^{-1})	EC$_{50}$ (mg kg^{-1})
E. andrei	Rep. 56d	<1.5	1.5	0.81 (0.39–1.24)	4.35 (3.43–5.28)
E. crypticus	Rep. 21d	20	40	21.3 (3.40–39.2)	33.5 (22.4–44.6)
F. candida	Rep. 28d	0.12	0.18	0.08 (0.05–0.12)	0.15 (0.12–0.18)
P. minuta	Rep. 28d	<0.08	0.08	0.04 (0.01–0.07)	0.11 (0.04–0.18)
S. curviseta	Rep. 28d	0.12	0.18	0.12 (0.08–0.16)	0.28 (0.22–0.34)

Rep. – reproduction.

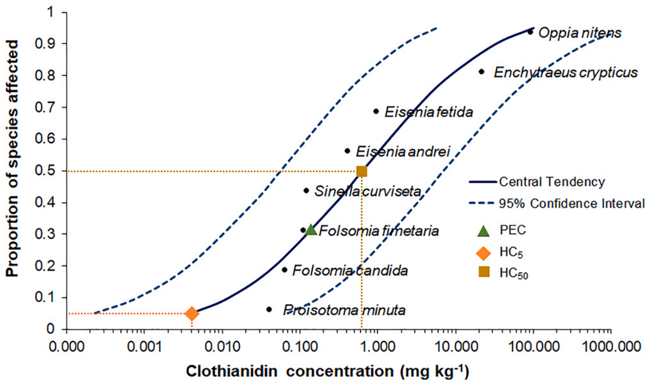

Fig. 2. Species sensitivity distribution (SSD$_{general}$) based on chronic toxicity values (EC$_{10}$, LOEC or NOEC) of eight species from four taxonomic groups exposed to clothianidin in soils. PEC: predicted environmental concentration. HC$_{x}$: hazardous concentrations for x% of soil species.

composed of five species from three taxonomic groups. The overall order of species sensitivity was similar for all approaches considered (Fig. 3). The HC$_5$ and HC$_{50}$ in the SSD$_{general}$ were estimated at 0.004 (0.0002–0.069) and 0.631 (0.056–7.037) mg kg^{-1}, respectively. The criteria chosen for data inclusion influenced the hazardous concentrations estimated in the alternative SSD approaches; the HC$_5$ values for the SSD$_{formulations}$, SSD$_{soil\ type}$ and SSD$_{standard}$ were 0.0023, 0.008 and 0.004 mg kg^{-1}, respectively (Table 3). When comparing the PEC (0.133 mg kg^{-1}) with the SSD$_{general}$, it is possible to infer that the expected level of clothianidin in the soil 28 days after sowing treated seeds will cause some chronic toxic effects in about 31% of the soil invertebrate species. All SSD approaches showed that the entire group of collembolans is expected to experience a 10% reduction in reproduction at concentrations below the PEC (Figs. 2 and 3). The HQ values are at least 16 times higher than the trigger value of 1 regardless of the approach considered (Table 3), indicating a significant/unacceptable risk of the exposure of soil organisms to the expected levels of clothianidin in the soil.

4. Discussion

4.1. Chronic toxicity assays

The exposure of earthworms and enchytraeids to pesticides normally occurs by passive diffusion of contaminated pore water through the body wall and intestinal uptake through the ingestion of contaminated soil particles (Katagi and Ose, 2015), whereas collembolans are mainly exposed through the absorption of pesticide molecules dissolved in the soil pore water (Ogungbemi and van Gestel, 2018). After being absorbed, clothianidin can trigger a series of mechanisms of toxic action on soil organisms. This molecule acts mainly in the central nervous system of exposed organisms by binding irreversibly to nicotinic acetylcholine receptors, which may cause ion flow deregulation, neuronal hyperexcitation, and ultimately the paralysis and death of exposed organisms (Goulson, 2013; Hilton et al., 2018). Furthermore, Liu et al. (2017) demonstrated that clothianidin can cause changes in the activity of antioxidant and detoxification enzymes and induce the generation of reactive oxygen species (ROS), as well as deregulate gene expression and cause DNA damage on earthworms. Some authors have suggested that when soil organisms are exposed to pesticides, they probably allocate more resources in detoxification processes to ensure survival, and consequently, other functions such as growth and reproduction could be hampered (Pelosi et al., 2014; Gunstone et al., 2021), which corroborates the changes in enzyme activity observed by Liu et al. (2017) as well

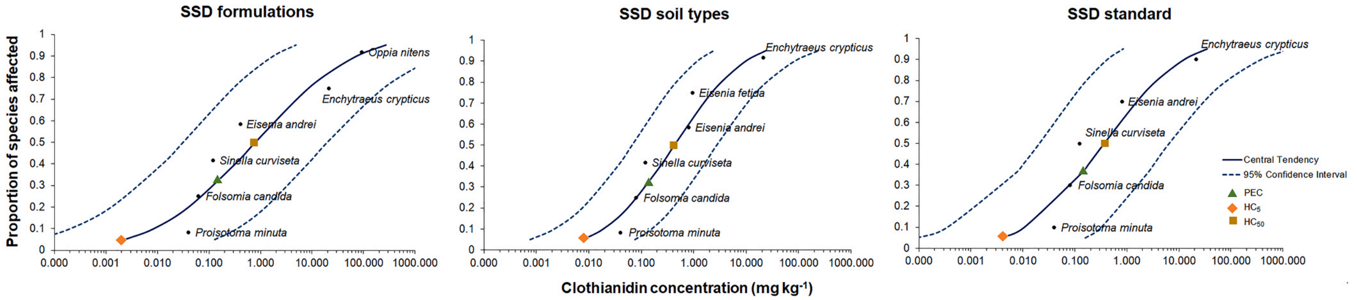

Fig. 3. Species sensitivity distributions (SSDs) generated based on three distinct criteria for data selection: $\text{SSD}_{\text{formulations}}$: data from tests with similar commercial formulations of clothianidin. $\text{SSD}_{\text{soil types}}$: data from tests with similar soil types. $\text{SSD}_{\text{standard}}$: data from tests with the same commercial formulations and soil type (i.e., only our toxic values).

as the impacts on reproduction found in our experiments.

A greater earthworms' sensitivity compared to enchytraeid (Fig. 1; Table 2) was also reported by De Lima e Silva et al. (2017) for two neonicotinoids (imidacloprid and thiacloprid), which attributed it to the differences in the metabolism and differences in the subunits with high affinity for neonicotinoids between the species. Also, Renaud et al. (2018) suggested that enchytraeids are more prone to avoid exposure to soil contaminants due to their smaller body size, by occupying soil microhabitats. On the other hand, avoiding exposure to contaminants is more difficult for *E. andrei* due to their large size, as well as their high skin area that can facilitate the internalization of the pesticide by dermal penetration (Renaud et al., 2018).

When comparing the toxicity observed in our experiments, collembolans were the most affected organisms (Table 2). Inhibition of collembolans' reproduction was observed at environmentally relevant clothianidin concentrations because levels close to the LOEC for these species were found in agricultural soils (Chowdhury et al., 2012; Ramasubramanian, 2013). Several studies have been shown high chronic sensitivity of collembolans to neonicotinoids (Alves et al., 2014; Van Gestel et al., 2017; De Lima e Silva et al., 2017, 2018, 2019; Renaud et al., 2018; Hennig et al., 2020; Bandeira et al., 2020a, 2020b). Neonicotinoids usually present a greater selectivity for the nicotinic acetylcholine receptors (nAChRs) of arthropods compared to other soil invertebrates such as Oligochaetes, which can be due to the phylogenetic proximity of arthropods to insects (Akeju, 2014; De Lima e Silva et al., 2019). This could explain the high sensitivity of collembolans to clothianidin.

4.2. *Species sensitivity distribution and ecological risk estimation*

To the best of our knowledge, there is still no well-defined guidance on how diverse toxicity data from the literature should be assembled for the generation of SSDs in soil. Despite that, researchers have argued that data selection from different studies should preferably consider tests carried out under similar conditions (i.e., temperature, photoperiod, chemical compound and soil type) (Silva et al., 2014; Li et al., 2018; Cândido et al., 2020). Combining data from different chemical compounds (i.e., commercial formulations and the pure a.i.) or different soil types should be performed with caution since it can increase uncertainty about the risk estimated via SSD. This is because the toxicity of some commercial formulations may vary significantly compared to its pure active ingredient (Nagy et al., 2020), as well as the same pollutant can have its toxicity greatly altered according to the type of soil where exposure occurs (Amorim et al., 2005a, 2005b; Bandeira et al., 2020a).

In our general SSD (Fig. 2), most values of the assessment endpoints from literature seem to be comparable with our data, i.e., EC_{10} values for *F. candida* (Ritchie et al., 2019) are within the overall range of sensitivity observed for the species (Table 1). Both values for *E. fetida* (Wang et al., 2015a; Ge et al., 2018) are close to each other and are also comparable with those observed for *E. andrei* (Table 1), despite the first being derived from tests with the pure a.i. The exception was the Ritchie et al. (2019) data for *E. andrei*, where large differences were observed between their EC_{10} value and ours (Table 1). This divergence may be related to the use of distinct soil types [natural loam soil (Ritchie et al., 2019) vs TAS (our study)] and particularly to other methodological differences between both studies, such as the number of organisms used and frequency of food supply. Also, their EC_{10} for *E. andrei* should be interpreted carefully, as the value presents a considerable uncertainty in its estimation, demonstrated by the large variation in the 95% confidence interval (Table 1). Despite these limitations, the value from Ritchie et al. (2019) for *E. andrei* was maintained in the SSD dataset because it may increase the representativeness of the outcomes.

The dataset used to construct the SSD covers chronic toxicity data of eight species from four taxonomic groups. With this, we met the minimum requirements proposed by the guidelines from Australia and New Zealand to allow the application of the SSD methodology, e.g, toxic data from at least five species and covering three distinct taxonomic groups (NEPC (National Environment Protection Council), 2011). In Europe, a standard methodology for SSD to in-soil organisms has not yet been defined, as there is still a need for clarity on how toxicity data should be combined (EFSA, 2017). In Brazil, so far there is still no technical guidance regulation establishing how SSDs to soil organisms should be elaborated, and ERA methodologies still must be consolidated. Therefore, although the general SSD elaborated in this work has some limitations [e.g., the use of different assessment endpoints (EC_{10}/LOEC/NOEC) from assays with different soil types and clothianidin formulations], which means that results must be interpreted carefully, this is a first attempt at applying the SSD methodology to improve the risk assessment of clothianidin in Brazil, and can provide a starting point for future researches to improve this SSD by the inclusion of toxicity data from a great number of species.

Fig. 2 shows that the entire taxonomic group of collembolans can be affected by the PEC since all species of collembolans have EC_{10} below PEC. Impacting Collembola population could be a threat for the ecosystem since they are involved in key processes, including the decomposition of dead leaves and roots, the regulation and dispersion of fungi and bacteria community by their feeding activity, and supporting the existence of predatory arthropods (such as spiders and beetles) serving as prey (Potapov et al., 2020).

According to Chahartaghi et al. (2009), parthenogenetic collembolan species (such as *F. candida*) colonize available habitats faster than sexual collembolans species. It indicates that, once chemical stress has ended, *F. candida* may have the ability to restore population size more quickly than the sexual species. For example, in a multigenerational toxicity experiment, Van Gestel et al. (2017) verified that *F. candida* populations were able to recover from thiacloprid exposure, with a similar number of juveniles being produced by the exposed and non-exposed groups in the second and third generations. On the other hand, the recovery of populations that reproduce sexually (such as *S. curviseta*, *P. minuta* and *F. fimetaria*) could be more difficult, since the offspring production is

Table 3

Risk assessment of clothianidin to non-target soil invertebrates for distinct SSD approaches and application scenarios. $SSD_{general}$: distribution based on toxicity data from similar commercial formulations. $SSD_{formulations}$: distribution based on data from tests with commercial formulations of clothianidin. $SSD_{soil\ types}$: distribution based on data from tests with similar soil types. $SSD_{standard}$: distribution based on data from tests with the same commercial formulations and soil type (i.e., only our toxic values).

Scenario	Application dose (g of a.i. ha^{-1})	Depth of incorporation	DT50	PEC[e] (mg kg^{-1})	$SSD_{general}$		$SSD_{formulations}$		$SSD_{soil\ types}$		$SSD_{standard}$	
					HC_5[f] (CI)	HQ (CI)	HC_5[f] (CI)	HQ (CI)	HC_5[f] (CI)	HQ (CI)	HC_5[f] (CI)	HQ (CI)
Corn seed treatment (Inside FS®)	72[a]	5 cm	155 days[d]	0.133	0.004 (0.0002–0.069)	33.2[g] (1.9–665)	0.0023 (0.00004–0.128)	57.8[g] (1.1–3325)	0.008 (0.0008–0.077)	16.6[g] (1.9–166)	0.004 (0.0001–0.159)	33.2[g] (0.8–1330)
Potato seed-piece treatment (Titan™)	162[b]	10 cm	155 days[d]	0.141		35.2[g] (2.0–705)		61.3[g] (1.1–3525)		17.6[g] (1.8–176)		35.2[g] (0.9–1410)
In-furrow application (Titan™)	180[c]	10 cm	155 days[d]	0.156		39[g] (2.3–780)		67.8[g] (1.2–3900)		19.5[g] (2.0–195)		39 g (1.0 –1560)

[a] Calculated by assuming a sowing density of 30 kg of corn seeds per hectare (ha), and a manufacturer recommended dose of 4 mL of the commercial formulation Inside FS (600 g of a.i. L^{-1}) per kg of seed.

[b] Calculated by assuming a sowing density of 1300 kg of potato seed pieces per ha, and a manufacturer recommended dose of 0.208 mL of the commercial formulation Titan™ (600 g of a.i. L^{-1}) per kg of seed-piece.

[c] Assuming a direct in-furrow application of Titan™ (600 g of a.i. L^{-1}) and a manufacturer recommended dosage of 2.7 mL applied on 100 m row length and 90 cm row spacing.

[d] Data from De Lima e Silva et al. (2019).

[e] The predicted environmental concentration (PEC) was calculated by assuming a soil density of 1000 kg m^{-3} and a homogeneous distribution of the active ingredient in the top-5 or 10 cm of the soil profile.

[f] Hazardous Concentration for 5% of soil species (HC_5).

[g] Hazard Quotient (HQ = PEC/HC_5); values greater than 1 indicate a significant/unacceptable risk of clothianidin to soil invertebrates.

more complex and depends on the females to find and collect the sperm packages (spermatophores) deposited in the soil by the males (Glime, 2017; De Lima e Silva et al., 2021).

While *F. candida* is unquestionable the most widely studied collembolans species in soil ecotoxicology due to their high sensitivity of several contaminants, it is highlighted that the sexually reproducing collembolans seem to represent more accurately the microarthropod community. According to Xu et al. (2009), in south China *S. curviseta* is an abundant species with a widespread occurrence, while *F. candida* is rarely found in the field. In the same context, *P. minuta* was found in 68% of the soil samples collected at forests of the Rio de Janeiro state, Brazil, while *F. candida* was found only in 2% of the samples (Buch et al., 2016). In addition to its good ecological representativeness, *P. minuta* also presented a high sensitivity to clothianidin, which in terms of EC_{10} and EC_{50} (and their 95% confidence limits) was equal to the standard collembola species *F. candida* (Table 2). This reinforces the importance of including a great variety of species with distinct reproductive strategies in pesticide risk assessment, as this may offer more precise values of environmental protection that could be safe for species with similar reproductive methods.

Residual levels of clothianidin ranging from 0.002 (De Perre et al., 2015) to 0.029 mg kg^{-1} (Botías et al., 2015) were already found 300 days after planting seeds treated with clothianidin. These values revealed that clothianidin can persist in the soil during long periods (normally > 100 days; Hashimoto et al., 2020), whereas after this time the residual concentrations hardly exceed 0.1 mg kg^{-1} (Jones et al., 2014; Limay-Rios et al., 2015). Although these residual concentrations are not expected to cause detrimental effects in most soil invertebrates, some more sensitive species (eg, collembolans) may experience slight impacts at these levels. Furthermore, higher clothianidin concentrations were found in other investigations. For instance, Chowdhury et al. (2012) detected levels of 0.25 ± 0.06 and 0.45 ± 0.09 mg kg^{-1}, respectively, after applications of 30 and 60 g a.i. ha^{-1}. Ramasubramanian (2013) found clothianidin concentrations of 0.443 (±0.017) and 0.879 (±0.031) mg kg^{-1} in soil, two hours after the application of doses of 50 and 100 g a.i. ha^{-1} (respectively) via soil drench. Zhang et al. (2016) also reported 2.06 mg kg^{-1} of the same a.i. in an agricultural soil. Based on our HC_{50} values, at exposure scenarios like these, more than 50% of soil invertebrate species would be negatively affected (Fig. 2).

Our HC_5 and HC_{50} results are in line with the findings of Wu et al. (2020), which constructed acute SSDs (LC_{50}-based) for six neonicotinoids in soil and found HC_5 and HC_{50} values for clothianidin of 0.008 and 0.714 mg kg^{-1}, respectively, which are in the same order of magnitude than our values. However, four of the six toxicity values used by Wu et al. (2020) in their acute SSD are from tests with insects (namely, the wasps *Neochrysocharis okazakii* and *Trichogramma chilonis*, the mosquito *Culex quinquefasciatus* and the honey bee *Apis mellifera*), whereas the unique representative of the non-target in-soil invertebrate species included in this study was *E. fetida*. Therefore, our results are more realistic for the soil compartment and provide more accurate hazardous concentrations for clothianidin to non-target soil invertebrates since the toxicity values used in our SSD are based on chronic exposure and are derived from tests with species that are better representants of soil fauna compared to insects, which spend only a limited fraction of their life cycle on the soil. Also, the predominant use of insect species in the composition of the SSD contributes to explain the low values of acute HC_5 and HC_{50} obtained by Wu et al. (2020), considering that neonicotinoids are intentionally designed to combat insects and, therefore, a high sensitivity of this group is expected.

An overall analysis of our alternative SSD approaches indicates that the criteria for data inclusion in the construction of SSDs can impact the HC_5 values as well as the uncertainty associated with the estimation (Table 3). The $SSD_{soil\ types}$ had the lowest confidence intervals among the alternative scenarios explored (Fig. 3), suggesting that selecting data from similar soil types could be a more suitable criterion, compared to the others considered by us. Despite that, no substantial changes in the order of species sensitivity could be observed by changing the selection criteria (Figs. 2 and 3). Also, the major outcomes for the three alternative approaches as well as for the "general" approach are the same: collembolans are the most sensitive group, which are expected to be impacted at concentrations below all PEC scenarios, and HQ values indicated a significant ecological risk of clothianidin (Table 3).

It suggests that, for this case, the selection criteria adopted to generate the $SSD_{general}$ seems to be appropriate, because even using some data from distinct formulations or soil types, it increased the representativeness of our outcomes, without changing the order/level of species sensitivity. Anyway, our findings also suggest that the criteria for data selection may have a considerable influence on the SSD distribution and HC_5-derived values (as observed via $SSD_{soil\ type}$ and $SSD_{formulations}$), therefore, data from similar conditions should be preferably selected to generate SSDs, when available. The approaches explored in this study may help researchers to establishing appropriate methods for combining data.

The risk analysis through the HQ approach revealed that our PEC is more than thirty times greater than the protective concentration for 95% of the species (HC_5, defined as PNEC), which revealed a significant/unacceptable risk of clothianidin to soil organism (EC, 2003). Additionally, considering that the formulation Titan™ (Ritchie et al., 2019) has a similar intended use as ours (seed treatment) but may have different doses and uses (e.g. can also be directly sprayed in the furrow), we did estimate additional PEC values and calculated the risk through the HQ approach for the distinct uses (Table 3). Despite that, the major outcomes and the ecological risk of clothianidin seems to be the same for the distinct considered uses, i.e., collembolans are still expected to be affected by all the PECs, and the HQ risk is also significant for all scenarios considered.

Our findings are somewhat worrisome because despite we have considered a single input of clothianidin in the soil for all the application scenarios (Table 3), successive clothianidin applications may occur in agricultural fields and the expected exposure levels to the a.i. could be even greater due to insecticide persistence and accumulation into the soil (Ge et al., 2018). In line with our findings, De Lima e Silva et al. (2019) found a significant risk of clothianidin (pure a.i.) to *E. andrei* and *F. candida*, with PEC being 8 and 27 times higher than the PNEC, respectively. Controversely, Wu et al. (2020) elaborated an acute SSD to assess the ecological risk of neonicotinoids and found a low risk of clothianidin to soil organisms, with deleterious effects being estimated to occur in less than 1% of the species. This is probably because the risk was calculated using residual concentrations of clothianidin (<0.02 mg kg^{-1}), which were measured in tomato and cucumber greenhouses, at least two years after the application of the insecticide in soil (Wu et al., 2020). The rate of pesticide application in greenhouses is generally much lower than in the open field. The maximum application rate (3.75 g ha^{-1} $year^{-1}$) adopted in greenhouses by Wu et al. (2020) is 19–45 times lower than the rate recommended for manufacturers of the seed treatment formulations considered in our risk scenarios. Also, the authors generated a SSD based on acute toxicity assays, which is no longer required in pesticide risk assessment in the EU due to its low efficiency in detecting the ecological risk for soil fauna (EFSA, 2017). This certainly also contributed to the absence of significant risk demonstrated by the authors.

Extrapolations from laboratory toxicity results to field conditions should be done carefully, especially because the chronic assays are designed as a worst-case scenario of exposure, where the toxicant is usually homogeneously spiked in soil samples, which does not represent accurately the way how contamination occurs in the field. Particularly for seed dressing formulations, the concentration of active compounds is expected to be higher in the soil regions surrounding the seeds, and decrease with increasing distance from the treated seeds, but normally remains in the first 5–10 cm of the zone around the seeds (Wood and Goulson, 2017; Raveton et al., 2007; Alford and Krupke, 2017), making our application method somewhat less unrealistic considering the PECs were derived based on the 0–5 or 0–10 cm soil layer.

At the field, soil organisms can avoid soil portions near the seeds highly concentrated with pesticides (Alves et al., 2013; Ge et al., 2018), which could mean that they would be exposed to concentrations lower than expected. Because our PEC calculations are based on a worst-case scenario (i.e., assuming a homogeneous distribution of the active compound throughout the top 5 or 10 cm of soil profile), we cannot rule out a possible overestimation in our risk estimative. However, the homogeneous pesticide spiking is widely accepted in the lab. assays as lower tiers of pesticide risk assessment (EFSA, 2017; Carniel et al., 2019). Therefore, our results should be interpreted as an initial warning of the ecological risk of clothianidin, but further research at the semi-field and field levels should be done to reduce the uncertainties associated with the methods used herein.

5. Conclusion

Clothianidin caused chronic effects in the five soil invertebrate species tested. However, the magnitude of the effects varied among species. All collembolans species were more sensitive (EC-based) to clothianidin than the oligochaetes *E. andrei* and *E. crypticus*. This reinforces the reasons why several species should be more often used in pesticide risk assessment. The SSD also revealed that the group of collembolans (*P. minuta* > *F. candida* > *F. fimetaria* > *S. curviseta*) was the most sensitive to clothianidin, followed by earthworms, enchytraeids and mites. The PECs estimated based on seed treatment and in-furrow application were greater than the protective concentration for 95% (HC_5) of the non-target soil species, being expected a negative effect on the whole collembolans group and a significant/unacceptable ecological risk at all the studied scenarios. Some limitations in terms of available data for different formulations, soil types, and a greater number of species/taxonomic groups were found in the generation of our general SSD. Despite that, interestingly the SSDs based on restrictive criteria provided similar results, suggesting that including data from distinct formulations and soils in a single SSD may be appropriate, when equivalent data are not available. Finally, our chronic SSD can be a starting point for future research focused on the ERA of clothianidin, as well as may provide regulatory threshold criteria for environmental policies for this insecticide in soils.

CRediT authorship contribution statement

Felipe Ogliari Bandeira: Conceptualization, Data curation, Formal analysis, Investigation, Writing - original draft. **Paulo Roger Lopes Alves**: Conceptualization, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing - review & editing. **Thuanne Braúlio Hennig**: Investigation. **Juliane Brancalione**: Investigation. **Diego José Nogueira**: Formal analysis, Writing - review & editing. **William Gerson Matias**: Conceptualization, Resources, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2021.126491.

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