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Abstract

Because of the wide use of pesticides in agriculture, there is still a need of higher-tier field studies to assess ecotoxicological effects of commercial formulations on a wider variety of non-target soil organisms such as soil annelids. We here tested the effects of different concentrations of two fungicide formulations, i.e., Cuprafor Micro® (composed of 500 $g.kg^{-1}$ copper oxychloride) and Swing Gold® (composed of 50 $g.l^{-1}$ epoxiconazole and 133 $g.l^{-1}$ dimoxystrobin) on two families of terrestrial oligochaetes (*Lumbricidae* and *Enchytraeidae*) after one month of exposure. We also assessed the feeding activity of soil organisms using the bait lamina method. Along with the feeding activity, the enchytraeid density, diversity and communities were not different in the control and the contaminated plots. By contrast, epigeic earthworms were absent and earthworm diversity and densities of anecic species decreased significantly in the plots contaminated at ten times the recommended dose of the Swing Gold® formulation. The copper fungicide (at 0.75 and 7.5 kg $Cu.ha^{-1}$) and the treatment with the pesticide mixture (Cuprafor Micro® at 0.75 kg $Cu.ha^{-1}$ and Swing Gold® at the recommended dose) did not affect *Oligochaeta* communities compared with the control, except the Shannon index for earthworms in the mixture of both fungicides. Responses of the two annelid families to the tested pesticides were different with higher effects observed on the diversity and the community structure of earthworms compared with enchytraeids. This study allowed detecting early changes on oligochaete populations after pesticide application.

Key words: Soil *Oligochaeta*, Pesticides, Ecotoxicology, Agroecosystems, Feeding activity

Introduction

The intensification of pesticide use in agriculture is a threat for the environment (Stoate *et al.*, 2001; DeLonge *et al.*, 2016). The resulting loss of biodiversity may impair soil functioning (e.g., nutrient cycling, soil structure) and the related ecosystem services (Altieri, 1999; Tilman *et al.*, 2002). Earthworms and enchytraeids (*Annelida: Oligochaeta*) are commonly found in a wide range of soils across the world. They respectively belong to the soil macrofauna (body diameter > 2 mm) and mesofauna (body diameter between 0.1 and 2mm) (Gobat *et al.*, 2004). Regarding their size, they play key ecological roles in soils at different scales (i.e., mesoscale for enchytraeids and macroscale for earthworms). Earthworms and enchytraeids contribute to the litter fragmentation and organic matter decomposition (Marinissen and Didden, 1997; Edwards, 2004). Moreover, they are involved in soil formation processes by creating soil porosity and enhancing soil aggregation by mixing mineral and organic particles (Topoliantz *et al.*, 2000; Lavelle and Spain, 2001). Both earthworms and enchytraeids have been recognized as indicators of soil quality and soil management (Paoletti, 1999; Pelosi and Römbke, 2016).

While the effects of pesticides on earthworms and enchytraeids are quite well documented under laboratory conditions (short term effects such as earthworm acute toxicity tests OECD 207, 1984; longer term effects such as reproduction tests on earthworms OECD 222, 2004 and enchytraeids OECD 220, 2016; Didden and Römbke, 2001; Amorim et al., 2005; Pelosi *et al.*, 2014), much less is known about these ecotoxicological impacts on annelid diversity and community structure under field conditions. Some authors reported negative effects of pesticides on earthworms in agricultural fields (Eijsackers *et al.*, 2005; Prado *et al.*, 2016; Datta *et al.*, 2016). However, other studies found opposite results mainly due to confounding factors, i.e., soil type, sampling season, type of alternative agricultural practices and crops (Lavelle and Spain, 2001; Marwitz *et al.*, 2014). Indeed, several agricultural practices might vary at the same time, making the identification of a causal relationship between exposure to contaminants and ecological damage very challenging (Pelosi *et al.*, 2014). Moreover, very few studies have been carried out with pesticides and enchytraeid populations under natural or semi-field conditions, i.e., Terrestrial Model Ecosystem (TME) (Maraldo *et al.*, 2006; Römbke *et al.*, 2009; Scholz-Starke *et al.*, 2013; Bandow *et al.*, 2016). Bandow *et al.* (2016) found negative effects of the pyrimethanil fungicide on several enchytraeid species, especially *Fridericia connata,* using TME. Thus, the enchytraeid response to pesticides under natural conditions has not been well established yet (Didden and Römbke, 2001; Scholz-Starke *et al.*, 2013) and needs to be more investigated. Finally, to our knowledge, only one study investigated the effects of pesticides on both earthworm and enchytraeid densities simultaneously under natural conditions (McColl, 1984).

While laboratory ecotoxicological tests of chemical substances on single species are routinely used, much less is known about the effects of commercial formulations of pesticides on oligochaete (enchytraeid and earthworms) diversity, community structure and the soil functioning under field conditions. Our aim was thus to study the patterns of diversity and community structure of earthworms and enchytraeids in response to pesticide exposure (i.e., two commercial formulations) under field conditions. We made the assumptions that the effects of pesticides on short-term (i.e., one month) on enchytraeid and earthworm communities would be negative and of the same magnitude for both organisms. We also hypothetized that lower density and diversity of oligochaetes would lead to lower soil functioning (i.e., feeding activity).

We investigated the density, diversity indices and some ecological and functional traits (i.e., ecological categories for earthworms, proportion of *r*-strategists for enchytraeids) of each family. We also explored the relationships between enchytraeid and earthworm populations. We finally assessed the feeding activity of soil organisms using the bait lamina method, making the assumption that lower–bait consumption rates would be observed in plots treated with pesticides. The two commercial formulations of fungicides were Cuprafor Micro® (copper oxychloride, used in organic agriculture) and Swing Gold® (composed of epoxiconazole and dimoxystrobin, used in conventional agriculture).

Material and methods

Study site and experimental design

The experiment was conducted and adapted from the ISO standard method for earthworm field studies (ISO 11268-3, 2014) in a meadow located in Versailles, France (48°48’31’’N, 2°05’26’’E) which had not received pesticides for more than 20 years. The climate was oceanic temperate, with a mean annual temperature of 11°C and a mean annual rainfall of 660 mm (weather station at 500 m from the study site, La lanterne, Versailles). The mean temperature between pesticide application and the sampling period (i.e. one month) was 11.1°C. The cumulated rainfall during this period was 54 mm. The soil was a deep Luvisol (FAO soil classification) developed on loess. The homogeneity of the physicochemical characteristics in the experimental site was verified by randomly sampling a soil core at seven different locations in the site. The main characteristics of the soil are loam texture (USDA), OM content 11 %, pH_{H2O} 7.5 and Cu_{tot} 25.2 $mg.kg^{-1}$. Physicochemical properties of the soil are more described in Bart et al. (2017).

Two commercial formulations of fungicides used in Europe (e-phy 2017 a,b) were tested: Cuprafor Micro® (Industrias Quimicas del Valles, composed of 500 $g.kg^{-1}$ copper oxychloride, $Cu_2Cl(OH)_3$) and Swing Gold® (BASF Agro SAS, dimoxystrobin 133 $g.l^{-1}$, epoxiconazole 50 $g.l^{-1}$).

Cuprafor Micro® is used to prevent spore germination by disrupting the enzyme systems of pathogens. It is an inorganic fungicide used as a foliar spray for pest control such as leaf spot and downy mildew (PPDB, 2017). Swing Gold® is a synthetic substance which is mainly used to control late season diseases (e.g. septoria, rusts) of cereals in conventional agriculture. Epoxiconazole (EPX) is a triazole fungicide and a sterol biosynthesis inhibitor with preventative and curative action (PPDB, 2017). Dimoxystrobin (DMX) is a stobilurin fungicide and a respiration inhibitor with protectant, curative and translaminar action.

The experimental trial consisted of four replicates of six treatments randomly located (24 plots, 100 m^2, Fig. 1): the control (T), Cuprafor Micro® at 0.75 kg $Cu.ha^{-1}$ (called C1) - equal to one of the three to four copper applications per year in an agronomical context (e-phy 2017a) - and ten times this concentration i.e., 7.5 kg $Cu.ha^{-1}$ (called C10), Swing Gold® at one (1.5 $l.ha^{-1}$, called D1) and ten (15 $l.ha^{-1}$, D10) times the recommended dose (e-phy 2017b), and a mixture of Cuprafor Micro® at 0.75 kg $Cu.ha^{-1}$ and Swing Gold® at the recommended dose (called M). The plots were treated in April 2016 using a manual sprayer (capacity of twenty liters). Before pesticide application, the vegetation was cut as short as possible and the residues were removed with a lawn mower. The pesticides were diluted within eight liters of water and applied homogeneously on each plot. A volume of eight liters of water was also spiked in the control plots.

Soil annelids

One month after pesticide application (i.e., in May 2016), earthworms were extracted by using an expellant solution of allyl isothiocyanate diluted with isopropanol (propan-2-ol) and water to obtain a 0.1 g L^{-1} solution. In each of the 24 plots, four sampling points were done. For each sampling point, twice 3.2 L of the expellant solution were poured in a metal frame of 0.16 m^2 surface (0.4 x 0.4 m) (Pelosi *et al.*, 2009). After twenty minutes during which emerging earthworms were retrieved, a block of soil (i.e., 40 x 40 x 20 cm) was excavated in the same squares and the last earthworms were extracted manually. Earthworms were stored in a 4 % formaldehyde solution. Adult and sub-adult individuals were identified at the species level (Sims and Gerard, 1999). Juveniles were also identified at the species level according to morphological characters of the adults and to the specific form they take in formalin in comparison with that of identified adults. In cases where species-level identification was impossible (e.g., no discrimination characters between juveniles of *Aporrectodea longa* and *Aporrectodea giardi*), juvenile individuals were allocated to species proportionally to the number of adults and sub-adults. All individuals were counted, weighted, and classified according to three ecological categories defined by Bouché (1977), i.e., epigeic, endogeic and anecic.

For enchytraeids, four soil cores were sampled in each plot using a split soil corer (diameter of 5 cm) at 10 cm depth. Each sample was transferred separately into a plastic bag and stored at 4°C. Enchytraeids were extracted using wet funnel extractors under a light from incandescent light bulbs (40 watts). Soil samples were heated up from 17°C to 43°C on their upper surface for 3 hours (O'Connor, 1955; Kobetičová and Schlaghamerský, 2003). All individuals were kept in Petri dishes with tap water and counted. Adult and sub-adult individuals were identified at the species level under a light microscope (up to 400x magnification) according to the key of Schmelz and Collado (2010). Not Identified (NI) enchytraeids (e.g., dead specimens) were also counted. The total enchytraeid density, the density of each species and the proportion of *r*-strategist species (Graefe and Schmelz, 1999) were determined.

Finally, soil temperature and moisture were checked at the experimental site to ensure earthworm sampling conditions. Soil temperature (i.e., 15.9 °C in average of all plots) was measured in the field with an electronic digital thermometer at 10 cm of soil depth. For soil moisture, soil cores were sampled with a metal cylinder (5 cm internal diameter) at two soil depths i.e., 10 cm for enchytraeids (i.e., 25.7 % in average of all plots) and 20 cm (i.e., 22.6 %) for earthworms. Soil moisture was then measured in the laboratory after drying soil samples for 72 hours at 105°C.

Feeding activity

The feeding activity of soil organisms was measured using the bait lamina method (ISO 18311, 2014). Briefly, PVC sticks (Terra Protecta GmbH, Berlin, Germany) containing 16 apertures, which are 5 mm apart from each other, were filled with cellulose powder (70 %), finely ground wheat bran (25 %) and activated carbon powder (5 %). In May 2016, in each plot, 16 sticks were inserted in a 4 × 4 grid within an area of about 30 cm × 30 cm. We determine the exposure time by regularly screening bait lamina strips inserted close to the study site in order to identify the duration in which at least 10 % of all baits were fed. After 10 days, the sticks were retrieved from the soil and fed (i.e., disappearance of at least half of the organic bait material) or not fed apertures were directly counted. The global rate and the vertical distribution of the feeding activity were calculated.

Statistical analyses

For each plot, measurement endpoints for the group of annelids (i.e., total density, species density, epigeic, anecic and endogeic density, proportion of *r*-strategist enchytraeids) 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 in diversity indices, i.e., species richness, Shannon and Pielou's evenness, and feeding activity between all treatments were assessed on log transformed data (log(x+1)) using parametric tests (one way ANOVA followed by a multiple comparison Dunnett test, Hothorn et al., 2017 (multcomp.glht)) if the homogeneity of variance (Bartlett-test, Snedecor and Cochran, 1989) and the normality of residuals (Shapiro test) were respected. Non-parametric tests (Kruskal-Wallis test followed by a multiple comparison kruskalmc test, Giraudoux, 2017 (pgirmess.kruskalmc)) were used if these conditions were not respected. At each multiple post-hoc test, adjusted p-values based on Bonferroni's corrections were applied (Bland and Altman, 1995). All statistical analyses were done with n = 4. The level of significance was fixed at $p < 0.05$. Minimum Detectable Differences (MDDs) were calculated for key species and ecological groups of earthworms according to Brock et al. (2015). They were expressed as percentage (% MDD, 4 replicates) of the control after back-transformation of the data.

The correlations between enchytraeid and earthworm variables, and between annelid variables and feeding activity were tested using Pearson or Kendall coefficient of correlation (for normal and non-normal distribution of the data, respectively). Given the high number of tests, Bonferroni's corrections to p-values were also applied. Relationships between earthworm and enchytraeid communities were assessed in the different treatments using Mantel tests (Mantel, 1967) using vegan (Oksanen *et al.*, 2017) on Bray-Curtis dissimilarity transformation matrices ($p < 0.05$, 23 permutations). All analyses were carried out with R statistical software (R Development Core Team, 2016).

Results

Enchytraeids

A total of 5 637 enchytraeids were collected from all plots. The mean density of total enchytraeids varied from 24 574 (in C10) to 36 733 ind.m^{-2} (in M) without any significant difference between treatments (Fig. 2 and Table 1). Similarly, no difference was observed for the diversity metrics (i.e., species richness, Shannon index, proportion of *r*-strategists, and evenness) between plots treated with or without pesticides. Species richness was positively correlated ($r = 0.348$, p-value = 0.025) with the enchytraeid density (Supplementary table 1). A total of 21 enchytraeid species were identified in the six treatments (Table 2). The most abundant species was the *r*-strategist *Enchytraeus buchholzi*, followed by *Fridericia galba* and then *Fridericia isseli* (Table 2). The density of each species was not significantly different between treatments (Table 2).

Earthworms

A total of 3 274 earthworms were collected from all plots. The mean density of total earthworms ranged from 127 (in D10) to 264 ind.m^{-2} (in C10) (Fig. 2) and the mean biomass ranged from 48.3 (in D10) to 93.6 g.m^{-2} (in C10) (Table 1). Density and biomass of earthworms were highly correlated (r = 0.941, p < 0.001). No significant difference was observed between treatments (Table 1). Contrarily, diversity metrics (i.e., species richness, Shannon index and Pielou's evenness) were significantly lower in the D10 than in the control (T) treatments. The same was found for the Shannon index in the M treatment compared with the control plots. The density and species richness of earthworms were positively correlated (r = 0.340, p-value = 0.027) (Supplementary table 1). A total of 12 earthworm species were identified in the six treatments (Table 3). The highest species richness was recorded in the T, C10, and D1 treatments with respectively 10, 11, and 10 species. The lowest species richness (i.e., 5 species) was recorded in the D10 treatment. The most abundant species was the endogeic *Aporrectodea icterica* followed by *Lumbricus terrestris* and then *Aporrectodea caliginosa* (Table 3).

The occurrence of the different ecological groups differed among treatments (Tables 1 and 3). The density of endogeic earthworms was not significantly different between treatments (Table 1) although *A. caliginosa* species was lower (- 81.4 % MDD) in D10 treatment compared with the control (Table 4). Epigeic earthworms were found in all treatments, except in D10. The anecic density was significantly lower in the D10 treatment compared with the control (Table 1). This can be mainly explained by lower *L. terrestris* (- 71.1 % MDD) and *Aporrectodea longa* (- 80.1 % MDD) densities in the D10 treatment than in the control plots (Table 4). The *A. longa* density was also lower (- 80.2 % MDD) in the mixture treatment compared with the control (Table 4).

Annelid community patterns

No significant correlation was observed between earthworms and enchytraeid species richness, density and functional groups (ecological categories for earthworms and percentage of *r*-strategists for enchytraeids) (Supplementary table 1). Moreover, mantel tests did not reveal any significant relationship between enchytraeid and earthworm communities in treated and non-treated soils, except a positive relationship between enchytraeid and earthworm communities in C10 (r = 0.743, p-value = 0.042) (Supplementary table 2). Earthworm and enchytraeid communities were not different in the control (T) and the other treatments (Supplementary table 3 and 4, respectively).

Feeding activity

The feeding rate varied from 16.7 % (in C10) to 24.1 % (in C1), but no significant difference was observed between treatments. In the first three centimeters of soil, the feeding rate was higher in C1 compared with the other treatments (Fig. 3). No relationship was found between the density of each annelid families and the feeding activity (r = 0.088, p-value = 0.551 for enchytraeids; r = -0.227 p-value = 0.123 for earthworms).

Discussion

Enchytraeid sensitivity to the tested fungicides

Soil annelid sensitivity to chemicals is generally considered as useful information to assess the effect of pesticides on the living compartment of the soil (Didden and Römbke, 2001). We here found that enchytraeids were not affected by Cuprafor Micro® and Swing Gold® whatever the fungicide concentrations (one and ten times the doses). For the commercial formulation of Swing Gold®, Bart *et al.* (2017) reported a LC50 (Lethal Concentration causing 50% mortality) at 11 times the recommended dose (RD) for *Enchytraeus albidus* (Henle, 1837) in the same soil (from the same experimental site), which is higher than the highest dose applied in our field study (10 times the recommended dose). The field conditions (i.e. vegetation cover playing a barrier role for pesticide migration into the soil) could explain why no differences were observed between the control and the plots treated with the Swing Gold®. Similarly, McColl (1984) found that enchytraeids were not affected by the nematicide oxamyl (12.7 l a.i ha^{-1}, equal to 85 times the recommended dose on maize, e-phy 2017c) after sampling every month for one year in a mixed ryegrass-clover. In our study, enchytraeid species densities were not significantly different in plots treated with pesticides compared to the control. This was not always the case as reported in previous studies (Moser *et al.*, 2007; Römbke *et al.*, 2009). Under field conditions (i.e., in a grassland), Römbke *et al.* (2009) found a change in community pattern and lower enchytraeid density (11 213 *vs.* 8 690 ind.m^{-2}) after two applications (interval of fourtheen days) of a mixture of the fungicide benomyl (8 kg.ha^{-1}, equal to 40 times the recommended dose on wheat, e-phy 2017d) and the insecticide chlorpyrifos (900 g.ha^{-1}). They found that densities of the genera *Buchholzia* (1 902 *vs.* 1 501 ind.m^{-2}), *Henlea* (301 *vs.* 198 ind.m^{-2}), *Fridericia* (3 274 *vs.* 2 452 ind.m^{-2}), and *Marionina* (2 435 *vs.* 1 860 ind.m^{-2}) were significantly reduced one month after pesticide application. Similarly, Moser *et al.* (2007) found that the fungicide carbendazim at 13 kg.ha^{-1} (equal to 65 times the recommended dose on wheat, e-phy 2017e) significantly affected enchytraeids four weeks after pesticide application, especially the genera *Fridericia* and *Henlea*.

Concerning the copper fungicide, our results are in accordance with Bart *et al.* (2017) who showed that Cuprafor Micro® did not induce lethal effect of enchytraeids (*E. albidus*) even at high concentration (5 000 mg Cu.kg^{-1} dry soil) after 14 days of exposure in laboratory. Maraldo *et al.* (2006) found a LC50 at 775 and over than 1 601 mg Cu.kg^{-1} dry soil in spiked and field contaminated soils under laboratory conditions, respectively. These differences with Bart *et al.* (2017)'s results could be explained by the soil type (loamy in Bart *et al.*, 2017 *vs.* sandy loam in Maraldo *et al.*, 2006), the species tested in laboratory (*E. albidus* in Bart *et al.,* 2017 *vs. E. crypticus* (Westheide & Graefe, 1992) in Maraldo *et al.*, 2006), and the tested copper formulations ($Cu_2Cl(OH)_3$ in Bart *et al.*, 2017 *vs.* $CuSO_4$ in Maraldo *et al.*, 2006). Under field conditions, some studies reported lower enchytraeid densities around 400 mg Cu.kg^{-1} dry soil (Yeates *et al.*, 1994; Maraldo *et al.*, 2006). These latter results could explain why we did not observe any difference in plots treated with copper, with a maximum dose applied at 7.5 kg.ha^{-1} (equal to 10 mg Cu.kg^{-1} dry soil if we consider the ten first soil centimeters). Moreover, Maraldo *et al.* (2006) observed that the diversity (2 to 9 species), the total number (3 to 67 individuals) and the species composition of enchytraeids (especially *Fridericia connata*, Bretsher, 1902, *Achaeta bohemica*, *Enchytronia parva*, *Marionina communis*, and *F. galba*) were highly affected by copper at concentrations ranging from 277 to 501 mg Cu.kg^{-1} dry soil, while other species such as *E. buchholzi* (*r*-strategist) and *Fredericia ratzeli* (Eisen, 1872) were found to high copper concentrations (over than 655 mg Cu.kg^{-1} dry soil). Five of the species recorded by Maraldo et al. (2016) were identified in our study (i.e., *A. bohemica*, *En. parva*, *M. communis*, *F. galba* and *E. buchholzi*) and all of them were not significantly affected by copper application.

Based on EFSA's opinion (EFSA PPR Panel, 2017), we found that effects of tested pesticides on enchytraeids are negligible (i.e., reduction up to 10 %) to small (i.e., reduction above 10 % and below 35 % four weeks after pesticide application) compared with the control. The magnitude of effects is considered to allow for internal recovery of enchytraeids populations and would have no consequences on the provision of ecosystem services (EFSA PPR Panel, 2017).

Earthworm sensitivity to the tested fungicides

The study of short term effects (i.e., one month) of pesticides on annelids allows detecting early changes on oligochaete populations and to compare results obtained under field and laboratory conditions. For earthworms, no significant effects were observed in the plots treated with the pesticides except with the Swing Gold® formulation at ten times the recommended dose. Less anecics – especially *L. terrestris* - were found in this treatment than in the control plots. It is worthwhile to note that numerous dead anecic and endogeic earthworms were observed in the D10 treatment few days after pesticide application (pers. obs., Fig. 4), showing the rapid lethal effect of the Swing Gold® on earthworms. Moreover, although the differences were not statistically different but partly shown with % MDDs, epigeic and some endogeic earthworms species (i.e., *L. castaneus* and *A. caliginosa*, Savigny, 1826) were negatively affected by the Swing Gold® formulation at ten times the recommended dose. In the literature, earthworms, and especially epigeics and anecics, are already shown to be directly affected by pesticides (Pelosi *et al.*, 2014) because they are directly exposed to them through their feeding activity at the soil surface (Edwards and Bohlen, 1996). 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. For the fungicide benomyl (1.0 kg a.i ha^{-1}, equal to 5 times the recommended dose on wheat, e-phy 2017c) studied under field conditions (i.e., in a grassland), Christensen and Mather (2004) found a significant increase in earthworm migration two days after the application, especially *L. terrestris*, *A. longa*, and *A. caliginosa* species. These results match well with our observations because the three species listed above were among the most affected by the Swing Gold® formulation at ten times the recommended dose. Similarly, Schnug *et al.* (2015) showed that the total density of earthworms and in particular the density of *A. caliginosa* significantly decreased one month after the application of the fungicide picoxystrobin at concentrations known to inhibit from 10 to 90 % the cocoon production of *Eisenia fetida* (Savigny, 1926) (i.e. from 250.9 to 592.9 $\mu mol.m^{-2}$). They also reported that the mixture of three biocides (the insecticide esfenvalerate, the fungicide picoxystrobin, and the bactericide triclosan) affected earthworm density, especially *A. caliginosa* and *Aporrectodea rosea* (Savigny, 1826) species densities. This trend was not observed in our study with the mixture of both pesticides, except the lower Shannon diversity index compared with the control plots. This can be mainly explained by lower densities of *L. castaneus* and *A. longa* species in this treatment compared to the control plots. In our case, binary (D treatment with DMX and EPX) and tertiary (M treatment with DMX, EPX and copper) mixtures of pesticides could be considered. The tested mixtures could have additive or synergistic effects on oligochaetes. For example, LC50 of *Eisenia foetida* with the single active substance of dimoxystrobin was 23.65 mg kg^{-1} (moderate effects) (PPDB, 2017). While, the LC50 of *Eisenia foetida* with the dimoxystrobin in the Swing Gold® formulation was 3.35 mg SA/kg (higher effect; Agritox, 2017).

Concerning the copper fungicide, no effect was observed on earthworm populations. Studies dealing with the effects of copper on earthworms showed contrasted results (Van Zwieten *et al.*, 2004; Owojori and Reinecke, 2010). This could be mainly explained by the mode of contamination (i.e., mine soils, spiked soils) and the number of tested contaminants (i.e., one versus several heavy metals) (Spurgeon and Hopkin, 1999; Eijsackers *et al.*, 2005; Tisher, 2008). These discrepancies may hinder the risk assessment linked to the use of copper on soil annelids. For example, Van Zwieten *et al.* (2004) investigated earthworm communities in two avocado orchards (New South Wales, Australia) and found a negative relationship between earthworm density and the long-term contamination of soils by Cu and Zn. They found from 155 to less than 1 earthworm per square meter in soil with 29 to 269 mg $Cu.kg^{-1}$ dry soil, respectively. Conversely, also under field conditions, Owojori and Reinecke (2010) did not found any significant effect on earthworms twenty days after an application of 25 kg $Cu.ha^{-1}$ (copper oxychloride, Effekto Virikop), equal to more than three times the maximum dose applied in our study site. These studies highlight that the effects of copper on soil biota are sometimes not immediate but can appear after several years of applications and accumulation in the soil. However, under controlled conditions Eijsackers *et al.* (2005) and Bart *et al.* (2017) showed, a significant decrease in biomass for the species *A. caliginosa* at 50 and 60 mg $Cu.kg^{-1}$ dry soil after fourteen and twenty days of exposure, respectively. According to the literature (Owojori and Reinecke, 2010) and our results, we assume that copper at the tested concentrations had no short-term impact on oligochaeta populations.

Based on EFSA's opinion (EFSA PPR Panel, 2017) and the percentage of MDD (Brock et al., 2015), we found medium to large effects (between 50 and 70 % for the anecic density, and between 70 and 90 % for the earthworm species *L. terrestris*, *A. longa and A. caliginosa*) of the Swing Gold® at ten times the recommended dose on earthworms. For magnitude of effects higher than 65%, this would probably not result in internal recovery in relevant time frame (i.e., six months) and could have consequences on the provision of ecosystem services (EFSA PPR Panel, 2017).

Relationships between Oligochaeta and feeding activity

In our study, the soil temperature and the soil moisture were close to optimal conditions (Puurtinen and Martikainen, 1997; Booth et al., 2000) for the development of earthworms (i.e., 15,9 °C and soil water content of 22,6 %) and enchytraeids (i.e., 15,9 °C and soil water content of 25,7 %). Moreover, earthworm (i.e., mean of 213 ind.m^{-2}) and enchytraeid (i.e., mean of 29 905 ind.m^{-2}) densities were in accordance with previous findings in grasslands (Beylich and Graefe, 2012; Domínguez and Bedano, 2016) and with ISO recommendations for earthworm field studies (i.e., > 100 earthworms.m^{-2}, ISO 11268-3, 2014). For example, Beylich and Graefe (2012) found between 91 to 886 earthworms.m^{-2} and between 9 218 and 75 274 enchytraeids.m^{-2} from twelve German grassland sites. They also studied earthworms and enchytraeids densities from seventeen cropland sites and found densities varying between 0 to 783 earthworms.m^{-2} and between 2 037 and 100 607 enchytraeids.m^{-2}. Because enchytraeids and earthworms have close habitats, interactions may occur, especially competition for food resources (Karaban and Uvarov, 2014; Domínguez and Bedano, 2016). However, no significant relationships for density, community structure and diversity were found between earthworms and enchytraeids in treated and non-treated soils. Similarly, Beylich and Graefe (2012) highlighted that the density of both families was not significantly correlated in various land uses: forest, wet grassland, grassland, and cropland. Under controlled conditions, Karaban and Uvarov (2014) reported that the inoculation of two individuals of the epigeic species *Lumbricus rubellus* negatively affected enchytraeid number (from 255 to 204 individuals per experimental unit) and especially litter enchytraeids (species not specified), which nearly disappeared. By contrast, positive effects of two *A. caliginosa* - an endogeic earthworm species – were observed on enchytraeid number (from 255 to 477 individuals per experimental unit) mainly explained by non-trophic relationships (i.e. burrowing activity, cast and mucus production). In our study, enchytraeid density, diversity and community structure did not change after pesticide application, even in the Swing Gold treatment (in D10) where lower density of anecics and the absence of epigeics were recorded. This suggested that no habitat competition occurred between earthworms and enchytraeids.

Concerning the feeding rate, few studies reported that pesticides such as glyphosate (an herbicide), chlorpyrifos or dimethoate (an insecticide) at recommended doses reduced the feeding activity under field conditions (Casabé *et al.*, 2007; Santos *et al.*, 2011). By contrast, other studies concluded that glyphosate or thiram (a fungicide) did not reduce the feeding activity and was even higher compared to the control (Larink and Sommer, 2002; Santos *et al.*, 2011). Although the feeding rate was not different between treatments in our study, this trend was also observed in C1 where a higher feeding activity in the three first centimeters of soil was recorded, compared to the other treatments. Numerous enchytraeids were observed in the bait lamina apertures (pers. obs.) in treated and non-treated soils after retrieving them from the soil. In accordance with Helling *et al.* (1998), enchytraeids could be therefore considered as among the main consumers of the baits in the topsoil. Topoliantz *et al.* (2000) also explained that enchytraeids may increase their activities when earthworm density is low. These small but numerous soil organisms could therefore maintain a biological activity and perform soil functions when earthworms are absent.

Our results showed that enchytraeid species were less affected than some earthworm species by the Swing Gold® formulation. This finding is in accordance with the review made by Jarratt and Thompson (2009) who reported in some cases similar but generally a lower sensitivity of enchytraeids than earthworms to pesticides. For example, Leitão *et al.* (2014), working under laboratory conditions with a natural soil treated with the fungicide azoxystrobin, found a EC50 (Effective Concentration reducing 50% of the reproduction) of 42.0 mg a.i. kg^{-1} dw soil for the enchytraeid species *E. crypticus* and 31.1 mg a.i. kg^{-1} dw soil for the earthworm species *Eisenia andrei* (Bouché, 1972). However, Jarratt and Thompson (2009) pinpointed the difficulty to accurately compare the sensitivity of enchytraeids and earthworms to chemicals using the existing literature due to confounding factors (e.g. exposure duration, doses, soil type, soil tillage, fertilization). Moreover, the sensitivity of enchytraeids and earthworms to pesticides is species-specific and it is thus difficult to conclude if one soil annelid family is more sensitive than the other. For example, Frampton *et al.* (2006) studied the soil fauna (e.g., earthworms, collembolan, enchytraeids, nematodes) species sensitivity to pesticides from a large number of publications including several ecotoxicological endpoints (e.g., LC50, No Observed Effect Concentration). They found that the enchytraeid species *F. ratzeli* and *E. albidus* were less sensitive than the earthworm species *E. fetida, E. andrei* and *L. rubellus* to the fungicide carbendazim. Conversely, the enchytraeid *Enchytraeus coronatus* (Nielsen and Christensen, 1959) was more sensitive to this pesticide than the earthworm species listed above.

Conclusion

The risk assessment of pesticide use is generally performed under laboratory conditions and not *in natura.* The strength of our study is to use various pesticide exposure conditions (i.e., one and ten times the recommended dose, mixture) and similar environmental field conditions for treated and non-treated plots, thus reducing confounding factors. Our study revealed contrasting patterns among annelid groups (i.e., earthworms and enchytraeids) in response to pesticide exposure and supports the idea that pesticides used in Europe and in the world can affect soil fauna communities on the short-term (i.e., one month). Moreover, we showed that the mixture of pesticides could have synergistic effects on soil organisms (i.e., lower Shannon index for earthworms) compared to the single use of the tested fungicides under field conditions. We also found that some oligochaete species were more affected than others by pesticides, especially the anecic earthworm *Lumbricus terrestris*. Longer-term sampling (e.g. after six and twelve months) would be needed to study annelid community dynamics (e.g. earthworm recovery) as suggested in the ISO norm for earthworm field studies. It would also be useful to study other non-target animals groups (e.g. microarthropods) and to measure other functional endpoints (e.g. organic matter decomposition, soil structuration) for a better assessment of pesticide effects on the biodiversity and the soil functioning. This study conducted under field conditions is a first step towards the environmental risk assessment of commercial formulations of pesticides.

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Compliance with Ethical Standards

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Conflict of interest: The authors (Joël Amossé, Sylvain Bart, Alexandre R.R. Péry, and Céline Pelosi) declare that they have no conflict of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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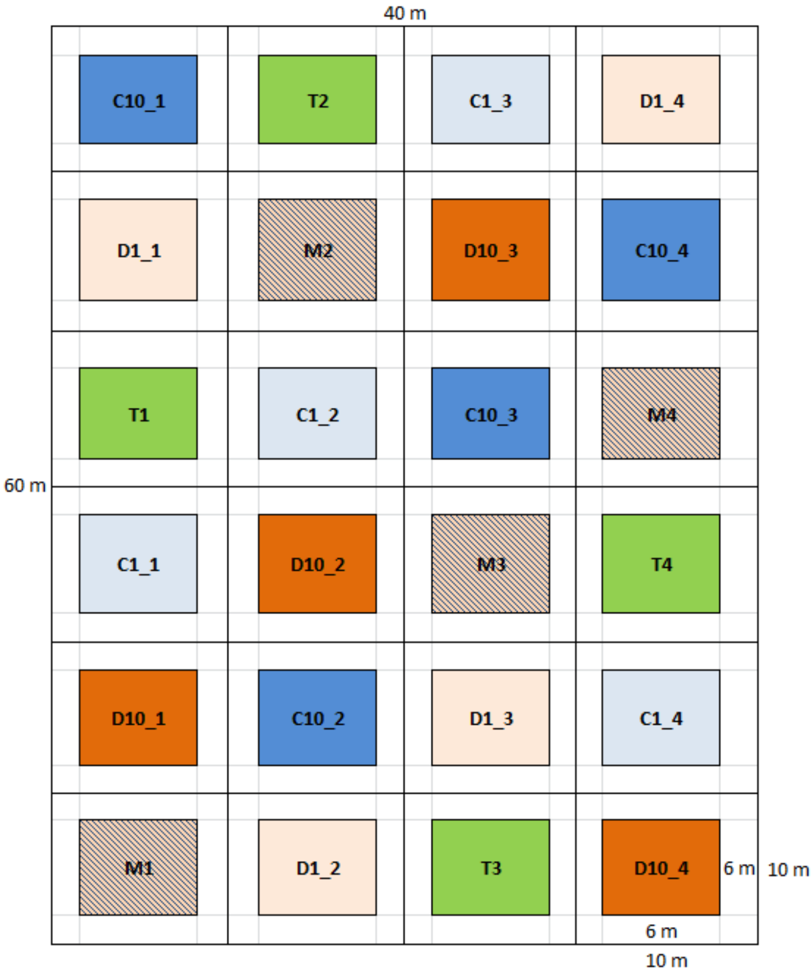

Fig. 1 Field experimental design with the six modalities (four replicates). Treatments are: control (T, green), Cuprafor Micro® at 0.75 kg $Cu.ha^{-1}$ (C1, light blue) and 7.5 kg $Cu.ha^{-1}$ (C10, dark blue), Swing Gold® at one (D1, light orange) and at ten (D10, dark orange) times the recommended dose, and a mixture of Cuprafor Micro® at 0.75 kg $Cu.ha^{-1}$ and Swing Gold® at the recommended dose (M, striped).

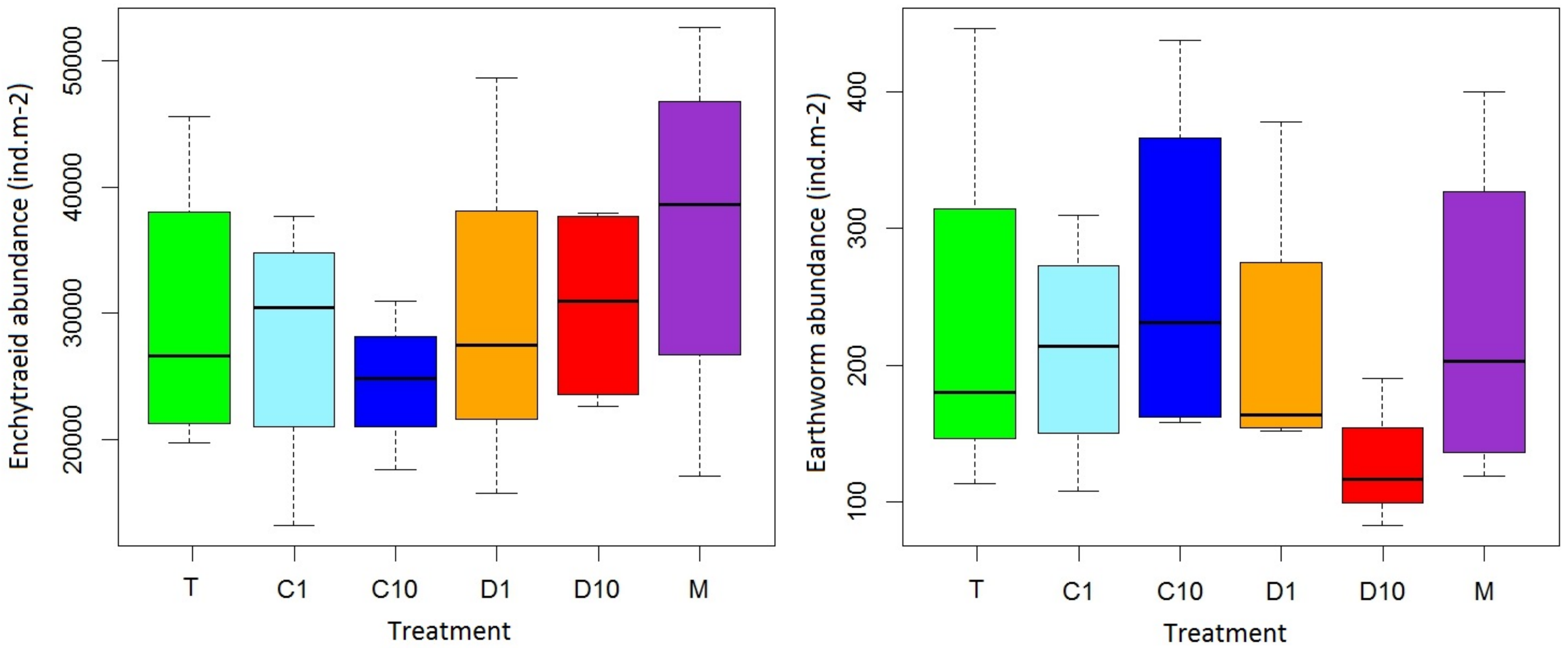

Fig. 2 Total densities of enchytraeids (on the left) and earthworms (on the right) per treatment. Treatments are: control (T), Cuprafor Micro® at 0.75 kg Cu.ha^{-1} (C1) and 7.5 kg Cu.ha^{-1} (C10), Swing Gold® at one (D1) and at ten (D10) times the recommended dose, and a mixture of Cuprafor Micro® 0.75 kg Cu.ha^{-1} and Swing Gold® at the recommended dose (M).

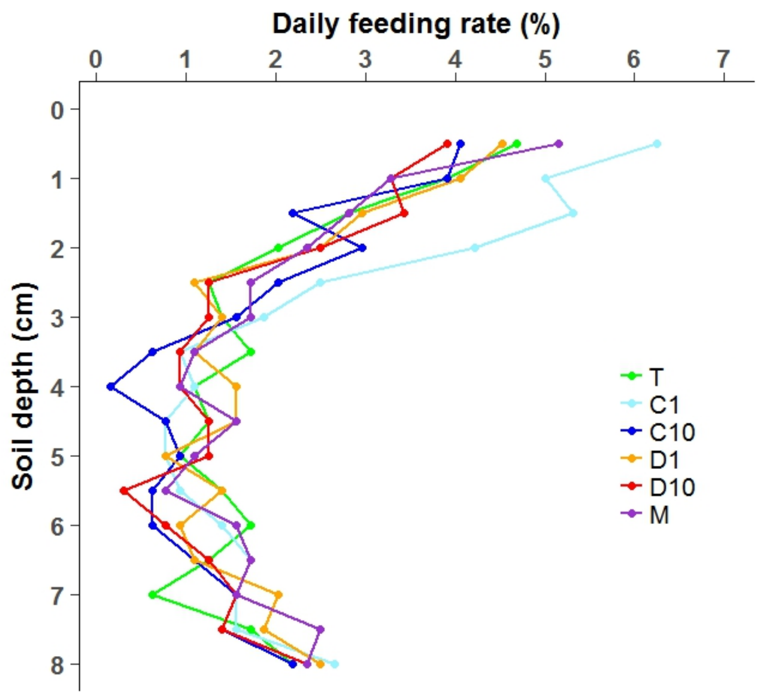

Fig. 3 Daily feeding activity (%) with soil depth (0.5 to 8 cm) from the four replicates of 16 sticks per treatment. Treatments are: control (T), Cuprafor Micro® at 0.75 kg Cu.ha^{-1} (C1) and 7.5 kg Cu.ha^{-1} (C10), Swing Gold® at one (D1) and at ten (D10) times the recommended dose, and a mixture of Cuprafor Micro® 0.75 kg Cu.ha^{-1} and Swing Gold® at the recommended dose (M).

Fig. 4 Dead endogeic earthworm at the soil surface after the application of the Swing Gold® at ten times the recommended dose

Table 1. Enchytraeid and earthworm densities, diversity metrics and community composition (n = 4, ± Standard Deviation) in the six treatments. For each row, asterisks indicate significant differences ('*' $p < 0.001$, '**' $p < 0.01$, '*' $p < 0.05$) between treatments with pesticides and the control. Treatments are: control (T), Cuprafor Micro® at 0.75 kg Cu.ha^{-1} (C1) and 7.5 kg Cu.ha^{-1} (C10), Swing Gold® at one (D1) and at ten (D10) times the recommended dose, and a mixture of Cuprafor Micro® 0.75 kg Cu.ha^{-1} and Swing Gold® at the recommended dose (M).**

Soil faunal group	Variable	T	C1	C10	D1	D10	M
Enchytraeids	Density (ind.m^{-2})	29667 ± 11519	27948 ± 10458	24574 ± 5430	29857 ± 13684	30653 ± 8163	36733 ± 14726
	Species richness	9.8 ± 1.0	10 ± 2.5	9.8 ± 1.7	10.5 ± 1	9.8 ± 1.5	10.0 ± 2.9
	Shannon index	6.61 ± 0.75	6.92 ± 1.38	6.32 ± 1.07	5.61 ± 1.21	6.06 ± 1.26	6.12 ± 1.91
	Evenness	0.83 ± 0.06	0.84 ± 0.03	0.81 ± 0.01	0.73 ± 0.07	0.79 ± 0.09	0.61 ± 0.11
	r-strategists (%)	25.8 ± 9.5	26.1 ± 5.3	33.5 ± 14.8	33.7 ± 22.1	32.0 ± 8.5	33.7 ± 20.1
Earthworms	Density (ind.m^{-2})	231 ± 147	211 ± 84	264 ± 131	214 ± 109	127 ± 46	231 ± 126
	Biomass (ind.m^{-2})	86.8 ± 32.4	79.5 ± 28.4	93.6 ± 34.7	78.9 ± 27.1	48.3 ± 14.0	83.1 ± 33.9
	Species richness	**7.0 ± 1.4**	5.8 ± 1.0	6.3 ± 1.7	7.8 ± 1.0	**2.8 ± 0.5 *****	5.5 ± 1.0
	Shannon index	**3.07 ± 0.74**	2.45 ± 0.28	2.48 ± 0.51	3.11 ± 0.61	**1.17 ± 0.08 *****	**2.13 ± 0.28 ***
	Evenness	**0.52 ± 0.09**	0.51 ± 0.14	0.55 ± 0.09	0.57 ± 0.09	**0.16 ± 0.05 *****	0.44 ± 0.04
	Epigeic (ind.m^{-2})	12.9 ± 13.0	10.2 ± 4.5	7.4 ± 3.9	8.2 ± 4.1	0 ± 0	3.1 ± 2.9
	Endogeic (ind.m^{-2})	184 ± 124	168 ± 70	211 ± 121	178 ± 94	124 ± 45	198 ± 107
	Anecic (ind.m^{-2})	**34 ± 13**	34 ± 18	47 ± 18	28 ± 17	**3 ± 1 *****	31 ± 17

Table 2. Enchytraeid density (ind.m^{-2}) per species (n = 4, ± Standard Deviation) in the different treatments. Nomenclature follows Schmelz and Collado (2010). Categorization as *r*-strategists follows Graefe and Schmelz (1999). For each species, asterisks indicate significant differences ('*' p < 0.001, '**' p < 0.01, '*' p < 0.05) between treatments with pesticides and the control. Treatments are: control (T), Cuprafor Micro® at 0.75 kg Cu.ha^{-1} (C1) and 7.5 kg Cu.ha^{-1} (C10), Swing Gold® at one (D1) and at ten (D10) times the recommended dose, and a mixture of Cuprafor Micro® 0.75 kg Cu.ha^{-1} and Swing Gold® at the recommended dose (M).**

Genus	Species	Authority	T	C1	C10	D1	D10	M	Mean density	*r*-strategists
Fridericia	*galba*	(Hoffmeister, 1843)	6462 ± 3233	3979 ± 2390	3470 ± 2033	4138 ± 2627	3470 ± 4398	4361 ± 3099	4313	
Fridericia	*ulrikae*	Rota & Healy, 1999	1592 ± 2221	0 ± 0	1528 ± 3056	446 ± 891	0 ± 0	1273 ± 1928	807	
Fridericia	*christeri*	Rota & Healy, 1999	1496 ± 1508	1655 ± 2166	2578 ± 2349	2515 ± 3248	2737 ± 3549	2578 ± 2358	2260	
Fridericia	*paroniana*	Issel, 1904	1623 ± 1881	1878 ± 2387	446 ± 555	1560 ± 1808	1082 ± 1254	3565 ± 5165	1692	
Fridericia	*isseli*	Rota, 1994	4488 ± 3971	4838 ± 3567	4234 ± 4918	1942 ± 1719	3342 ± 1214	4870 ± 3523	3952	
Fridericia	*nix*	Rota, 1995	0 ± 0	191 ± 127	64 ± 127	127 ± 147	95 ± 191	64 ± 74	90	
Fridericia	*maculata*	Issel, 1905	0 ± 0	0 ± 0	223 ± 446	95 ± 191	0 ± 0	0 ± 0	53	
Fridericia	*tuberosa*	Rota, 1995	700 ± 1088	1783 ± 2069	1114 ± 1262	1432 ± 1522	3024 ± 3912	32 ± 64	1348	
Fridericia	*viridula*	Issel, 1904	127 ± 255	1846 ± 3051	0 ± 0	0 ± 0	0 ± 0	0 ± 0	329	
Enchytraeus	*buchholzi*	(Vejdovský, 1879)	7417 ± 5976	6112 ± 2735	6303 ± 1027	4966 ± 1422	8149 ± 4028	9517 ± 2307	7056	X
Enchytraeus	*lacteus*	Nielsen & Christensen, 1961	0 ± 0	0 ± 0	0 ± 0	0 ± 0	64 ± 127	0 ± 0	11	X
Enchytraeus	*bulbosus*	Nielsen & Christensen, 1963	668 ± 774	382 ± 764	127 ± 255	159 ± 191	955 ± 1178	796 ± 872	515	X

Enchytraeus	*christenseni*	Dózsa-Farkas, 1992	0 ± 0	0 ± 0	923 ± 1846	0 ± 0	95 ± 191	0 ± 0	170	X
Buchholzia	*fallax*	Michaelsen, 1887	159 ± 241	382 ± 329	64 ± 74	64 ± 74	159 ± 122	32 ± 64	143	
Buchholzia	*appendiculata*	(Buchholz, 1962)	127 ± 147	796 ± 1592	223 ± 301	6080 ± 11906	255 ± 509	95 ± 122	1263	X
Enchytronia	*parva*	Nielsen & Christensen, 1959	2355 ± 873	1655 ± 1003	1337 ± 767	1082 ± 535	5029 ± 3726	2578 ± 1464	2339	
Marionina	*Argentea*	(Michaelsen, 1889)	255 ± 509	191 ± 382	414 ± 351	3279 ± 6473	0 ± 0	3629 ± 6677	1295	
Marionina	*communis*	Nielsen & Christensen, 1959	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	95 ± 191	16	
Achaeta	*unibulba*	Graefe, Dozsa-Farkas & Christensen, 2005	191 ± 382	32 ± 64	0 ± 0	95 ± 191	0 ± 0	95 ± 191	69	
Achaeta	*iberica*	Graefe, 1989	1592 ± 788	1814 ± 1227	1114 ± 1533	1464 ± 1896	1369 ± 1493	2546 ± 2473	1650	
Achaeta	*pannonica*	Graefe, 1989	0 ± 0	0 ± 0	0 ± 0	0 ± 0	382 ± 764	0 ± 0	64	
	NI		411 ± 282	340 ± 265	414 ± 217	414 ± 122	446 ± 221	605 ± 282	438	

Table 3. Earthworm density (ind.m^{-2}) per species (n = 4, ± Standard Deviation) in the different treatments. Nomenclature follows Bouché (1972) and Sims and Gerard (1999). Categorization with ecological categories follows Bouché (1977). For each species, asterisks indicate significant differences ('*' p < 0.001, '**' p < 0.01, '*' p < 0.05) between treatments with pesticides and the control. Treatments are: control (T), Cuprafor Micro® at 0.75 kg Cu.ha^{-1} (C1) and 7.5 kg Cu.ha^{-1} (C10), Swing Gold® at one (D1) and at ten (D10) times the recommended dose, and a mixture of Cuprafor Micro® 0.75 kg Cu.ha^{-1} and Swing Gold® at the recommended dose (M).**

Genus	Species	Authority	T	C1	C10	D1	D10	M	Mean density	Ecological category
Lumbricus	*castaneus*	Savigny, 1826	11.7 ± 10.9	10.2 ± 4.5	7.4 ± 3.9	7.0 ± 4.7	0.0 ± 0.0	1.6 ± 2.2	6.3	epigeic
Lumbricus	*rubellus*	Hoffmeister, 1843	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.8	0.0 ± 0.0	0.0 ± 0.0	0.1	epigeic
Dendrobaena	*mammalis*	Savigny, 1826	1.2 ± 2.3	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.9	0.0 ± 0.0	1.3 ± 3.1	0.6	endogeic
Allolobophora	*chlorotica*	Savigny, 1826	4.7 ± 4.0	1.6 ± 2.0	3.9 ± 4.7	5.5 ± 5.3	0.4 ± 0.8	5.5 ± 7.0	3.6	endogeic
Allolobophora	*icterica*	Savigny, 1826	158.6 ± 111.1	157.8 ± 59.7	202.0 ± 121.7	141.8 ± 63.0	123.4 ± 45.6	183.6 ± 96.8	161.2	endogeic
Allolobophora	*muldali*	Omodeo 1956	7.8 ± 15.6	0.0 ± 0.0	1.2 ± 2.3	4.7 ± 9.4	0.0 ± 0.0	0.0 ± 0.0	2.3	endogeic
Octolasion	*cyaneum*	Savigny, 1826	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1	endogeic
Aporrectodea	*caliginosa*	Savigny, 1826	12.5 ± 15.0	8.2 ± 9.5	2.3 ± 2.0	25.4 ± 25.9	0.0 ± 0.0	8.6 ± 5.8	9.5	endogeic
Aporrectodea	*rosea*	Savigny, 1826	0.4 ± 0.8	0.0 ± 0.0	0.8 ± 1.6	0.8 ± 1.6	0.0 ± 0.0	0.0 ± 0.0	0.3	endogeic
Arporrectodea	*longa*	Ude, 1885	8.2 ± 5.9	9.0 ± 6.3	10.9 ± 6.4	4.3 ± 3.2	0.8 ± 0.9	1.6 ± 3.1	5.8	anecic
Aporrectodea	*giardi*	Ribaucourt, 1901	3.5 ± 2.7	1.2 ± 1.5	0.8 ± 1.6	7.0 ± 5.3	0.8 ± 1.6	5.9 ± 5.6	3.2	anecic
Lumbricus	*terrestris*	Linnaeus, 1758	**22.3 ± 13.2**	23.4 ± 13.8	34.8 ± 13.8	16.8 ± 12.7	**1.2 ± 0.8 *** **	23.0 ± 14.7	20.2	anecic

Table 4. Percentage of minimum detectable difference (% MDD) relative to the control for key earthworm variables. Treatments with pesticides are: Cuprafor Micro® at 0.75 kg $Cu.ha^{-1}$ (C1) and 7.5 kg $Cu.ha^{-1}$ (C10), Swing Gold® at one (D1) and at ten (D10) times the recommended dose, and a mixture of Cuprafor Micro® 0.75 kg $Cu.ha^{-1}$ and Swing Gold® at the recommended dose (M).

	% MDD				
Variable	**C1**	**C10**	**D1**	**D10**	**M**
Epigeic density (ind.m^{-2})	- 33.4	- 65.9	- 56.7	- 148.7	- 115.3
***L. castaneus* density (ind.m^{-2})**	- 20.2	- 54.6	- 61.0	- 140.1	- 123.9
Endogeic density (ind.m^{-2})	- 9.2	+ 18.1	- 3.6	- 31.3	+ 8.8
***A. caliginosa* density (ind.m^{-2})**	- 33.1	- 66.8	+ 167.1	**- 81.4**	- 27.3
Anecic density (ind.m^{-2})	- 1.2	+ 37.5	- 16.9	- 55.1	- 10.3
***A. longa* density (ind.m^{-2})**	+ 12.2	+ 43.0	- 47.5	**- 80.1**	**- 80.2**
***L. terrestris* density (ind.m^{-2})**	+ 5.7	+ 60.9	- 25.5	**- 71.1**	+ 3.9