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Highlights

- Effects on earthworms of two currently used fungicides were tested in the field.
- Ecotoxicological effects and recovery were assessed in a two-year experiment.
- The mixture of fungicides at field application rate negatively affected earthworms.
- *A. caliginosa*, *A. chlorotica* and anecic species were the most sensitive taxa.
- Earthworm communities recovered at best one year after the fungicide application.

Abstract

Recent EFSA (European Food Safety Authority) reports highlighted that the ecological risk assessment of pesticides needed to go further by taking more into account the impacts of chemicals on biodiversity under field conditions. We assessed the effects of two commercial formulations of fungicides separately and in mixture, i.e., Cuprafor Micro® (containing 500 g kg^{-1} copper oxychloride) at 4 (C1, corresponding to 3.1 mg kg^{-1} dry soil of copper) and 40 kg ha^{-1} (C10), and Swing® Gold (50 g L^{-1} epoxiconazole EPX and 133 g L^{-1} dimoxystrobin DMX) at one (D1, 5.81 10^{-2} and 1.55 10^{-1} mg kg^{-1} dry soil of EPX and DMX, respectively) and ten times (D10) the recommended field rate, on earthworms at 1, 6, 12, 18 and 24 months after the application following the international ISO standard no. 11268-3 to determine the effects on earthworms in field situations. The D10 treatment significantly reduced the species diversity (Shannon diversity index, 54% of the control), anecic abundance (29% of the control), and total biomass (49% of the control) over the first 18 months of experiment. The Shannon diversity index also decreased in the mixture treatment (both fungicides at the recommended dose) at 1 and 6 months after the first application (68% of the control at both sampling dates), and in C10 (78% of the control) at 18 months compared with the control. *Lumbricus terrestris*, *Aporrectodea caliginosa*, *Aporrectodea giardi*, *Aporrectodea longa*, and *Allolobophora chlorotica* were (in decreasing order) the most sensitive species to the tested fungicides. This study not only addressed field ecotoxicological effects of fungicides at the community level and ecological recovery, but it also pinpointed some methodological weaknesses (e.g., regarding fungicide concentrations in soil and statistics) of the guideline to determine the effects on earthworms in field situations.

Keywords: Soil annelids, Ecotoxicology, Agroecosystems, Risk assessment procedures, Guidelines

1. Introduction

Since the 1960ies (Carson, 1962), the awareness that pesticides might harm human health and the environment has been growing. Before the placing of pesticides on the market, risk assessment procedures according to standard test guidelines are used to assess the potential of pesticides to impact non-target organisms. The results of these tests are used for registration nationally and internationally (Werner and Hitzfeld, 2012). Pesticide risk assessment uses a tiered approach, with a number of steps involving laboratory and field tests of increasing complexity and realism (Maund and Mackey, 1998). Ecotoxicology, described as a multidisciplinary science that integrates toxicology and ecology (Walker et al., 2012), benefited from the ecotoxicological tests required for risk assessment as new concepts and tools were developed.

However, despite these advances and although ecotoxicology theoretically addresses the impact of chemicals on organisms from molecules to ecosystems (Walker et al., 2012), ecotoxicological studies for regulatory purposes or from scientific literature mainly use standardized laboratory conditions that are recommended in international standard test guidelines (e.g. ISO 11268-2, 2012) and used in environmental risk assessment, aiming at a worst-case scenario: single model species, direct acute and chronic effects of one chemical, adult individuals (except for the collembola reproduction test, where juveniles are also used, OECD, 2009) and artificial soils for terrestrial organisms. Moreover, in laboratory assays from scientific literature, the tested concentrations are often not realistic (very high), and the substances and products are not always applied as in the field (i.e., incorporation in soil versus spraying). The duration of laboratory tests is often short, ranging from some days to weeks. Finally, very few studies have assessed the impact of pesticides after their market authorization under natural conditions on non-target communities, be it terrestrial or aquatic. For instance, less than 1% of the studies on pesticides and freshwater invertebrates were performed in the field at the community level (Beketov and Liess, 2012). As a consequence, after 60 years of ecotoxicology, and despite the development of useful tools such as biomarkers (Forbes et al., 2006), it is still difficult to assess the effects of pesticides on communities and ecosystems under “real” field conditions considering species sensitivities, interactions with the environment, toxic effects of chemical mixtures, and recovery (EFSA PPR Panel, 2017; EFSA Scientific Committee, 2016a, b; Werner and Hitzfeld, 2012).

When working under natural field conditions, we face increasing complexity due to spatiotemporal variability of environmental conditions, and a high number of biotic and abiotic factors operating at the same time (Werner and Hitzfeld, 2012). This complexity may hinder the interpretation of the results when assessing the impact of pesticides under natural conditions (Winqvist et al., 2011). This is one of the main reasons why some studies reported contrasting results regarding the effects of one or several pesticide products on non-target organisms in this context (Bengtsson et al., 2005; Hole et al., 2005). To limit confounding factors and properly address the consequences of pesticide use on soil communities under natural conditions, a guideline exists for earthworms to assess potential effects in field situations (ISO 11268-3, 2014). It is commonly used as a higher-tier tests in environmental risk assessments for registration of plant protection products when unacceptable risk is identified with lower-tier tests performed according to OECD guideline no. 222 under laboratory conditions on *Eisenia fetida* Savigny, 1826 or *E. andrei* Bouché, 1972. However, field data on the effects of chemicals on earthworms generated according to standard guidelines are scarce because most registered active substances did not need to be tested in the field (i.e. higher-tier tests) during the registration process at the European level and data used for plant protection product registration are not easily accessible due to data protection. Moreover, data on post-registration effects monitoring are very scarce in the scientific literature, and mostly performed with semi-field studies and terrestrial model ecosystems (TME) (e.g. Förster et al., 2011; Moser et al., 2007; Santos et al., 2011; Scholz-Starke et al., 2013). These useful approaches address an intermediary level between laboratory and field studies and help to assess potential effects of pesticides on the soil community structure (i.e. species composition and abundance) that could compromise the soil functioning. They enable a standardization of the exposure and habitat conditions and they are more ecologically realistic than laboratory testing such as OECD guideline no. 222. However, they do not capture all ecological interactions that may occur and they cannot assess multi-season effects of chemicals. Moreover, these confined systems do not allow the assessment of chemical effects on external recovery (e.g. dispersal, recolonization) (EFSA Scientific Committee, 2016a). Compared with TME studies, field studies are generally carried out for a longer period (i.e. one year and longer). They represent a higher level of complexity because they are performed under realistic conditions (e.g. pedoclimatic context). Field

studies allow a relevant assessment of community dynamics (i.e., species sensitivities and recovery) under realistic exposure conditions.

With their soft bodies and their soil ingestion mode, earthworms are exposed to pesticides. Depending on their functional traits (e.g., habitat preferences, behavior, metabolism efficiency) (Pelosi et al., 2013a; Pey et al., 2014; Velki and Hackenberger, 2012), the different species of earthworms are differently exposed and sensitive to pesticides (Pelosi et al., 2013b). Earthworms are used for several decades as model organisms in soil ecotoxicology tests both in the scientific literature (Pelosi et al., 2014) and in the reports for pesticide registration (e.g. OECD 207, 1984; OECD 222, 2016). They are reliable indicators of land use and management (Paoletti, 1999) and they create habitats for themselves and for other organisms, influencing and even controlling their activities through physical and biochemical processes (Jones et al., 1994, 1997). They are the main contributors of organic matter and soil structure transformation by soil ingestion, transport and excretion (Liu et al., 2019). After a disturbance, and because they are animals of relatively low mobility (Eijsackers, 2011), the recovery of earthworm communities can be slow which may impair the soil functioning (Potter et al., 1990).

The present study aimed to assess the effects of two commercial products of fungicides currently used in different farming systems (i.e. Cuprafor Micro® made of copper oxychloride and used in organic farming; Swing® Gold made of dimoxystrobin (DMX) and epoxiconazole (EPX), and used in conventional farming) on earthworm communities under field conditions over two years. This study is the continuation of a previous study conducted by Amossé et al. (2018). They showed that Swing® Gold at 10 × the recommended field rate (RR) drastically reduced anecic and epigeic earthworm diversity and abundances one month after application. In contrast, the copper fungicide (at 0.75 and 7.5 kg Cu ha^{-1}) and the treatment with the mixture of both fungicides (Cuprafor Micro® at 0.75 kg Cu ha^{-1} and Swing® Gold at the RR) did not affect earthworm communities compared with the control (no fungicides), except for the Shannon index that decreased in the mixture treatment. Considering these results and the lack of knowledge regarding the pesticide effects over a long period in field trials, we assessed the effects of these fungicides on the different earthworm species present in this field and the recovery of the earthworm community. The experimental design presented by Amossé et al. (2018) was thus followed with the investigation of the earthworm abundance, biomass, and diversity at 1, 6,

12, 18 and 24 months after the first fungicide applications (MAFA). We tested the following hypotheses: 1) the two fungicides have different effects (amplitude and duration) on the different earthworm ecological groups and species, 2) the mixture of the two fungicides has stronger effects than those observed for each fungicide applied separately. We also measured fungicide concentrations (i.e. total contents of Cu, DMX and EPX) in the soil, and we found some shortcomings of using the international guideline to determine the effects of chemicals on earthworms in field situations.

2. Materials and methods

2.1. Study site and experimental design

The experiment was conducted for a period of two years - between April 2016 and April 2018 – and followed the ISO standard method for the study of the effects of pollutants on earthworms under field conditions (ISO 11268-3, 2014). The study site was a meadow located in Versailles, France (48°48'31''N, 2°05'26''E) that had not received pesticides for more than 20 years. Detailed information about the experimental site is described in Amossé et al. (2018).

Two commercial formulations of fungicides 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}). They were chosen for their potential negative effects on earthworm populations (Bart et al., 2017; Pelosi et al., 2016) and because they were used in different farming systems, either organic or conventional farming, respectively.

The experimental trial consisted of four replicates of six treatments randomly located (24 plots, see Amossé et al., 2018). In accordance with the guideline (ISO 11268-3, 2014), the area of each plot was 100 m² (10 m x 10 m), and the whole 100m² area was treated with fungicides. Samples were taken exclusively in the central zone of 36 m² (6 x 6m). No fungicide was applied in the control (T) treatment. Jones and Belling (1967) and Sun et al. (2019) found that Cu remained in the upper 5 cm of the soil, so the RR for Cuprafor Micro® was calculated by considering a penetration depth of 5 cm. Two concentrations of copper were tested (RR and 10 RR, Table 1). The application pattern of Cuprafor Micro® was chosen to be realistic so we used multiple applications as is most often done in field conditions (see Table 1). For the Swing® Gold fungicide, to calculate the RR, we considered that

the was mainly found in the top 10 cm of soil (Chabauty et al., 2016; McDonald et al., 2013). We tested two concentrations of Swing® Gold (D1 and D10, Table 1) in one application in April 2016. Finally, a mixture of both fungicides at the RR (abbreviated M) was also tested: Cuprafor Micro® at 4 kg Cu ha^{-1} (with four applications as mentioned in Table 1) and Swing® Gold at 1.5 l ha^{-1} applied once in April 2016. Although it is not really common to use these two fungicides simultaneously in the field, the M treatment was used for scientific interest. At the same time, this situation is relevant in case of a change in land use on a copper-polluted soil, although the speciation of copper is different between recent and old pollution. Finally, copper is mostly used in organic farming but it can also be used in conventional farming. Then, in some perennial crops such as orchards or vineyards, copper and organic fungicides can be used the same year or in alternance every other year to prevent from the different fungal diseases and avoid resistance to a single chemical.

The plots were treated with the fungicides by using a manual sprayer (capacity of twenty liters). Before each fungicide application, the vegetation was cut as short as possible and the grass residues were removed with a lawn mower. The fungicides were diluted within eight liters of water and applied homogeneously on each plot. A volume of eight liters of water was also sprayed on the control plots. As recommended in the guideline (ISO 11268-3, 2014), the soil surface was examined two days after each application to assess dead or alive earthworms.

The D10 treatment was chosen in the present study as a toxic reference instead of carbendazim which is recommended in the guideline ISO 11268-3 (2014) because this latter pesticide is forbidden in Europe since 2014 and is thus hardly commercially available. Moreover, according to EFSA reports, carbendazim is slightly less toxic to earthworms (no observed effect concentration (NOEC) reproduction of 1 mg as/kg soil dry weight, EFSA Scientific Report, 2010) than EPX (NOEC reproduction of 0.167 mg as/kg soil dry weight, EFSA Scientific Report, 2008) and DMX (NOEC reproduction < 0.0887 mg as/kg soil dry weight, EFSA Scientific Report, 2005) under laboratory conditions. Finally, although no field data was available on Swing® Gold at this concentration as a comparison, the expected effect was a decrease in population by 40 to 80% (ISO 11268-3, 2014). As Bart et al. (2017) found an estimated LC50 of 7.0 10^{-3} mL kg^{-1} dry soil of Swing® Gold (6.3 times the

RR) for *Aporrectodea caliginosa*, the tested D10 concentration was assumed to reach the expected effect on earthworm populations, or at least on this species.

2.2. Analyses of fungicide residues in soil

Soil samples for fungicide analyses were collected just before annelid sampling (i.e., in May 2016, November 2016, April 2017, October 2017 and April 2018). Three soil cores (5 cm in diameter, 10 cm depth) were collected at days 5, 26, 209, 363, 544, and 727 after the first application of fungicides in each of the 24 plots, pooled and homogenized to have one soil sample per plot. DMX and EPX were also measured 9 days after application between 10 and 30 cm deep to assess the penetration of these compounds in the soil. Although copper is known to be mainly retained in the first 5 cm of soil (Jones and Belling, 1967; Sun et al., 2019), the depth of 10 cm was chosen for soil analyses because it is recommended in the guideline (ISO 11268-3, 2014). Moreover, we wanted to ensure homogeneity between treatments (Swing® Gold and Cuprafor micro®). Finally, as the field trial was a meadow, the root system was very dense in the first 5 cm, making it difficult to sample the soil to a depth of only 5 cm.

For total copper analyses, soils were air-dried and sieved to < 2 mm. An aliquot was then ground in order to pass through a 200 µm mesh. For that, 0.5 g of soil were weighed in Teflon containers, and digested by HF/HNO_3 (1:3, v:v) and microwave heating (CEM MarsXpress, Matthews, NC, USA). After complete digestion, the excess of 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 (water resistivity = 18 MΩ cm) was prepared by Milli-Q water system (Millipore). Total Cu content in 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 certified reference materials (TMDA-70.2, Environment Canada).

For Swing® Gold, total contents of the two main active substances, i.e., DMX and EPX, were analyzed in the soil samples collected in treated and non-treated plots. After homogenization, fresh soil was sieved at 5 mm and stored at -40°C. Before analysis, freeze-dried soil was manually ground

with a mortar. Triplicate soil subsamples of 5 g were placed in 50 mL polypropylene tube (Falcon BD) and 10 mL of methanol was added to each tube. The tubes were shaken on an orbital shaker (10 min, 300 rpm) and sonicated for 20 min, before being centrifuged for 10 min at 1300 *g* and 20 °C. After collecting 7 mL of supernatant, the soil was again extracted by 10 mL of methanol and shaken, sonicated and centrifuged as described previously. Then, 10 mL of supernatant was collected and mixed with the first extract. The D1 samples were analysed without further preparation. For D10, an aliquot was diluted by a factor of 10 using a water/acetonitrile mixture (80:20, v/v) prior to analysis, to allow these samples to be within the calibration curve range. The samples containing low fungicide concentrations (less than 0.002 mg kg^{-1}, i.e. controls) were further diluted by 200 mL of water and submitted to purification-concentration, by solid phase extraction on HR-XA cartridges (500 mg, Macherey-Nagel) preconditioned successively with 5 mL of methanol, acetonitrile and water. After percolation of the diluted extracts, cartridges were rinsed with 5 mL of water, dried under vacuum and eluted by 6 mL of a 95:5 (v/v) acetonitrile/formic acid mixture. Samples were then evaporated under a N_2 stream, dissolved in 3 mL of 8:2 (v/v) water/acetonitrile and analysed. Analyses were performed on an ultra-high-performance liquid chromatograph (Acquity UPLC, Waters) coupled through an electrospray interface to a triple quadrupole mass spectrometer (TQD, Waters). The analytical conditions were as described by Nélieu et al. (2016), except the UPLC gradient (95/5 to 45/55 of water/acetonitrile, each containing 0.1% acetic acid) and the introduction of conditions to detect DMX (cone voltage 23 V, MRM transitions 327>205 at 10 eV for quantitation and 327>116 at 17 eV for confirmation). In control soils spiked with DMX and EPX, the extraction yields were estimated as 93-110% with a low matrix effect due to electrospray ionization. The limit of detection (according to a signal-to-noise ratio of 3) was 0.03 µg kg^{-1} and 0.05 µg kg^{-1} for DMX and EPX, respectively, and the limit of quantification (validated by accuracy profile methodology) was 0.28 µg kg^{-1} and 0.22 µg kg^{-1} for DMX and EPX, respectively. None of those pesticides was detected in analytical blanks. Considering all sampling dates of D1, D10 and M plots, the relative standard deviation of triplicate analysis presented a mean of 4.2% and 6.0% for DMX and EPX, respectively.

2.3. Earthworms

Earthworms were sampled before setting up the trial by randomly choosing 10 sample locations in the study site. Earthworms were then sampled at 1, 6, 12, 18, and 24 months after the first application, called 1, 6, 12, 18 and 24 MAFA (i.e., in May 2016, November 2016, April 2017, October 2017, and April 2018, respectively). Four samples were taken per sampling date on each of the 24 plots of the trial. The sampling method combined an expellant solution of allyl isothiocyanate (AITC) on a 40x40 cm square, followed by hand sorting (40x40x20 cm-depth block of soil) (Pelosi et al., 2009). For the expellant solution, AITC was first diluted with isopropanol (propan-2-ol) to obtain a 5 g L^{-1} solution. This solution was then diluted with water to give a concentration of 0.1 g L^{-1} (Pelosi et al., 2009). Emerging 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, individuals were allocated to species level using a pro rata distribution corresponding to adult and sub-adult proportions. All individuals were counted, weighed wet after preservation, and classified according to three ecological groups defined by Bouché (1977), i.e., epigeic, endogeic, and anecic.

2.4. Soil parameters

The main soil physical and chemical properties were as follows (n=7, ± SE): pH 7.5 ± 0.2, organic matter content 32.6 ± 1.7 g kg^{-1}, C/N ratio 12.7 ± 0.3, sand 29% ± 1.3, silt 48% ± 1.5, and clay 23% ± 0.6. The soil bulk density was measured using the volumetric cylinder method (Al-Shammary et al., 2018). It was 1.29 ± 0.03 g cm^{-3} (n=6, ± SE). The soil temperature was measured twice in each plot at all sampling periods with an electronic digital thermometer at 10 cm depth. The mean temperature of all plots combined were 15.9°C in May 2016, 7.9°C in November 2016, 13.1°C in April 2017, 14.4°C in October 2017, and 12.4°C in April 2018. The soil moisture was assessed by sampling two soil cores (5 cm internal diameter, 0-20 cm depth) on each plot at all sampling periods. The two samples were pooled, homogenized, and dried for 72 hours at 105°C in a stove. The mean soil water content (0-20

cm soil depth) was of 22.6% (±1.6), 19.9% (±2.1), 13.7% (±0.5), 21.7% (±0.6), and 29.1% (±0.4) at 1, 6, 12, 18, and 24 MAFA respectively.

2.5. Statistical analyses

For each plot at each sampling period, earthworm variables (i.e., total abundance and biomass, abundance of epigeic, anecic and endogeic earthworms) were calculated from the sum of the four samples and expressed as individuals m^{-2}. Statistical differences between treatments were assessed on log transformed data (log(x+1)) using parametric tests (one-way ANOVA and then two-sided Dunnett's t-tests) if the conditions of homogeneity of variance (Bartlett test) and normality (Shapiro test) of residuals were met. Non-parametric tests (Kruskal-Wallis tests) were used if these conditions were not met. The level of significance was fixed at $p < 0.05$.

The Shannon diversity index (H) (Hill, 1973) was calculated in each sample to represent the diversity of species, based on their proportions, in the various treatments as a function of time:

$$H = \Sigma [(pi) \times \ln(pi)],$$

where pi is the proportion of the species i of the total sample for each plot.

The principal response curve (PRC) method (Van den Brink and Ter Braak, 1999) was used to test the effects of the different treatments of fungicides (i.e. C1, C10, D1, D10, and M) at 1, 6, 12, 18, and 24 MAFA on the abundance of each earthworm species. The PRC method is a constrained ordination method developed for the analysis of community data. One key aspect of the PRC is that it allows interpretation down to the species level (Van den Brink and Ter Braak, 1998). It is designed to study the effects of chemicals (or other stressors) on the community structure over time compared to an untreated control, resulting in an easily interpretable graphical representation. The abundance data (number of individuals per species) were transformed by log(2x + 1) as usually recommended for PRC analyses (Van den Brink et al., 1995, 2000), because the smallest non-null abundance was 1 (Van den Brink et al., 1995). The Hellinger transformation, where abundances are expressed as relative abundances in each sample, was also tested. Total abundance over time and each species' abundance in each treatment were also observed to identify the main features of the data. The significance of the

PRC overall and of each PRC axis was assessed with Monte Carlo permutation tests (9999 permutations, $p < 0.05$) of time series residuals (Freedman and Lane, 1983). Statistical significance of the effects at each date was also tested with 9999 Monte Carlo permutations of residuals. When a significant effect of a treatment was observed at a sampling date, a Principal Component Analysis (PCA) was performed. A 2-sided Dunnett's test was then used to compare the mean PCA scores of each treatment with the control at each sampling date where a significant effect of the treatment was observed. Other environmental factors were not included in the PRC analysis, but care was taken to ensure that replicates of each treatment covered the whole range of environmental conditions in the experiment as well as possible, so that the variability due to slight differences in environmental conditions was included in the residual variability. All calculations were carried out with R 3.2.3 (R Core Team, 2016), vegan package 2.4-1 (Oksanen et al., 2016), FactoMineR package (Husson et al., 2014), multcomp (Hothorn et al., 2008) and reshape (Wickham, 2007).

3. Results

3.1. Fungicide residues in soils

Total contents of DMX and EPX were below the limit of quantification in the control treatment at all sampling dates (i.e., 1, 6, 12, 18, and 24 MAFA) (data not shown). Five days after the application, the soil content in DMX and EPX was observed to be about one fourth of the nominal concentration (Figure 1). A minor part of the pesticides penetrated more deeply into the soil as we found 2.2 to 6.3% and 1.8% to 4.8% for DMX and EPX, respectively, between 10 and 30 cm deep, 9 days after application. One month after the application of the Swing® Gold fungicide, the mean of the total DMX content ranged from 26.4 ± 10.7% SD (in D10) to 35.5 ± 17.6% SD (in M) of the applied initial amount, with high variations between replicates, as shown by the standard errors (Figure 1). A similar trend was observed for the mean of the total EPX content that ranged from 23.1 ± 11.2% SD (in D10) to 29.2 ± 18.9% SD (in M) of the applied concentration. The total DMX concentration was reduced by a factor of 3.2, 4.6, 14.3 and 22.4 (on average between the three treatments with Swing® Gold) between 1 and 6, 6, 12, 18, 24 MAFA respectively (Figure 1). EPX was less degraded than DMX within the two years of experiment as the total EPX concentration was reduced by a factor of 1.9, 2.1,

4.7, and 7.7 (on average between the three treatments with Swing® Gold) between 1 and 6, 12, 18, 24 MAFA respectively (Figure 1).

Total Cu contents in the soil were not significantly different (two-sided Dunnett's t-tests, $p < 0.05$) between the control and treatments C1 or M, whatever the period (i.e. 1, 6, 12, 18, and 24 MAFA) (Figure 2). However, in the C10 treatment, once the whole quantity of Cuprafor Micro® fungicide was applied (i.e., from 6 MAFA), the Cu concentration was significantly 1.8 times higher in C10 (on average over these four periods 6, 12, 18, and 24 MAFA) than in the control treatment (Figure 2).

3.2. Earthworms

Before setting up the trial, the total earthworm abundance was 288 individuals m^{-2} (± 135 ind. m^{-2} SD) with the following 12 species (and % in the community) of epigeic earthworms: *Lumbricus castaneus* (Savigny, 1826) (7%) and *Lumbricus rubellus* (Hoffmeister, 1843) (1%); anecic earthworms: *Lumbricus terrestris* (Linnaeus, 1758) (11%), *Aporrectodea giardi* (Ribaucourt, 1901) (8%), and *Aporrectodea longa* (Ude, 1885) (2%); endogeic earthworms: *Allolobophora icterica* (Savigny, 1826) (63%), *Aporrectodea caliginosa* (Savigny, 1826) (4%), *Allolobophora chlorotica* (Savigny, 1826) (2%), *Allolobophora muldali* (Omodeo 1956) (1%), *Dendrobaena mammalis* (Savigny, 1826) (< 1%), *Octolasion cyaneum* (Savigny, 1826) (< 1%), *Aporrectodea rosea* (Savigny, 1826) (< 1%).

At 1, 6, 12, 18, and 24 MAFA, the total abundance of earthworms ranged from 127 (in D10) to 264 ind. m^{-2} (C10), from 56 (D10) to 157 ind. m^{-2} (C10), from 67 (D10) to 158 ind. m^{-2} (T), from 88 (D10) to 198 ind. m^{-2} (C10) and from 120 (D10) to 208 ind.m^{-2} (M), respectively (Figure 3a). The earthworm abundance per treatment also varied between sampling time points, mainly between the first sampling (1 MAFA) and the four others (Figure 3a). Earthworm abundance was not significantly different (two-sided Dunnett's t-tests, $p < 0.05$) between treatments at each sampling period. However, although no difference was significant, a trend to a decrease in earthworm abundance was observed in the D10 treatment compared with the control (T) at all sampling periods with the abundance in the D10 treatment representing 55%, 44%, 42%, 53%, and 82% of the abundance in T at 1, 6, 12, 18, and 24 MAFA, respectively. Moreover, the total earthworm biomass (Figure 3b) in the D10 treatment was significantly lower than in the control, representing 56% and 44% of the control at 1 and 6 MAFA,

355 respectively. The Shannon diversity index was also significantly lower in D10 compared with the
356 control (the D10 treatment representing only 39%, 49%, and 59% of the control, respectively) at 1, 6
357 and 18 MAFA (Figure 4). A lower Shannon diversity index and significant differences were also
358 found for the mixture treatment (M) at 1 and 6 MAFA, and in the C10 treatment at 18 MAFA
359 compared with the control. The proportion of juveniles (all treatments combined) represented 73% (±
360 4% SD), 75% (± 3% SD), 66% (± 2% SD), 52% (± 3% SD), and 44% (± 6% SD) of the total
361 abundance at 1, 6, 12, 18, and 24 MAFA, respectively. Although variations between sampling time
362 points were found, the proportion of juveniles between treatments at each sampling period was very
363 similar.
364 Regarding ecological groups of earthworms, the abundance of anecics was significantly lower in the
365 D10 treatment compared with the control at 1, 6, and 12 MAFA, with the anecic abundance in the D10
366 treatment representing only 9%, 20%, and 27 the abundance in T, respectively (Table 2a). Anecic
367 abundance also decreased in the C10 treatment compared with the control, representing only 12% of
368 the control at 12 MAFA. No significant difference was found for the endogeic and epigeic
369 earthworms. However, a trend to a decrease in endogeic abundance was systematically observed in the
370 D10 treatment compared to the control at all sampling periods (Table 2b). Similarly, no epigeics were
371 recorded in D10 at 1 MAFA, while a mean abundance of 17 ind. m^{-2} was found in the control
372 treatment at the same sampling period (Table 2c). These results were in line with the field
373 observations in the D10 treatment, where some earthworms (epigeics, anecics and endogeics) were
374 found dead at the soil surface two days after the spraying of the Swing® Gold fungicide.
375 The study of earthworm community patterns with PRC analysis on log-transformed abundance data
376 (Figure 5a) revealed that 21% of the total variance of the earthworm species community data was
377 explained by the time and 25% by treatments with fungicides. Overall, the PRC showed statistically
378 significant effects of the fungicide treatments (9999 Monte Carlo permutations, $p = 0.0085$). The first
379 axis of the PRC analysis represented a large part (51%) of the treatment effects on earthworm
380 abundance and it was statistically significant (9999 Monte Carlo permutations, $p = 0.0161$). No
381 significant effects of the fungicide treatments were observed on subsequent axes. Earthworm
382 community in the Swing® Gold treatment at ten times the RR was significantly different to the control

(two-sided Dunnett's t-test, $p < 0.05$) at 1, 6, and 18 MAFA. All taxa weights were positive (on the x axis, Figure 5a), reflecting a global pattern of decrease in abundance followed by slow recovery, mostly in D10. The highest positive taxa weights - reflecting a decrease in the respective taxon in plots treated with fungicides - was calculated for *L. terrestris* (0.53), *A. caliginosa* (0.47), *A. giardi* (0.39), *A. longa* (0.37), and *A. chlorotica* (0.35). These earthworm species showed the strongest decrease in abundance mainly explained by the Swing® Gold treatment at ten times the RR. The PRC analysis with the Hellinger transformation showed similar results (Figure 5b). However, at 18 MAFA, no significant effect of D10 was noticed. Interestingly, this approach showed that in D10, the relative abundance *A. icterica*, the most abundant taxa by far (64% to 99% of collected individuals in each sample), increased at the expense of all other earthworm taxa, although the previous analysis showed that this species also was negatively affected by the D10 treatment. *A. icterica* was thus obviously less affected than the other species of earthworms in our experiment.

4. Discussion

4.1. Impacts of the tested fungicides on earthworm communities over two years

This study revealed negative effects of two fungicides commonly used in organic or conventional farming on earthworm communities. The slower degradation of EPX than DMX measured in the soil of the field trial was in accordance with the field DT90 of these active substances (2960 and 365 days, respectively; PPDB, 2020). Overall, the strongest effects (e.g., mortality of earthworms observed at the soil surface and a decrease in earthworm abundance and diversity) were measured after the Swing® Gold application at ten times the RR, notably on anecic species (i.e. *Lumbricus terrestris*, *Aporrectodea giardi*, and *Aporrectodea longa*) and two endogeic species (i.e. *Aporrectodea caliginosa* and *Allolobophora chlorotica*). These significant effects found at short term (i.e. one month after application) in Amossé et al (2018) persisted for at least twelve months after the fungicide application. Our observations are in accordance with EFSA scientific report (2005) highlighting an initial adverse effect of the BAS 507 01F commercial formulation (i.e. Swing® Gold) (two applications at the RR) on earthworm abundance and biomass, with partial or full recovery compared with the control by the end of the studies (duration of 12 to 36 months). It is worth specifying that the field studies with

earthworms reported in EFSA scientific report (2005) were conducted because a high long-term risk to earthworms was identified in a first-tier risk assessment under laboratory conditions (NOEC reproduction 56 days <0.0887 mg DMX a.s. kg^{-1} soil). Moreover, under laboratory conditions, Pelosi et al. (2016) and Bart et al (2019, 2020) reported negative impacts of these fungicides (EPX applied alone or EPX+DMX in the Swing® Gold formulation) on the biochemical response to oxidative stress of *A. icterica*, and on the growth and reproduction of *A. caliginosa* at realistic concentrations. The Swing® Gold fungicide at three times the RR (0.465 mg DMX kg^{-1} and 0.174 mg EPX kg^{-1}) decreased the cocoon production of *A. caliginosa* by 63%, and the hatching success significantly decreased by 16% at the RR (0.155 mg DMX kg^{-1} and 0.0581 mg EPX kg^{-1}) (Bart et al., 2020). The latter fungicide was also reported to negatively affect the growth of *A. caliginosa*, with NEC (no effect concentration) values estimated at 0.387 mg kg^{-1} and 0.128 mg kg^{-1} for the DMX and the EPX, respectively (Bart et al., 2019).

The EFSA report (2005) related fluctuations in earthworm numbers and biomass following the application of the BAS 507 01F commercial formulation (i.e. Swing® Gold) but it did not address the different species of earthworms or ecological groups, which can potentially reveal ecotoxicological effects obscured by the total abundance. For instance, in our study, earthworm diversity was negatively affected by recommended rates of Cuprafor Micro® and Swing® Gold in the mixture (M) at the beginning of the experiment (i.e., at 1 and 6 MAFA) while no significant effects were measured on the total abundance or biomass. These effects could be due to additional or synergic mixture effects, which are poorly known and scarcely assessed, especially under field conditions (Schnug et al., 2015). Similarly, anecic abundance was lower in the C10 treatment at 12 MAFA and in the D10 treatment at 1 MAFA compared to the control, but no significant effects were measured on the total abundance or biomass.

The measured soil copper concentration (45 mg kg^{-1} dry soil) was realistic as this metal accumulates in soils. Although the effects of copper on earthworms are well documented under laboratory conditions (Bart et al., 2017, 2019; Eijsackers et al., 2005) and in long-term contaminated sites by anthropic agricultural or industrial activities (e.g., Mirmonsef et al., 2017; Owojori and Reinecke, 2010; Van Zwieten et al., 2004), little is known about the effects of recent copper applications (less than two

years) on soil fauna under field conditions. The lower anecic abundance found in the C10 treatment compared to the control could be explained by the dispersal of earthworms since it has been shown that *L. terrestris* was able to avoid pesticides (Slimak, 1997). Similarly, Wentsel and Guelta (1988) found significant effects of a brass powder (mix of 70% Cu and 30% Zn) on the avoidance (threshold value at 26 mg Cu kg^{-1} dry soil after 7 days) of the earthworm species *L. terrestris* in climatic chambers (15 x 50 cm). Moreover, although done with an endogeic species, the study of Bart et al. (2017) showed an avoidance (effect concentration for 50% of *A. caliginosa* individuals (EC50) of 51.2 mg Cu kg^{-1} after 48 hours) with the same commercial formulation (i.e. Cuprafor Micro®) and the same soil as in the field experiment. The different copper applications over time in the present field experiment (from April to September 2016), the gradual increase in Cu concentration in the soil and its inherent persistence might explain the longer-terms effects of copper on earthworm communities compared with the Swing® Gold. Highlighting these effects was only possible because we considered the different ecological categories of earthworms, since compensation between species noticeably took place (i.e., effect on the diversity but not on the total abundance). According to the PRC analysis with the Hellinger transformation, *A. icterica* could have benefited from the decrease in the abundance of other species.

4.2. Species sensitivity and recovery

We here revealed the highest sensitivity of the three anecic species present in the trial, *L. terrestris*, *A. giardi,* and *A. longa*. Two endogeic species were also found to be sensitive to the tested fungicides, *A. caliginosa* and *A. chlorotica*. Römbke et al. (2004) found that the genus *Lumbricus* and in particular *L. terrestris* and *L. rubellus* were more affected by the fungicide carbendazim than other species belonging to the *Aporrectodea* genus in a TME ring-testing and in field-validation studies carried out in different European countries (i.e. Amsterdam (NL), Flörsheim (DE), Bangor (UK), and Coimbra (PT)). Big anecic earthworms such as *A. giardi, A. longa,* or *L. terrestris* which feed at the soil surface and live in the subsoil, are highly exposed to the bioavailable fraction of pesticides and would inevitably take longer time to recover (Mombo et al., 2018; Pelosi et al., 2014). The results on *A. caliginosa* observed in this study were in line with previous studies revealing its sensitivity to

pesticides (Bart et al. 2018; Pelosi et al., 2013b) and metals (Khalil et al., 1996; Maity et al., 2018) in general, and to these contaminants in particular (Bart et al., 2017, 2019, 2020). The sensitivity of *A. chlorotica* (the green morph, as identified in the present study) to pesticides has already been highlighted in a field-based study (Pelosi et al., 2013b), and related to its habitat as it is commonly found in the top 5 cm of the soil.

Once earthworm populations are affected, their recovery in the field mainly depends on the persistence of the test compound (Kattwinkel et al., 2015) but also on the species' reproductive and recolonization traits (e.g., life cycle, number of offspring, dispersal capacity, distribution) (Liess and Von der Ohe 2005). Here we observed a recovery of earthworm populations at the end of the two years of experiment for both tested fungicides. The recovery could have been internal, which depends upon surviving of individuals in the stressed ecosystem, and originates from an increase in populations through reproduction once the pesticides have dissipated (EFSA Scientific Committee, 2016a). This assumption is unlikely for copper due to its inherent persistence in soils. However, the proportion of juveniles was stable between treatments, suggesting that the reproduction was also stable in the different treatments of the experimental trial. The recovery might thus have been partly internal in our study.

The recovery can also be external, being dependent on the immigration of individuals from neighboring areas by active or passive dispersal (EFSA PPR Panel, 2017; Marinissen and van den Bosch, 1992; Pelosi et al., 2015). This process can be longer than for internal recovery since earthworms do not disperse much when conditions are favorable (Mathieu et al., 2010). In our case, passive dispersal by agricultural machinery was rather unlikely as the meadow was used only for this experiment. Thus, no machines entered the trial during the two years of experiment except the lawn mower before fungicide applications. Earthworms could also have progressively colonized the plots from the edge of the trial but we did not observe more individuals in the border plots than in the middle of the trial during the experiment. Thus, we assume that the recovery was mainly internal in our trial. In realistic agricultural situations, successive applications of pesticides with different modes of action and dissipation time may hinder the recovery of earthworms, either internal or external. At the same time, a tolerance or adaptation processes by earthworms to chemicals can occur, as shown by

Givaudan et al. (2014) with the metabolism efficiency (detoxification enzyme) of *A. caliginosa* and *A. chlorotica* exposed to commercial formulations made of epoxiconazole and glyphosate (Opus® and RoundUp Flash®, respectively).

4.3. Methodological shortcomings of the standard test guideline

This study allowed underlining methodological shortcomings in the use of the international guideline to determine the effects on earthworms in field situations (ISO 11268-3, 2014). Although the application was similarly done in all the plots and the fungicides were homogeneously sprayed within the plots, we faced difficulties to retrieve the active substances of fungicide at the desired concentrations. Despite the precautions taken during application and although this is not mentioned in the guideline, we could have placed Petri dishes of known diameter on the soil to collect the fungicides during spraying in order to measure the concentrations applied to the soil per unit area. For quality assurance measures, the ISO guideline requires to achieve a range from 50% to 150% of the nominal concentration. Here, at best one third of the applied organic Swing® Gold fungicide was measured in soils after the application. Fungicides might have entered the soil deeper than 10 cm but we found only 2.2 to 6.3% and 1.8% to 4.8% for DMX and EPX, respectively, between 10 and 30 cm deep (9 days after application). This issue to retrieve the applied concentrations in soil can also be due to the interception by the vegetation. We could have calculated an interception factor by the vegetation before the application as it is recommended in the guideline (ISO 11268-3, 2014).

Moreover, the fungicide concentrations varied in the different plots (i.e., replicates) of the trial, probably due to heterogeneity in soil physicochemical conditions within the experimental trial. We scrupulously followed the recommendations of the guideline concerning the selection of the experimental site but nothing is mentioned in the current version of the ISO guideline about the assessment of the heterogeneity of the trial site before the start of the experiment. Although this heterogeneity does not necessarily influence ecotoxicological effects (due to a random design), it can make the results more variable and thus non-significant.

For copper, the geochemical background (around 25 mg kg^{-1}) prevented us from measuring a difference in copper concentration between the control and the treatments at the RR of copper (i.e., in

C1 and M treatments). In addition, high variability in soil copper concentration between replicates of the same treatment was observed, probably due to the same environmental factors as mentioned above that can hinder highlighting significant effects of the fungicides while trends are identified.

This latter issue can also be related to the analyses that are used to assess ecotoxicological effects. The ISO guideline recommends "traditional" univariate tests that here did not allow revealing significant differences while strong negative impacts were found (e.g., at least 30% of reduction in endogeic and epigeic abundance in the D10 treatment). The current guideline does not provide information about the statistical power of the field study test or the effect size that should be detectable. For this purpose, we recommend the systematic use of the minimal detectable difference (MDD), an easy-to-use indicator allowing to evaluate differences that are statistically relevant as they consider the statistical test used and data distribution in the field study. Moreover, the MDD calculation allows the assessment of risks considering the different effect classes and specific protection goals as proposed by the EFSA's opinion (EFSA PPR Panel, 2017). In our study, the MDD (Duquesne et al., 2020) of our Dunnett's tests ranged from 74,3% to 83,9%, using power calculations for t tests with R software and transposing the MDDs obtained to the Dunnett's test with 6 groups of 4 samples with a bootstrap approach. A pre-sampling before the beginning of the study is also recommended in order to get an overview of soil organism distribution in the field and to balance sampling size and statistical relevance.

Other improvements of the current guideline can be proposed. If no full recovery is observed within a year, restrictions of use may be applied to the tested product in the context of its authorization for market distribution. The duration of the field study may also be extended until the full recovery is observed. New endpoints could be also added such as diversity indices and measurements related to the soil functioning. In our study, we showed that the Shannon index provided useful indication on earthworm community dynamics compared with earthworm species treated alone. Regarding the soil functioning, soil fauna recovery can be rapid (i.e., within a few months) but soil function recovery such as organic matter decomposition can be much longer.

The guideline also underlines the usefulness of multivariate analyses such as the PRC method. Indeed, as seen in the present study, PRC analyses are promising representational tools, which can convey

different important features of the community response depending on how the data is transformed prior to PRC analysis. In this analysis, the use of two different data transformations reflect both the general decrease in abundance and the decrease in diversity pinpointed by other ecological indices. Thus, it would be necessary to go further on new relevant statistical analyses such as PRC analysis to depict ecotoxicological effects of pesticides in field approaches.

Conclusion

This study highlighted negative ecotoxicological effects of two commonly used fungicides at realistic and high application rates on key beneficial organisms. The mixture of both fungicides at the recommended field rate had detrimental effects on the earthworm community while no effects were detected when used alone. Moreover, the two fungicides affected differently the earthworm species, the ecological categories, and the recovery. At the highest concentrations, the Swing® Gold fungicide had direct effects after application while the copper fungicide had longer term effects on earthworm communities. Once again, *A. caliginosa* was shown to be one of the most sensitive species to pesticides, as well as earthworm species living in close contact with the soil surface (e.g., anecic species, *A. chlorotica*). More studies on the relative sensitivity of earthworms could help better understanding the effects of anthropogenic stressors on ecosystem functioning. Cairns (1988) underlined the importance to put more “eco” into ecotoxicology by using more environmentally realistic tests. Although many useful tools have been developed since the 1980’s, field studies dealing with sublethal exposure of non-target organisms to pesticides, species sensitivity, and the time during which animals are affected by contaminants are still very scarce (Kattwinkel et al., 2015; Mirmonsef et al., 2017).

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