Long-Term Effects of Imidacloprid, Thiacloprid, and Clothianidin on the Growth and Development of Eisenia andrei

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Abstract: Recently, the high toxicity of neonicotinoids to the survival and reproduction of adult earthworms has become apparent in standard 56-day toxicity tests. The persistence of some neonicotinoids and/or their repeated application may lead to long-term exposure, possibly also affecting other parts of the life cycle of earthworms. The present study aimed at providing insight into the sublethal effects of imidacloprid, thiacloprid, and clothianidin on juvenile *Eisenia andrei* exposed for 16 weeks in Lufa 2.2 soil. Significant effects on growth and maturation were observed for all compounds. Exposure to 0.125 mg imidacloprid/kg dry soil and 0.03125 and 0.0625 mg thiacloprid/kg dry soil significantly affected the growth of the earthworms, while significant maturation effects were observed at 0.03125 mg/kg dry soil for imidacloprid and thiacloprid and 0.25 mg clothianidin/kg dry soil. The 16-week no-observed-effect concentrations (NOECs) found in the present study were lower than previously reported NOECs for effects on earthworm reproduction. Predicted environmental concentrations after a single application exceeded the observed NOECs for effects on earthworm maturation in the case of imidacloprid and thiacloprid and for effects on earthworm growth in the case of thiacloprid and clothianidin. *Environ Toxicol Chem* 2022;41:1686–1695. © 2022 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Neonicotinoids; *Eisenia andrei*; Life-cycle effects; Soil ecotoxicology

INTRODUCTION

Since their introduction in the late 1980s, neonicotinoids have become the most widely used insecticides in the world (Zhu et al., 2017). Neonicotinoids are broad-range insecticides, binding almost irreversibly to the nicotinergic acetylcholine receptors (nAChR) of an organism (Simon-Delso et al., 2015). Neonicotinoids act agonistically on nAChRs, blocking the central nerve cell receptors, leading to paralysis and eventually to death (Zhang et al., 2000).

Neonicotinoids contain a nitro or cyano group, causing them to interact with the cationic nAChR subsites uniquely found in insects (Thany, 2010). This, as well as poor passage through the blood-brain barrier, causes high specificity to insects, with relatively little effect on mammals (Rose, 2012; Tomizawa & Casida, 2004). Application routes include soil treatment; foliar spraying; soil drenching, through irrigation water; trunk injection; seed dressing; and seedling dipping (Goulson, 2013; Jeschke et al., 2011). Seed dressing and seedling dipping minimize drift of the insecticides to neighboring environments and limit exposure of nontarget invertebrates not directly feeding on the treated crops. The mobile properties of the neonicotinoids facilitate their easy uptake by the plant and their systemic action, supporting these applications.

However, even with decreased drift, these insecticides end up in the soil, and their systemic properties cause accumulation in pollen and nectar. This exposes nontarget soil organisms and pollinators to these compounds (Goulson, 2013). Recent research has shifted to the long-term effects of neonicotinoids on pollinators, because of the major lethal and sublethal effects they have even at low doses (Baines et al., 2017; European
Food Safety Authority, 2018; Goulson, 2013; Henry et al., 2012; Iwasa et al., 2004; Simon-Delso et al., 2015; Whitehorn et al., 2012; Zhu et al., 2017). Woodcock et al. (2016) suggested that sublethal effects can eventually cascade to cause biodiversity decline in bees.

Recently, neonicotinoids were also shown to be highly toxic to nontarget soil invertebrates, especially springtails but also earthworms. Both lethal and sublethal effects (reproduction) were shown in earthworms and springtails at concentrations close to those expected on normal application of these insecticides (de Lima et al., 2017, 2020).

To assess the environmental risk of pesticides to earthworms, Organisation for Economic Co-operation and Development (2016) guidelines recommend assessing the effects on the survival of adult earthworms following exposure for up to 28 days, with an assessment of reproduction after 56 days. However, from the knowledge that neonicotinoids bind almost irreversibly to nAChRs (Abbink, 1991; Buckingham et al., 1997; Zhang et al., 2000) and suggested effects on other sensitive species, it may be expected that neonicotinoid toxicity will increase with time of exposure (Sánchez-Bayo & Tennekes, 2020). From that perspective, the standard short-term toxicity test may not be adequate in assessing life-cycle effects on sublethal endpoints.

This is also due to the fact that 28- or 56-day tests focus on adult survival and reproduction, respectively, and not on maturation and life-cycle effects. Because of the high persistence of several neonicotinoids and indications that (some) neonicotinoids are endocrine-disrupting compounds, effects on maturation and life-cycle effects need to be determined to adequately assess their environmental risk (Baines et al., 2017). Proposed endocrine-disrupting effects and effects on hormone regulation also call for evaluation of the maturation effects of these compounds (Baines et al., 2017; Danish Environmental Protection Agency, 2018).

The aim of the present study was to assess the life-cycle effects of imidacloprid, thiacloprid, and clothianidin on the earthworm Eisenia andrei, Bouché, 1972, a common model species in soil ecotoxicology. Tests were done with juvenile earthworms, and survival, growth, and sexual development were monitored over an exposure period of 16 weeks, which was expected to be sufficient to see earthworm maturation (Van Gestel et al., 1991).

### MATERIALS AND METHODS

#### Test organisms

For the toxicity tests, juvenile E. andrei were taken from a synchronized culture kept at the Amsterdam Institute for Life and Environment, Section Ecology & Evolution at the Vrije Universiteit Amsterdam. All animals used were 0–4 weeks old. The culture was fed horse dung, free of pharmaceuticals, and kept in a climate room at 20 °C.

#### Test compounds and soil

Natural standard Lufa 2.2 soil (Lufa Speyer) was used, which has approximately 1.6% organic carbon, a water holding capacity (WHC) of 45% (w/w), and a pH (0.01 M CaCl₂) of 5.6. In the tests, the soil was moistened to a moisture content of 23%, which corresponds to 50% of its WHC. Thiacloprid and imidacloprid (purity >98%) were provided by Bayer CropScience. Analytical-grade clothianidin was purchased from Sigma-Aldrich.

#### Toxicity test

Five exposure concentrations were used: 0.03125, 0.0625, 0.125, 0.25, and 0.5 mg/kg dry soil for imidacloprid and thiacloprid and 0.0625, 0.125, 0.25, 0.5, and 1.0 mg/kg dry soil for clothianidin. These concentrations were chosen based on earlier research on reproduction effects on E. andrei (de Lima e Silva et al., 2017, 2020). A control was also included.

Soil spiking was done by dissolving the neonicotinoids in Milli-Q. The solution was mixed in with dry Lufa 2.2 soil using a hand-mixer, to ensure homogenous distribution of the pesticides. Clothianidin and imidacloprid, despite having a long half-life (148–6931 and 28–1250 days, respectively [Goulson, 2013]), can be susceptible to biodegradation, while exposure concentrations may also be influenced by the test organisms themselves because of increased aeration and mixing of the soil by bioturbation and incorporation of the food into the soil (Hussain et al., 2016; Parte & Kharat, 2019). Therefore, the earthworms were transferred to freshly spiked soil after 8 weeks for imidacloprid and clothianidin, to ensure a more constant exposure concentration over the entire 16-week experiment. Because of its short half-life (10–12 days in Lufa 2.2 soil [van Gestel et al., 2017]), for thiacloprid freshly spiked soil was prepared every 2 weeks of the experiment.

Juvenile earthworms were exposed to the three neonicotinoids for 16 weeks, during which growth and maturation were monitored. For the first 8 weeks, the worms were kept in approximately 300 g of moist soil in 600-ml jars, after which they were transferred to 1-L jars containing approximately 600 g of moist soil. Each jar contained 10 juvenile earthworms. Each test concentration and control had four replicate jars.

Prior to the start of the experiment, the earthworms were acclimatized for 24 h in noncontaminated Lufa 2.2 soil, moistened at 50% of the WHC, after which they were rinsed, blotted dry on a tissue paper, weighed, and randomly allocated to the test jars. The individual earthworm masses (mean ± SD, n = 240) at the start were 30.1 ± 16.5, 27.1 ± 11.7, and 30.8 ± 18.3 mg for the tests with imidacloprid, thiacloprid, and clothianidin, respectively.

Ground dried horse dung (<2 mm) was used as a food source, after hydration to a 3:1 water to horse dung ratio. To prevent the earthworms from residing in a clump of manure, avoiding exposure to the neonicotinoids, the food addition was built up over the length of the experiment. In the first 6 weeks the earthworms were fed 3.5 g of horse dung per 2 weeks. After 6 weeks, this was increased to 4 g/2 weeks; and after 8 weeks, 5 g of food was added per 2 weeks. The food was distributed over three small holes made in the soil to further prevent the earthworms from residing in the horse dung.
The food was covered with a layer of soil to prevent fungal growth. Every week, the water loss was replenished by weighing the jar and adding water to restore to the original weight. After 4 weeks of exposure, the earthworms were extracted from the soil by hand sorting, after which sexual maturity was determined according to Van Rhee (1970). Individuals with tubercles of puberty but without a (fully grown) clitellum were considered subadults, and those with a fully grown clitellum were considered adults. The worms were then rinsed with water, blotted dry on tissue paper, and weighed (scale \( d = 0.0001 \) g), after which they were returned to the soil, to which fresh food was added. This procedure was repeated every 2 weeks.

After 16 weeks, the earthworms were placed on wet filter paper for 24 h to void their gut contents. Half of the earthworms were used to determine dry weight, by weighing the worms before and after freeze-drying. For thiacloprid, the remaining earthworms were stored for assessing bioaccumulation.

Soil samples were taken at \( t = 0 \) from freshly spiked soil and from soil in which earthworms resided after 4 and 8 weeks for imidacloprid and clothianidin. For thiacloprid soil was sampled every 2 weeks (on renewing soil). Soil samples were frozen at \(-20^\circ C\) for chemical analysis, to determine the actual neonicotinoid exposure concentrations.

During the 16-week test, in several replicates very small individuals were observed to show no growth whatsoever. Large differences were also observed between the absolute growth of individuals between the controls of the different test compounds. It seemed that these individual earthworms had different average starting weights. Therefore, a fully crossed experimental design was set up for 28 days to check for the influence of starting weight on the effect of the chemical exposures. The experimental setup and results can be found in the Supporting Information (SI-1).

**Chemical analysis**

Imidacloprid extraction was conducted in duplicate soil samples at the Instituto de Recursos Naturales y Agrobiología de Salamanca. A volume of 12 ml of methanol was added to 6 g of sample. The mixture was sonicated in glass tubes for 1 h, and then constant agitation was maintained in a rotatory shaker at \( 20^\circ C \) for 24 h. The samples were centrifuged at 300 rpm for 7 min and the extracts filtered with Minisart NY 25 filters (Sartorius Stedim Biotech) to remove particles >0.45 \( \mu \)m. A volume of 8 ml of extract was evaporated to dryness under a nitrogen steam at 25 \( ^\circ C \) in an EVA-EC2-L evaporator (VLM). The residue was dissolved in 0.75 ml of methanol with 1% formic acid and transferred to glass vials for analysis.

Imidacloprid was determined by high-performance liquid chromatography equipped with a model e2695 multisolvant delivery and autosampler system attached to a ZQ mass spectrometer (MS) detector (Waters). The detection was carried out by monitoring the positive molecular ion (mass to charge ratio) 256.2.

Chemical analysis of thiacloprid and clothianidin in soil samples and thiacloprid in earthworms was performed by Groen Agro Control using liquid chromatography–tandem MS, applying certified chemical analytical procedures and quality control measures. The detection limit was 0.01 mg/kg dry weight for all three neonicotinoids in soil and earthworms.

The bioaccumulation factor (BAF) for thiacloprid was calculated as the ratio of the concentration in earthworms (milligrams per kilogram fresh wt) to soil (milligrams per kilogram dry soil).

**Statistical analysis**

Von Bertalanffy growth curves (Equation 1) were fitted to the effects of the tested neonicotinoids on earthworm growth rate.

\[
m_t = m_0 + (m_{end} \times (1 - e^{-\gamma(t-t_0)})^\beta)\]

In Equation 1, \( m_t \) is the average individual earthworm mass (milligrams) at time \( t \) (weeks), \( m_0 \) is the measured average individual mass at the start of the exposure, \( m_{end} \) is the modeled average final individual mass, \( \gamma \) is the Von Bertalanffy growth rate (per week), \( t \) is the time in weeks, and \( t_0 \) is time point 0. \( m_{end} \) and \( \gamma \) were calculated using the “Solver” function in Microsoft Excel. Significance of differences (\( \alpha < 0.05 \)) between Von Bertalanffy growth parameters for different neonicotinoid exposure levels and controls was statistically compared using a generalized likelihood ratio test.

G tests were used to determine the significance of difference in maturation between treatments and controls. Earthworm dry weights were compared using Student t tests.

**RESULTS**

Shortly after spiking, for all test compounds the measured concentrations were lower than the nominal ones, generally ranging between 70% and 98% of nominal concentrations except for the imidacloprid spiking at 0.0125 mg/kg dry soil, where it was only 36%–59% (see Supporting Information, Table SI-1). Imidacloprid concentrations showed quite some fluctuation over time but overall decreased to 40%–80% of the measured initial concentration after 8 weeks. The concentrations of clothianidin and especially thiacloprid consistently decreased with time to 59%–67% and 32%–35% of measured initial concentrations after 8 and 2 weeks, respectively. All effects are expressed on the basis of nominal concentrations.

In all exposures, >90% of the earthworms survived; therefore, no data were omitted from the further analysis of growth and maturation effects. The full Von Bertalanffy curves fitted for the growth of \( E. \) andrei exposed to thiacloprid over the 16-week test period are shown in Figure 1; the curves for the other compounds can be found in Supporting Information, Figure SI-2. The mean earthworm masses (±SE) recorded at the
different sampling times on which these growth calculations are based are shown in Supporting Information, Table SI-2.

Although starting from the same initial mass and ruling out interaction effects between starting weight and growth (see Supporting Information, SI-1), the final control weight was substantially higher for the earthworms from the thiacloprid test than for the tests on the other compounds. The measured final weight of the thiacloprid control group was 584 ± 22.1 mg (mean ± SE), whereas the controls of the imidacloprid and clothianidin tests both only reached a final weight of 229 ± 10.9 and 229 ± 11.5 mg (mean ± SE), respectively.

The percentage dry weight of the earthworms in control and neonicotinoid-spiked soils did not differ significantly (p > 0.05) within or between any of the exposures and amounted to 29.8 ± 0.5%, 34.8 ± 1.2%, and 30.0 ± 0.4% (mean ± SE) for imidacloprid, thiacloprid, and clothianidin, respectively.

The Von Bertalanffy growth parameters for the earthworms exposed to the three neonicotinoids are shown in Table 1. For all test compounds, a dose-related decrease in earthworm growth rate was observed. For imidacloprid, the modeled earthworm growth rate was significantly lower compared to the control at 0.25 mg/kg (p < 0.05) and 0.5 mg/kg (p < 0.001). The modeled final mass was significantly lower compared to the control at 0.125 mg/kg imidacloprid (p < 0.05), with no significant effect on final mass at 0.25 and 0.5 mg/kg, despite a significantly decreased growth rate. At all test concentrations of thiacloprid, both the modeled growth rate and the final weight were significantly (p < 0.001) lower compared to the control. For clothianidin, the modeled earthworm growth rate was significantly reduced compared to the control at the lowest test concentration (p < 0.01), at 0.125 mg/kg (p < 0.05), and at 0.5 mg/kg (p < 0.001). At 1 mg/kg clothianidin, the modeled final mass was significantly (p < 0.001) lower than in the control.

After 16 weeks of exposure, the no-observed-effect concentrations (NOECs) for growth effects of imidacloprid, thiacloprid, and clothianidin were 0.0625, < 0.03125, and < 0.0625 mg/kg dry soil, respectively (Table 2). These NOECs for growth relate to the significance of effects on either one of the Von Bertalanffy growth parameters reported in Table 1.

Earthworms exposed for 16 weeks to thiacloprid at 0.25 and 0.5 mg/kg dry soil (nominal test concentrations) contained 0.97 and 1.3 mg thiacloprid/kg fresh body weight, corresponding to BAFs of 3.9 and 2.6, respectively. The bioaccumulation of clothianidin and imidacloprid was not determined.

**FIGURE 1**: Effect of thiacloprid on the growth of the earthworm *Eisenia andrei* exposed in Lufa 2.2 soil over a 16-week period. Points show the mean earthworm weights (milligrams) recorded for each exposure concentration and sampling time; lines show the fit of the Von Bertalanffy growth model to the data.

**TABLE 1**: Von Bertalanffy parameters for the growth of *Eisenia andrei* exposed for 16 weeks to three neonicotinoids in Lufa 2.2 soil

| Concentration (mg/kg) | Imidacloprid | Thiacloprid | Clothianidin |
|-----------------------|--------------|-------------|--------------|
|                       | m<sub>end</sub> | γ           | m<sub>end</sub> | γ         | m<sub>end</sub> | γ       |
| 0                     | 285.7        | 0.156       | 910.2        | 0.114     | 285.5        | 0.151   |
| 0.03125               | 284.0        | 0.154       | 719.1        | 0.112***  | NA           | NA      |
| 0.0625                | 278.3        | 0.155       | 674.6        | 0.107***  | 261.8        | 0.149** |
| 0.125                 | 272.0*       | 0.144       | 661.1**      | 0.106***  | 265.5        | 0.146   |
| 0.25                  | 261.8        | 0.145*      | 625.8**      | 0.100**   | 278.9        | 0.147   |
| 0.5                   | 225.5        | 0.131***    | 546.0**      | 0.092**   | 247.3        | 0.139*** |
| 1                     | NA           | NA          | NA           | NA        | 247.5**      | 0.127   |

*p < 0.05, **p < 0.01, ***p < 0.001. m<sub>end</sub> = modeled average final mass (milligrams) per individual; γ = growth rate coefficient (per week); NA, not analyzed (concentration not tested for this compound).
TABLE 2: No-observed-effect concentrations (NOECs) and 10% effect concentration values for the effect of imidacloprid, thiacloprid, and clothianidin (pure active substance) on the reproduction of *Eisenia andrei* after 8 weeks and NOECs for growth and maturation effects after 16 weeks of exposure (present study) in Lufa 2.2 soil (or artificial soil)*

| Compound/effect measure | Reproduction (NOEC) | Reproduction (EC10) | Growth (NOEC) | Maturation (NOEC) | PEC | MEC |
|-------------------------|---------------------|---------------------|---------------|-------------------|-----|-----|
| Imidacloprid            | 0.20–0.30<sup>b</sup> | 0.30<sup>c</sup> | 0.0625 | <0.03125 | 0.04<sup>d</sup> | 0.01–1.0<sup>e,f,g</sup> |
| Thiacloprid             | 0.125<sup>a</sup>–0.30<sup>b</sup> | 0.70<sup>c</sup> | <0.03125 | <0.03125 | 0.08<sup>e</sup> | 0.00081<sup>h</sup> |
| Clothianidin            | – | 0.10<sup>c</sup> | <0.0625 | 1.0 | 0.08<sup>e</sup> | 0.018–0.05<sup>i,j</sup> |

*<sup>a</sup>Wang et al. (2015).
<sup>b</sup>de Lima e Silva et al. (2017).
<sup>c</sup>de Lima e Silva et al. (2020).
<sup>d</sup>Bandiera et al. (2019).
<sup>e</sup>Silva et al. (2019).
<sup>f</sup>Jones et al. (2014).
<sup>g</sup>Zhou et al. (2021).
<sup>h</sup>Zhou et al. (2021).
<sup>i</sup>Zhou et al. (2022).
<sup>j</sup>Xu et al. (2016).

NOECs for growth relate to the significance of effects on either one of the Von Bertalanffy growth parameters reported in Table 1. Also given is the predicted environmental concentration after only one application/yr and the measured environmental concentrations after single or multiple applications. All concentrations are in milligrams per kilogram dry soil.

**DISCUSSION**

The aim of the present study was to assess the life-cycle effects of imidacloprid, thiacloprid, and clothianidin on the earthworm *E. andrei*. All compounds affected the growth of the earthworms at sublethal concentrations, while imidacloprid and thiacloprid also had a dose-dependent effect on their sexual development (maturation). This shows the importance of including growth and maturation studies in the risk assessment of neonicotinoids.

**Growth effects**

Significant differences in growth rate as well as final mass were observed between the different tests (Table 1). Where the mass of the control earthworms from the thiacloprid test averaged 584 mg after 16 weeks, the earthworms exposed to clothianidin and imidacloprid both had average individual masses of 229 mg after 16 weeks. This is most likely explained by our experimental setup. In the clothianidin and imidacloprid tests, the earthworms were transferred to freshly spiked soil just once over the course of the experiment, after 8 weeks. Because of the short half-life of thiacloprid, 10–12 days in Lufa 2.2 soil (van Gestel et al., 2017), and the aim to keep exposure concentrations as constant as possible, the earthworms were transferred to freshly spiked soil every second week. The repeated transfer to new soil may have had an unintended side effect by creating a better environment for the earthworms in the thiacloprid tests. The observed differences may be due to self-toxicity effects in the clothianidin and imidacloprid groups (Kaplan et al., 1980), caused by microbial growth in the casts shed by the earthworms, causing weight loss effects in their culture. This effect is in line with the effects observed in our study.

The growth of the earthworms was negatively and dose-relatedly affected by all three neonicotinoids. Both the final mass and growth rate of the earthworms were affected. This might be related to a decrease in energy reserves, as was found for the land snail *Cornu aspersum* (formerly named *Helix aspersa*) by Radwan and Mohamed (2013); but it requires more research to determine the exact mechanism behind these effects. Because the dry weight as a percentage of wet weight did not differ between earthworms exposed to the neonicotinoids and the controls, increased water intake or major edema formation can be ruled out as the cause of the growth effects.

One assumption to explain the decreased growth rate is a decrease in food intake due to toxic effects on earthworm behavior, which was seen especially at the higher concentrations of all compounds. A decreased bioturbation was
observed, with less fecal matter on the surface of the soil in the test jars as well as a higher level of algal growth, especially at higher concentrations of clothianidin and imidacloprid. These observations, as well as a lack of response to touching and handling, are indicative of latent behavior and might explain a lower food intake and therefore the slower growth of the exposed earthworms. Although other studies also suggest an effect on energy reserves (see, e.g.
Radwan & Mohamed, 2013; Rezac et al., 2021), from our data it is not possible to prove or disprove this as the mechanism behind the growth effects.

**Maturation effects**

Maturation effects were observed on exposure to all three neonicotinoids. The most observed effect was an earlier onset of maturation, which was significant at all imidacloprid exposure concentrations, at 0.25 mg/kg clothianidin, and at 0.03125, 0.0625, and 0.125 mg/kg thiacloprid (Figure 2). The only significant delay in the onset of maturation was observed at the highest concentration of thiacloprid.

Although the exact mechanism of maturation effects is unclear, hormonal effects of neonicotinoids have been suggested for several mammalian species (Berheim et al., 2019; Caron-Beaudoin et al., 2018; Danish Environmental Protection Agency, 2018; El Sawsany et al., 2017). This might offer an explanation for the observed effects because the supposedly affected hormones are sex hormones like testosterone and estrogen. However, there seems to be a discrepancy between studies on the possible hormonal effects, with some authors finding increased levels or shifts in sex hormone levels and others reporting no such effects (Kapoor et al., 2011; Mesnage et al., 2018).

A second explanation for the observed effects may be a trade-off between growth and reproduction, being affected by the stress the earthworms experienced (Hirshfield & Tinkle, 1975). This would cause the earthworms to invest more in fast maturation to become adult because of the experienced stress. Although a similar trade-off between earthworm weight change and cocoon production was observed by van Gestel et al. (1992), no hard evidence was found to support this. It therefore remains unclear what caused the effect observed in the present study.

The results on thiacloprid may be explained by hormesis, “a term used by toxicologists to refer to a biphasic dose response to an environmental agent characterized by a low dose stimulation or beneficial effect and a high dose inhibitory or toxic effect” (Mattson, 2008). This effect can explain the earlier onset of maturation on exposures to imidacloprid and thiacloprid and can even explain the delayed maturation at the highest concentration of thiacloprid. However, this is not relevant for the observed earlier onset of maturation at the clothianidin concentration of 0.125 mg/kg dry soil because in the case of hormesis we would expect to see a negative effect at higher concentrations, which we did not. The same goes for the imidacloprid exposure. Even though strong effects were observed in the earlier onset of maturation, the large range of concentrations at which these effects were observed does not comply with what is expected if this was indeed a hormetic effect.

Because maturation is a complex biological process, it might very well be a combination of several of these effects, an entirely different effect, or a compound-specific effect. We recommend more research on the growth and maturation effects of neonicotinoids, linking to molecular and biochemical effects and exploring the possibility of developing an adverse outcome pathway.

**Consequences for neonicotinoid risk assessment**

Despite being relevant fitness indicators, earthworm growth and maturation under neonicotinoid influence have not yet been thoroughly investigated. The present study shows that both the final mass and growth rate of earthworms are affected at concentrations lower than the effect levels observed in reproduction tests.

The NOECs observed after 16 weeks of sublethal exposure are lower than reported values for effects on earthworm reproduction for all compounds tested. Imidacloprid exposure led to a NOEC almost three to five times lower for growth and seven to 10 times lower for maturation compared to reproduction (Table 2). Thiacloprid affected growth and maturation at concentrations four to 10 times lower than the previously reported NOEC and >22 times lower than the previously reported 10% effect concentration (EC10) value from 8-week reproduction tests (Table 2). The clothianidin concentration affecting earthworm maturation did not differ greatly from previously reported EC10 values for reproduction effects, although growth effects were exhibited at lower concentrations. The NOEC for growth effects was higher than the EC10 values of 0.10 and 0.0031 mg/kg dry soil for reproduction effects reported by de Lima e Silva et al. (2020) and Ritchie et al. (2019), respectively. The latter authors, however, tested a commercial formulation, making it possibly difficult to compare their EC10 values with our data, although de Lima e Silva et al. (2020) did not find differences in toxicity between pure compounds and commercial formulations.

Observed NOECs for maturation effects of imidacloprid, growth and maturation effects of thiacloprid, and growth effects of clothianidin are lower than the predicted environmental concentrations (PECs) based on single applications of these compounds and close to, or within the range of concentrations measured in field soils (Table 2). This suggests that already at normal application rates, these neonicotinoids may affect the growth and sexual development of earthworms in field soils. The present study used E. andrei, which may not be the most sensitive or field-representative earthworm species because it is a compost worm. But the results of our study, showing that growth and sexual development are relevant indicators of effects on long-term exposure, seem equally valid for more field-relevant earthworms species.

The results of our study stress the importance of life-cycle testing. Short-term tests provide good insight into the effects on the reproductive output of healthy adult individuals but are not able to detect the effects observed at much lower concentrations in maturation tests, which were twice the test duration. Effects on growth and maturation in the long run inevitably influence reproductive output as well, even at concentrations below PECs after a single application (Bart et al., 2019).

Besides the effects mentioned above, the present study revealed several other reasons to include life-cycle tests in the risk assessment of neonicotinoids. Despite being infamous for their
inability to metabolize a vast array of xenobiotics, the effects of imidacloprid on the earthworms seemed to diminish over time, with the difference in earthworm mass being larger after 4 weeks compared to 8 weeks (see Supporting Information, Table SI-2). Imidacloprid metabolism has been shown in bees and mayflies through cytochrome P450s (CYP450) but, to the best of our knowledge, not for any larger soil organism (Yang et al., 2013; Zhu et al., 2017). Usually, CYP450s provide Phase I metabolism of xenobiotics by hydroxylation, demethylation, nitro reduction, cyano hydrolysis, and dechlorination (Simon-Delso et al., 2015). Therefore, we performed a quantitative polymerase chain reaction (qPCR) analysis on earthworms exposed to imidacloprid, to test for up-regulation of CYP450s. Up-regulation of these genes would provide evidence of Phase I metabolism of imidacloprid in the earthworms and explain a diminishing effect of imidacloprid exposure over time.

Because no full genome sequence of *E. andrei* was available, a full genome sequence of the closely related species *Eisenia fetida* was used, applying a method validated by Van Ommen Kloek et al. (2014; see also Gong et al., 2010). Using this genome sequence, we performed a qPCR analysis among five different CYP450s. The results showed a significant P450 up-regulation at 0.4 mg imidacloprid/kg dry soil (Kolmogorov-Smirnov test, p < 0.05), a concentration where also effects on growth became evident; P450 up-regulation was not significant at 0.2 mg imidacloprid/kg dry soil. A full description of the experiment can be found in the Supplementary Information (SI-2). The present study, to the best of our knowledge, provides the first evidence of significant CYP450 up-regulation in response to imidacloprid exposure in earthworms. These findings have implications for the results found in short- and long-term tests on neonicotinoid exposure and require more research to determine its ecotoxicological relevance.

Another implication found was the observed bio-accumulation of thiacloprid in *E. andrei*. After 16 weeks of exposure to thiacloprid at 0.25 and 0.5 mg/kg dry soil, BAFs of 3.9 and 2.6 were found, respectively (n = 5). This confirms the potential of neonicotinoids to bioaccumulate in earthworms, as also demonstrated by Chevillot et al. (2017). The bio-accumulation of neonicotinoids over time needs to be investigated to make conclusive statements on the effect of bioaccumulation on differences observed between short-term and long-term exposures.

**CONCLUSION**

The present study shows the relevance of determining the sublethal effects on earthworms of long-term exposure to imidacloprid, thiacloprid, and clothianidin. Exposure of juvenile *E. andrei* in the present study showed lower NOECs for sublethal effects on growth and maturation than previously reported NOECs for reproduction effects. The NOECs from the present study also are close to or even below concentrations measured in field soils. Although short-term tests may provide useful insight into the (acute) effects on reproduction, the present study stresses the importance of long-term effects on growth and maturation, which may very well affect reproductive output as well. The results of the present study therefore have implications for the risk assessment of neonicotinoids but may also ask for further refinement of the tools used for assessing the risk of pesticides in general.

**Supporting Information**—The Supporting Information is available on the Wiley Online Library at https://doi.org/10.1002/etc.5345.

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**Data Availability Statement**—Data may be requested by email to the corresponding author (kees.van.gestel@vu.nl).

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