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Toward a harmonized methodology to analyze field side effects of two pesticide products on earthworms at the EU level

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Abstract

Before plant protection product (PPP) marketing authorization, a risk assessment for nontarget soil organisms (e.g., earthworms) is required as part of Regulation (EC) No. 1107/2009. Following a stepwise approach, higher tier earthworm field studies are needed if they cannot demonstrate low long-term risk based on laboratory studies. The European guidance for terrestrial ecotoxicology refers to ISO guideline 11268-3 as a standard to conduct earthworm field studies. Assessment of such studies may be challenging, as no European harmonized guidance is available to properly analyze the accuracy, representativeness, and appropriateness of experimental designs, as well as the statistical analysis robustness of results and their scientific reliability. Following the ISO guideline 11268-3, a field study was performed in 2016–2017 (Versailles, France). An assessment of the first year of this field study was performed in agreement with the quality criteria provided in 2006 in the guidance document published by de Jong and collaborators and recommendations by Kula and collaborators that allows describing the protocol and results of earthworm field studies. Not only did we underline the importance of a detailed analysis of raw data on the effects of pesticides on earthworms in field situations, but we also provided recommendations to harmonize protocols for assessing higher tier field studies devoted to earthworms to advance a better assessment of PPP fate and ecotoxicity. In particular, we provided practical field observations related to the study design, pesticide applications, and earthworm sampling. Concurrently, in addition to the conventional earthworm community study, we propose carrying out an assessment of soil function (i.e., organic matter decomposition, soil structuration, etc.) and calculating diversity indices to obtain information about earthworm community dynamics after the application of PPPs. Finally, through field observations, any relevant observation of external and/or internal recovery should be reported. *Integr Environ Assess Manag* 2023;19:254–271. © 2022 The Authors. *Integrated Environmental Assessment and Management* published by Wiley Periodicals LLC on behalf of Society of Environmental Toxicology & Chemistry (SETAC).

KEYWORDS: Copper compounds, dimoxystrobin, earthworm field study, plant protection products, regulatory risk assessment

INTRODUCTION

The key role of earthworms (*Annelida oligochaeta*) in continental ecosystems is well established (Liu et al., 2019; van Groenigen et al., 2015). These organisms represent a significant part of soil macrofauna and are common in a wide range of soils. Earthworms play a key role in the breakdown of organic matter, allowing a better incorporation of nutrients into the soil, which maintains soil fertility. They are often described as soil ecosystem engineers (Jones et al., 1994; Le Bayon et al., 2017; Singh et al., 2016), as their activity increases nutrient availability for other organisms, such as microorganisms and plants. They are also involved in the maintenance of soil structure, allowing for better water drainage and a more stable soil structure that helps improve sustainable productivity in agricultural lands. Earthworms are also regarded as good bioindicators of soil health, quality, and pollution (Calisi et al., 2013; Cortet et al., 1999; Paoletti, 1999; Pérès et al., 2008).

This article contains online-only Supporting Information.

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For many decades, plant protection products (PPPs; i.e., pesticides) have been used widely in agriculture to prevent and/or manage damage caused by pathogens, insect pests, and weeds. The ecotoxicological impact of these pesticides on a wide range of organisms is well documented (Berny, 2007; Köhler & Triebskorn, 2013), and earthworms are known to be sensitive soil organisms (Gunstone et al., 2021; Pelosi et al., 2014). However, the impact of pesticide applications on the annelid community and population structure in the field is less documented. Recently, we carried out a two-year field experiment to assess the impact of two fungicides on earthworm communities and their recovery (Amossé et al., 2020). In this study, we highlighted the negative effects of dimoxystrobin (DMX) and epoxiconazole (EPX)-based and copper-based products on earthworm abundance and biomass (mainly on one ecological category of earthworms, i.e., anecics) 12 months after the first application. Full recovery of the population was observed after one additional year without the application of PPPs. More interestingly, this study also allowed us to highlight several methodological shortcomings of field studies performed according to the International Organisation for Standardization (ISO) 11268-3 guideline (1999, revised in 2014). The issues identified were related mainly to product applications, interception by the vegetation (and thus the residues of compounds reaching the soil), and statistical analyses of the data. Furthermore, from the analysis of our results, we proposed improvements by considering diversity indices and measurements related to soil functioning. However, the data from this field study were not fully analyzed using regulatory guidance documents.

Following Regulation (EC) No. 1107/2009, risk assessment is mandatory before PPPs are placed on the market. Regulatory risk assessment includes a risk characterization for the soil compartment and nontarget organisms. Regulations (EU) Nos. 283/2013 and 284/2013 (Commission Regulation EU, 2013a, 2013b) set the data requirements for active substances and PPPs, respectively. The ecotoxicological regulatory risk assessment typically follows a tiered approach (conservative assumptions to a more realistic, worst-case approach) based on a broad range of organism tests, following the latest guidelines. Regarding soil organisms, risk assessment ranges from worst-case situations (i.e., conservative estimates and toxicity laboratory studies) to more realistic assessments (i.e., field studies). In the case when an exposure of earthworms following the application of PPPs cannot be excluded, the guidance document for terrestrial organisms (European commission, 2002) recommends conducting a regulatory risk assessment following a two-tier risk assessment scheme. The first tier is based on comparison of sublethal toxicity endpoints (no observed effect concentration [NOEC] and effective concentration at 10% [EC_{10}]) from laboratory chronic studies (with representative species *Eisenia fetida or Eisenia andrei*) performed in controlled conditions to predict environmental concentrations in soil (PEC_{soil}). If no acceptable risk is identified in the first tier of the risk assessment scheme, refinement of the risk assessment by carrying out field studies must be performed to assess the potential effects on the soil communities in more realistic conditions. Such studies allow investigating the impact of pesticides on abundance, biomass, and species diversity after the application of pesticide products. The guidance document on terrestrial ecotoxicology (European Commission, 2002) refers mainly to the methodology provided in the ISO guideline 11268-3 (1999, 2014) to conduct earthworm field studies without further recommendations regarding a methodology to analyze the results. The ISO guideline for earthworm field studies provides the experimental setting recommendation for selecting the field, plot size, and margin between them, as well as earthworm sampling and extraction methodology. An update of the ISO guideline was also published in 2014, introducing new validity criteria, that is, a minimal density of earthworms collected before application in preapplication plots (100 and 60 individuals m^{-2} in grasslands and arable soils, respectively). Quality assurance is also required to achieve a range from 50% to 150% of the targeted nominal soil concentration for the product applied during the study. However, no key element is given about how data retrieved from the field study should be summarized properly and analyzed statistically. High variability between replicates in abundance, biomass, and diversity, due mainly to the heterogeneous spatial distribution of populations, is commonly observed in ecotoxicological field studies with earthworms (Römbke et al., 2020). Such variability usually leads to nonrobust statistical analysis and a misleading interpretation of the results. The lack of recommendation about the application of relevant statistical analysis in the reference documents, such as the ISO guideline 11268-3, does not provide a consistent framework for harmonized risk assessment.

Furthermore, since the publication of the ISO guideline in 1999, further guidance has been provided to help regulators assess earthworm field studies. In 2006, some technical recommendations for the update of the ISO 11268-3 guideline (Kula et al., 2006) and guidance for summarizing the results of earthworm field studies (de Jong et al., 2006) were published to improve and harmonize the assessment of earthworm communities in field studies. However, since its publication, de Jong's guidance has not been widely used to summarize and assess earthworm field studies in a regulatory context.

The aim of the present study was to use a field study (Amossé et al., 2020) based on the ISO guideline 11268-3 (2014) and the de Jong guidance (de Jong et al., 2006) to propose potential improvements to the ISO guideline. Furthermore, recommendations made from the present study could help in the development and/or revision of guidelines for earthworm field studies. The de Jong guidance provides all the experimental details and results of field studies of earthworms. The European Food Safety Authority (EFSA) recommended the systematic use of this guidance to assess the reliability of earthworm field studies (European Food Safety Authority [EFSA], 2019), but it has only been used for regulatory purposes and, to our knowledge, it has never

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been utilized in a scientific publication. This could, however, help create a link between scientific research and regulatory initiatives. The field study was carried out near the Versailles castle in France between April 2016 and April 2017. In Amossé et al. (2020), the tested substances were chosen for their potential negative effects on earthworm populations and because they were used in different farming systems, either organic or conventional farming. At the field level, the results published in our recent studies (Amossé et al., 2020) revealed significant effects on earthworm biomass and abundance in plots treated with the highest tested rate (i.e., 10 times the authorized application rates mentioned on the label of the product in France) of Swing Gold one month after the application. The soil dissipation of DMX and EPX and their bioavailability to earthworms were studied at the same time (Nélieu et al., 2020). Following the recommendations provided by the guidance published by de Jong et al. (2006), different items must be checked for the description of field studies (e.g., purity of the substance, test site, mode of application, dosage, test design, sampling, etc.), and for the reporting of the results (e.g., actual concentration, type of endpoint, statistical comparison, etc.). The aim of the present study was to provide new recommendations to improve the ISO 11268-3 guideline through practical observations and statistics to be applied. In addition, our study aims to provide a basis for future research using tools available from regulatory initiatives that provide a clear framework for earthworm field studies.

MATERIAL AND METHODS

Guidelines ISO 11268-3 (2014).

Test substances

The formulated products registered in France under the following names were considered: Swing Gold product (product authorization number 2090171 in France), a concentrated suspension containing DMX (133 g L^{-1}) and EPX (50 g L^{-1}); Cuprafor Micro product (product authorization number 9400346 in France), a product composed of copper oxychloride at 500 g Cu kg^{-1}. More information is provided in Supporting Information: Table S1. In Amossé et al. (2020), the tested substances were chosen for their potentially negative effects on earthworm populations and because they were used in different farming systems, either organic or conventional farming.

Test site and maintenance

The test was performed from April 2016 to April 2017 in a meadow located near the Versailles Castle (INRAE experimental station, 48°48′22.8″N 2°05′27.4″E), France. For detailed information on soil characteristics, see Amossé et al. (2020). The meadow had not been fertilized or treated with pesticides for more than 20 years (INRAE internal communication, March 2016). Before the first treatment, the upper layer soil concentrations of DMX and EPX were under the limits of quantification at the beginning of the experiment, meaning that existing residues in the soil were negligible compared with the applied concentrations.

Weather data (rainfall and air temperature) were collected from a weather station close to the site (La lanterne, Versailles, INRAE, 48,80°N 2,09°E; Altitude 118 m; 0.3 km from trial). No climatic barrier is expected between the experimental site and the station.

Pesticide applications, replicates

The first pesticide application took place on 13 April 2016, using hand horticultural sprayers (20 L capacity). Solutions were first diluted in 8 L of water and applied homogeneously (two applications) on plots of 100 m^2. Before each treatment, the vegetation was cut as short as possible, and plant residues were removed with a lawn mower. A volume of 8 L of water was also sprayed in control plots. The trial consisted of four replicates, each including five treatments randomly located (four replicates, 20 plots of 100 m^2; Supporting Information: Figure S1). Treatments were:

- Control (called T);
- Cuprafor Micro at 4 and 40 kg Cu ha^{-1} $year^{-1}$ (called C1 and C10, respectively, corresponding to the recommended dose in France and 10 times the recommended dose.) To mimic the use by farmers, four applications were performed as follows: April (0.75 or 7.5 kg Cu ha^{-1}), May (0.75 or 7.5 kg Cu ha^{-1}), June (1.25 or 12.5 kg Cu ha^{-1}), and September (1.25 or 12.5 kg Cu ha^{-1}) 2016;
- Swing Gold at one (1.5 L ha^{-1}, called D1) and 10 (15 L ha^{-1}, D10) times the recommended dose in France in April 2016. The Swing Gold formulation contains 133 g L^{-1} DMX (strobilurin, inhibiting fungal respiration) and 50 g L^{-1} EPX (triazole, inhibiting sterol biosynthesis). The recommended doses correspond to 199.5 and 75 g ha^{-1} DMX and EPX, respectively.
- The mixture (called M) was composed of Cuprafor Micro (0.75 kg Cu ha^{-1} in April, 0.7 kg Cu ha^{-1} in May, 1.25 kg Cu ha^{-1} in June, and 1.25 kg Cu ha^{-1} in September 2016) and Swing Gold at the recommended dose (1.5 L ha^{-1}). The results are not presented here (see Amossé et al., 2020 for details).

Earthworm sampling

Earthworm sampling took place two days before the first pesticide application and 28, 203, and 370 days after the first pesticide application. Earthworm sampling followed the ISO standard method for earthworm field studies (ISO 11268-3, 2014). On each sampling occasion, earthworms were sampled with an expellant solution of allyl isothiocyanate diluted with isopropanol and water to obtain a 0.1 g L^{-1} solution. For detailed information on the sampling protocol, see Amossé et al. (2020). Earthworm numbers from the four samples were summed and expressed as density (ind m^{-2}). For each treatment, the mean densities of the earthworm species were calculated from the four replicates. Earthworms were stored in formaldehyde (4% solution). They were identified at the species level and counted. Juveniles were identified at the species level according to morphological characteristics similar to those of adults. The species were classified according to three ecological categories, epigeic, endogeic, and anecic, defined by Bouché (1977).

Analytical measurement

Soil. For copper residue analyses, soil samples were collected in treated and untreated plots 5, 26, 40, 70, 75, 152, 159, 209, and 363 days after the first treatment. Three soil cores (5 cm in diameter at 10 cm depth) were collected per plot, pooled, and homogenized, removing plant residues and large grains. The soils were air-dried and sieved to <2000 µm, and then an aliquot was ground to pass through a 200-µm mesh for total Cu analysis. For this, 0.5 g of ground soil samples were weighed in Teflon containers and digested by HF/HNO_3 (1:3, v:v) and microwave heating (CEM MarsXpress). After acidic digestion with hydrofluoric acid, the excess acid was evaporated, and the samples were diluted to 50 ml with 1% HNO_3. All the reagents used were of analytical grade, and deionized water of high purity (water resistivity = 18 MΩ cm) was prepared by a Milli-Q water system (Millipore). The total Cu content in the solution was determined by flame atomic absorption spectrophotometry (FAAS, Varian SpectrAA 220, quantification limit 0.04 mg Cu L^{-1} equal to 4 mg Cu kg^{-1} of soil) following quality control assured by triplicate samples, running blanks, and using a certified reference material (TMDA-70.2; Environment Canada).

For Swing Gold, the contents of the two active substances, that is, DMX and EPX, were analyzed in the soil samples collected 5, 26, 209, and 363 days after the application. After homogenization, fresh soil was sieved at 5 mm and stored at −40 °C until analysis. The extraction and analytical methods were described by Nélieu et al. (2020). Briefly, triplicate soil subsamples were extracted by sonication in methanol, and then, the extracts of samples containing the lowest concentrations (i.e., pretreatment and controls) were further purified and concentrated by solid phase extraction. The analysis was performed by ultrahigh-performance liquid chromatography coupled through an electrospray interface to a triple quadrupole mass spectrometer in multiple reaction monitoring mode using two transitions per compound (for quantitation and confirmation purposes). The global recoveries, considering both extraction yields and matrix effects caused by electrospray ionization, were estimated as 93%–110%. The limit of detection (LOD; according to a signal-to-noise ratio of 3) was 0.03 and 0.05 µg kg^{-1} for DMX and EPX, respectively, and the limit of quantification (LOQ; validated by accuracy profile methodology) was 0.28 and 0.22 µg kg^{-1} for DMX and EPX, respectively.

Earthworms. The most abundant earthworm species, *Aporrectodea icterica* (Savigny 1826), found in the site was sampled in each plot with the digging method. Three adults and/or subadults were collected 5, 26, 209, and 363 days after the application of pesticides. Earthworms were relieved of their gut contents for 48 h before being frozen and stored at −20 °C and −80 °C for total copper and Swing Gold (i.e., total DMX and EPX contents) analyses, respectively.

The total Cu content in earthworms was quantified after worm lyophilization and dry weight recording. Digestion of earthworms was performed in a concentrated HNO_3 solution (Normapur; VWR-Prolabo) in a microwave system (CEM; MarsXpress). Typically, approximately 500 mg of earthworm (2–3 individuals) was digested with 3 ml of HNO_3, and the digests were recovered in a final volume of 25 ml with ultrapure water (18 MΩ cm, Milli-Q water; Millipore). Blanks were used to ensure the absence of contamination during the mineralization procedure. The total copper concentration in earthworms was determined by FAAS (Varian SpectrAA 220) following quality assurance procedures for the soil.

As described by Nélieu et al. (2020), to determine the DMX and EPX concentrations in earthworms, the earthworms were first ground in water and extracted by sonication in a 1:2 water/acetonitrile mixture. The samples were then submitted to two purification steps by QuEChERS and solid phase extraction on Florisil cartridges. The UHPLC–MS/MS analysis was performed on the soil. The LOQ (according to a signal-to-noise ratio of 7) was 0.02 ng DMX g^{-1} earthworm fresh weight and 0.05 ng EPX g^{-1} earthworm fresh weight.

Statistical evaluation

For each plot, annelid variables (i.e., total density, species density, and epigeic, anecic, and endogeic density) were calculated from the sum of the four samples and expressed as density (ind m^{-2}). Mean values of each variable were then averaged on the four replicates of each treatment. The differences between treatments were assessed on log transformed data ($\log(x+1)$) using parametric tests (one-way ANOVA and Dunnett tests) if the homogeneity of variance (Bartlett test; Snedecor & Cochran, 1989) and the normality of residuals (Shapiro test) were respected. Nonparametric tests (Kruskal–Wallis tests) were used if these conditions were not respected. All statistical analyses were performed with $n = 4$. The level of significance was fixed at $p < 0.05$. The analyses were carried out with R statistical software (R Core Team 2017, package version 1.6.7).

RESULTS

Environmental conditions

Total natural precipitation fluctuated throughout the study period as relatively wet months alternated with dry periods. A summary of rainfall and temperature data is given in Supporting Information: Table S2. The water content in the 0–10 cm soil layer, measured by sampling soil cores and drying them at 105 °C for 24 h, was approximately 25.7%, 26.8%, and 14% in May 2016, November 2016, and

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April 2017, respectively. The soil temperature using a thermometer (0–10 cm) was 15.9 °C, 7.9 °C, and 13.1 °C in May 2016, November 2016, and April 2017, respectively.

Pesticide residue analysis

Soil. Data on pesticide residues in soils are summarized in Table 1. Residues of the DMX and EPX concentrations in the soil—expressed as the percentage of the nominal dose—after 5, 26, 209, and 363 days are shown in Figure 1. Concentrations of total copper in the soil before and after each Cuprafor Micro application (T0, 1 month, 2 months, 5 months) and at 6 and 12 months after the first application are shown in Figure 2.

Earthworms. Mean pesticide residues in earthworm tissues are shown in Table 2. Relationships between DMX, EPX, and copper in soils and in soil organisms are presented in Supporting Information: Figure S2. Copper concentrations in earthworms over time are presented in Figure 3.

Pesticide impacts on earthworms

In all, 12 species were identified in the meadow before the establishment of the experiment: three anecic (*Lumbricus terrestris*, *Aporrectodea longa*, and *Aporrectodea giardi*), six endogeic (*Aporrectodea caliginosa*, *Aporrectodea rosea*, *A. icterica*, *Allolobophora chlorotica*, *Allolobophora muldali*, and *Octalasion cyaneum*), and three epigeic species (*Lumbricus castaneus*, *Lumbricus rubellus*, and *Dendrobaena mammalis*). The most abundant species were *A. icterica* and *L. terrestris*, with percentages of occurrence of 62.7% and 11.3%, respectively (see Supporting Information: Figure S3 for more details). Before pesticide application, the mean density of earthworms was 288 ind m^{-2}. One, 6, and 12 months after pesticide application, the mean earthworm densities were 212, 128, and 124 ind m^{-2}, respectively.

Five days after pesticide application, numerous dead earthworms (i.e., large anecic earthworms) were found at the soil surface in the Swing Gold treatment at 10 times the recommended rate of application. Moreover, dead individuals (anecics and endogeics) were found when digging the soil in the 0–20 cm soil layer in the Swing Gold treatment at 10 times the agronomic dose.

Density. The mean density of earthworms per sampling date is given for adults (Supporting Information: Table S3), juveniles (Supporting Information: Table S4), and the total earthworm community (Table 3). Relative differences to the control are given in parentheses. Changes in total earthworm density over time are presented in Figure 4, based on the absolute density (Figure 4A) and changes relative to the control treatment (Figure 4B). Figures were prepared based on the data given in Table 3. A summary of significant differences in density of the identified species and classes is given in Table 4.

TABLE 1 Mean residues ($n = 4$ plots, ±standard deviation) of Swing Gold (total contents of DMX and EPX) and Cuprafor Micro (total Cu content) in the different treatments in the 0–10 cm soil layer

Treatment (g as ha^{-1})	Time (DAT)[a]	Residue (mg kg^{-1} dwt)	Residue (% of nominal)[b]
Control Cu	0	25.2 ± 3.8	–
Control DMX	5	<LOQ[c]	–
Control EPX	5	<LOQ[c]	–
D1 (1.5 L ha^{-1}) DMX	5	0.020 ± 0.008	13.3 ± 5.4
D1 (1.5 L ha^{-1}) EPX	5	0.007 ± 0.004	12.4 ± 7.3
D10 (15 L ha^{-1}) DMX	5	0.378 ± 0.194	25.2 ± 12.9
D10 (15 L ha^{-1}) EPX	5	0.120 ± 0.077	19.9 ± 12.8
Control Cu	5	25.8 ± 6.9	–
C1 (0.75 kg Cu ha^{-1})	5	26.5 ± 5.0	–
C10 (7.5 kg Cu ha^{-1})	5	31.0 ± 6.4	–
Control DMX	26	<LOQ[c]	–
Control EPX	26	<LOQ[c]	–
D1 DMX	26	0.045 ± 0.026	29.9 ± 17.1
D1 EPX	26	0.016 ± 0.011	27.3 ± 18.1
D10 DMX	26	0.395 ± 0.160	26.4 ± 10.7
D10 EPX	26	0.138 ± 0.067	23.1 ± 11.2
Control Cu	26	24.7 ± 7.2	–
C1	26	25.2 ± 3.2	–
C10	26	30.6 ± 5.7	–
Control Cu	40	24.5 ± 7.8	–
C1 (0.75 kg Cu ha^{-1})	40	24.1 ± 3.8	–
C10 (7.5 kg Cu ha^{-1})	40	28.6 ± 5.1	–
Control Cu	70	25.4 ± 8.6	–
C1	70	25.0 ± 3.6	–
C10	70	31.7 ± 7.8	–
Control Cu	75	22.9 ± 7.7	–
C1 (1.25 kg Cu ha^{-1})	75	24.1 ± 3.8	–
C10 (12.5 kg Cu ha^{-1})	75	32.2 ± 9.0	–
Control Cu	152	22.0 ± 5.3	–
C1	152	22.9 ± 4.1	–
C10	152	25.6 ± 9.0	–
Control Cu	159	22.0 ± 5.6	–
C1 (1.25 kg Cu ha^{-1})	159	23.8 ± 4.5	–
C10 (12.5 kg Cu ha^{-1})	159	35.2 ± 6.4	–
Control DMX	209	<LOQ[c]	–
Control EPX	209	<LOQ[c]	–

(*Continued*)

TABLE 1 (*Continued*)

Treatment (g as ha^{-1})	Time (DAT)[a]	Residue ($mg\ kg^{-1}$ dwt)	Residue (% of nominal)[b]
D1 DMX	209	0.015 ± 0.004	9.9 ± 2.7
D1 EPX	209	0.007 ± 0.001	12.5 ± 2.0
D10 DMX	209	0.132 ± 0.057	8.8 ± 3.8
D10 EPX	209	0.101 ± 0.046	16.9 ± 7.6
Control Cu	209	21.0 ± 5.0	–
C1	209	24.4 ± 4.7	–
C10	209	41.4 ± 9.0	–
Control DMX	363	<LOQ[c]	–
Control EPX	363	<LOQ[c]	–
D1 DMX	363	0.008 ± 0.002	5.3 ± 1.6
D1 EPX	363	0.006 ± 0.002	9.6 ± 4.1
D10 DMX	363	0.102 ± 0.023	6.8 ± 1.5
D10 EPX	363	0.132 ± 0.072	22.0 ± 12.0
Control Cu	363	22.0 ± 6.4	–
C1	363	24.0 ± 3.9	–
C10	363	37.7 ± 9.2	–

Notes: D1 and D10 for 1 and 10 times the recommended dose of Swing Gold, respectively; C1 and C10 for 1 and 10 times the recommended dose of Cuprafor Micro, respectively. Modified from Amossé et al. (2020).
Abbreviations: DMX, dimoxystrobin; EPX, epoxiconazole.
[a]Days after treatment.
[b]Nominal is based on the total amount applied on the surface of three cores and the dry weight of the soil.
[c]In soil, limit of detection (LOD; according to signal-to-noise ratio): 0.00003 $mg\ kg^{-1}$ for DMX, 0.00005 $mg\ kg^{-1}$ for EPX; limit of quantification (LOQ; validated by accuracy profile methodology): 0.00028 $mg\ kg^{-1}$ for DMX, 0.00022 $mg\ kg^{-1}$ for EPX.

Biomass. The mean biomass of earthworms per sampling date is given for adults (Supporting Information: Table S5), juveniles (Supporting Information: Table S6), and the total earthworm community (Table 5). Relative differences to the control are given in parentheses. Changes in the biomass of earthworms over time are presented in Figure 5, based on absolute weights (Figure 5A) and changes relative to the control (Figure 5B). A summary of significant differences in biomass of the identified species and classes is given in Table 6.

Overall, the results demonstrated that the tested fungicides had no effect on earthworm populations at the recommended dose under field conditions over one year. However, a single application of Swing Gold at 10 times the recommended dose significantly affected anecic (total and juveniles) earthworm densities, especially the species *L. terrestris*. Highly significant effects (>50%) were observed on total anecic densities in the D10 treatment compared with the control treatment, 1, 6, and 12 months after pesticide application, with earthworm densities decreasing by 91%, 79%, and 73%, respectively. The total density of earthworms and total density of endogeic earthworms were also affected in the D10 treatment, with decreases of −56% and −50% and −58% and −56% compared with the control after 6 and 12 months of exposure, respectively. Earthworm recovery was not observed in the D10 treatment within a year. Moreover, anecic earthworm density decreased significantly in the C10 treatment compared with the control (−88%) 12 months after pesticide application.

Detailed results of interest to ecotoxicologists can be monitored in such field studies, such as earthworm abundance and biomass during the study period (Supporting Information: Figures S4 and S5) or correlations between

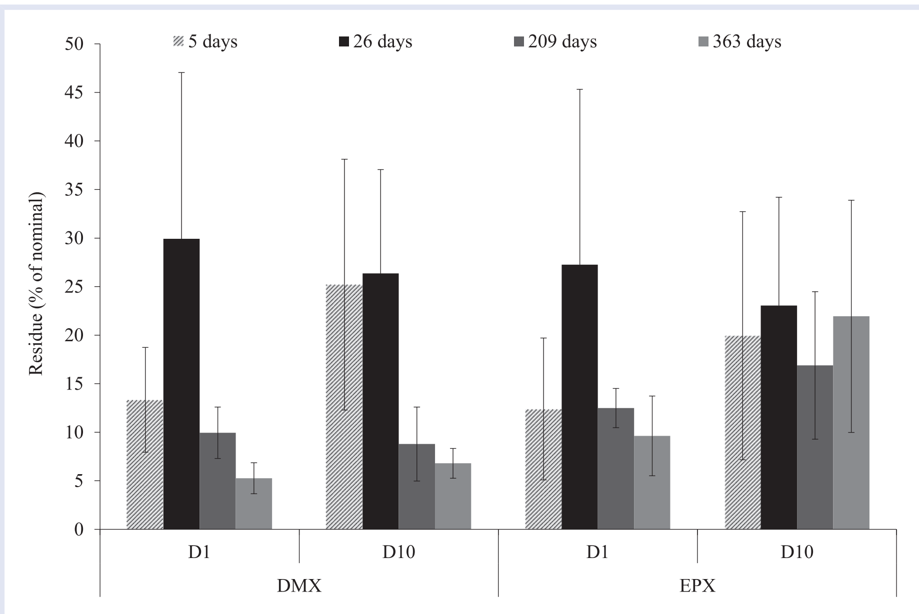

FIGURE 1 Mean residues of dimoxystrobin (DMX) and epoxiconazole (EPX; total contents, $n = 4$ plots, and standard deviation) in sampled soils, in % of the nominal dose. Modified from Amossé et al. (2020)

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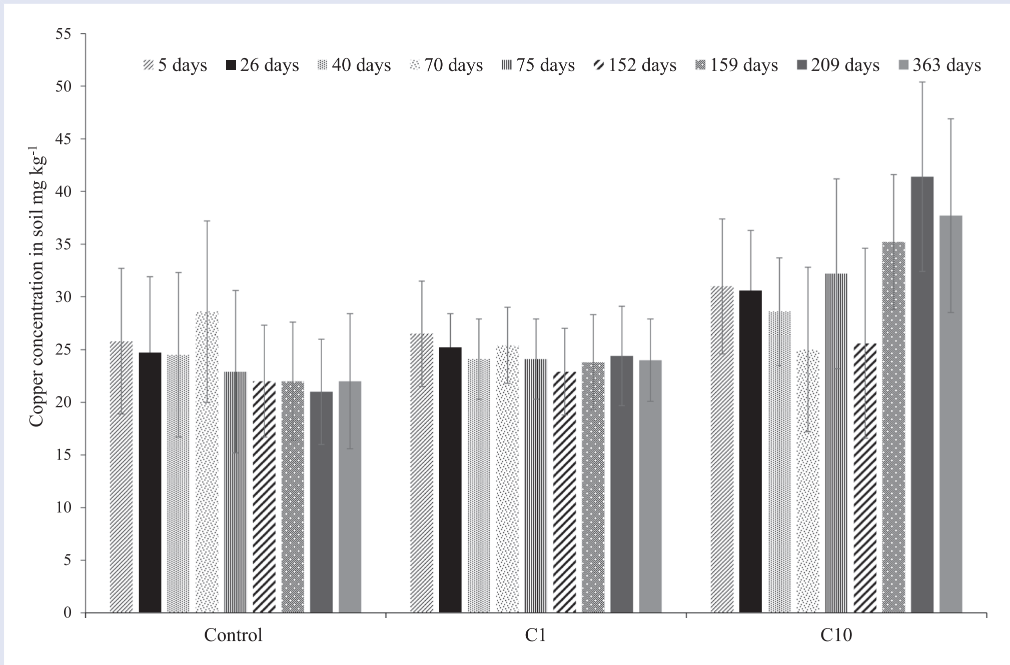

FIGURE 2 Mean residues of copper (total contents, $n = 4$ plots, and standard deviation) in sampled soils, in mg kg^{-1}. Modified from Amossé et al. (2020)

pesticide residues in soils and in earthworms (Supporting Information: Figure S2).

DISCUSSION AND RECOMMENDATIONS

Field study to assess the effects of Cuprafor Micro and Swing Gold on earthworm populations

Pesticides in soils and in soil organisms. The total Cu content increased significantly (ANOVA, $p < 0.05$) in the soil in the C10 treatment over the control at t6 and t12 after the four applications of Cuprafor Micro (i.e., 21.0 ± 5.0 and 41.4 ± 9.0 mg Cu kg^{-1} in the T and C10 treatments, respectively, at t6). The measured soil concentrations after 12 months following four applications of copper are in line with the estimated predicted environmental concentrations in soil (PEC_{soil}) corresponding to an annual applied rate of 4 and 40 kg ha^{-1} (27.86 and 51.8 mg kg^{-1} dry soil, respectively). The PEC_{soil} was calculated considering a default soil density of 1.5 g cm^{-3} (FOCUS, 1997), homogenization in the 10 cm soil depth,

TABLE 2 Mean residues (total contents, $n = 4$ plots, ±standard deviation) of Swing Gold (DMX and EPX) and copper in the species *Aporrectodea icterica*, calculated from the fresh (ng g^{-1} wwt) and dry (mg kg^{-1} dwt) earthworm biomass measured, respectively

		Residues in earthworms at each sampling interval (DAT)[a]			
Treatment	0	5	26	209	363
Control Cu	10.3 ± 0.9	10.7 ± 1.8	10.0 ± 1.5	12.6 ± 3.0	15.9 ± 2.6
Control DMX	<LOQ[b]	0.82 ± 0.41	0.40 ± 0.36	0.08 ± 0.10	<LOQ[b]
Control EPX	<LOQ[b]	0.14 ± 0.06	<LOQ[b]	0.06 + 0.08	<LOQ[b]
D1 DMX		29.00 ± 7.45	5.13 ± 2.75	3.11 ± 2.91	1.21 ± 0.78
D1 EPX		2.83 ± 1.24	0.31 ± 0.16	1.39 ± 1.65	0.25 ± 0.07
D10 DMX		130.42 ± 76.44	32.21 ± 11.41	22.83 ± 9.04	19.39 ± 8.80
D10 EPX		15.65 ± 9.96	1.55 ± 0.96	10.76 ± 7.00	8.08 ± 8.73
C1		9.9 ± 1.0	9.5 ± 0.6	13.2 ± 1.7	14.1 ± 2.3
C10		11.7 ± 1.6	10.0 ± 0.9	10.8 ± 3.1	15.0 ± 3.5

Abbreviations: DMX, dimoxystrobin; EPX, epoxiconazole.
[a]Days after treatment.
[b]In earthworms, limit of quantification (LOQ; according to signal-to-noise ratio): 0.02 ng g^{-1} for DMX, 0.05 ng g^{-1} for EPX.

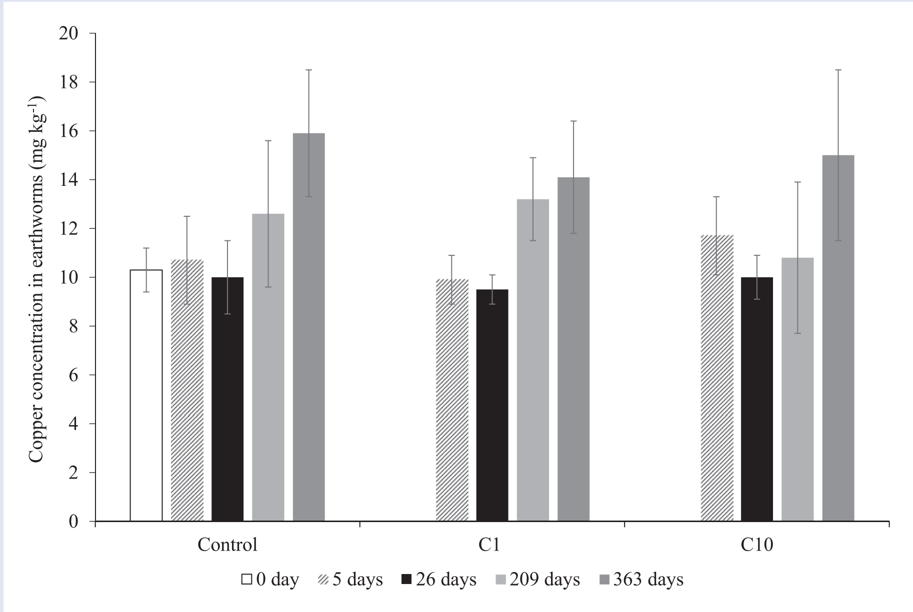

FIGURE 3 Mean residues of copper in earthworms ($n = 4$ plots, with standard deviation) in mg Cu kg^{-1}

and no soil dissipation of copper between applications (SANCO).

Not surprisingly, no bioaccumulation of copper in earthworms was observed between treated and untreated plots at 4 and 40 kg Cu ha^{-1}, and internal Cu body concentrations were in the range of those classically observed (Ma, 2005). Indeed, copper, as an essential nutrient, is known to be regulated and thus poorly accumulated by earthworms,

TABLE 3 Density of total earthworms over time, values represent mean number of earthworms m^{-2}

Class	Sampling time (month(s))	Treatment				
		Control	D1	D10	C1	C10
Epigeics	Pre-appl	23.8				
	1	12.9	8.2 (−36)	0.0	10.2 (−21)	7.4 (−42)
	6	0.0	0.0	0.0	0.0	0.0
	12	1.6	5.9 (+369)	2.7 (+75)	1.2 (−25)	1.2 (−25)
Endogeics	Pre-appl	202.5				
	1	184.0	178.1 (−3)	123.8 (−33)	167.6 (−9)	210.5 (+14)
	6	100.8	109.4 (+9)	50.4 (−50)	108.2 (+7)	123.0 (+22)
	12	127.3	83.2 (−35)	56.3 (−56)	99.6 (−22)	135.9 (+7)
Anecics	Pre-appl	62.5				
	1	34.0	28.1 (−17)	2.7 (−92)	33.6 (−1)	46.5 (+37)
	6	28.5	29.3 (+3)	5.9 (−79)	44.1 (+55)	33.6 (+18)
	12	29.3	18.8 (−36)	7.8 (−73)	22.3 (−24)	3.5 (−88)
Total earthworms	Pre-appl	288.8				
	1	230.9	214.5 (−7)	126.6 (−45)	211.3 (−8)	264.5 (+15)
	6	129.3	138.7 (+7)	56.3 (−56)	152.3 (+18)	156.6 (+21)
	12	158.2	107.8 (−32)	66.8 (−58)	123.0 (−22)	140.6 (−11)

Notes: Values between brackets are relative differences to the control (in %). Modified from Amossé et al. (2020).

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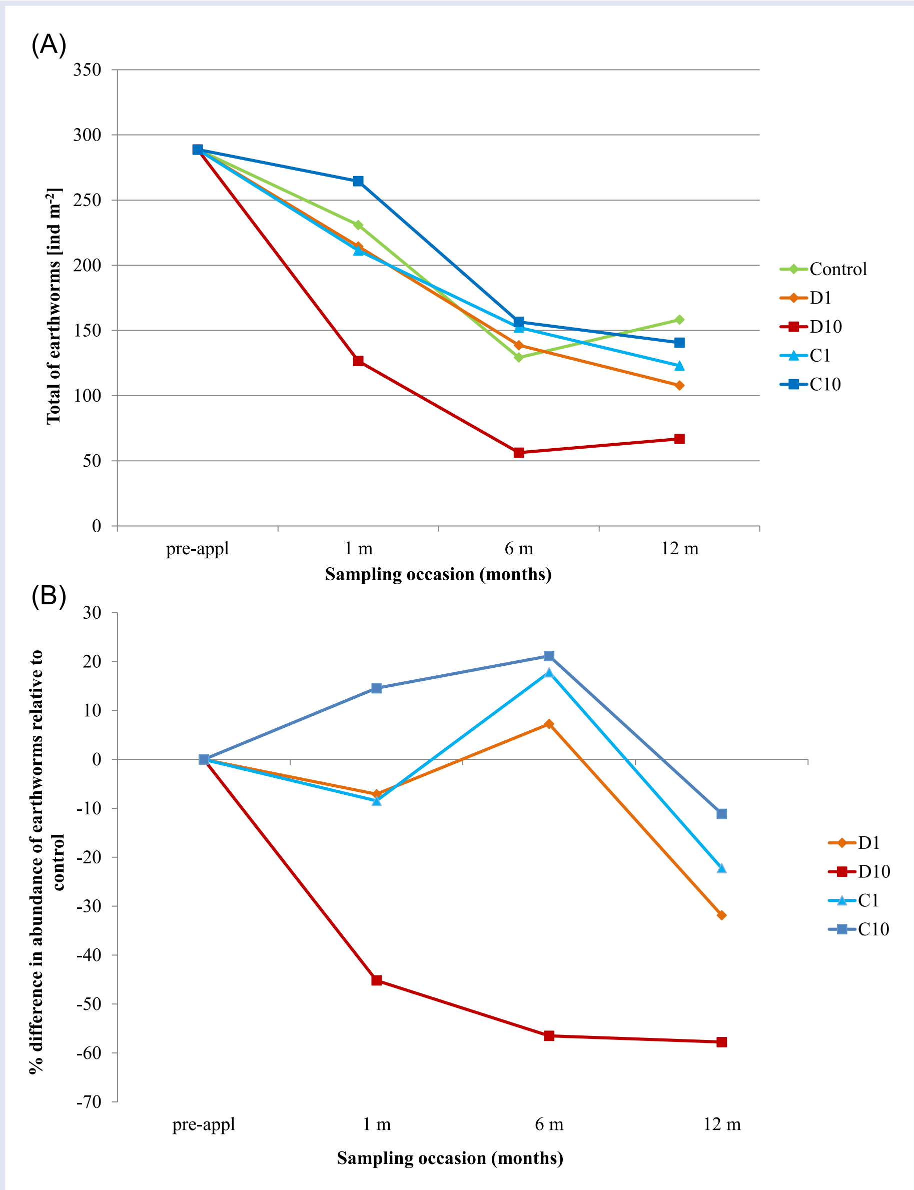

FIGURE 4 (A) Total density (absolute) of earthworms on the different sampling occasions. Modified from Amossé et al. (2020). (B) Total density of earthworms on the different sampling occasions, relative to control (*X*-axis)

especially in the range of the total copper soil contents of our study (Marinussen et al., 1997). For example, the first soil copper addition corresponded to 3.1 mg Cu kg^{-1} soil for C1, which must be compared with the initial soil copper content originating from the pedological background. Indeed, copper from geogenic origin was established at 17 mg Cu kg^{-1} soil in the control plots, with some variability from 17 to 28 mg Cu kg^{-1} soil observed in the field from the bottom to the top of the parcel. Moreover, we verified that most of the added copper was confined in the uppermost 10 soil centimeters, and the avoidance of contaminated zones by earthworms might have limited their exposure (Ballabio et al., 2018). Bart et al. (2017) found a significant effect of Cuprafor Micro on the avoidance (EC_{50} of 51.2 mg Cu kg^{-1} after 48 h) of the endogeic earthworm species *A. caliginosa* under controlled conditions. Finally, if we exclude the potential differences in earthworm exposure, the bioavailability of copper could be reduced in our studied site because of the presence of large organic matter in the grassland. Indeed, the grassland rhizosphere is known to be rich in organic ligands complexing with copper, thus reducing its bioavailability (Duan et al., 2016).

The two organic fungicides DMX and EPX were initially absent in the soil where the experiment took place. Their concentrations remained under the LOQ in the sampled soil from control plots throughout the experiment. Five days after the application of Swing Gold, the total DMX and EPX contents in the soil were evaluated to be far below the amount of pesticide applied in the D1 and D10 treatments (Table 1; Figure 1). This could be explained in minor part by leaching (for 4%–7%, according to analysis up to 30-cm depth). The main reason for the low initial concentration in

TABLE 4 Significant differences with the control treatment $p < 0.05$ (Dunnett or Kruskal–Wallis tests) in earthworm density are indicated in gray with the trend observed (↓ indicates significant decrease, ↑ indicates significant increase)

Species	Sampling time (month(s))	Treatment			
		D1	D10	C1	C10
Lumbricus terrestris	1		↓		
	6		↓		
	12				↓
Anecic juveniles	1		↓		
	6				
	12		↓		↓
Total anecics	1		↓		
	6				
	12		↓		↓

the 0–10 cm soil layer may be caused by the foliar interception of fungicides, and the vegetation cover acts as a barrier for pesticide migration in the soil. One month after the application of Swing Gold, the DMX and EPX concentrations in the soil increased in the D1 treatment, which could be attributed partially to the wash-off of residues indicating leaching and photodegradation from the leaf surface or runoff from close areas. Overall, the DMX and EPX contents in the soil reached approximately 23.1%–35.5% of the initial amount of pesticide applied at one month. Important heterogeneity was observed between the four replicates for DMX and EPX in the D1 and D10 treatments at all sampling times (the high standard deviations are not the result of analytical uncertainty; Nélieu et al., 2020). Such heterogeneity and difference between nominal and observed concentrations are not surprising under field conditions. A decrease in DMX concentration can be observed six months and one year after application, respectively; the decrease remains insignificant for EPX in the D1 treatment, and no tendency was observed for EPX in the D10 treatment. The dissipation of both pesticides seems to be higher in this experiment than expected according to their field DT50, as proposed in the respective EFSA conclusions (European Food Safety Authority [EFSA], 2005, 2008). The respective EFSA journal indicates for DMX a field DT50 in soils ranging from 2 to 39 days (EFSA, 2005) and for EPX a DT50 in the field ranging from 1 to 226 days (EFSA, 2008). The pesticide residues found in the soil were tentatively related to residues analyzed in earthworms (Supporting Information: Figure S2). Globally, the concentration in earthworms remained lower (DMX) or largely lower (EPX) than that measured in soil. The only exception was observed for DMX five days after application in the D1 treatment, where an earthworm to soil ratio of

TABLE 5 Mean biomass of total earthworms over time, values represent g m^{-2}

Class	Sampling time (month(s))	Treatment				
		Control	D1	D10	C1	C10
Epigeics	Pre-appl	2.8				
	1	1.6	0.8 (−51)	0.0	1.1 (−34)	0.7 (−57)
	6	0.0	0.0	0.0	0.0	0.0
	12	0.11	0.4 (+273)	0.436 (+225)	0.108 (−28)	0.109 (−18)
Endogeics	Pre-appl	69.1				
	1	60.6	53.7 (−11)	45.7 (−25)	54.1 (−11)	62.7 (+3)
	6	25.7	29.9 (+16)	14.7 (−43)	31.4 (+22)	31.0 (+20)
	12	42.82	31.0 (−26)	20.877 (−51)	37.7 (−12)	47.655 (+11)
Anecics	Pre-appl	78.1				
	1	24.6	24.4 (−1)	2.6 (−89)	24.3 (−1)	30.2 (+23)
	6	21.2	27.4 (+29)	6.0 (−72)	39.7 (+87)	30.2 (+43)
	12	27.34	14.04 (−49)	4.3 (−84)	15.9 (−42)	3.30 (−88)
Total earthworms	Pre-appl	149.9				
	1	86.8	78.9 (−9)	48.3 (−44)	79.5 (−8)	93.6 (+8)
	6	46.9	57.3 (+22)	20.7 (−56)	71.0 (+51)	61.2 (+30)
	12	70.28	46.15 (−34)	25.45 (−64)	53.71 (−24)	50.95 (−28)

Notes: Values between brackets are relative differences to the control (in %).

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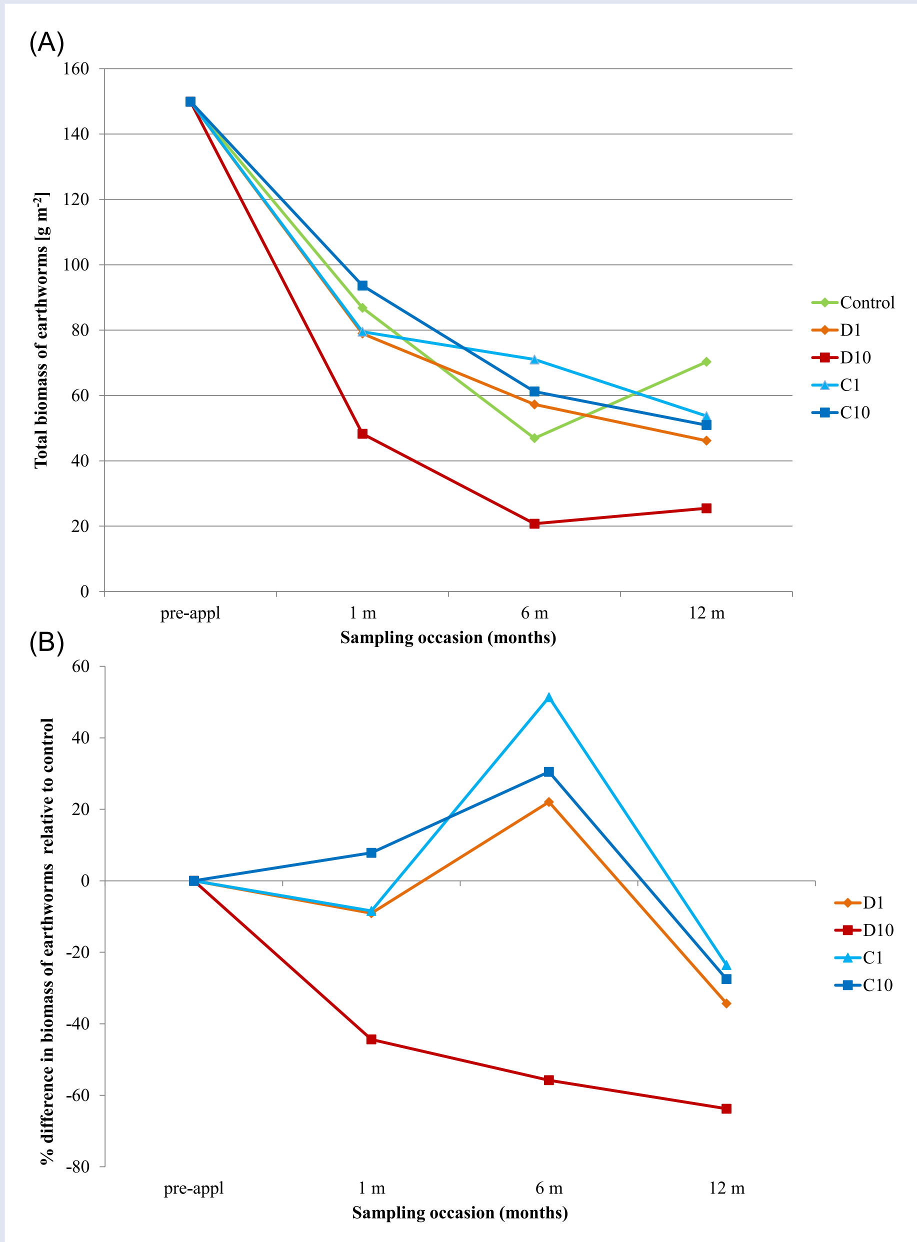

FIGURE 5 (A) Total biomass (absolute) of earthworms on the different sampling occasions. Modified from Amossé et al. (2020). (B) Total biomass of earthworms on the different sampling occasions, relative to control (*X*-axis)

1.53 ± 0.43 revealed that some DMX bioaccumulation occurred. Early and transitory bioaccumulation may also have occurred in some earthworms in the D10 treatment but caused earthworm death; thus, it was not observed in the living sampled earthworms.

The actual low concentrations measured from soil analyses can add some uncertainty regarding the exposure of earthworms to the active substances. However, observation of dead animals at the surface of the soil in a short period after the application of Swing Gold confirms the exposure of earthworms. This is also further supported by the observation of effects above 50% on abundance and biomass throughout the study duration in the D10 treatment, which should be regarded as the toxic reference for this field study. In addition, these observations allowed us to provide recommendations to avoid this issue being repeated. These recommendations need to be considered further before setting up such trials.

Effects of tested pesticides on earthworm populations. The response of earthworms to the two tested products varied over time. First, negative effects of Swing Gold (i.e., the D10 treatment) on earthworms were observed in very short terms (i.e., five days) in the presence of dead earthworms (i.e., anecics) on the soil surface, highlighting the rapid lethal effect of this fungicide. This trend was confirmed 1, 6, and

TABLE 6 Significant differences with the control treatment $p < 0.05$ (Dunnett or Kruskall–Wallis tests) in earthworm biomass are indicated in gray with the trend observed (↓ indicates significant decrease, ↑ indicates significant increase)

Species	Sampling time (month(s))	Treatment D1	D10	C1	C10
Total biomass	1				
	6		↓		
	12		↓		
Total juveniles	1				
	6		↓		
	12		↓		
Lumbricus terrestris	1				
	6				
	12				↓
Anecic juveniles	1		↓		
	6		↓		
	12		↓		↓
Total anecics	1		↓		
	6		↓		
	12		↓		↓

12 months after pesticide application with a clear decrease in anecic (especially the species *L. terrestris*) density in the Swing Gold treatment at 10 times the recommended dose, with an effect up to and above 50% on abundance that was not significantly different from the control. Moreover, although the differences were not significantly different, densities of epigeic earthworms and some endogeic species (i.e., *A. caliginosa* and *A. chlorotica*, Savigny, 1826) were negatively affected by the Swing Gold product at 10 times the recommended dose. For endogeic earthworms, a clear trend was also observed, with, for instance, a strong decrease in abundance (33%, 50%, and 56% decrease in abundance related to the control at 1, 6, and 12 months, respectively, in the D10 treatment) from the beginning to the end of the experiment. It has already been observed that earthworms, especially epigeics and anecics, may be affected directly by pesticides (Pelosi et al., 2014) because they are directly exposed to them through their feeding activity near the soil surface (Edwards, 2004). Moreover, the results found in the present study are in accordance with the LC50 of 6.3 times the recommended dose calculated by Bart et al. (2017) for the species *A. caliginosa* under laboratory conditions.

A significant effect of Cuprafor Micro on anecic earthworms at t12 months was observed, with abundances ranging from 29 in the T treatment to 4 ind m^{-2} in the C10 treatment. Endogeic abundance and biomass were higher in the C10 treatment than in the C1 treatment regardless of the sampling occasion (Supporting Information: Figure S4a, b), which was not the case for anecic earthworms (Supporting Information: Figure S5a,b). This underlines the difference in species sensitivity and a potential compensation between species that are differently sensitive to the tested pesticides. The effects of copper on earthworms are well documented under laboratory conditions (Bart et al., 2020; Eijsackers et al., 2005) and long-term contaminated sites (Mirmonsef et al., 2017; Owojori & Reinecke, 2010; Van Zwieten et al., 2004), but studies have exhibited contrasting results. For instance, Van Zwieten et al. (2004) reported that earthworm number and biomass were influenced by the level of copper in soils of avocado orchards on the north coast of New South Wales, Australia. In contrast, Owojori and Reinecke (2010), in outdoor microcosm experiments, highlighted that copper, on its own, did not have a significant effect on the measured earthworm parameters (i.e., survival, weight change, and cocoon production of *A. caliginosa*). This could be explained mainly by the mode of contamination (i.e., mine soils, spiked soils) and the number of tested contaminants (i.e., one vs. several heavy metals; Eijsackers et al., 2005; Spurgeon & Hopkins, 1999; Tisher, 2008). However, little is known about the effects of copper addition over one year under field conditions. The low anecic density found in the C10 treatment could be explained by the dispersal of earthworms (Christensen & Mather, 2004). For example, Wenstel and Guelta (1988) found significant effects of brass powder (mix of 70% Cu and 30% Zn) on the avoidance (threshold value at 26 mg Cu kg^{-1} dry soil after seven days) of the earthworm species *L. terrestris* in climatic chambers (15 × 50 cm). In our case, the different copper applications over time (i.e., 0.75, 0.75, 1.25, and 1.25 kg Cu ha^{-1} in April, May, June, and September 2016) and the gradual increase in Cu_{tot} in the soil (means of 28.6, 41.4, and 37.7 mg Cu kg^{-1} in C10 at t1, t6, and t12, respectively) may explain the longer term effects of copper on earthworm communities compared with Swing Gold. Therefore, our results and previous studies highlight that the effects of copper on soil biota are not immediate and can appear after several months or years of copper applications and accumulation in the soil. However, under controlled conditions, Eijsackers et al. (2005) and Bart et al. (2017) demonstrated a significant decrease in biomass for the species *A. caliginosa* at 50 and 60 mg Cu kg^{-1} dry soil after 14 and 20 days of exposure, respectively. The concentrations tested in these two studies were, however, higher than the maximum dose applied at our experimental site.

Assessment of the field study according to de Jong et al.'s (2006) recommendations

In this section, we propose to evaluate and discuss our experimental design and our results based on each item (total of 10) identified by de Jong et al. (2006). We also propose some improvements to the ISO guideline 11268-3 (2014).

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Item 1. Substance. Tested products (i.e., Swing Gold and Cuprafor Micro) have been reported in detail in the article. They are already well characterized because they are used in the European Union (E-phy, 2017a, 2017b). No vehicle other than the product was used.

Item 2. Test site. The location of the experimental site has been referenced in detail with geographical coordinates. The meadow has not been fertilized or treated with pesticides for more than 20 years, allowing a very low influence of the field history on the results of the study. The soil properties measured (i.e., particle-size distribution, organic carbon content, pH, water holding capacity in the A-horizon) followed ISO 11268-3. Concerning the substances to be measured, their contents must be determined before the first application. Indeed, in our study, the initial copper concentration (pedological background) was characterized through a first sampling across the experimental plot. We noticed a slight spatial heterogeneity of the copper concentration from 17 to 28 mg Cu kg^{-1} of soil (thereafter seen in the control plots), which cannot be neglected for such low contents. For the organic molecules, we verified that their initial contents were close to zero before the applications. Concerning the site vegetation, the cover has not been described before pesticide application. In particular, we found that moss coverage was heterogeneous and may have influenced pesticide migration into the soil.

Recommendation 1: The site should be as representative as possible for EU agricultural climatic and soil conditions. This recommendation is not new and should be regarded as a reminder, as similar advice was already expressed in 2006 by Kula and collaborators: "When selecting a study site, regional soil properties and climatic conditions should be taken into consideration." Furthermore, to ease fieldwork, the site should be near institutes (e.g., mowing, earthworm sampling).

Recommendation 2: A thorough investigation of the initial concentrations of nonbiodegradable compounds would ease the interpretation of the results. For transition metals, the localization of the site is important to avoid potential disturbances such as natural atmospheric deposition, anthropogenic atmospheric deposition, and bedrock.

Recommendation 3: A clear method for the description of vegetation is needed, as it can influence pesticide fate and behavior and thus the effects on soil fauna. Moreover, vegetation cover changed according to the season and the pesticide used (i.e., reduction in moss coverage one year after copper application).

Item 3. Application. The fungicide products were applied according to the conditions of use for agricultural practices: period (i.e., spring), amounts of pesticides (i.e., 1 and 10 times the recommended dose in kg ha^{-1} or L ha^{-1}), and route of exposure (i.e., foliar adsorption). Substances were diluted in tap water (8 L 100 m^{-2}) before application and respected the dilution recommended for meadows in ISO 11268-3 (i.e., between 400 and 800 L ha^{-1}). In this study, the use of a manual sprayer was considered more adapted to the surface to be treated (100 m^2) and allowed avoiding soil compaction compared with heavy machinery. Moreover, pesticide application with a manual sprayer generated less drift, and two passages were performed to allow homogeneous application. However, the application of the pesticides with the manual sprayer might not have been homogeneous in each plot. Moreover, it would have been necessary to calibrate the manual sprayer and to include a soil collector, such as Petri dishes, to check the applied rate. It was decided to mow the meadow with residue exportation 1 week before pesticide application to avoid compact grass cover and pesticide uptake by vegetation residues.

Recommendation 1: For "small" surfaces (i.e., 100 m^2), manual sprayers should be recommended for the reasons listed above. For larger surfaces, tractors equipped with boom sprayers could be a better option to reflect agricultural practices and ensure appropriate application.

Recommendation 2: A detailed method for the evaluation of pesticide application (e.g., Petri dishes laid at the soil surface) in the field could be strongly recommended to better assess the amount of pesticide applied and homogeneity of application. These tests could be performed before the experimental trial.

Recommendation 3: Measurement of pesticides in vegetation (e.g., plant material) over time could help to better characterize the actual application rate and to interpret the results.

Item 4. Test design. The test design followed ISO 11268-3 with a random plot design, plots of 100 m^2, a treated edge strip of 2 m, four replicates (allowing statistical analyses and a better interpretation of results), dose–response (1 and 10 times the recommended application rate) and duration (i.e., one year to assess earthworm recovery). Moreover, four blocks were added to overcome the shade of trees, decreasing daylight length, soil temperature, and thus biological activity. A negative control (without pesticide application) was added but not the toxic reference with carbendazim at 6–10 kg ha^{-1}. Indeed, the use of this latter active substance has not been approved in Europe since 2014 and is therefore not commercially available. Moreover, according to EFSA reports, carbendazim is slightly less toxic to earthworms (NOEC for reproduction of 1 mg as kg^{-1} soil dry weight; EFSA Scientific Report, 2010) than EPX (NOEC reproduction of 0.167 mg as kg^{-1} soil dry weight; EFSA Scientific Report, 2008); and DMX (NOEC reproduction <0.0887 mg as kg^{-1} soil dry weight; EFSA Scientific Report, 2005) under laboratory conditions. Therefore, Swing Gold at 10 times the recommended application rate was considered a toxic reference, as Bart et al. (2017) found an LC50 of 6.3 times the recommended application rate, and we found a statistically significant reduction in earthworm density of more than 50% on at least one of the sampling occasions. This fulfilled the expected effect of a toxic reference toward the population (population decrease by 40%–80%) provided in the ISO guideline (ISO 11268-3, 2014).

Recommendation 1: More consideration should be given in the ISO 11268-3 guideline regarding the heterogeneity of soil conditions and characteristics in the experimental field. The norm should mention that the plots (replicates) have to be placed as much as possible to overcome spatial heterogeneity. Moreover, if possible (enough space for earthworm sampling for the duration of the trial), each plot should be initially (before the application of the chemicals) characterized for earthworm communities and soil properties to finely characterize the plot and the trial heterogeneity.

Recommendation 2: Edge effects (e.g., in our case shadow cast by surrounding trees) of the experimental site should be considered because it has an effect on soil fauna recolonization and thus on the measured recovery.

Recommendation 3: The active substance or product used as toxic reference can be adapted (i.e., not always the carbendazim) if the decrease in earthworm population respects ISO 11268-3 (i.e., decrease >50% in abundance or biomass on at least one sampling date). In this case, preliminary tests are needed to ensure that the active substance or product identified could be considered a toxic reference.

Item 5. Biological system. The test was fitted to the ISO 11268-3 guideline with a mean earthworm density greater than or equal to 100 ind m^{-2} and a mixed community of species (i.e., 12 species) belonging to the three ecological categories (epigeics, endogeics, and anecics) before the application of the two products. Moreover, dominant and representative species (i.e., *A. icterica* and *L. terrestris*) of agricultural areas were found in sufficient densities (10% of the total population). Adults and juveniles were also recorded at each sampling period at sufficient densities.

Recommendation 1: Earthworm density varies over time with possible lower density at different sampling periods. The best conditions for earthworm sampling must be considered to properly assess the effects of the tested substances and not a climate effect or another confounding effect, such as drought. Generally, seasonal fluctuations in earthworms are characterized by a decrease in abundance and diversity during summer and larger numbers in spring and autumn. Furthermore, for earthworms, the main activity periods are spring and autumn. For instance, during these periods, most of the endogeic earthworms may be found in the upper soil layer. Exposure to products and active substances is maximized under these conditions. These periods might vary owing to annual climatic variability, local soil conditions, and particularly soil humidity.

Item 6. Sampling. No other pesticide product was used before or during the test or in the surroundings of the plot. The earthworm sampling area per replicate was 0.16 m^2, with four replicates per plot (0.64 m^2). This sampling area is in accordance with ISO 11268-3, and a relatively high density of earthworms was found in the meadow. The vegetation was cut before each earthworm sampling. Sampling periods respected ISO 11268-3 recommendations (i.e., pretreatment <2 weeks, 1, 6, and 12 months after treatment). The expellant solution (i.e., allyl isothiocyanate diluted with isopropanol) was used before the hand-sorting method according to sampling recommendations.

Earthworms were classified as:

(i) adults and juveniles; (ii) species level for adults and according to morphological criteria for juveniles; (iii) classification of individuals according to the three ecological categories (i.e., epigeic, endogeic, and anecic).

The sampled earthworms were analyzed, and the results were expressed as:

(i) total density (ind m^{-2}) and biomass (g m^{-2}); (ii) density and biomass for each species; (iii) density and biomass of adults and juveniles; (iv) species diversity.

Pesticide residues were measured in the soil before and after each pesticide application. Local climatic conditions were also measured on site: soil moisture and soil temperature, amount of precipitation, and air temperature before and during earthworm sampling.

Recommendation 1: Close monitoring of climatic conditions must be performed throughout the test. Sampling periods can be managed if climatic conditions are not appropriate (e.g., irrigation is needed during dry seasons). If a sampling point must be delayed because of suboptimal climatic conditions, this should be reported clearly in the study report.

Recommendation 2: As already highlighted by Kula et al. (2006), irrigation within three days following application of the product should be considered if insufficient rainfall occurs. This would lead to optimal exposure of earthworms.

Recommendation 3: Systematic earthworm surface monitoring of the plot should be performed in the following hours or days to monitor potential mortality or behavior induced by the application of active substances or products, such as earthworms escaping the plots. If observations are made, they should be quantified and recorded.

Item 7. Application. As mentioned in ISO 11268-3, soil pesticide residue analyses are only required to confirm the application rate in the uppermost 10 soil centimeters to ensure the exposure of earthworms. The expected rate of the nominal concentration in the soil was set between 50% and 150%. In our case, we reached between 10% and 35% in the soil of the nominal amounts applied of EPX and DMX regardless of the sampling period, which is lower than expected. This could be explained by interception by vegetation cover, which acts as a barrier preventing pesticides from reaching the soil. Moreover, the tested products (i.e., Swing Gold and Cuprafor Micro) are known to be foliar fungicides, and active substances were probably intercepted mainly by plants. The degradation of DMX and EPX is not likely, as the whole study demonstrated a certain stability of the soil concentration of the substances throughout the study.

Recommendation 1: Pesticide residue analyses and extraction yields would be required for all field studies. Where available, relevant and validated extraction methods of pesticides in the soil and in soil organisms should be proposed in the ISO 11268-3 guideline.

Recommendation 2: Moreover, the percentage of the nominal amount applied in the soil should be adapted according to active substances, the route of exposure (i.e., foliar application or soil treatment), and the land use chosen for the field study (meadow, type of crop). In a regulatory risk assessment context, this would need verification and validation.

Recommendation 3: It is difficult to unravel a high geogenic soil metal content from an added inorganic pesticide content when applied at low doses to decipher potential effects on soil fauna. The natural background of trace elements at the experimental site should thus be systematically measured and compared with the range of applied pesticide contents. This issue is also suggested in a statement released by EFSA in 2021 (EFSA Panel of the Plant Protection Products and their Residues [EFSA PPR Panel], 2021).

Item 8. Endpoint. Statistical tests followed the recommendations of de Jong et al. (2006). Statistical differences ($p < 0.05$, one-sided Dunnett or Kruskal–Wallis tests) were found between plots treated with or without pesticide (e.g., anecic density). However, statistical tests need to be fit for purpose according to the studied variables regarding the European and Mediterranean Plant Protection Organization (EPPO; 2003) and EFSA opinions (2017) reports. For example, clear effects of pesticides were observed on a few earthworm variables at one or several sampling occasions (e.g., >50% earthworm density reduction in D10 at each sampling period). According to the EPPO standard (2003), different risk levels exist:

- No effects >30%–50%: low risk;
- Effects >50% observed during a study but with full recovery within one year: minimum risk;
- Effects >50% without full recovery after one year: high risk.

In our case, no full recovery was observed, classifying the effect of the D10 treatment as high risk.

Recommendation 1: Selected statistical tests need to be adapted to the experimental design for regulatory environmental risk assessment. For example, the minimal detectable difference (MDD) should be developed and tested, as should the magnitude effects proposed by EFSA opinions (EFSA PPR Panel, 2017). Such MDD analysis was performed and presented by Amossé et al. (2020).

Recommendation 2: To improve the statistical power and to retrieve robust NOEC from field studies, the number of replicates at the plot level for the control and test treatments might be increased (up to six). This opinion is further supported by a recent report published by UBA (Römbke et al., 2020).

Recommendation 3: If full recovery is not observed within a year, the duration of the field study may be extended until full recovery is observed. For this purpose, it appears essential to carefully assess the proportion of juveniles in the different treatments. This can help determine the level of recruitment in each treatment, which would constitute the main part of the internal recovery that might be observed later in the field.

Recommendation 4: Other endpoints could also be considered, such as the assessment of soil functions (e.g., with litterbags; Organisation for Economic Co-operation and Development [OECD], 2006). In the literature, soil faunal recovery can be rapid (i.e., within a few months), but the duration of the recovery of soil functions can be much longer (i.e., 1–2 years). Such endpoints should also be assessed to better understand the potential disruption of soil functioning following the application of pesticide products.

Recommendation 5: Diversity indices (e.g., Shannon index) could be relevant as an endpoint other than species diversity and could inform earthworm community dynamics. Recently, Amossé et al. (2020) illustrated the usefulness of the Shannon index. Earthworm diversity was negatively affected by the application of pesticide products at the recommended application rates, whereas no significant effects were measured on the total earthworm abundance or biomass. This can be the result of compensation between species that are differently sensitive to the tested pesticides. Diversity indices can thus allow highlighting differences in species sensitivity that cannot be seen with total abundance or biomass.

Item 9. Elaboration of results. Differences between treatments and control were expressed with absolute and relative data at each sampling period. The relative difference allows the comparison between treatments and control over time.

Recommendation 1: These graphs should be added in each evaluation report as they show the effect (or the absence of effect) of substances compared with the control.

Recommendation 2: The principal response curve (PRC) of substance effects on earthworm communities would be useful, as mentioned in the ISO 11268-3 guideline and as recommended by Kula (Kula et al., 2006). It would allow the analysis of earthworm community changes and the determination of which earthworm species (i.e., the most affected species by pesticides) are involved in these modifications over time (Amossé et al., 2020).

Item 10. Classification of effects. The small percentage (below 50% of the nominal) of active substances determined from soil sampling compared with the expected ones should be regarded as a factor adding uncertainty to the results that lower the reliability of this field study. Recommendations made above would have brought relevant information to reduce this uncertainty. Therefore, based on the evaluation of all other items in the description and results sections, our study can be classified as "less reliable"

(Reliability index [RI] = 2), although the substance used as a toxic reference was not carbendazim.

CONCLUSIONS AND PERSPECTIVES

The regulatory risk assessment of pesticide use is performed at the first tier under laboratory conditions and not *in natura*. Thus, earthworm field studies may be needed to refine the risk assessment. However, because of high biological variability, such higher tier studies are complex to carry out and assess. Clear guidance allowing for a harmonized regulatory assessment at the European level is needed. Indeed, based on the observations made during the past decade, several aspects of the current ISO 11268-3 guideline (2014) need amendments, especially regarding study design and statistical analysis. To our knowledge, there is no other published paper of earthworm field study following the ISO 11263-3 guideline so scrupulously and generating abundance and biomass data that may be compared with the registration reports assessed in the frame of the regulation (Regulation (EC) No. 1107/2009, 2009). In the present field study, we identified key points and shortcomings that should be better presented or considered to ease harmonization of the assessment of side effects of pesticides on earthworms.

For instance, we identified difficulties in retrieving the active substances of fungicide at the desired concentrations. Although not mentioned in the ISO 11268-3 guideline (2014), the correctness of the application could have been confirmed by the use of Petri dishes of known diameter on the soil surface to collect the fungicides during spraying to measure the fluxes and the concentrations applied to the soil per unit area. This would also further confirm the exposure of earthworm populations during field studies. The use of an interception factor to account for foliar interception could have also been calculated as recommended in the ISO 11268-3 guideline (2014). In addition, the heterogeneity in soil physicochemical conditions in the experimental trial should have been better scrutinized, but no information is provided in the ISO guideline about full characterization and assessment of the heterogeneity of the trial site before application of the products. Although this heterogeneity does not necessarily influence the results of the study due to the use of a random design, it may explain the high variability observed in field studies and thus lead to incorrect conclusions, as this might decrease the robustness of the statistical analysis.

Another major issue is related to the statistical analyses commonly used to assess field studies. The ISO guideline mainly recommends univariate tests that are known to be not robust enough to properly detect significant differences. Other univariate tests were also recommended by Kula et al. (2006). However, as emphasized by Amossé et al. (2020), no information is provided about the statistical power of the field study test or the size effect that should be detectable. To overcome this issue, we recommend the use of the MDD, an indicator that defines relevant differences between means of a treatment and the control that must exist to detect a statistically significant effect (Brock et al., 2015). An MDD analysis was performed in the Amossé et al. (2020) study. The MDD ranged from 74.3% to 83.9% for Dunnett's tests. This is quite low and further demonstrates the need for amendment of the study design and for other robust statistical analysis. Furthermore, the guideline points out the usefulness of multivariate analyses such as the PRC method, and the same recommendation was also made by Kula et al. (2006). Thus, it would be necessary to systematically provide these statistical analyses to accurately assess the ecotoxicological effects of pesticides in field studies. One way to improve the robustness of the NOEC retrieved from field studies might be to increase the number of replicates at the plot level (Römbke et al., 2020). However, the feasibility of this option may be difficult to undertake in the field for practical reasons and in some cases not sufficient to overcome the natural variation in population size.

The final important issue is the acceptable duration for soil faunal recovery after pesticide application. If the continuous effects of pesticides on soil fauna are observed over one year under field conditions, we propose to study the longer term effects of pesticides until soil fauna recovery occurs (e.g., over two years). To gain knowledge of the factors influencing recolonization in the field, we propose adding special care to the characterization of juveniles, with reproduction being the main part of internal recovery. Additional monitoring related to the behavior of earthworms at the soil surface following the application of products and active substances should also be undertaken. The measurements of several parameters (e.g., pesticide residues) and functional endpoints (e.g., feeding activity, soil structuration, organic matter decomposition) are also needed to better assess impacts on soil fauna and soil functioning. Moreover, from our results, we demonstrated that diversity indices (here, we used the Shannon index, but others can be used, such as evenness) provided relevant information on earthworm community dynamics compared with earthworm species considered alone or at the population level. A posteriori tests carried out under laboratory conditions with species found in the field (e.g., *A. caliginosa*) and tested pesticides could inform on mechanisms (i.e., avoidance, reproduction) that affect soil fauna.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DISCLAIMER

The views expressed in this study are those of the individual authors and do not necessarily reflect the views or policies of any company, organization, or government agency, and no official endorsement should be inferred. Mention of trade names or commercial products does not constitute endorsement or recommendation for use. The authors have no competing financial interests to declare.

DATA AVAILABILITY STATEMENT

Data, associated metadata, and calculation tools are available from the corresponding author Franck Brulle (franck.brulle@anses.fr).

SUPPORTING INFORMATION

The supporting information file consists of additional tables and figures showing more data analyzed through the use of the de Jong et al. (2006) guidance document. It provides a more complete picture of the earthworm field study we performed.

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