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Hazard/Risk Assessment

Variation in the Chemical Sensitivity of Earthworms from Field Populations to Imidacloprid and Copper

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Abstract: The chemical risk of pesticides for nontarget soil macroorganisms has mainly been assessed using the compost earthworm *Eisenia fetida*. However, *E. fetida* does not occur in agroecosystems, and it is generally less sensitive than other earthworm species. Thus, the extrapolation of its response to pesticides to other earthworm species may lead to uncertainties in risk assessment. Because toxicity data for other earthworms are scarce, we assessed the chemical sensitivity of five species (*Allolobophora chlorotica*, *Aporrectodea caliginosa*, *Aporrectodea longa*, *Aporrectodea rosea*, and *Lumbricus rubellus*) from different habitats (forests, wetlands, and grasslands), as well as *E. fetida*, to imidacloprid and copper in single-species acute toxicity tests. In addition, we examined the relationship between earthworm traits (ecotype and weight), habitat characteristics (ecosystem type and soil pH), and chemical sensitivity. The lower limits of the hazardous concentration affecting 5% (HC5) of species were 178.99 and 0.32 mg active ingredient/kg dry weight for copper and imidacloprid, respectively. Some concentrations that have been measured in European agroecosystems for both pesticides were above the HC5s, indicating toxic risks for these organisms. Furthermore, soil pH from the sampling habitat played a significant role, with earthworms sampled from extremely acidic soils being less sensitive to copper than earthworms from neutral soils. In addition, endogeic earthworms were more sensitive to imidacloprid than epigeic earthworms. This may translate to changes in soil functions such as bioturbation, which is mainly carried out by endogeic earthworms. Our results suggest that risk assessment should include a wider range of earthworms covering different habitats and ecosystem functions to achieve a better protection of the biological functions carried out by these key soil organisms. *Environ Toxicol Chem* 2023;42:939–947. © 2023 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Earthworms; Risk assessment; Species sensitivity distribution

INTRODUCTION

Earthworms are ecosystem engineers (Jones et al., 1994; Jouquet et al., 2006), playing key roles in pedogenesis (Edwards, 2004; Lee & Foster, 1991), soil structure (Bernier, 1998; Kavdir & İlay, 2011), and soil fertility (Edwards & Bohlen, 1996). In addition to their ecological relevance, earthworms are bioindicators of soil pollution caused by toxic substances such as heavy metals (Suthar et al., 2008) and pesticides (Pelosi et al., 2014). The compost earthworm *Eisenia fetida* (SAVIGNI, 1826) has been used as a standard organism for the risk assessment of pesticides on nontarget soil macroorganisms in the European Union, regulated by the European Commission (EC) under legislation 1107/2009 (European Union, 2011) and guidance document SANCO/10329/2002 (Santé et Consommateurs Directorate General Health and Consumers, 2002). *Eisenia fetida* is considered to be a species complex (Römbke et al., 2016) consisting of at least *E. fetida* and *Eisenia andrei* (BOUCHÉ, 1972). This species meets the basic requirements for being a standard test organism because it is easy to rear under laboratory conditions (Paradise, 2001). Consequently, several standardized guidelines have been developed to assess the acute (International Organization for Standardization [ISO], 2012; Organisation for Economic Co-operation and Development [OECD], 1984), as well as chronic, and sublethal effects (ISO, 2008; OECD, 2016) of pesticides and other chemicals on *E. fetida* under laboratory conditions.

Pesticide testing on *E. fetida* is well established for regulatory purposes (Edwards, 2004). However, its use as the

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only earthworm representative has often been criticized because of the lack of realism in terms of extrapolating its responses to chemicals to other species with different functional roles found in the field (Edwards, 2002). Therefore, current risk assessment leads to uncertainties when *E. fetida* responses are extrapolated to different ecosystems inhabited by earthworms with variable chemical sensitivity (Forbes et al., 2021). In addition, the European Food Safety Authority (EFSA) is aiming to shift the focus of the pesticide risk-assessment protection goals on biodiversity and ecosystem services by integrating multiple stressors, scales, and environmental compartments (Devos et al., 2019; EFSA Scientific Committee, 2016). Hence, ecotoxicological data need to be linked with ecosystem functions and services to identify sensitive communities and protect their ecological roles (Faber et al., 2019; Maltby et al., 2017). However, toxicity information for soil organisms is generally scarce (Frampton et al., 2006; Weyers et al., 2004) and, specifically for earthworms, mainly comes from tests with *E. fetida* (Forbes et al., 2021; Pelosi et al., 2014), making up almost 80% of soil acute earthworm toxicity studies (US Environmental Protection Agency [USEPA], 2022). Therefore, a data gap to future risk assessment has been recognized where toxicity data derived from multiple field earthworm species is required to establish links with their ecosystem functions (Forbes et al., 2021).

The present study aimed to provide information on the sensitivity of six earthworm species toward acute chemical exposure. For this purpose, we conducted single-species tests based on the soil test described in OECD guideline 207 (OECD, 1984) using earthworms sampled from different habitats, such as grasslands, forests, and wetlands, as well as *E. fetida*. Insecticides and fungicides are typically the most toxic pesticides for earthworms (Pelosi et al., 2014). Hence, we chose the insecticide imidacloprid and a copper-based fungicide (copper II sulfate pentahydrate) for the present study. Also, both substances have been tested on other earthworm species in addition to *E. fetida* and, therefore, provide further data which may be compared and used to complement our results (see Capowiez et al., 2005; Haque & Ebing, 1983). Imidacloprid acts on the nervous system, blocking nicotinic acetylcholine receptors (Talcott, 2013), and is known to be toxic for earthworms at low soil concentrations (i.e., 0.2 mg/kg; Zang et al., 2000). Copper acts by denaturing proteins and enzymes in an organism's cells (Dalecki et al., 2017), and long-term use causes its accumulation in soil (Fagnano et al., 2020). Thus, copper can be present at high concentrations, >200 mg/kg, in soils treated with this fungicide, especially in vineyards (Komárek et al., 2010; Steinmetz et al., 2017), which may pose a risk for earthworms (Streit, 1984). Thus, we aimed to derive species sensitivity distributions (SSDs) of earthworms for both copper and imidacloprid and the hazardous concentrations which affect 5% of the species (HC5), together with their lower 95% confidence limit (Newman et al., 2000; Posthuma et al., 2001). In addition, we explored relationships between earthworm chemical sensitivity, their biological traits, and ecosystem characteristics.

MATERIALS AND METHODS

Source of earthworms and ecosystem characterization

Adult earthworms from the species *Allolobophora chlorotica* (SAVIGNY, 1826), *Aporrectodea caliginosa* (SAVIGNY, 1826), *Aporrectodea longa* (UDE, 1885), *Aporrectodea rosea* (SAVIGNY, 1826), and *Lumbricus rubellus* (HOFFMEISTER, 1843) were collected by hand in winter and fall of 2020 around Landau in der Pfalz, Germany (Supporting Information, Table S1). Sampling sites, located outside of agricultural areas, were selected to cover the major ecosystems of the region, that is, grasslands, forests, and wetlands (Supporting Information, Table S1), including both acidic and neutral soils. The same ecosystems were sampled for imidacloprid and copper tests, and dominant species at the sampling time were collected (Supporting Information, Table S1). Live organisms were first identified to species in situ, which was confirmed prior to the test following the identification keys of Bährmann & Müller (2015) and Krück (2018). Earthworms were then stored in 1-L polypropylene containers (18 cm length × 13.2 cm width × 6.8 cm height) with approximately 700 g of natural soil (i.e., soil collected from their ecosystem of origin; Supporting Information, Table S1), which had been transported to the laboratory and stored for acclimatization in a climate chamber (16 ± 1 °C, 65 ± 10% humidity, 600 ± 200 lux, and 16:8-h light: dark cycle) for 1 week prior to the ecotoxicological assessment. In addition, the pH of the natural soil (Supporting Information, Table S1) was measured at the laboratory in 0.01 M $CaCl_2$ following ISO (2005). Natural soil pH was classified according to the *Soil Survey Manual* (Soil Science Division Staff, 2017) as follows: extremely acidic (3.5–4.4), slightly acidic (6.1–6.5), and neutral (6.6–7.3). Because *E. fetida* is not a typical soil species (Krück, 2018), this species was obtained from a domestic compost pile (Supporting Information, Table S1) as well as from a laboratory culture (ECT Oekotoxikologie, Flörsheim/Main, Germany).

Pesticides

The insecticide imidacloprid (Kohinor® 70 WG; Leu+Gygax, Birmenstorf, Switzerland; 70% active ingredient [a.i.]) and the fungicide copper II sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$; Centrum Metal Odczynniki Chemiczne, Falenty, Polen; 25% a.i.) were used in the present study. For the ecotoxicological assessment, the substances were weighed to the nearest 0.01 mg (AT261 DeltaRange® 205 g/0.01 mg; Metler Toledo) and diluted in ultrapure water, and stock solutions were created using serial dilutions.

Ecotoxicological assessment

The mortality tests were based on OECD guideline 207 (OECD, 1984), with the following adaptations. Instead of artificial soil, we used the standard soil LUFA 2.2 (Landwirtschaftliche Untersuchungs- und Forschungsanstalt, Speyer, Germany; Supporting Information, Table S2) as the test substrate, which is widely used as a standard soil for the ecotoxicological assessment of soil invertebrates (Løkke & van Gestel, 1998). In addition, the test temperature was decreased from 20 °C to 16 °C, which is a more typical temperature for field situations (Lowe & Butt, 2005). Range-finding tests for both substances were done based on previously reported *E. fetida* median lethal concentrations (LC50s): 2.26 mg/kg for imidacloprid (Wang et al., 2019) and 643 mg/kg for copper (Neuhauser et al., 1985). For the tests, geometric series with seven concentrations of imidacloprid (ranging from 0 to 5.41 mg a.i./kg; Supporting Information, Table S3) or copper (ranging from 0 to 1075.6 mg a.i./kg; Supporting Information, Table S3) were tested on earthworms from the same species and ecosystem, with one experimental unit per concentration. An experimental unit consisted of approximately 690 g of moist LUFA soil (Supporting Information, Table S2), spiked with 20 ml (to achieve a final soil moisture of ~20%; Supporting Information, Table S3) of the desired test concentration together with 10 earthworms. Ultrapure water was used for the control. After spiking, the soil was thoroughly mixed, homogenized, and transferred to 1-L polypropylene containers (18 cm length × 13.2 cm width × 6.8 cm height). At the beginning of the test, the earthworms were weighed (Supporting Information, Table S1) to the nearest mg (PA214® 210 g/0.0001 g; OHAUS) and introduced to the soil immediately after spiking. Test boxes were closed with perforated lids to allow gas exchange and stored in randomized positions in a climate chamber under the same conditions as for the acclimatization period. The LUFA soil pH (ISO, 2005) and moisture (ISO, 1993) were measured at the beginning and end of the test (Supporting Information, Table S3). Survival was assessed by testing the organism's reaction to a gentle mechanical stimulus on Days 7 and 14 after the chemical application. In addition, approximately 10 g of soil were sampled on Days 0, 7, and 14, and stored at −20 °C to analyze pesticide concentrations.

Imidacloprid concentrations were quantified by Eurofins Umwelt Südwest (Speyer, Germany). Briefly, 20 ml of acetone was added to a 5-g dried soil sample, shaken for 60 min, and centrifuged. Then an aliquot of 200 µl was taken, evaporated to dryness, and reconstituted with 500 µl methanol and 500 µl water. The sample was filtrated, and the imidacloprid concentration was quantified via high-performance liquid chromatography–tandem mass spectrometry using a recovery standard. Copper contents were extracted at the iES Landau as follows: 10 ml of aqua regia (HNO_3 + 3 HCl, 65% and 32% suprapure assay, respectively; Carl Roth, Germany) was added to a 5-g dried soil sample. Samples were digested using microwave-induced (Mars Xpress; CEM, Germany) aqua regia at 800 watts and 60 min of digestion phase at 175 °C. Then, samples were diluted 1:10 with Milli-Q water and quantified with inductively coupled plasma atomic emission spectroscopy (700 Series; Agilent, Germany). Measured copper concentrations were consistently up to 30% lower than nominal concentrations (Supporting Information, Table S4), indicating incomplete recovery from the soil matrix. Imidacloprid measured concentrations (Supporting Information, Table S4) varied around nominal concentrations, which were always included within the confidence intervals of measurements. Following the majority of existing studies (USEPA, 2022), nominal concentrations of the pesticides at the beginning of the test were measured and are reported throughout our study.

Data analysis

Following Ritz et al. (2019), LC50s were calculated (Supporting Information, Table S3) after 7 (when possible) and 14 days of exposure for all tested species by fitting binomial dose–response models to the data. Model fits were compared using the Akaike information criterion, and the best-fit model was selected (Supporting Information, Figures S1–S28). The intraspecific differences in LC50s (Supporting Information, Table S5) were assessed via pairwise comparisons of multiple binomial dose–response curves (Ritz et al., 2019). Furthermore, SSDs (Posthuma et al., 2001) were fitted for both pesticides using the 14-day LC50 values for all examined species and literature data from comparable studies (i.e., soils with similar organic matter content; Supporting Information, Table S6) because 7-day LC50s were not available for all tests (Supporting Information, Table S3). If multiple LC50 values from the same species were available, the geometric mean LC50 was calculated (Supporting Information, Table S6). Values of HC5 were derived from these distributions, and parametric bootstrap 95% confidence intervals (CIs), from 1000 iterations, were calculated to obtain the lower limits of the HC5. In addition, potential associations between earthworm chemical sensitivity in terms of LC50, habitat (grassland, wetland, forest), natural soil pH, fresh weight, and ecotype (endogeic, epigeic, anecic) were analyzed via analysis of covariance (ANCOVA). *Eisenia fetida*, an epigeic compost earthworm rarely found in nature (Krück, 2018), was excluded from these calculations. Because *Aporrectodea longa* was the only anecic species tested for both pesticides, anecic and endogeic earthworms were merged into one category, "nonepigeic," for analysis. In addition, soil pH classes "slightly acidic" and "neutral" (Soil Science Division Staff, 2017) were combined into one category for the analysis because pH values were close to 6.5, which is the limit between these classes. All statistical analyses and figures were created with R Ver 4.2.1 for Windows together with the add-on packages "drc" (Ritz et al., 2015), "multcomp" (Hothorn et al., 2008), "plotrix" (Lemon, 2006) for dose–response modeling, "fitdistrplus" (Delignette-Muller & Dutang, 2015), "reshape2" (Wickham, 2007), "ggplot2" (Wickham, 2016), "ggpubr" (Kassambara, 2020) for the SSD, and "car" (Fox & Weisberg, 2019) for the ANCOVA.

RESULTS

Acute toxicity

In total, 14 tests were run for each pesticide, with six species of earthworms from the genera *Allolobophora*, *Aporrectodea*, *Eisenia*, and *Lumbricus* (Supporting Information, Table S3). Earthworm 14-day LC50s (Supporting Information, Table S3) for imidacloprid ranged between 0.72 and 3.53 mg a.i./kg dry weight, and values for copper ranged from 199.99 to 433.09 mg a.i./kg dry weight. Intraspecific differences (Supporting Information, Table S5) showed that *Aporrectodea caliginosa* collected from an extremely acidic grassland (4.24 pH; Supporting Information, Table S1), for imidacloprid (Figure 1A), and an extremely acidic forest (4.16 pH; Supporting Information, Table S1), for copper (Figure 1B), were significantly less sensitive than *Aporrectodea caliginosa* sampled from the other ecosystems. Furthermore, laboratory-raised *E. fetida* were significantly less sensitive than *E. fetida* collected in the field for both chemicals (Figure 1C,D).

SSDs

Additional LC50 values were included from the literature to fit the SSDs (Supporting Information, Table S6). *Allobophora chlorotica* tested in the present study was the most sensitive species to acute imidacloprid and copper exposure. The HC5 (95% CI) derived from the SSDs for imidacloprid (Figure 2A) and copper (Figure 2B) were 0.70 (0.32–1.47) and 201.51 (178.49–234.07) mg a.i./kg dry weight, respectively.

Earthworm sensitivity and habitat characteristics

Chemical sensitivity to imidacloprid differed significantly between epigeic and nonepigeic earthworms ($F_{(1,6)} = 17.45$, $p < 0.01$; Table 1). The LC50s of nonepigeic earthworms were generally twice as low as those from epigeic earthworms (Figure 3). Earthworm sensitivity to copper increased significantly with increasing soil pH of their ecosystem of origin ($F_{(1,6)} = 11.66$, $p = 0.01$; Table 1). Thus, earthworms obtained from extremely acidic soils were approximately twice as resistant to copper than those sampled in neutral soils (Figure 4).

DISCUSSION

SSDs and implications for risk assessment

The European Union (EU) pesticide risk assessment for soil organisms defines a safety factor of 5 (EFSA Panel on Plant Protection Products and Their Residues et al., 2017) to assess the acceptable risk of a substance (Regulation EU 546/2011 [EU, 2011]). For an acceptable risk, the differences in the sensitivity of the standard test organism *E. fetida* and other earthworm species (i.e., $LC50_{E.\ fetida}/LC50_{other\ species}$) should be lower than the safety factor of 5 (Frampton et al., 2006). Although *E. fetida*, tested in the present study, was not the most sensitive species to imidacloprid (Figure 2A) and copper (Figure 2B), the ratio of the $LC50_{E.\ fetida}$ to the most sensitive species in our study, *Allobophora chlorotica*, was less than the safety factor for both substances (Supporting Information, Table S3; Figure 2A,B). Still, the species tested in our study, including *E. fetida*, were very sensitive to copper and imidacloprid, which are known to be toxic compounds for

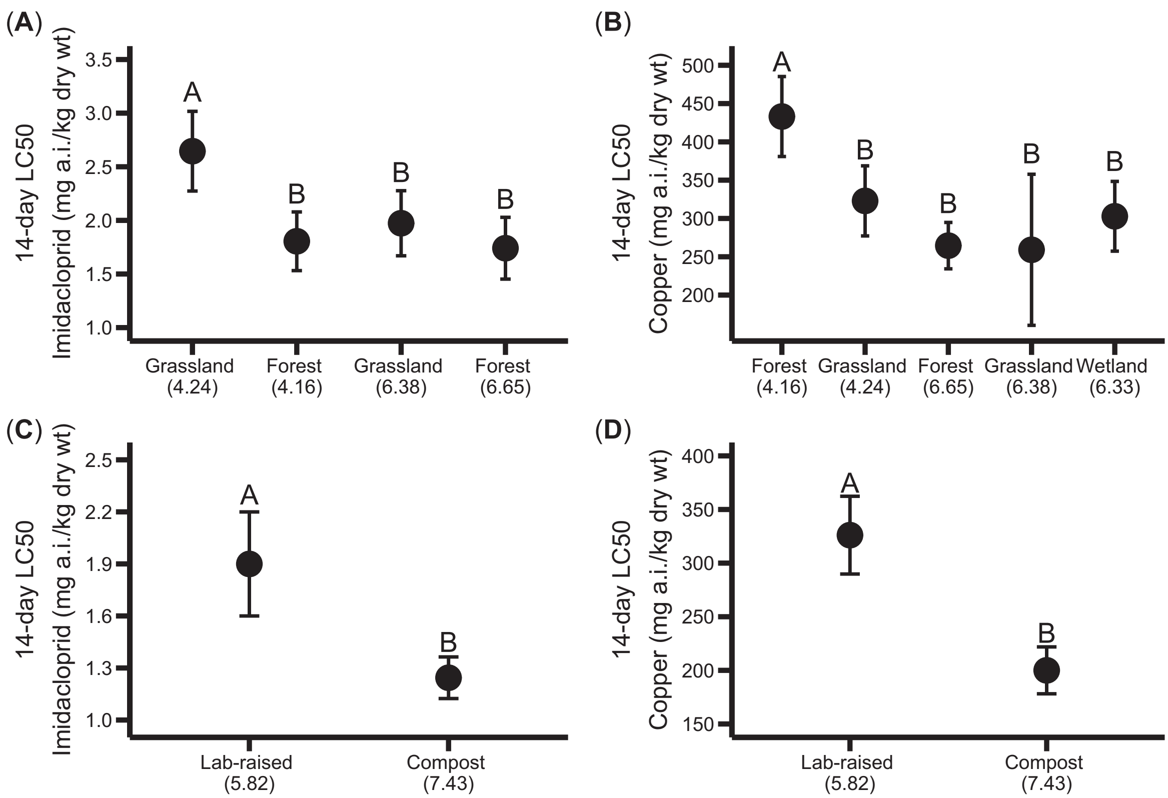

FIGURE 1: Intraspecific variation of earthworm chemical sensitivity for *Aporrectodea caliginosa* exposed to imidacloprid (**A**) and copper (**B**) and *Eisenia fetida* exposed to imidacloprid (**C**) and copper (**D**). Black points represent the 14-day median lethal concentration and whiskers their respective 95% confidence interval. For *Aporrectodea caliginosa* the *x*-axis shows the habitat of origin (soil pH). Different letters show significant differences ($p < 0.05$). LC50 = median lethal concentration; a.i. = active ingredient.

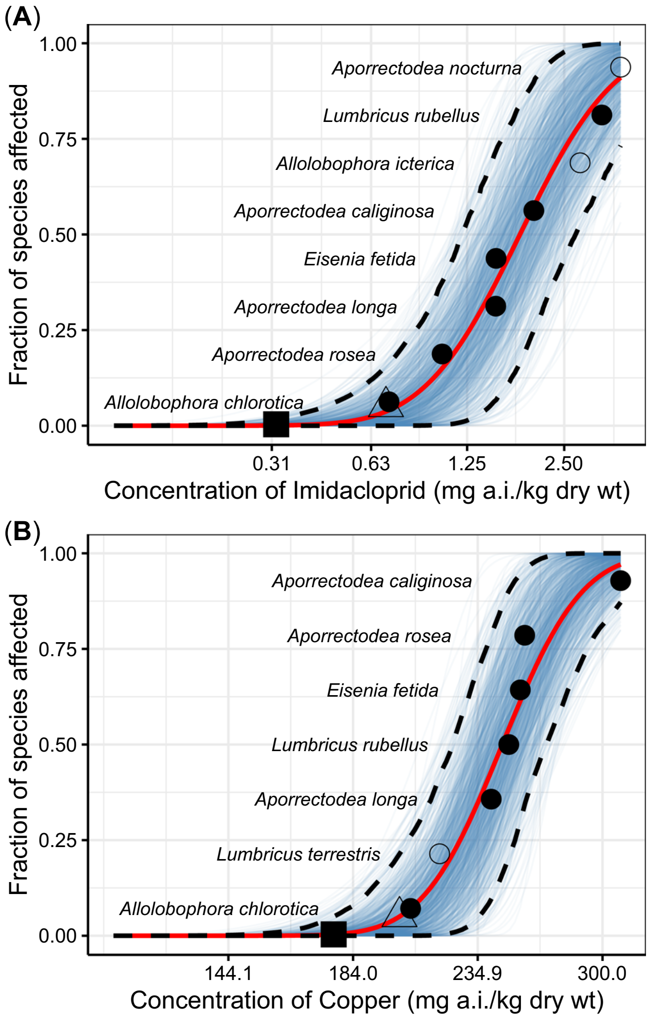

FIGURE 2: Species sensitivity distributions for imidacloprid (**A**) and copper (**B**) calculated from earthworm species sensitivity (red line). Black points (data from the present study) and open points (literature) represent the 14-day median lethal concentration values of earthworm species. Species names are aligned by sensitivity in ascending order from bottom to top on the *y*-axes, with the most sensitive at the bottom. Dashed lines enclose the parametric bootstrap (95% confidence interval; 1000 iterations). Blue transparent lines display all parametric bootstrap samples. The open triangle marks the hazardous concentration affecting 5% and the black square its lower limit. a.i. = active ingredient.

earthworms (see Streit, 1984; Wang et al., 2012). Short et al. (2021) exposed different earthworm species to imidacloprid and found that the safety factor proposed by the EFSA did not cover the most sensitive species tested, *Amynthas gracilis*

TABLE 1: Comparison between earthworm traits, habitat characteristics, and chemical sensitivity for imidacloprid and copper

	LC50 imidacloprid			LC50 copper		
Covariate	df	*F*	*p*	df	*F*	*p*
Ecotype	1	17.45	**0.005**	1	0.01	0.91
Weight	1	0.23	0.65	1	0.04	0.85
Habitat	2	1.54	0.29	2	0.31	0.74
pH	1	1.1	0.34	1	11.66	**0.01**

Statistically significant differences ($p < 0.05$) are printed in bold.
LC50 = median lethal concentration.

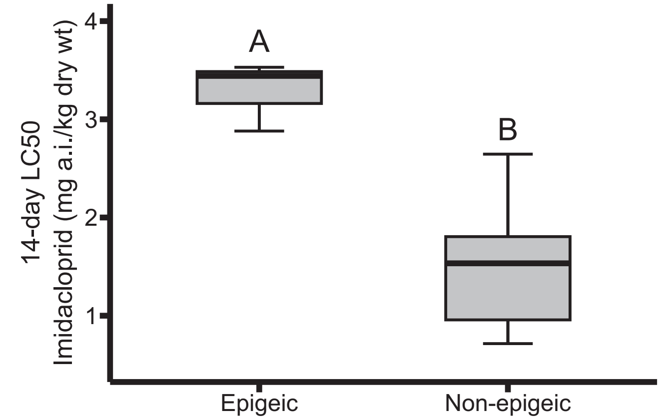

FIGURE 3: Comparison between epigeic and nonepigeic earthworm imidacloprid median lethal concentrations. Different letters show significant differences ($p < 0.05$). LC50 = median lethal concentration; a.i. = active ingredient.

(KINBERG, 1866). In addition, Frampton et al. (2006) performed a pesticide analysis using SSDs and soil invertebrates. In addition to oligochaetes, mainly arthropods reacted very sensitively to insecticides, as expected. They also concluded that *E. fetida* was not the most sensitive soil organism, and in most cases, the safety factor did not cover the range of chemical acute sensitivities of all species analyzed. These findings question the strong reliance of the current risk-assessment framework on *E. fetida* and underline the need to test pesticides on more ecologically relevant and sensitive soil organisms (Forbes et al., 2021).

The HC5s derived for imidacloprid (Figure 2A) and copper (Figure 2B) are useful as a proxy for potential mortality risk for earthworms under field conditions when compared with measured and recommended field concentrations. To be more conservative, the lower limit of the CI of the HC5 is often considered to achieve a higher level of protection. In European vineyards, concentrations of copper in topsoil and subsoil were

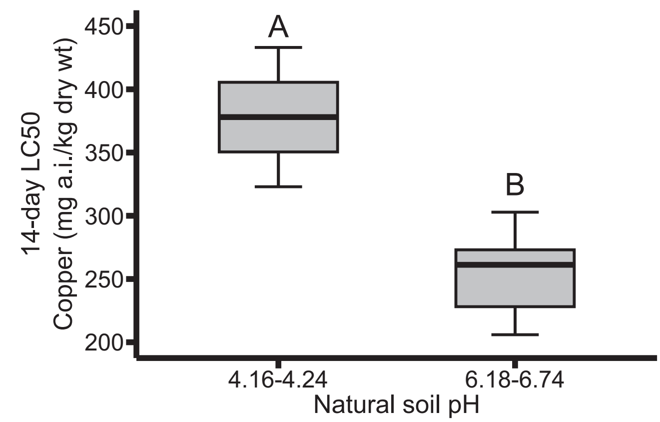

FIGURE 4: Comparison between copper median lethal concentrations of earthworms sampled in extremely acidic (4.16–4.24), slightly acidic (6.18–6.38), and neutral (6.65–6.74) soils. Different letters show significant differences ($p < 0.05$). LC50 = median lethal concentration; a.i. = active ingredient.

reported up to 600 mg/kg (Komárek et al., 2010) and even 1600 mg/kg in the study area, around Landau in der Pfalz, Germany (Steinmetz et al., 2017), which is characterized by long-term intensive viniculture. This shows that the soils in some areas that are heavily contaminated with copper, especially vineyards, may exert acute toxicity to earthworms.

Regarding imidacloprid, concentrations in agricultural soils were reported up to 0.65 mg/kg after 1 month of application in crops (Donnarumma et al., 2011). The most sensitive species, that is, *Allobophora chlorotica* and *Aporrectodea rosea* (Figure 2A; Supporting Information, Table S3), may have a survival risk in such soils. Nevertheless, because imidacloprid use is currently restricted to greenhouses in the EU (Regulation EU 2018/783 [EC, 2018]), a low risk for earthworms populations can be expected. In a recent monitoring study, the maximum concentration of imidacloprid found was 0.06 mg/kg (Silva et al., 2019). However, sublethal endpoints, such as reproduction, are more relevant for risk assessment than acute endpoints because they are typically affected at much lower concentrations than the observed LC50 (Neuhauser & Callahan, 1990). For example, *E. fetida* reproduction was negatively affected at a concentration of 0.87 mg imidacloprid/kg soil, while its acute LC50 was 2.26 mg imidacloprid/kg soil (Wang et al., 2019). Nevertheless, information on sublethal effects for other substances and earthworm species is scarce compared with acute data; only approximately 16% of earthworm toxicity studies addressed sublethal endpoints (USEPA, 2022). Although laboratory culturing of field earthworm species may be challenging, the mineral dweller *Aporrectodea caliginosa* promises to be a good candidate for evaluating the chronic effects of pesticides (Bart et al., 2018). Moreover, the update of the ISO 11268-2 (ISO, 2023) incorporates environmentally relevant species, for example, *Aporrectodea caliginosa* and *Dendrodrilus rubidus* (SAVIGNY, 1826), for testing pollutant effects on earthworm reproduction.

Intraspecific variation in chemical sensitivity

The differences in chemical sensitivity among populations of *Aporrectodea caliginosa* could be partially related to the organisms' ecosystem of origin (Figure 1A,B; Supporting Information, Table S5). *Aporrectodea caliginosa* is a species complex, often divided into different species (see Sims & Gerard, 1985) or subspecies (see Briones, 1996). Although differences in this classification are rather phenotypic than taxonomic (Bart et al., 2018), organisms used in the present study were identified morphologically (cf., Krück, 2018). Thus, the sensitivity differences obtained (Figure 1A,B; Supporting Information, Table S5) may be between different species that could not be morphologically separated. In this context, DNA barcoding probably will reveal *Aporrectodea caliginosa* cryptic species, which should be considered in future studies (Römbke et al., 2016). Furthermore, the chemical sensitivity of *E. fetida* differed by origin, with individuals from a laboratory culture being less sensitive than those from compost (Figure 1C,D; Supporting Information, Table S5). Laboratory-raised organisms fulfilled the standardization recommendations of the acute OECD guideline (OECD, 1984), for example, adult, weight, age, whereas only adult earthworms of unknown age were considered from compost. Moreover, laboratory test organisms were cultured in a moderately acid substrate (pH 5.82; Supporting Information, Table S1), whereas compost earthworms were raised in a slightly alkaline substrate (pH 7.43; Supporting Information, Table S1). The different substrates may have influenced their chemical response because pH appears to affect earthworm sensitivity to pesticides (see next section). Including additional earthworm species in risk assessment would be confronted with the challenge of standardization, with field organisms potentially failing to live and reproduce under laboratory conditions (Fründ et al., 2010). Nevertheless, the inclusion of field earthworms in standardized guidelines, such as the ISO 11268-2 (ISO, 2023), will help to improve risk assessment of soil organisms.

Earthworm sensitivity and habitat characteristics

Abiotic soil characteristics, such as soil type, pH, and moisture, influence earthworm biodiversity (Edwards & Bohlen, 1996). Furthermore, our results show that soil pH appears to affect earthworm sensitivity to pesticides (Figure 4 and Table 1). We are not aware of other studies that investigated the relationship between earthworm pesticide sensitivity and habitat characteristics and can only speculate on the reasons for our results. Ontogenetic traits acquired during earthworm development may explain the observed differences with soil pH (see Briones & Álvarez-Otero, 2018). For example, a reduced sensitivity to copper in earthworms from highly acidic soil may be an adaptation to low pH values (<5.5), in which toxic metals such as copper are mobilized (Fernández-Calviño et al., 2008). Moreover, recent studies have used toxicogenomic analysis to investigate and explain why some species are more sensitive to a certain compound (cf., Short et al., 2021). Pesticide uptake in earthworms is mainly through direct contact and oral ingestion (see Short et al., 2021; Streit, 1984). Uptake varies among different species, as do their toxicokinetic and toxicodynamic traits; and these dynamics mainly determine organism sensitivity to pesticides (Ashauer & Jager, 2018). Thus, toxicogenomic experiments combined with earthworm populations from different habitats could clarify the differences in earthworm sensitivity and habitat relationships observed in the present study.

The variation in earthworm sensitivity seen in the present study may affect soil functions such as bioturbation, that is, reworking of soil performed by soil organisms (Meysman et al., 2006). Nonepigeic earthworms contribute considerably to this process (Lee & Foster, 1991). Furthermore, anecic and endogeic earthworms are more sensitive than epigeic earthworms (Figure 3), especially to insecticides (Pelosi et al., 2014), which may result in a reduction in populations and cast production (Lal et al., 2001) and affect ecosystem functioning. Identifying sensitive traits and thresholds for safeguarding ecological functions would require further studies considering earthworm ecological groups and species within these groups (Forbes et al., 2021).

CONCLUSION

Our results confirm that the standard test organism *E. fetida* is not the most sensitive earthworm species (Frampton et al., 2006; Pelosi et al., 2013). Protecting the ecosystem services and functions provided by these soil invertebrates would require the inclusion of more ecologically relevant and sensitive earthworms in risk assessment (Forbes et al., 2021; ISO, 2023). While the sensitivities of earthworms showed no clear differences between ecosystem types, they varied with soil pH. The protection of a region-specific soil community and its ecological roles would require considering the soil characteristics of their habitat of origin. Furthermore, the higher sensitivity to imidacloprid shown by soil-inhabiting compared with epigeic earthworms could affect ecosystem services, such as bioturbation, if sensitive species are lost.

Supporting Information—The Supporting Information is available on the Wiley Online Library at https://doi.org/10.1002/etc.5589.

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Disclaimer—The authors declare no conflict of interest.

Author Contributions Statement—**Tomás Duque**: Conceptualization; Formal analysis; Investigation; Methodology; Writing—original draft; Writing—review & editing. **Rufat Nuriyev**: Investigation. **Jörg Römbke**: Conceptualization; Methodology; Writing—review & editing. **Ralf B. Schäfer**: Conceptualization; Funding acquisition; Methodology; Resources; Supervision; Writing—review & editing. **Martin H. Entling**: Conceptualization; Funding acquisition; Methodology; Resources; Supervision; Writing—review & editing.

Data Availability Statement—R codes for toxicity data calculations are available from Figshare: https://doi.org/10.6084/m9.figshare.21119884. Data, associated metadata, and calculation tools are available from the corresponding author (duque@uni-landau.de).

REFERENCES

Ashauer, R., & Jager, T. (2018). Physiological modes of action across species and toxicants: The key to predictive ecotoxicology. *Environmental Science: Processes & Impacts*, *20*, 48–57. https://doi.org/10.1039/c7em00328e

Bährmann, R., & Müller, H. J. (2015). Lumbricidae-regenwürmer. In G. Köhler (Ed.), *Bestimmung wirbelloser Tiere* (pp. 5–7). Springer Spektrum. https://doi.org/10.1007/978-3-642-55395-0

Bart, S., Amossé, J., Lowe, C. N., Mougin, C., Péry, A. R. R., & Pelosi, C. (2018). *Aporrectodea caliginosa*, a relevant earthworm species for a posteriori pesticide risk assessment: Current knowledge and recommendations for culture and experimental design. *Environmental Science and Pollution Research*, *25*, 33867–33881. https://doi.org/10.1007/s11356-018-2579-9

Bernier, N. (1998). Earthworm feeding activity and development of the humus profile. *Biology and Fertility of Soils*, *26*, 215–223. https://doi.org/10.1007/s003740050370

Briones, M. J. I. (1996). A taxonomic revision of the *Allolobophora caliginosa* complex (Oligochaeta, Lumbricidae): A preliminary study. *Canadian Journal of Zoology*, *74*(2), 240–244. https://doi.org/10.1139/z96-030

Briones, M. J. I., & Álvarez-Otero, R. (2018). Body wall thickness as a potential functional trait for assigning earthworm species to ecological categories. *Pedobiologia*, *67*, 26–34. https://doi.org/10.1016/j.pedobi.2018.02.001

Capowiez, Y., Rault, M., Costagliola, G., & Mazzia, C. (2005). Lethal and sublethal effects of imidacloprid on two earthworm species (*Aporrectodea nocturna* and *Allolobophora icterica*). *Biology and Fertility of Soils*, *41*, 135–143. https://doi.org/10.1007/s00374-004-0829-0

Dalecki, A. G., Crawford, C. L., & Wolschendorf, F. (2017). Copper and antibiotics: Discovery, modes of action, and opportunities for medicinal applications. In R. K. Poole (Ed.), *Advances in microbial physiology* (Vol. *70*, 1st ed., pp. 193–260). Academic. https://doi.org/10.1016/bs.ampbs.2017.01.007

Delignette-Muller, M. L., & Dutang, C. (2015). fitdistrplus: An R package for fitting distributions. *Journal of Statistical Software*, *64*(4), 1–34. https://doi.org/10.18637/jss.v064.i04

Devos, Y., Munns, W. R., Forbes, V. E., Maltby, L., Stenseke, M., Brussaard, L., Streissl, F., & Hardy, A. (2019). Applying ecosystem services for pre-market environmental risk assessments of regulated stressors. *EFSA Journal*, *17*(S1), Article e170705. https://doi.org/10.2903/j.efsa.2019.e170705

Donnarumma, L., Pulcini, P., Pochi, D., Rosati, S., Lusco, L., & Conte, E. (2011). Preliminary study on persistence in soil and residues in maize of imidacloprid. *Journal of Environmental Science and Health—Part B Pesticides, Food Contaminants, and Agricultural Wastes*, *46*(6), 469–472. https://doi.org/10.1080/03601234.2011.583848

Edwards, C. A. (2002). Assessing the effects of environmental pollutants on soil organisms, communities, processes and ecosystems. *European Journal of Soil Biology*, *38*(3–4), 225–231. https://doi.org/10.1016/S1164-5563(02)01150-0

Edwards, C. A. (Ed.). (2004). *Earthworm ecology* (2nd ed.). CRC. https://doi.org/10.1201/9781420039719

Edwards, C. A., & Bohlen, P. J. (1996). *Biology and ecology of earthworms* (3rd ed.). Springer.

EFSA Panel on Plant Protection Products and Their Residues, Ockleford, C., Adriaanse, P., Berny, P., Brock, T., Duquesne, S., Grilli, S., Hernandez-Jerez, A. F., Bennekou, S. H., Klein, M., Kuhl, T., Laskowski, R., Machera, K., Pelkonen, O., Pieper, S., Stemmer, M., Sundh, I., Teodorovic, I., Tiktak, A., … Smith, R. (2017). Scientific opinion addressing the state of the science on risk assessment of plant protection products for in-soil organisms. *EFSA Journal*, *15*(2), Article 4690. https://doi.org/10.2903/j.efsa.2017.4690

EFSA Scientific Committee. (2016). Guidance to develop specific protection goals options for environmental risk assessment at EFSA, in relation to biodiversity and ecosystem services. *EFSA Journal*, *14*(6), Article 4499. https://doi.org/10.2903/j.efsa.2016.4499

European Commission. (2018). Commission implementing regulation (EU) 2018/783 of 29 May 2018 amending Implementing Regulation (EU) No 540/2011 as regards the conditions of approval of the active substance imidacloprid. *Official Journal of the European Union, L132*, 31–34.

European Union. (2011). Commission Regulation (EU) No 546/2011 of 10 June 2011 implementing Regulation (EC) No 1107/2009 of the European Parliament and of the Council as regards uniform principles for evaluation and authorisation of plant protection products. *Official Journal of the European Union*, *L155*, 127–175.

Faber, J. H., Marshall, S., Van den Brink, P. J., & Maltby, L. (2019). Priorities and opportunities in the application of the ecosystem services concept in risk assessment for chemicals in the environment. *Science of the Total Environment*, *651*(1), 1067–1077. https://doi.org/10.1016/j.scitotenv.2018.09.209

Fagnano, M., Agrelli, D., Pascale, A., Adamo, P., Fiorentino, N., Rocco, C., Pepe, O., & Ventorino, V. (2020). Copper accumulation in agricultural soils: Risks for the food chain and soil microbial populations. *Science of the Total Environment*, *734*, Article 139434. https://doi.org/10.1016/j.scitotenv.2020.139434

Fernández-Calviño, D., Pateiro-Moure, M., López-Periago, E., Arias-Estévez, M., & Nóvoa-Muñoz, J. C. (2008). Copper distribution and acid-base mobilization in vineyard soils and sediments from Galicia (NW Spain). *European Journal of Soil Science*, *59*(2), 315–326. https://doi.org/10.1111/j.1365-2389.2007.01004.x

Forbes, V. E., Agatz, A., Ashauer, R., Butt, K. R., Capowiez, Y., Duquesne, S., Ernst, G., Focks, A., Gergs, A., Hodson, M. E., Holmstrup, M., Johnston, A. S. A., Meli, M., Nickisch, D., Pieper, S., Rakel, K. J., Reed, M., Roembke, J., Schäfer, R. B., … Roeben, V. (2021). Mechanistic effect modeling of earthworms in the context of pesticide risk assessment: Synthesis of the FORESEE workshop. *Integrated Environmental Assessment and Management*, *17*(2), 352–363. https://doi.org/10.1002/ieam.4338

Fox, J., & Weisberg, S. (2019). *An R companion to applied regression* (3rd ed.). SAGE.

Frampton, G. K., Jänsch, S., Scott-Fordsmand, J. J., Römbke, J., & Van Den Brink, P. J. (2006). Effects of pesticides on soil invertebrates in laboratory studies: A review and analysis using species sensitivity distributions. *Environmental Toxicology and Chemistry*, *25*(9), 2480–2489. https://doi.org/10.1897/05-438R.1

Fründ, H. C., Butt, K., Capowiez, Y., Eisenhauer, N., Emmerling, C., Ernst, G., Potthoff, M., Schädler, M., & Schrader, S. (2010). Using earthworms as model organisms in the laboratory: Recommendations for experimental implementations. *Pedobiologia*, *53*(2), 119–125. https://doi.org/10.1016/j.pedobi.2009.07.002

Haque, A., & Ebing, W. (1983). Toxicity determination of pesticides to earthworms in the soil substrate. *Journal of Plant Disease and Protection*, *90*(4), 395–408.

Hothorn, T., Bretz, F., & Westfall, P. (2008). Simultaneous inference in general parametric models. *Biometrical Journal*, *50*(3), 346–363. https://doi.org/10.1002/bimj.200810425

International Organization for Standardization. (1993). *Soil quality—Determination of dry matter and water content on a mass basis—Gravimetric method* (ISO 11465:1993). https://www.iso.org/standard/20886.html

International Organization for Standardization. (2005). *Soil quality—Determination of pH* (ISO 10390:2005). https://www.iso.org/standard/40879.html

International Organization for Standardization. (2008). *Soil quality—Avoidance test for determining the quality of soils and effects of chemicals on behaviour—Part 1: Test with earthworms* (Eisenia fetida and Eisenia andrei) (ISO 17512-1:2008). https://www.iso.org/standard/38402.html

International Organization for Standardization. (2012). *Soil quality—Effects of pollutants on earthworms—Part 1: Determination of acute toxicity to* Eisenia fetida/Eisenia andrei (ISO 11268-1:2012). https://www.iso.org/standard/53527.html

International Organization for Standardization. (2023). Soil quality—*Effects of pollutants on earthworms—Part 2: Determination of effects on reproduction of* Eisenia fetida/Eisenia andrei *and other earthworm species* (ISO 11268-2:2023). https://www.iso.org/standard/79045.html

Jones, C. G., Lawton, J. H., & Shachak, M. (1994). Organisms as ecosystem engineers. *In Ecosystem management* (pp. 130–147). Springer. https://doi.org/10.1007/978-1-4612-4018-1_14

Jouquet, P., Dauber, J., Lagerlöf, J., Lavelle, P., & Lepage, M. (2006). Soil invertebrates as ecosystem engineers: Intended and accidental effects on soil and feedback loops. *Applied Soil Ecology*, *32*(2), 153–164. https://doi.org/10.1016/j.apsoil.2005.07.004

Kassambara, A. (2020). *ggpubr: "ggplot2" based publication ready plots*. https://cran.r-project.org/package=ggpubr

Kavdir, Y., & İlay, R. (2011). Earthworms and soil structure. In A. Karaca (Ed.), *Biology of earthworms* (pp. 39–50). Springer. https://doi.org/10.1007/978-3-642-14636-7_3

Komárek, M, Čadková, E., Chrastný, V., Bordas, F., & Bollinger, J. C. (2010). Contamination of vineyard soils with fungicides: A review of environmental and toxicological aspects. *Environment International*, *36*(1), 138–151. https://doi.org/10.1016/j.envint.2009.10.005

Krück, S. (2018). *Bildatlas zur regenwurmbestimmung*. Natur+Text.

Lal, O. P., Palta, R. K., & Srivastava, Y. N. S. (2001). Impact of imidacloprid and carbofuran on earthworm castings in okra field. *Annals of Plant Protection Sciences*, *9*(1), 137–138.

Lee, K. E., & Foster, R. C. (1991). Soil fauna and soil structure. *Australian Journal of Soil Research*, *29*(6), 745–775. https://doi.org/10.1071/SR9910745

Lemon, J. (2006). Plotrix: A package in the red light district of R. *R-News*, *6*(4), 8–12.

Løkke, H., & van Gestel, C. A. M. (1998). *Handbook of soil invertebrate toxicity tests*. John Wiley & Sons.

Lowe, C. N., & Butt, K. R. (2005). Culture techniques for soil dwelling earthworms: A review. *Pedobiologia*, *49*(5), 401–413. https://doi.org/10.1016/j.pedobi.2005.04.005

Maltby, L., van den Brink, P. J., Faber, J. H., & Marshall, S. (2017). Advantages and challenges associated with implementing an ecosystem services approach to ecological risk assessment for chemicals. *Science of the Total Environment*, *621*, 1342–1351. https://doi.org/10.1016/j.scitotenv.2017.10.094

Meysman, F. J. R., Middelburg, J. J., & Heip, C. H. R. (2006). Bioturbation: A fresh look at Darwin's last idea. *Trends in Ecology and Evolution*, *21*(12), 688–695. https://doi.org/10.1016/j.tree.2006.08.002

Neuhauser, E. F., & Callahan, C. A. (1990). Growth and reproduction of the earthworm *Eisenia fetida* exposed to sublethal concentrations of organic chemicals. *Soil Biology and Biochemistry*, *22*(2), 175–179. https://doi.org/10.1016/0038-0717(90)90083-C

Neuhauser, E. F., Loehr, R. C., Milligan, D. L., & Malecki, M. R. (1985). Toxicity of metals to the earthworm *Eisenia fetida*. *Biology and Fertility of Soils*, *1*, 149–152. https://doi.org/10.1007/BF00301782

Newman, M. C., Ownby, D. R., Mézin, L. C. A., Powell, D. C., Christensen, T. R. L., Lerberg, S. B., & Anderson, B. A. (2000). Applying species-sensitivity distributions in ecological risk assessment: Assumptions of distribution type and sufficient numbers of species. *Environmental Toxicology and Chemistry*, *19*(2), 508–515. https://doi.org/10.1002/etc.5620190233

Organisation for Economic Co-operation and Development. (1984). Test No. 207: Earthworm, acute toxicity tests. *OECD Guidelines for the Testing of Chemicals*. https://doi.org/10.1787/9789264070042-en

Organisation for Economic Co-operation and Development. (2016). Test No. 222: Earthworm reproduction test (*Eisenia fetida/Eisenia andrei*). *OECD Guidelines for the Testing of Chemicals*. https://doi.org/10.1787/9789264264496-en

Paradise, C. J. (2001). A standardized soil ecotoxicological test using red worms (*Eisenia fetida*). *The American Biology Teacher*, *63*(9), 662–668. https://doi.org/10.1662/0002-7685(2001)063[0662:ASSETU]2.0.CO;2

Pelosi, C., Barot, S., Capowiez, Y., Hedde, M., & Vandenbulcke, F. (2014). Pesticides and earthworms. A review. *Agronomy for Sustainable Development*, *34*, 199–228. https://doi.org/10.1007/s13593-013-0151-z

Pelosi, C., Joimel, S., & Makowski, D. (2013). Searching for a more sensitive earthworm species to be used in pesticide homologation tests—A meta-analysis. *Chemosphere*, *90*(3), 895–900. https://doi.org/10.1016/j.chemosphere.2012.09.034

Posthuma, L., II, Suter, G. W. & Traas, T. P. (Eds.). (2001). *Species sensitivity distributions in ecotoxicology* (1st ed.). CRC. https://doi.org/10.1201/9781420032314

R: A language and environment for statistical computing (Version 4.2.1) [Computer software]. (2022). R Foundation for Statistical Computing. https://www.r-project.org/

Ritz, C., Baty, F., Streibig, J. C., & Gerhard, D. (2015). Dose–response analysis using R. *PLOS ONE*, *10*(12), Article e0146021. https://doi.org/10.1371/journal.pone.0146021

Ritz, C., Jensen, S. M., Gerhard, D., & Streibig, J. C. (2019). *Dose–response analysis using R* (1st ed.). Chapman and Hall/CRC. https://doi.org/10.1201/b21966

Römbke, J., Aira, M., Backeljau, T., Breugelmans, K., Domínguez, J., Funke, E., Graf, N., Hajibabaei, M., Pérez-losada, M., Porto, P. G., Schmelz, R. M., Vierna, J., Vizcaíno, A., & Pfenninger, M. (2016). DNA barcoding of earthworms (*Eisenia fetida/andrei* complex) from 28 ecotoxicological test laboratories. *Applied Soil Ecology*, *104*, 3–11. https://doi.org/10.1016/j.apsoil.2015.02.010

Santé et Consommateurs Directorate General Health and Consumers. (2002). *Guidance document on terrestrial ecotoxicology under Council Directive 91/414/EEC* (SANCO/10329/2002 rev 2 final). European Commission.

Short, S., Robinson, A., Lahive, E., Etxabe, A. G., Hernadi, S., Gloria Pereira, M., Kille, P., & Spurgeon, D. J. (2021). Off-target stoichiometric binding identified from toxicogenomics explains why some species are more sensitive than others to a widely used neonicotinoid. *Environmental Science & Technology*, *55*(5), 3059–3069. https://doi.org/10.1021/acs.est.0c05125

Silva, V., Mol, H. G. J., Zomer, P., Tienstra, M., Ritsema, C. J., & Geissen, V. (2019). Pesticide residues in European agricultural soils—A hidden reality unfolded. *Science of the Total Environment*, *653*, 1532–1545. https://doi.org/10.1016/j.scitotenv.2018.10.441

Sims, R. W., & Gerard, B. M. (1985). Systematics. In D. M. Kermacj & R. S. K. Barnes (Eds.), *Earthworms: Keys and notes for the identification and study of the species* (pp. 41–147). Brill and Backjuys.

Soil Science Division Staff. (2017). Examination and description of soil profiles. *Soil survey manual* (Handbook 18, pp. 83–234). US Department of Agriculture. Government Printing Office.

Steinmetz, Z., Kenngott, K. G. J., Azeroual, M., Schäfer, R. B., & Schaumann, G. E. (2017). Fractionation of copper and uranium in organic and conventional vineyard soils and adjacent stream sediments studied by sequential extraction. *Journal of Soils and Sediments*, *17*, 1092–1100. https://doi.org/10.1007/s11368-016-1623-y

Streit, B. (1984). Effects of high copper concentrations on soil invertebrates (earthworms and oribatid mites): Experimental results and a model. *Oecologia*, *64*, 381–388. https://doi.org/10.1007/BF00379137

Suthar, S., Singh, S., & Dhawan, S. (2008). Earthworms as bioindicator of metals (Zn, Fe, Mn, Cu, Pb and Cd) in soils: Is metal bioaccumulation affected by their ecological category? *Ecological Engineering*, *32*(2), 99–107. https://doi.org/10.1016/j.ecoleng.2007.10.003

Talcott, P. A. (2013). Miscellaneous herbicides, fungicides, and nematocides. In M. E. Peterson & P. A. Talcott (Eds.), *Small animal toxicology* (3rd ed., pp. 401–408). W. B. Saunders. https://doi.org/10.1016/B978-1-4557-0717-1.00028-4

US Environmental Protection Agency. (2022). *ECOTOX knowledgebase*. https://cfpub.epa.gov/ecotox/search.cfm

Wang, X., Zhu, X., Peng, Q., Wang, Y., Ge, J., Yang, G., Wang, X., Cai, L., & Shen, W. (2019). Multi-level ecotoxicological effects of imidacloprid on earthworm (*Eisenia fetida*). *Chemosphere*, *219*, 923–932. https://doi.org/10.1016/j.chemosphere.2018.12.001

Wang, Y., Cang, T., Zhao, X., Yu, R., Chen, L., Wu, C., & Wang, Q. (2012). Comparative acute toxicity of twenty-four insecticides to earthworm, *Eisenia fetida*. *Ecotoxicology and Environmental Safety*, *79*, 122–128. https://doi.org/10.1016/j.ecoenv.2011.12.016

Weyers, A., Sokull-Klüttgen, B., Knacker, T., Martin, S., & Van Gestel, C. A. M. (2004). Use of terrestrial model ecosystem data in environmental risk assessment for industrial chemicals, biocides and plant protection products in the EU. *Ecotoxicology*, *13*, 163–176. https://doi.org/10.1023/B:ECTX.0000012412.44625.69

Wickham, H. (2007). Reshaping data with the reshape package. *Journal of Statistical Software*, *21*(12), 1–20. https://doi.org/10.18637/jss.v021.i12

Wickham, H. (2016). *ggplot2: Elegant graphics for data analysis* (2nd ed.). Springer. https://doi.org/10.1007/978-3-319-24277-4

Zang, Y., Zhong, Y., Luo, Y., & Kong, Z. M. (2000). Genotoxicity of two novel pesticides for the earthworm, *Eisenia fetida*. *Environmental Pollution*, *108*(2), 271–278. https://doi.org/10.1016/S0269-7491(99)00191-8

