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1 A two years field experiment to assess the impact of two fungicides on earthworm communities and
2 their recovery

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19 Highlights

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

25

26 **Abstract**

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

47

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

49

50 **1. Introduction**

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

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

78 When working under natural field conditions, we face increasing complexity due to spatiotemporal
79 variability of environmental conditions, and a high number of biotic and abiotic factors operating at
80 the same time (Werner and Hitzfeld, 2012). This complexity may hinder the interpretation of the
81 results when assessing the impact of pesticides under natural conditions (Winqvist et al., 2011). This is
82 one of the main reasons why some studies reported contrasting results regarding the effects of one or
83 several pesticide products on non-target organisms in this context (Bengtsson et al., 2005; Hole et al.,
84 2005). To limit confounding factors and properly address the consequences of pesticide use on soil
85 communities under natural conditions, a guideline exists for earthworms to assess potential effects in
86 field situations (ISO 11268-3, 2014). It is commonly used as a higher-tier tests in environmental risk
87 assessments for registration of plant protection products when unacceptable risk is identified with
88 lower-tier tests performed according to OECD guideline no. 222 under laboratory conditions on
89 *Eisenia fetida* Savigny, 1826 or *E. andrei* Bouché, 1972. However, field data on the effects of
90 chemicals on earthworms generated according to standard guidelines are scarce because most
91 registered active substances did not need to be tested in the field (i.e. higher-tier tests) during the
92 registration process at the European level and data used for plant protection product registration are
93 not easily accessible due to data protection. Moreover, data on post-registration effects monitoring are
94 very scarce in the scientific literature, and mostly performed with semi-field studies and terrestrial
95 model ecosystems (TME) (e.g. Förster et al., 2011; Moser et al., 2007; Santos et al., 2011; Scholz-
96 Starke et al., 2013). These useful approaches address an intermediary level between laboratory and
97 field studies and help to assess potential effects of pesticides on the soil community structure (i.e.
98 species composition and abundance) that could compromise the soil functioning. They enable a
99 standardization of the exposure and habitat conditions and they are more ecologically realistic than
100 laboratory testing such as OECD guideline no. 222. However, they do not capture all ecological
101 interactions that may occur and they cannot assess multi-season effects of chemicals. Moreover, these
102 confined systems do not allow the assessment of chemical effects on external recovery (e.g. dispersal,
103 recolonization) (EFSA Scientific Committee, 2016a). Compared with TME studies, field studies are
104 generally carried out for a longer period (i.e. one year and longer). They represent a higher level of
105 complexity because they are performed under realistic conditions (e.g. pedoclimatic context). Field

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

108 With their soft bodies and their soil ingestion mode, earthworms are exposed to pesticides. Depending
109 on their functional traits (e.g., habitat preferences, behavior, metabolism efficiency) (Pelosi et al.,
110 2013a; Pey et al., 2014; Velki and Hackenberger, 2012), the different species of earthworms are
111 differently exposed and sensitive to pesticides (Pelosi et al., 2013b). Earthworms are used for several
112 decades as model organisms in soil ecotoxicology tests both in the scientific literature (Pelosi et al.,
113 2014) and in the reports for pesticide registration (e.g. OECD 207, 1984; OECD 222, 2016). They are
114 reliable indicators of land use and management (Paoletti, 1999) and they create habitats for themselves
115 and for other organisms, influencing and even controlling their activities through physical and
116 biochemical processes (Jones et al., 1994, 1997). They are the main contributors of organic matter and
117 soil structure transformation by soil ingestion, transport and excretion (Liu et al., 2019). After a
118 disturbance, and because they are animals of relatively low mobility (Eijsackers, 2011), the recovery
119 of earthworm communities can be slow which may impair the soil functioning (Potter et al., 1990).

120 The present study aimed to assess the effects of two commercial products of fungicides currently used
121 in different farming systems (i.e. Cuprafor Micro® made of copper oxychloride and used in organic
122 farming; Swing® Gold made of dimoxystrobin (DMX) and epoxiconazole (EPX), and used in
123 conventional farming) on earthworm communities under field conditions over two years. This study is
124 the continuation of a previous study conducted by Amossé et al. (2018). They showed that Swing®
125 Gold at 10 × the recommended field rate (RR) drastically reduced anecic and epigeic earthworm
126 diversity and abundances one month after application. In contrast, the copper fungicide (at 0.75 and
127 7.5 kg Cu ha⁻¹) and the treatment with the mixture of both fungicides (Cuprafor Micro® at 0.75 kg Cu
128 ha⁻¹ and Swing® Gold at the RR) did not affect earthworm communities compared with the control
129 (no fungicides), except for the Shannon index that decreased in the mixture treatment. Considering
130 these results and the lack of knowledge regarding the pesticide effects over a long period in field trials,
131 we assessed the effects of these fungicides on the different earthworm species present in this field and
132 the recovery of the earthworm community. The experimental design presented by Amossé et al. (2018)
133 was thus followed with the investigation of the earthworm abundance, biomass, and diversity at 1, 6,

134 12, 18 and 24 months after the first fungicide applications (MAFA). We tested the following
135 hypotheses: 1) the two fungicides have different effects (amplitude and duration) on the different
136 earthworm ecological groups and species, 2) the mixture of the two fungicides has stronger effects
137 than those observed for each fungicide applied separately. We also measured fungicide concentrations
138 (i.e. total contents of Cu, DMX and EPX) in the soil, and we found some shortcomings of using the
139 international guideline to determine the effects of chemicals on earthworms in field situations.

140

141 **2. Materials and methods**

142 2.1. Study site and experimental design

143 The experiment was conducted for a period of two years - between April 2016 and April 2018 – and
144 followed the ISO standard method for the study of the effects of pollutants on earthworms under field
145 conditions (ISO 11268-3, 2014). The study site was a meadow located in Versailles, France
146 (48°48'31''N, 2°05'26''E) that had not received pesticides for more than 20 years. Detailed
147 information about the experimental site is described in Amossé et al. (2018).

148 Two commercial formulations of fungicides were tested: Cuprafor Micro® (Industrias Químicas del
149 Valles, composed of 500 g kg⁻¹ copper oxychloride, Cu₂Cl(OH)₃) and Swing® Gold (BASF Agro
150 SAS, dimoxystrobin 133 g L⁻¹, epoxiconazole 50 g L⁻¹). They were chosen for their potential negative
151 effects on earthworm populations (Bart et al., 2017; Pelosi et al., 2016) and because they were used in
152 different farming systems, either organic or conventional farming, respectively.

153 The experimental trial consisted of four replicates of six treatments randomly located (24 plots, see
154 Amossé et al., 2018). In accordance with the guideline (ISO 11268-3, 2014), the area of each plot was
155 100 m² (10 m x 10 m), and the whole 100m² area was treated with fungicides. Samples were taken
156 exclusively in the central zone of 36 m² (6 x 6m). No fungicide was applied in the control (T)
157 treatment. Jones and Belling (1967) and Sun et al. (2019) found that Cu remained in the upper 5 cm of
158 the soil, so the RR for Cuprafor Micro® was calculated by considering a penetration depth of 5 cm.

159 Two concentrations of copper were tested (RR and 10 RR, Table 1). The application pattern of
160 Cuprafor Micro® was chosen to be realistic so we used multiple applications as is most often done in
161 field conditions (see Table 1). For the Swing® Gold fungicide, to calculate the RR, we considered that

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

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

179 The D10 treatment was chosen in the present study as a toxic reference instead of carbendazim which
180 is recommended in the guideline ISO 11268-3 (2014) because this latter pesticide is forbidden in
181 Europe since 2014 and is thus hardly commercially available. Moreover, according to EFSA reports,
182 carbendazim is slightly less toxic to earthworms (no observed effect concentration (NOEC)
183 reproduction of 1 mg as/kg soil dry weight, EFSA Scientific Report, 2010) than EPX (NOEC
184 reproduction of 0.167 mg as/kg soil dry weight, EFSA Scientific Report, 2008) and DMX (NOEC
185 reproduction < 0.0887 mg as/kg soil dry weight, EFSA Scientific Report, 2005) under laboratory
186 conditions. Finally, although no field data was available on Swing® Gold at this concentration as a
187 comparison, the expected effect was a decrease in population by 40 to 80% (ISO 11268-3, 2014). As
188 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

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

191

192 2.2. Analyses of fungicide residues in soil

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

204 For total copper analyses, soils were air-dried and sieved to < 2 mm. An aliquot was then ground in
205 order to pass through a 200 µm mesh. For that, 0.5 g of soil were weighed in Teflon containers, and
206 digested by HF/HNO₃ (1:3, v:v) and microwave heating (CEM MarsXpress, Matthews, NC, USA).
207 After complete digestion, the excess of acid was evaporated and the samples were diluted to 50 mL
208 with 1% HNO₃. All the reagents used were of analytical grade and deionized water (water resistivity =
209 18 MΩ cm) was prepared by Milli-Q water system (Millipore). Total Cu content in solution was
210 determined by flame atomic absorption spectrophotometry (FAAS, Varian SpectrAA 220,
211 quantification limit 0.04 mg Cu L⁻¹ equal to 4 mg Cu kg⁻¹ of soil) following quality control assured by
212 triplicate samples, running blanks, and using certified reference materials (TMDA-70.2, Environment
213 Canada).

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

217 with a mortar. Triplicate soil subsamples of 5 g were placed in 50 mL polypropylene tube (Falcon BD)
218 and 10 mL of methanol was added to each tube. The tubes were shaken on an orbital shaker (10 min,
219 300 rpm) and sonicated for 20 min, before being centrifuged for 10 min at 1300 g and 20 °C. After
220 collecting 7 mL of supernatant, the soil was again extracted by 10 mL of methanol and shaken,
221 sonicated and centrifuged as described previously. Then, 10 mL of supernatant was collected and
222 mixed with the first extract. The D1 samples were analysed without further preparation. For D10, an
223 aliquot was diluted by a factor of 10 using a water/acetonitrile mixture (80:20, v/v) prior to analysis, to
224 allow these samples to be within the calibration curve range. The samples containing low fungicide
225 concentrations (less than 0.002 mg kg⁻¹, i.e. controls) were further diluted by 200 mL of water and
226 submitted to purification-concentration, by solid phase extraction on HR-XA cartridges (500 mg,
227 Macherey-Nagel) preconditioned successively with 5 mL of methanol, acetonitrile and water. After
228 percolation of the diluted extracts, cartridges were rinsed with 5 mL of water, dried under vacuum and
229 eluted by 6 mL of a 95:5 (v/v) acetonitrile/formic acid mixture. Samples were then evaporated under a
230 N₂ stream, dissolved in 3 mL of 8:2 (v/v) water/acetonitrile and analysed. Analyses were performed on
231 an ultra-high-performance liquid chromatograph (Acquity UPLC, Waters) coupled through an
232 electrospray interface to a triple quadrupole mass spectrometer (TQD, Waters). The analytical
233 conditions were as described by Nélieu et al. (2016), except the UPLC gradient (95/5 to 45/55 of
234 water/acetonitrile, each containing 0.1% acetic acid) and the introduction of conditions to detect DMX
235 (cone voltage 23 V, MRM transitions 327>205 at 10 eV for quantitation and 327>116 at 17 eV for
236 confirmation). In control soils spiked with DMX and EPX, the extraction yields were estimated as 93-
237 110% with a low matrix effect due to electrospray ionization. The limit of detection (according to a
238 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
239 limit of quantification (validated by accuracy profile methodology) was 0.28 µg kg⁻¹ and 0.22 µg kg⁻¹
240 for DMX and EPX, respectively. None of those pesticides was detected in analytical blanks.
241 Considering all sampling dates of D1, D10 and M plots, the relative standard deviation of triplicate
242 analysis presented a mean of 4.2% and 6.0% for DMX and EPX, respectively.

243

244 2.3. Earthworms

245 Earthworms were sampled before setting up the trial by randomly choosing 10 sample locations in the
246 study site. Earthworms were then sampled at 1, 6, 12, 18, and 24 months after the first application,
247 called 1, 6, 12, 18 and 24 MAFA (i.e., in May 2016, November 2016, April 2017, October 2017, and
248 April 2018, respectively). Four samples were taken per sampling date on each of the 24 plots of the
249 trial. The sampling method combined an expellant solution of allyl isothiocyanate (AITC) on a 40x40
250 cm square, followed by hand sorting (40x40x20 cm-depth block of soil) (Pelosi et al., 2009). For the
251 expellant solution, AITC was first diluted with isopropanol (propan-2-ol) to obtain a 5 g L⁻¹ solution.
252 This solution was then diluted with water to give a concentration of 0.1 g L⁻¹ (Pelosi et al., 2009).
253 Emerging earthworms were stored in a 4% formaldehyde solution. Adult and sub-adult individuals
254 were identified at the species level (Sims and Gerard, 1999). Juveniles were also identified at the
255 species level according to morphological characters of the adults and to the specific form they take in
256 formalin in comparison with that of identified adults. In cases where species-level identification was
257 impossible, individuals were allocated to species level using a pro rata distribution corresponding to
258 adult and sub-adult proportions. All individuals were counted, weighed wet after preservation, and
259 classified according to three ecological groups defined by Bouché (1977), i.e., epigeic, endogeic, and
260 anecic.

261

262 2.4. Soil parameters

263 The main soil physical and chemical properties were as follows (n=7, ± SE): pH 7.5 ± 0.2, organic
264 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% ±
265 0.6. The soil bulk density was measured using the volumetric cylinder method (Al-Shammary et al.,
266 2018). It was 1.29 ± 0.03 g cm⁻³ (n=6, ± SE). The soil temperature was measured twice in each plot at
267 all sampling periods with an electronic digital thermometer at 10 cm depth. The mean temperature of
268 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
269 in October 2017, and 12.4°C in April 2018. The soil moisture was assessed by sampling two soil cores
270 (5 cm internal diameter, 0-20 cm depth) on each plot at all sampling periods. The two samples were
271 pooled, homogenized, and dried for 72 hours at 105°C in a stove. The mean soil water content (0-20

272 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,
273 6, 12, 18, and 24 MAFA respectively.

274

275 2.5. Statistical analyses

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

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

$$285 H = \sum [(p_i) \times \ln(p_i)],$$

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

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

299 PRC overall and of each PRC axis was assessed with Monte Carlo permutation tests (9999
300 permutations, $p < 0.05$) of time series residuals (Freedman and Lane, 1983). Statistical significance of
301 the effects at each date was also tested with 9999 Monte Carlo permutations of residuals. When a
302 significant effect of a treatment was observed at a sampling date, a Principal Component Analysis
303 (PCA) was performed. A 2-sided Dunnett's test was then used to compare the mean PCA scores of
304 each treatment with the control at each sampling date where a significant effect of the treatment was
305 observed. Other environmental factors were not included in the PRC analysis, but care was taken to
306 ensure that replicates of each treatment covered the whole range of environmental conditions in the
307 experiment as well as possible, so that the variability due to slight differences in environmental
308 conditions was included in the residual variability. All calculations were carried out with R 3.2.3 (R
309 Core Team, 2016), vegan package 2.4-1 (Oksanen et al., 2016), FactoMineR package (Husson et al.,
310 2014), multcomp (Hothorn et al., 2008) and reshape (Wickham, 2007).

311

312 **3. Results**

313 3.1. Fungicide residues in soils

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

327 4.7, and 7.7 (on average between the three treatments with Swing® Gold) between 1 and 6, 12, 18, 24
328 MAFA respectively (Figure 1).

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

334

335 3.2. Earthworms

336 Before setting up the trial, the total earthworm abundance was 288 individuals m^{-2} (± 135 ind. m^{-2} SD)
337 with the following 12 species (and % in the community) of epigeic earthworms: *Lumbricus castaneus*
338 (Savigny, 1826) (7%) and *Lumbricus rubellus* (Hoffmeister, 1843) (1%); anecic earthworms:
339 *Lumbricus terrestris* (Linnaeus, 1758) (11%), *Aporrectodea giardi* (Ribaucourt, 1901) (8%), and
340 *Aporrectodea longa* (Ude, 1885) (2%); endogeic earthworms: *Allolobophora icterica* (Savigny, 1826)
341 (63%), *Aporrectodea caliginosa* (Savigny, 1826) (4%), *Allolobophora chlorotica* (Savigny, 1826)
342 (2%), *Allolobophora muldali* (Omodeo 1956) (1%), *Dendrobaena mammalis* (Savigny, 1826) ($< 1\%$),
343 *Octolasion cyaneum* (Savigny, 1826) ($< 1\%$), *Aporrectodea rosea* (Savigny, 1826) ($< 1\%$).

344 At 1, 6, 12, 18, and 24 MAFA, the total abundance of earthworms ranged from 127 (in D10) to 264
345 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)
346 to 198 ind. m^{-2} (C10) and from 120 (D10) to 208 ind. m^{-2} (M), respectively (Figure 3a). The earthworm
347 abundance per treatment also varied between sampling time points, mainly between the first sampling
348 (1 MAFA) and the four others (Figure 3a). Earthworm abundance was not significantly different (two-
349 sided Dunnett's t-tests, $p < 0.05$) between treatments at each sampling period. However, although no
350 difference was significant, a trend to a decrease in earthworm abundance was observed in the D10
351 treatment compared with the control (T) at all sampling periods with the abundance in the D10
352 treatment representing 55%, 44%, 42%, 53%, and 82% of the abundance in T at 1, 6, 12, 18, and 24
353 MAFA, respectively. Moreover, the total earthworm biomass (Figure 3b) in the D10 treatment was
354 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% (\pm
360 4% SD), 75% (\pm 3% SD), 66% (\pm 2% SD), 52% (\pm 3% SD), and 44% (\pm 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

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

395

396 **4. Discussion**

397 4.1. Impacts of the tested fungicides on earthworm communities over two years

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

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

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

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

439 years) on soil fauna under field conditions. The lower anecic abundance found in the C10 treatment
440 compared to the control could be explained by the dispersal of earthworms since it has been shown
441 that *L. terrestris* was able to avoid pesticides (Slimak, 1997). Similarly, Wentsel and Guelta (1988)
442 found significant effects of a brass powder (mix of 70% Cu and 30% Zn) on the avoidance (threshold
443 value at 26 mg Cu kg⁻¹ dry soil after 7 days) of the earthworm species *L. terrestris* in climatic
444 chambers (15 x 50 cm). Moreover, although done with an endogeic species, the study of Bart et al.
445 (2017) showed an avoidance (effect concentration for 50% of *A. caliginosa* individuals (EC50) of 51.2
446 mg Cu kg⁻¹ after 48 hours) with the same commercial formulation (i.e. Cuprafor Micro®) and the same
447 soil as in the field experiment. The different copper applications over time in the present field
448 experiment (from April to September 2016), the gradual increase in Cu concentration in the soil and its
449 inherent persistence might explain the longer-terms effects of copper on earthworm communities
450 compared with the Swing® Gold. Highlighting these effects was only possible because we considered
451 the different ecological categories of earthworms, since compensation between species noticeably took
452 place (i.e., effect on the diversity but not on the total abundance). According to the PRC analysis with
453 the Hellinger transformation, *A. icterica* could have benefited from the decrease in the abundance of
454 other species.

455

456 4.2. Species sensitivity and recovery

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

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

472 Once earthworm populations are affected, their recovery in the field mainly depends on the persistence
473 of the test compound (Kattwinkel et al., 2015) but also on the species' reproductive and recolonization
474 traits (e.g., life cycle, number of offspring, dispersal capacity, distribution) (Liess and Von der Ohe
475 2005). Here we observed a recovery of earthworm populations at the end of the two years of
476 experiment for both tested fungicides. The recovery could have been internal, which depends upon
477 surviving of individuals in the stressed ecosystem, and originates from an increase in populations
478 through reproduction once the pesticides have dissipated (EFSA Scientific Committee, 2016a). This
479 assumption is unlikely for copper due to its inherent persistence in soils. However, the proportion of
480 juveniles was stable between treatments, suggesting that the reproduction was also stable in the
481 different treatments of the experimental trial. The recovery might thus have been partly internal in our
482 study.

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

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

498

499 4.3. Methodological shortcomings of the standard test guideline

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

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

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

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

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

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

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

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

556

557 **Conclusion**

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

573

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592

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