

## Environmental Toxicology

Toxicity in Neonicotinoids to *Folsomia candida* and *Eisenia andrei*

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**Abstract:** We compared the toxicity of the neonicotinoids imidacloprid, thiacloprid, thiamethoxam, acetamiprid, and clothianidin in terms of the survival and reproduction of 2 species of soil invertebrates, *Folsomia candida* and *Eisenia andrei*. Tests were performed using LUFA 2.2 natural soil, following standard protocols aimed at answering 2 questions: 1) Is there a difference in the toxicity between pure compound and its formulation? and 2) Is there a difference in the sensitivity of the species exposed to the same compound? For *E. andrei*, formulations and pure compounds had similar toxicity to both endpoints tested. For *F. candida*, acetamiprid and imidacloprid had different toxicities, with acetamiprid being 4 times more toxic to survival (median lethal concentration [LC50] 0.12 mg active substance [a.s.]/kg dry soil) and imidacloprid being 4 times more toxic to reproduction of the springtail (median effect concentration [EC50] 0.25 mg a.s./kg dry soil) than their commercial formulations. The most toxic compound to *E. andrei* was acetamiprid (LC50 0.80 and EC50 0.35–0.40 mg a.s./kg), and the most toxic to *F. candida* was clothianidin (LC50 0.07 and EC50 0.05 mg a.s./kg). Estimated risk ratios indicated that only one application/yr of clothianidin in the formulation Poncho® may pose a threat to the populations of springtails and earthworms. *Environ Toxicol Chem* 2020;39:548–555. © 2019 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals, Inc. on behalf of SETAC.

**Keywords:** Soil ecotoxicology; Soil invertebrates; Risk assessment

## INTRODUCTION

Almost 70 yr ago, in the United States, different types of insecticides (DDT, dieldrin, malathion, parathion, and many others) were applied to urban and rural environments on a large scale. Without a full comprehension of their toxic effects on humans and the local biodiversity, authorities such as the US Department of Agriculture began an intense campaign aiming to eradicate certain invasive species considered to pose a risk to the productivity and quality of crops and woodlands. This decision had disastrous results, including the reduction or extinction of populations of nontarget species of vertebrates and invertebrates, and the development of different types of cancer in humans (Carson 1962). As a consequence, in the years following 1970, regulations concerning the use of insecticides were developed and implemented in the United States and Europe.

Currently, the most used plant protection products worldwide belong to the chemical family of the neonicotinoids, which is divided into 2 major classes: N-cyanoamidines (thiacloprid and acetamiprid) and N-nitroguanidines (imidacloprid, thiamethoxam, clothianidin, and dinotofuran; Jeschke et al. 2011). Most of the scientific literature on these insecticides concerns the toxicity of imidacloprid used as a pure compound, with mainly earthworms as surrogates. Only a few studies, such as those of Drobne et al. (2008), De Lima et al. (2017), and Renaud et al. (2018), have tested different neonicotinoids and/or different species. Within the earthworm tests, different endpoints (burrowing activity, acute toxicity, cast production, avoidance, biomarkers, survival, and reproduction), and soil types are used (natural soil, artificial soil [Organisation for Economic Co-operation and Development; OECD]), tropical artificial soil, and LUFA 2.2 natural soil, making it difficult to compare the results (Luo et al. 1999; Capowiez et al. 2003, 2006a, 2006b, 2010; Dittbrenner et al. 2011a, 2011b, 2012; Wang et al. 2012; Feng et al. 2015; Wang et al. 2016; De Lima et al. 2017). Other tests assessed mixture toxicity (Gomez-Eyles et al. 2009), or the effect of soil properties on the toxicity of a single compound (Alves et al. 2013; Wang et al. 2015; Ogungbemi and van Gestel 2018; Renaud et al. 2018). These differences in test design, species, types of soil, and, specifically, different types of neonicotinoids tested (active substance or commercial formulation), prevent a

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proper comparison of the toxicity of different neonicotinoids to a single species, for example. One attempt was made by De Lima et al. (2018), who assessed the toxicity of thiamethoxam and its commercial formulation Actara® to 3 generations of the springtail *Folsomia candida*, using the same endpoints, and under the same abiotic conditions (soil type and concentration range). Their results showed similar toxicity of the pure compound and the commercial formulation, indicating that the formulating agents had little effect on the toxicity to the springtails of the active substance. That study is one of the few in the scientific literature to compare the toxicity of pure neonicotinoids and their respective formulations within the same species.

Thus, with the aim of increasing our knowledge of the toxicity of these insecticides, and comparing such toxicity between pure compounds and the corresponding commercial formulations, we tested 5 pure neonicotinoids (imidacloprid, thiacloprid, thiamethoxam, acetamiprid, and clothianidin) and the respective formulations (Confidor®, Calypso®, Actara®, and Mospilan®) of the first 4, using the same concentration range, soil type, and other abiotic factors. Two species of soil invertebrates were chosen to perform the tests, the springtail *F. candida* and the earthworm *Eisenia andrei*. Our main goals were to: 1) facilitate comparison between the toxicity of pure neonicotinoids and their respective commercial formulations, and 2) assess the sensitivity to the same active substance in terms of survival and reproduction in *E. andrei* and *F. candida*.

## MATERIALS AND METHODS

### Test soil, chemicals, and treatments

All tests used natural standard LUFA 2.2 soil (LUFA Speyer), which has approximately 1.6% organic carbon, a water-holding capacity of 45%, and a soil pH (0.01 M CaCl<sub>2</sub>; measured in a preliminary test) ranging between 5.03 and 5.87. Properties of the neonicotinoids tested are presented in Table 1. Pure imidacloprid and thiacloprid (Pestanal, purity 98%) were provided by Bayer CropScience. Thiamethoxam, acetamiprid, and clothianidin (purity 97–98%) were purchased from Fluka Sigma-Aldrich. The formulations Confidor, Calypso, Actara, and Mospilan were purchased in Brazil to compare with the first 4 neonicotinoids, respectively, but no commercial formulation

could be obtained for clothianidin. Stock solutions for all the neonicotinoids were prepared in Milli-Q water, to spike the chemicals into the test soil and at the same time bring the moisture content to 50% of the water-holding capacity. The nominal concentrations for all compounds tested were as follows: control and 0.04, 0.12, 0.37, 1.1, 3.3, and 10.0 mg active substance (a.s.)/kg dry soil.

### Toxicity tests

*Folsomia candida* used for age synchronization were obtained from cultures at the Department of Ecological Science, Vrije Universiteit Amsterdam, The Netherlands. Adult individuals were transferred to 125-mL translucent plastic boxes with an approximately 2-cm layer of plaster of Paris mixed with activated charcoal (10:1), moistened with water, and left for a period of 2 to 3 d to lay eggs, under a 12:12-h light:dark cycle at 20 °C and 75% relative humidity. Toxicity tests were performed following OECD test guideline 232 for collembolan reproduction (Organisation for Economic Co-operation and Development 2009). Ten juveniles of 10 to 12 d old were introduced into replicate 100-mL glass jars (5 replicates) with 30 g moist soil. Two additional jars filled with 15 g moist soil each, without animals, were used for chemical analysis. All the test jars were weighed at the start of the experiment, so water loss could be checked and replenished on a weekly basis, and covered with a black plastic lid, loosely attached to the rim to allow for aeration. At the start and every week, approximately 1 g of dry baker's yeast (Instant Yeast from Algist Bruggeman) was added as a food source. After 28 d, excess demineralized water was added, and the contents of the jars were transferred to a beaker, allowing animals to float and be photographed with a Nikon camera, model COOLPIX P510; a posterior analysis was performed using ImageJ, a Java-based processing program adjusted for counting the animals.

Following OECD test guideline 222 (Organisation for Economic Co-operation and Development 2004), clitellate *E. andrei* collected from a 2-mo age-synchronized culture from the Department of Ecological Science, Vrije Universiteit Amsterdam were separated and acclimatized for 24 h in noncontaminated LUFA 2.2 soil moistened at 50% of its water-holding capacity. After that, groups of 10 animals were randomly selected, washed, blotted dry on tissue paper, and weighed; then the individuals were introduced into 800-mL glass jars (4 replicates), with approximately 600 g moist soil. For all treatments, soil samples were taken from replicate jar number 4 for posterior chemical analysis. Finely ground horse manure (10 g moist), added through a small central hole and covered with soil to avoid mold, was provided as a food source. The jars were covered with opaque metal lids, loosely attached to the rim to allow for some aeration. To assess survival, on day 28 adults from each replicate were removed from the soil, washed, weighed, and counted; then the soil, including cocoons, was carefully returned to the jars, and the organisms were incubated for another 28 d to assess reproduction. On day 56, test jars were transferred to a water bath (Julabo TW12) at 60 °C to extract and count the juveniles emerging from the soil.

**TABLE 1:** Chemical properties of the pure neonicotinoids and the respective formulations tested<sup>a</sup>

Neonicotinoid	Water solubility (mg/L) <sup>b</sup>	Log K <sub>ow</sub> <sup>c</sup>	Formulation tested	% of Active substance (a.s.) in formulation
Thiamethoxam	4100	−0.13	Actara®	25
Acetamiprid	2950	0.8	Mospilan®	20
Thiacloprid	184	1.26	Calypso®	40
Clothianidin	340	0.95	n.t.	
Imidacloprid	610	0.57	Confidor®	17.7

<sup>a</sup>The data were obtained from the Pesticide Property Database (University of Hertfordshire 2018).

<sup>b</sup>At 20 °C.

<sup>c</sup>Octanol/water partition coefficient at pH 7, 20 °C.

n.t. = no formulation was tested for this compound.

All tests for both species were incubated in a climate room at  $20 \pm 2^\circ\text{C}$ , with 75% relative humidity and a 16:8-h dark:light photoperiod. To reduce possible variations, the tests with pure compounds and their commercial formulations used the same batch of synchronized individuals, and started and finished on the same day for *F. candida* and 1 d apart for *E. andrei* due to technical constraints.

## Chemical analysis

Soil samples (5 g) were taken from all the concentrations tested, including control, for both pure compound and formulation. There were 2 sampling time points for *F. candida* (days 0 and 28) and 2 or 3 times points for *E. andrei* (days 0, 28, and/or 56). The concentrations we chose to analyze, with the exception of controls, were based on the observed toxic response for both species. To check whether thiamethoxam was converted into clothianidin, an additional analysis for the presence of clothianidin was performed. All soil samples were kept in glass vials at  $-20^\circ\text{C}$  prior to analysis, which was performed in 2 steps: quantitative and qualitative. Quantitative analysis was performed using the Quick, Easy, Cheap, Effective, Rugged, Safe (QuEChERS) extraction method, followed by liquid chromatography–tandem mass spectrometry with a reverse-phase chromatography to identify the active substance (qualitative analysis). Samples were analyzed by the commercial certified analytical laboratory Groen Agro Control in Delfgauw, The Netherlands. The detection limit was 0.01 mg a.s./kg dry soil.

## Data analysis

Using measured concentrations in the test soils, the half-life (DT50) was estimated assuming first-order degradation kinetics without a lag phase as follows:

$$C = C_0 \times e^{-kT} \quad (1)$$

where  $C$  is the total measured concentration in mg a.s./kg dry soil at day 28 or 56,  $C_0$  is the measured concentration at time 0 (mg a.s./kg dry soil),  $k$  is the degradation rate constant ( $\text{d}^{-1}$ ), and  $t$  is the time in days.

The DT50 was derived as

$$\ln(2)/k \quad (2)$$

The weight change of adult earthworms was calculated as:

$$\text{WV}(\%) = [(W28/x) - (W0/10)] \times 100 / (W0/10) \quad (3)$$

where WV stands for weight change, W28 and W0 indicate the weights of all earthworms (in g) from each test jar on days 28 and 0, and  $x$  is the number of survivors on day 28. Analyses of variance, followed by Tukey's post hoc test (95% confidence level) and linear regression (Rstatistics), were performed, respectively, to compare weight changes in treatments with controls and to verify how much concentration influenced changes in weight (in percentage).

All dose–response data were analyzed using the package dose–response curve (drc) in the software Rstatistics, applying a

general model fitting function/dose–response model (drm, function log–logistic; Ritz and Streibig 2005). The median lethal concentrations (LC50s; to determine the effects on adult survival) and the effect concentration,  $x\%$  (EC $x$ ) values; to address the effects on reproduction were expressed on the basis of nominal soil concentrations.

The predicted environmental concentration (PEC) was calculated based on doses provided by the pesticide producers for application on potatoes or sugar beets. It was assumed that 50% of the dose reached the soil surface and was homogeneously distributed over the top 5-cm layer of the soil, using the formula

$$\text{mg/kg} = (\text{kg/ha}) \times 1.33 \quad (4)$$

where the value 1.33 was derived assuming a soil bulk density of  $1500 \text{ kg/m}^3$  (so, 750 000 kg of soil in the top 5 cm of 1 ha).

The risk ratio was calculated as

$$(\text{PEC/PNEC}) \quad (5)$$

where the predicted no-effect concentration (PNEC) was based on EC10 values obtained from tests with the commercial formulation divided by an assessment factor of 10 to account for extrapolation from laboratory to field. A neonicotinoid was considered to pose a threat to the species considered when the risk ratio was  $>1$ .

## RESULTS

Nominal and measured concentrations in test soils and recovery rates for the active substance following dosing with the pure compound or the commercial formulation are presented in the Supplemental Data, Table S1. At the start of the tests, recovery rates were approximately 50% for both species, with some variation (Supplemental Data, Table S1). After 28 d, recovery rates for the N-cyanoamidines were 2 to 17%, and those for the N-nitroguanidines were 30 to 80%, with the exception of Confidor® (*F. candida* test). After 56 d, for the tests performed with *E. andrei*, recovery rates dropped to 1 to 4% for the N-cyanoamidines, but were stable for the N-nitroguanidines (Supplemental Data, Table S1). For the tests performed with thiamethoxam, a low concentration of clothianidin (0.09 mg a.s./kg) was detected after 56 d, indicating that this compound was converted during the experiment.

In most toxicity tests, control performance of both species met validity criteria (Supplemental Data, Table S2). Exceptions were the reduced number of juveniles (mean) produced in control jars for thiamethoxam and clothianidin tests on *E. andrei*, and the high coefficient of variation (52%) for the thiacloprid test on *F. candida*.

Weight loss in most of the earthworm exposures was significant compared with the controls ( $p < 0.00$ ), with the exception of thiamethoxam ( $p > 0.05$ ; Supplemental Data, Table S3). Concentration explained 30 to 50% of the weight variation, and for most compounds the lowest-observed-effect concentration based on earthworm weight loss was 1.1 mg a.s./kg dry soil.

For *E. andrei*, the toxicities of pure neonicotinoids and their respective formulation to both endpoints, survival (LC50) and reproduction (EC50), were similar, with overlapping confidence intervals (Table 2). The least toxic compounds, to both endpoints, were thiamethoxam (a.s.) and thiacloprid (a.s.), with the first presenting no toxicity at the highest concentration tested (10 mg a.s./kg dry soil). The most toxic neonicotinoid was acetamiprid (a.s.), being 10 times more toxic to the survival of the earthworms compared with thiacloprid.

For the tests performed with *F. candida*, there were some toxicity differences between the pure compounds acetamiprid (a.s.) and imidacloprid (a.s.) and their respective formulation. Acetamiprid was a factor of 3 more toxic to survival, and imidacloprid was a factor of 4 more toxic to reproduction compared with their respective commercial formulations (Table 2). The least toxic neonicotinoid to *F. candida* was thiacloprid (a.s.), with LC50s of 4.3 to 5.0 mg a.s./kg dry soil and EC50s of 1.5 to 2.0 mg a.s./kg dry soil. The most toxic compound was clothianidin, with an LC50 of 0.07 mg a.s./kg dry soil and an EC50 of 0.05 mg a.s./kg dry soil. Sensitivity of the 2 species to the same neonicotinoid varied, but acetamiprid was one of the most toxic and thiacloprid the least toxic to both species.

Estimated DT50 values for the degradation of the N-cyanoanilines compounds in soil from the tests with both species varied between 5 and 10 d. Neonicotinoids belonging to the N-nitroguanine family had a higher DT50, which varied between 150 and 400 d for the earthworm tests, and between 60 and 90 d for the springtail tests (Table 3). When PNEC values were compared with the respective PEC values for springtails, there was an indication of risk after one application of acetamiprid (as Mospilan) and clothianidin (in the formulation Poncho). The PNEC values derived from the tests with Calypso and Poncho were below their respective PEC values for both species, with PNECs for Poncho being 8 times lower for *E. andrei* and approximately 2 to 7 times lower for *F. candida*.

## DISCUSSION

The present study showed that most of the neonicotinoids tested are toxic to both earthworms and springtails, with low concentrations ( $\leq 1.0$  mg a.s./kg) being sufficient to cause a reduction in survival and/or reproduction. There was no difference in the toxicity of different neonicotinoids to earthworms, when dosed as pure active substance or the corresponding commercial formulation; however, there were some differences for *F. candida*. Based on the comparison between PEC and PNEC values, the acetamiprid, thiacloprid, and especially clothianidin (in their commercial formulations as Mospilan, Calypso, and Poncho, respectively) may already pose a risk to populations of earthworms and/or springtails after a single application and even more so when applied frequently during a growing season.

Soil samples were analyzed to confirm the concentrations of the neonicotinoids spiked on the first day of the tests. The recovery rate for imidacloprid spiked as Confidor was low,

**TABLE 2:** Median lethal concentration (LC50) and effect concentration (ECx) values for the effect of neonicotinoids, pure and as commercial formulations, on the survival and reproduction of *Eisenia andrei* and *Folsomia candida* in LUFA 2.2 soil<sup>a</sup>

Neonicotinoids	Endpoints (mg a.s./kg dry soil)							
	<i>Eisenia andrei</i>				<i>Folsomia candida</i>			
	LC50	EC50	EC20	EC10	LC50	EC50	EC20	EC10
Thiamethoxam	>10	>10	n.g.	n.g.	0.32 (0.30–0.40)	0.20 (0.20–0.30)	0.10 (0.10–0.15)	0.10 (0.05–0.10)
Actara <sup>®</sup>	>10	>10	n.g.	n.g.	0.33 (0.30–0.40)	0.30 (0–0.60)	0.25 (0–0.50)	0.20 (0–0.60)
Acetamiprid	0.80 (0.30–1.0)	0.40 (0–0.90)	0.30 (0.10–0.60)	0.30 (0–0.80)	0.12 (0.10–0.15)	0.10 (0.08–0.12)	0.06 (0.03–0.10)	0.05 (0.01–0.10)
Mospilan <sup>®</sup>	0.80 (0.30–1.0)	0.35 (0.20–0.50)	0.30 (0–1.0)	0.30 (0–1.0)	0.42 (0.30–0.50)	0.20 (0–0.30)	0.06 (0–0.15)	0.03 (0–0.10)
Thiacloprid <sup>b</sup>	8.0 <sup>c</sup>	1.50 (0.70–2.0)	1.0 (0.50–1.5)	0.70 (0.20–1.0)	5.0 (4.0–7.0)	1.5 (0.50–3.0)	0.90 (0.40–1.5)	0.65 (0.15–1.2)
Calypso <sup>®</sup>	7.0 (6.5–7.5)	1.0 (0.50–2.0)	0.50 (0–1.0)	0.30 (0–0.80)	4.30 (3.0–6.0)	2.0 (0.8–3.0)	0.80 (0–1.5)	0.50 (0–1.0)
Clothianidin	2.5 (1.0–4.0)	0.70 (0–2.0)	0.20 (0–1.0)	0.10 (0–0.70)	0.07 (0.04–0.08)	0.05 (0.05–0.06)	0.04 (0.03–0.04)	0.03 (0.02–0.03)
Imidacloprid <sup>a</sup>	1.0 (0–2.5)	0.50 (0–1.0)	0.35 (0.30–0.50)	0.30 (0–0.60)	0.55 (0.06–1.0)	0.25 (0.20–0.30)	0.15 (0.05–0.30)	0.10 (0.01–0.20)
Confidor <sup>®</sup>	0.80 (0.60–1.0)	0.60 (0.30–0.80)	0.35 (0.20–0.50)	0.25 (0.10–0.40)	1.0 (0.30–2.0)	1.0 (0.80–1.2)	0.80 (0.10–1.5)	0.75 (0–2.0)

<sup>a</sup>All values are based on nominal exposure concentrations. Confidence intervals (95%) are in parentheses.

<sup>b</sup>De Lima et al. (2017).

<sup>c</sup>No reliable confidence interval could be generated.

a.s. = active substance; n.g. = no value could be generated.



**TABLE 3:** Risk assessment of neonicotinoids in soil, based on data from toxicity tests with *Eisenia andrei* and *Folsomia candida*

Formulation	Active substance (a.s.)	Recommended application dose <sup>b</sup>	Dose of active substance (kg/ha)	DT50 (d) <i>E. andrei</i> <sup>c</sup>	DT50 (d) <i>F. candida</i> <sup>c</sup>	PEC (mg/kg) <sup>d</sup>	PNEC (mg/kg) <i>E. andrei</i> <sup>e</sup>	PNEC (mg/kg) <i>F. candida</i> <sup>e</sup>	Risk ratio <i>E. andrei</i> <sup>f</sup>	Risk ratio <i>F. candida</i> <sup>f</sup>
Actara <sup>®</sup>	Thiamethoxam	100 g/ha	0.025	290	88	0.02	— <sup>g</sup>	0.02	— <sup>g</sup>	1
Mospilan <sup>®</sup>	Acetamiprid	206 g/ha	0.0412	5	7	0.03	0.03	0.003	1	9
Calypso <sup>®</sup>	Thiacloprid	250 mL/ha	0.12	10	5	0.08	0.03	0.05	3	1.5
Confidor <sup>®</sup>	Imidacloprid	300 mL/ha	0.053	388	87	0.04	0.03	0.07	1	0.5
Poncho <sup>®a</sup>	Clothianidin	300 mL/ha	0.12	155	58	0.08	0.01	0.003	8	27

<sup>a</sup>Data were calculated using information provided by the manufacturer of Poncho; however, we did not test this compound.<sup>b</sup>Dosages recommended by the producers.<sup>c</sup>The degradation time (DT50) was calculated based on our tests, performed on LUFA 2.2 natural soil.<sup>d</sup>The predicted environmental concentration (PEC) for only one application/yr.<sup>e</sup>The predicted no-effect concentration (PNEC) values were based on the effect concentration, 10% (EC10) values obtained in our toxicity tests, divided by a factor of 10.<sup>f</sup>Risk ratio = PEC/PNEC.<sup>g</sup>Actara was not toxic to *E. andrei* at the concentrations tested.

being only 9% for the tests performed with *F. candida* (Supplemental Data, Table S1). This low recovery might have been due to different factors, such as: 1) despite being thoroughly mixed in with the soil, the sample may have been derived from a spot with a lower concentration due to soil heterogeneity; 2) some samples were kept in the freezer for approximately 3 yr prior to analysis, which may have contributed to some degradation of the compound (Gupta et al. 2008; Bonmatin et al. 2015; Li et al. 2018); and finally, 3) because Confidor was the only sample with a low recovery rate, a problem with transport and analysis cannot be excluded. Different factors can contribute to the recovery of a compound, making it difficult to base the toxic dose response on measured concentrations, and therefore we used nominal concentrations in our analysis. In addition, the response of the test organisms to Confidor showed a toxicity similar to that of imidacloprid (Table 2), indicating that the desired concentration was present in the soil. For the tests performed with thiamethoxam (*E. andrei*), an additional analysis for the presence of clothianidin was performed. According to Nauen et al. (2003), thiamethoxam can be converted into clothianidin in insect haemolymphs (fifth instar of *Spodoptera frugiperda*) and cotton plant tissues. Residues of clothianidin were confirmed in soil samples taken at the end of the test (56 d), indicating that thiamethoxam was indeed converted into clothianidin in our earthworm tests. Ritchie et al. (2019) also found residues of clothianidin in the tests performed with thiamethoxam, using *E. andrei* as a surrogate. In their results, concentration of clothianidin increased over time. Hilton et al. (2019) showed degradation of thiamethoxam into clothianidin in soil under aerobic laboratory conditions. Zhou et al. (2013) suggested that conversion in soil might be mainly due to the cleavage of the oxadiazine ring by microorganisms. More research is needed to assess whether this conversion can contribute to the toxicity of the parent compound. Even though clothianidin was one of the most toxic neonicotinoids to earthworms (EC50 0.70 mg a.s./kg dry soil), the concentration detected in our soil samples was low, 0.09 mg a.s./kg dry soil, and not high enough to cause a toxic response (Table 2).

Control performance of both test organisms in most of the tests met OECD criteria, with some exceptions (Supplemental Data, Table S2). In the controls of the thiamethoxam tests, only 18.8 juvenile earthworms were produced, whereas in the test with Actara, an average of 61.0 juveniles was found, even though both tests were performed with the same batch of individuals. In the tests with *F. candida* on thiacloprid (a.s.), the coefficient of variation for the number of juveniles was high (52.0%). One of the reasons for this variation might relate to individual differences, with some adults reproducing more than others. According to Crouau and Cazes (2003), the reproduction rate of individual springtails may vary within the same batch, causing differences in the number of juveniles produced. Because in all cases consistent dose–response curves were obtained, these deviations seemed not to have affected the outcome of our tests.

Weight change proved to be a sensitive endpoint for the earthworm tests, with no-observed-effect concentrations

(Supplemental Data, Table S3) often close to the EC10s for effects on reproduction (Table 2). This parameter could therefore be used as additional information to support the analysis of the toxicity of the neonicotinoid insecticides to this species.

Thiamethoxam (a.s.) was one of the least toxic neonicotinoids to the survival and reproduction of *E. andrei* (Table 2). Alves et al. (2013) tested the commercial formulation Cruiser® 350 FS; their results showed no effect of thiamethoxam on earthworm survival (14 d) up to 1000 mg a.s./kg dry soil and an EC50 for effect on reproduction of 792 mg a.s./kg, which is a factor of 80 higher than our maximum concentration (10 mg a.s./kg dry soil). Acute toxicity tests (14 d) presented by the Australian National Registration Authority for Agricultural and Veterinary Chemicals (2001), using thiamethoxam and the formulation Cruiser 350 FS, also showed no toxicity to survival (LC50 > 1000 mg a.s./kg dry soil), and no toxicity was found when *E. andrei* were exposed to Actara 70 WS in a 28-d test (National Registration Authority for Agricultural and Veterinary Chemicals 2001). Ritchie et al. (2019) also tested thiamethoxam as Actara to *E. andrei*, and their results showed no toxicity to survival; however, the compound was toxic to reproduction, with an EC50 of 3.0 mg a.s./kg dry soil. This EC50 is lower than the value we found, which might be the result of differences in density of biomass/soil between the tests—2 adults/jar into 50 g of soil, in contrast to our density, which was 10 adults/jar into 600 g of soil. Thiacloprid was also found to have low toxicity to survival and reproduction of the earthworms. According to Feng et al. (2015), the LC50 for the test performed with thiacloprid on *E. fetida* was 8.43 mg/kg, very similar to our results (Table 2). They did not present results for toxicity to reproduction, but at sublethal concentrations (1.0 and 3.0 mg/kg), which are close to our EC50s (0.5–1.0 mg a.s./kg dry soil), thiacloprid caused inhibition of some detoxification enzymes (superoxide dismutase, peroxidase, and catalase). In contrast to thiamethoxam (a.s.) and thiacloprid (a.s.), acetamiprid (a.s.) was the most toxic neonicotinoid to *E. andrei*. The Results from Wang et al. (2012), Ge et al. (2018), and Wang et al. (2015) for acute toxicity tests (14 d) on acetamiprid performed with *E. fetida* showed LC50s ranging from 1.12 to 2.70 mg a.s./kg and EC50s of approximately 0.30 mg a.s./kg dry soil. Renaud et al. (2018) also tested acetamiprid as the formulation Epik® 20SG and found, for *E. andrei*, an LC50 (28 d) of 0.85 mg a.s./kg and an EC50 of 0.32 mg a.s./kg. Despite differences in duration of exposure (14 d compared with 56 d), type of soil (natural or artificial), and species used (*E. fetida* and *E. andrei*), these results are similar to ours (Table 2), indicating that acetamiprid is very toxic to the survival and reproduction of earthworms.

Few reports in the scientific literature have been devoted to the toxicity of neonicotinoids to *F. candida*; most studies tested imidacloprid and/or thiacloprid (Alves et al. 2014) using different endpoints, such as reproduction and the activity of detoxification enzymes (Sillapawattana and Schäffer 2016). Renaud et al. (2018), Mabubu et al. (2017), and van Gestel et al. (2017) tested the toxicity of thiacloprid to springtails and showed LC50s between 3.0 and 9.0 mg a.s./kg, with the latter being almost a factor of 2 higher than ours. This difference in

toxicity could be related to variation between the treatments, which led to a wide confidence interval for this endpoint (5.6–14 mg/kg dry soil). Despite showing low toxicity to *F. candida* when compared with other neonicotinoids, thiacloprid was approximately 3 times more toxic to reproduction than to survival, following a trend of the tests performed with *E. andrei* (Table 2). Clothianidin was approximately 100 times more toxic than thiacloprid, with an LC50 of 0.07 mg a.s./kg dry soil. Ritchie et al. (2019) tested the commercial formulation Titan® (clothianidin) to *F. candida*, and their results are similar to ours, indicating that toxicity of the pure active substance (a.s.) is not affected by formulating agents.

*Eisenia andrei* and *F. candida* had different sensitivities when toxicities for the same neonicotinoid were compared, but in general *F. candida* was the most sensitive species. Springtails are generally more sensitive than earthworms because they are phylogenetic relatives of the Insecta group, and are therefore more susceptible to the action of these chemicals. Nonetheless, *E. andrei* proved to be quite sensitive to the insecticides tested, despite belonging to the Annelid group. Alexander (2002) tested imidacloprid on 2 species of aquatic invertebrates, *Epeorus longimanus* (Arthropod) and *Lumbriculus variegatus* (Annelida), with results showing the arthropod to be the most sensitive. De Lima et al. (2017) assessed the toxicity of imidacloprid (a.s.) and thiacloprid (a.s.) to 5 different species of soil invertebrates belonging either to the annelids or arthropods, and their results showed that species belonging to the same phylum had different sensitivities to the same compound, indicating that this response could be species specific. Scott-Dupree et al. (2009) tested the effects of clothianidin and imidacloprid on different species of bees (arthropods), and their results showed that *Bombus impatiens* was less sensitive to clothianidin and imidacloprid compared with *Osmia lignaria* and *Megachile rotundata*. Based on these and our results, it is expected that the sensitivity to neonicotinoids of nontarget organisms would be species specific, with soil invertebrates belonging to different taxonomic groups, such as *E. andrei*, presenting a relatively high sensitivity to these compounds.

In light of the widespread use of neonicotinoids in agricultural fields (Jeschke et al. 2011; Goulson 2013) and their potential for accumulating in the soil (Bonmatin et al. 2015; Simon-Delso et al. 2015), a more comprehensive analysis of our data was performed, aiming to evaluate possible environmental risks of these insecticides to soil invertebrates. The PECs of acetamiprid (Mospilan) and clothianidin (applied as Poncho) were above their respective PNECs, derived from EC10s, for springtails. For acetamiprid applied as Mospilan, the expected concentration in the environment was even equal to the EC10 (0.03 mg a.s./kg dry soil). For clothianidin (Poncho), the PEC was approximately 3 times higher than the EC10 of *F. candida* (0.03 mg a.s./kg dry soil), and close to the LC50 (0.07 mg a.s./kg dry soil). Acetamiprid belongs to the N-cyanoamidine group (Jeschke et al. 2011), with relatively short half-lives between 5 and 10 d (present results). This may allow for some potential recovery of the springtail populations in the field. However, clothianidin belongs to the N-nitroguanidines (Jeschke et al. 2011), which have longer

half-lives, with the DT50 for clothianidin in LUFA 2.2 natural soil being approximately 58 d (Goulson 2013; Bonmatin et al. 2015), posing considerable risk to springtail populations after a single application/yr. None of the neonicotinoids had a PEC higher than the EC10s for *E. andrei*; however, thiacloprid (Calypso) and clothianidin (Poncho) present a considerable risk to earthworm populations in the field, with the PEC for thiacloprid being 3 times higher than the PNEC, and that for clothianidin being 8 times higher. When we assessed the risk of the commercial formulations tested, the most toxic neonicotinoid for both species was clothianidin applied as Poncho, which can pose a considerable risk to populations of earthworms and springtails in the field after one single application/yr (Table 3). This scenario is quite worrying because different studies refer to the high persistence of neonicotinoids in soil and their presence in agricultural soils. Silva et al. (2019) recently showed the presence of multiple residues of pesticides, including imidacloprid (N-nitroguanidine), in 58% of 317 soil samples from European agricultural fields. Even though clothianidin is banned from application as a seed dressing and its use is restricted by the European Union (2013), its high persistency in soil and the fact that it is among the most used neonicotinoids mean that low clothianidin concentrations can still pose a risk to populations of soil invertebrates in the foreseeable future.

## CONCLUSIONS

Our results showed, in general, that active neonicotinoid substances cause toxicity to the species tested and that commercial formulations were never more toxic than the pure compounds. The most sensitive species was *F. candida* when toxicities to the same neonicotinoid were compared, but *E. andrei* proved to be quite sensitive as well, even though it is not phylogenetically related to the Insecta group. The least toxic neonicotinoid for both species was thiacloprid, whereas clothianidin was the most toxic. We have presented a preliminary and still fairly conservative risk assessment for neonicotinoids based on their application in commercial formulations, and have shown that neonicotinoids may already pose a risk to populations of soil invertebrates in the field after a single application.

**Supplemental Data**—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.4634.

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**Data Availability Statement**—Data, associated metadata, and calculation tools may be requested from the corresponding author (claudiadelimaesilva@gmail.com). The raw data are stored in a data repository at the Department of Ecological Science of the Vrije Universiteit, Amsterdam, The Netherlands, and can be made available on request to the corresponding author.

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