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1 **An energy-based model to analyze growth data of earthworms exposed to two**
2 **fungicides.**

3

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26 **Abstract**

27 The pesticide risk assessment for earthworms is currently performed using
28 standardized tests, the model species *Eisenia fetida*, and the analyses of the data obtained are
29 performed with *ad hoc* statistical tools. We assessed the impact of two fungicides on the
30 entire growth pattern of the earthworm species *Aporrectodea caliginosa*, which is highly
31 representative of agricultural fields. Individuals of three different ages (from hatching to 56
32 days old) were exposed to Cuprafor micro[®] (copper oxychloride) and Swing[®] Gold
33 (dimoxystrobin and epoxiconazole). Data were analyzed with an energy-based toxicodynamic
34 model coupled with a toxicokinetic model. The copper fungicide caused a drastic growth
35 inhibition once the No Effect Concentration (NEC), estimated at 65 mg kg⁻¹ of copper, was
36 exceeded. The Swing[®] Gold negatively affected the growth with NEC values estimated at
37 0.387 mg kg⁻¹ and 0.128 mg kg⁻¹ for the dimoxystrobin and the epoxiconazole in this
38 fungicide formulation, respectively. The time-profile of the effects on *A. caliginosa*
39 individuals was fully accounted for by the model, whatever their age of exposure.
40 Furthermore, toxicity data analyses, supported by measurements of fungicide concentrations
41 in earthworm at the end of the experiment, allowed bettering understanding of the
42 mechanisms of action of the fungicides towards earthworm growth.

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49 **Keywords:** Ecotoxicology, *Lumbricidae*, Growth, Risk assessment, Toxicokinetic-
50 toxicodynamic modelling.

51 **Introduction**

52 Pesticides used in agroecosystems can harm biodiversity and biological activity
53 (Bengtsson et al. 2005; Hole et al. 2005). Among non-target soil organisms that can be
54 impacted by pesticides, earthworms are commonly used as biological indicators of chemical
55 stress (OECD, 1984) because they are key soil organisms, involved in nutrient cycling, soil
56 water regulation and aeration (Blouin et al. 2013; Bertrand et al. 2015; Bart et al., 2019a).
57 During the last decades, different ecotoxicological laboratory tests have been developed such
58 as the acute toxicity test (ISO 2012a) or the reproduction tests (ISO 2012b; OECD 2004). In
59 the ISO and OECD tests, the recommended species is *Eisenia fetida fetida* or *Eisenia fetida*
60 *andrei* and they are often used to assess the impacts of pesticides or other chemicals on
61 earthworms. Contrarily, growth tests with earthworms are poorly documented while some
62 authors reported growth to be a very sensitive endpoint (Springett and Gray 1992; Booth et al.
63 2000; Booth and O'Halloran 2001). Authors highlighted the need to develop a test system for
64 measuring key demographic traits in juvenile earthworms, especially growth (Spurgeon et al.
65 2004). To move towards a more realistic and relevant assessment of the environmental risks
66 of pesticide use, some issues have to be overcome.

67 First, the model species (i.e. *E. fetida fetida* or *andrei*) do not generally inhabit mineral
68 soils (Lowe and Butt 2007) and are therefore rarely found in cultivated fields where pesticides
69 are applied. To complement the use of *E. fetida* in pesticide risk assessment procedures, the
70 earthworm species *Aporrectodea caliginosa* s.s. (Sims and Gerard 1999) was recently
71 proposed as a relevant species to be used in soil ecotoxicology tests (Klobucar et al. 2011;
72 Bart et al. 2018). This species is one of the dominant species in agroecosystems in temperate
73 areas (Boström and Lofs-Holmin 1996; Boag et al. 1997; Curry et al. 2008; Amossé et al.,
74 2018) and is found to be more sensitive to pesticides and metabolites than *E. fetida* (Pelosi et
75 al. 2013). The second issue in currently used risk assessment procedures is data analyses. *Ad*

76 *hoc* statistics are used to test differences between effects measured in polluted and unpolluted
77 soils or to calculate no observed effect concentrations (NOEC), lowest observed effect
78 concentrations (LOEC), or effective concentrations (EC_x). However, these parameters cannot
79 easily be extrapolated for other exposure durations than the one used for the test, and do not
80 account for the kinetics of the toxicant in soil. To go towards a better understanding of the
81 mechanisms of toxicants on life cycle parameters, energy-based models were proposed to
82 analyze toxicity data (Kooijman and Bedaux 1996). These models are based on the dynamic
83 energy budget (DEB) theory (Kooijman 1986, 2000, 2010) which partitions the use of energy
84 between growth, maintenance, and reproduction. Effects models, called DEBtox models,
85 assume that the use of energy by an organism described in the DEB model can be unbalanced
86 by a toxicant. The effect is described as an impact on one of the parameters of the energy-
87 based model and the magnitude of the effects is assumed to be related to the internal
88 concentration of the toxicant of the organism. The exposure concentration and internal
89 concentration of the organisms are related throughout time by a toxicokinetic model. The
90 DEBtox models are toxicokinetic-toxicodynamic models (TK-TD) and have proved their
91 reliability in the analysis of data from growth and reproduction tests (Péry et al. 2002; Jager et
92 al. 2004; Goussen et al. 2013). These models also allow estimating a no effect concentration
93 (NEC) which is a threshold for toxicity that does not depend on the time of exposure. The
94 NEC can be used to compare ecotoxicity of toxicants avoiding time dependency issues of
95 classical parameters such as LOEC, NOEC or EC_x (Baas et al. 2010; Heckmann et al. 2010;
96 Jager et al. 2014).

97 We here tested the impact of two widely used commercial formulations of fungicides,
98 that are of interests for the pesticide risk assessment (Bart et al. 2017), on the growth of the
99 earthworm *A. caliginosa*. The exposure was performed at three different ages to reveal
100 potential differences in earthworm sensitivity over their development. Data were analyzed

101 with an energy-based model, calibrated for this species (see supplementary material and Bart
102 et al., 2019b), in order to understand the mechanisms of action and the time-dependence of
103 the two different fungicides on *A. caliginosa* growth. To support the understanding of the
104 toxicity mechanisms provided by the model, we performed concentration measurements in
105 earthworms at the end of the growth experiment.

106

107 **Material and methods**

108 **Soil, animals and pesticides.**

109 All experiments were performed using a loamy soil texture (Based on the texture
110 definition of the Food and Agriculture Organization of the United Nations (FAO)), sampled
111 from a permanent grassland in Versailles (48°48' N, 2°5' E) where no pesticides have been
112 applied for more than 20 years. The soil was collected from the top 0-20 cm, air-dried and
113 crushed to pass a 2 mm mesh. Its main physico-chemical characteristics were: pH 7.5, organic
114 matter 32.6 g kg⁻¹, C/N 12.7, 29% sand, 48% silt, 23% clay, and 25.2 mg kg⁻¹ of copper (see
115 Bart et al. 2017 for more details).

116 *Aporrectodea caliginosa* s.s used in this experiment were bred in the laboratory from
117 individuals initially collected from an agricultural field in Estrée-Mons, France (49°52' N, 3°01'
118 01' E) one year before this study, and determined according to Sims and Gerard (1999). The
119 earthworms were bred in the same soil described above. To get cohorts of hatchlings, cocoons
120 were collected in the breeding culture by wet sieving the soil through a 1-mm mesh size (Bart
121 et al. 2018), and incubated at 20 °C in Petri dishes on wet filter papers (Holmstrup et al.
122 1991). Cocoons were checked every two days and new hatchlings were collected and stored in
123 the breeding soil at 4 °C for a maximum of 1 week, to slow their development. This procedure
124 allowed synchronizing cohorts of individuals to the same level of development (Bart et al.
125 2018).

126 The first studied fungicide was Swing[®] Gold (BASF Agro SAS, dimoxystrobin 133 g
127 L⁻¹, epoxiconazole 50 g L⁻¹), used to protect cereal crops in conventional farming. The
128 French Recommended Dose (RD) for this product is 1.5 L ha⁻¹ on wheat (E-phy 2017a). The
129 RD in laboratory was calculated as 1.16 10⁻³ mL kg⁻¹ of dry soil (corresponding to 150 µg kg⁻¹
130 of dimoxystrobin and to 60 µg kg⁻¹ of epoxiconazole) with a soil density of 1.29 and
131 considering that the active compounds of this fungicide are mainly found in the top 10 cm of
132 soil (McDonald et al. 2013; Chabauty et al. 2016). We tested the following concentrations:
133 0.33, 1, and 3 times the RD, abbreviated SG0.33, SG1, and SG3, respectively. These
134 concentrations were assumed to be sub-lethal considering the LC50 estimated at 7.0 10⁻³ mL
135 kg⁻¹ for *A. caliginosa* (Bart et al. 2017), or 6.03 times the RD.

136 The second studied fungicide was Cuprafor micro[®], used to prevent spore germination
137 in organic farming mainly. The French RD for this product is 10 kg ha⁻¹ for potato crops and
138 in vineyards (E-phy 2017b). The RD in laboratory was calculated as 15.5 mg kg⁻¹
139 (corresponding to 7.75 mg kg⁻¹ of copper) of dry soil with a soil density of 1.29 and
140 considering that copper is mainly found in the top 5 cm of soil (Couto et al. 2015). We tested
141 the following concentrations: 3.33, 10, and 30 times the RD abbreviated Cu3.33, Cu10 and
142 Cu30, corresponding respectively to 25.8, 77.5, and 232.5 mg kg⁻¹ of copper. These
143 concentrations were assumed to be sublethal (Ma 1984; Spurgeon et al. 2004; Bart et al. 2017;
144 PPDB 2018).

145 In all experiments, the dry soil was spiked with aqueous solutions of the fungicides,
146 and the soil water holding capacity was adjusted concomitantly at 70% of the Water Holding
147 Capacity (WHC).

148

149 **Growth experiment**

150

151 In order to monitor the growth, the weight of individuals was measured using an analytical
152 balance (± 0.1 mg). The impact of fungicides was tested exposing earthworm juveniles at
153 three different ages: just after hatching, after 28 days of growth in a control soil and after 56
154 days of growth in a control soil (see Fig. S1 in supplementary material). These three ages
155 were named age 1, age 2 and age 3 respectively (A1, A2 and A3) and individuals weighed 12
156 ± 3 mg, 90 ± 15 mg and 300 ± 40 mg when their exposure began at the three ages,
157 respectively. Under control condition of the experiment, *A. caliginosa* reach maturity (i.e.
158 apparition of the clitellum) and are able to reproduce after 85 ± 10 days of growth and at a
159 weight of 575 ± 125 mg.

160 Earthworms were placed individually in 1 L plastic vessels (15 x 10 x 7 cm) with 400 g of
161 soil (dry mass). Seven replicates (each replicate corresponded to one individual) were used
162 per age of exposure and fungicide concentration, including a control without fungicide. All
163 the vessels were stored in a climate room at 15 ± 1 °C. Individuals were fed with horse dung in
164 *ad libitum* conditions, as presented in the supplementary material. Individuals were weighed
165 at least every 14 days and the experiment was stopped when individuals had reached maturity,
166 characterized by the apparition of a fully developed clitellum. For the individuals who
167 stopped to grow, without reaching the adult stage, we stopped the experiment after around 35,
168 70 and 98 days without growth for individuals exposed at Age 3, 2 and 1 respectively. At the
169 end of the experiment, all individuals were placed in petri dishes on damp filter paper for 48 h
170 in the dark at 15 ± 1 °C to void gut contents (Hartenstein et al. 1981). Then, they were
171 weighted and frozen at -80°C for fungicide analysis.

172

173 For each treatment, the soil was renewed every 28 days to avoid unsuitable conditions for
174 earthworm growth (e.g. soil compaction). All the soils for a given fungicide treatment were
175 prepared at the same time (see Fig. S1), to ensure a comparable evolution of pesticide
176 concentrations and environmental available fraction. Swing[®] Gold fungicide concentration in

177 the soil with earthworms and horse dung and in the new soil was monitored at each soil
178 renewal (see supplementary material for more details). Moreover, in order to characterize the
179 exposure concentration of dimoxystrobin (DMX) and epoxiconazole (EPX), the total
180 concentrations and the environmental available fraction of the active substances were
181 monitored over the time of the experiment, every 28 days.

182

183 **Fungicide analyses.**

184 *The copper fungicide* - The soil was sampled just after the soil preparation soils to verify the
185 contamination level. The copper concentration in earthworms at the end of the experiment
186 was also measured in order to quantify the accumulation. Details of the chemicals analyses in
187 soil and earthworms are available in the supplementary material.

188 *The Swing[®] Gold fungicide* - The total soil concentration and of the environmentally available
189 fraction of DMX and EPX were measured over time (0, 28, 56 and 84 days after the
190 contamination) in 4 of the 7 replicates to take into account their dissipation, which changed
191 the earthworm's exposure. These measured concentrations were used in the toxicokinetic
192 model. The DMX and EPX concentration in earthworms at the end of the experiment was also
193 measured in order to quantify the accumulation. Details of the chemicals analyses in soil and
194 earthworms are available in the supplementary material.

195

196 **The energy-based model.**

197

198 We used a growth model shortly presented in the supplementary material and fully
199 presented in Bart et al., (2019b). This model is based on the DEB theory (Dynamic Energy
200 Budget) (Kooijman 1986, 2000, 2010). Under *ad libitum* conditions and according to the

201 assumptions of isomorphism and neglected energy costs of maintenance, the growth is
202 expressed with the following equation:

203
$$\text{If } l < Cs, \text{ then } \frac{d}{dt}l = a(1 - b) \quad (1)$$

204
$$\text{If } l > Cs, \text{ then } \frac{d}{dt}l = a \quad (1)$$

205 Where l is the cubic root of the wet weight of the organisms, Cs is the Critical size
206 (below which the individual cannot access all the food) a is a constant and b is a food
207 accessibility factor to take into account that when individuals are too small, they cannot
208 access the whole food quantity. In our experimental conditions, the parameter values were
209 optimized with the control treatment as follows $Cs = 3.99$, $a = 0.075$ and $b = 0.13$.

210

211 **The toxicokinetic/toxicodynamic (TK/TD) model.**

212

213 The effect model is based on the energy-based model mentioned above. We assumed that
214 the exposure to the toxicant increases the energy cost of growth. As in the DEBtox model
215 (Kooijman and Bedaux 1996), we assumed that there is a threshold for effect, a no effect
216 concentration (NEC), and that the effects are proportional to the difference between the
217 internal concentration and the NEC value. The toxicokinetic of the internal concentration (C_i)
218 was deduced from the exposure concentration with a one compartment model:

219

$$\frac{dC_i}{dt} = Ku \times ce(t) - Ke \times C_i(t) \quad (2)$$

220 where Ku and Ke are the uptake and the elimination rate, ce and C_i are the external
221 and internal concentrations of the toxicant, respectively. However, because we do not have

222 access to the internal concentration, it was scaled by the bio-concentration factor as explained
 223 in a previous study (Péry et al. 2001), leading to the following equation:

$$\frac{dci(t)}{dt} = Ke (ce(t) - ci(t)) \quad (3)$$

$$\text{with } ci = Ci \frac{Ke}{Ku} \quad (4)$$

224 Where ci is proportional to the internal concentration, but corresponds to an external
 225 concentration. Moreover, the individuals had a measurable growth during the experiment
 226 which led to a dilution by growth (the earthworm increased biomass reduced the internal
 227 concentration). We accounted for this in the toxicokinetic model (Kooijman and Bedaux,
 228 2010). The elimination rate is assumed to be proportional to the ratio of the surface area to the
 229 volume, and thus inversely proportional to the length for an isomorphic organism as explained
 230 theoretically (Kooijman and Bedaux, 2010) and shown experimentally (Sijm and van der
 231 Linde 1995; Sijm et al. 1995). This is why the elimination rate must be divided by a scaled
 232 length if the body size changes leading to the following equation:

$$\frac{dci(t)}{dt} = \frac{Ke (ce(t) - ci(t))}{l} - \frac{3a ci}{l} \quad (5)$$

234
 235 In the case of an increase in the growth costs, we assumed that the costs of building a
 236 cell are multiplied by a factor $1 + e (ci(t) - NEC)$, e being a constant and accounting for the
 237 level of toxicity as soon as the NEC is exceeded by the scaled internal concentration, leading
 238 to the following equation:

239 If $l < Cs$, then $\frac{d}{dt} l = \frac{a(1-b)}{1 + e (ci - NEC)}$ (6)

240 If $l > Cs$, then $\frac{d}{dt} l = \frac{a}{1 + e (ci - NEC)}$ (6)

241

242 All the parameters (Ke , NEC and e) were simultaneously calibrated for each fungicide
243 (including the three different ages of exposure).

244

245

246 **Model calibration and statistical analyses.**

247 The differential equations were implemented in the software R Core Team (2015), and
248 solved with the package deSolve (Soetaert et al. 2010). The model was fitted to the data, for
249 all concentrations and ages of exposure for each fungicide, using the least square method.
250 The bootstrap method⁴⁵ was used for the estimation of the confidence intervals of the
251 parameters. The R script is available on request to the corresponding author.

252

253 **Results**

254 **The copper fungicide**

255 The copper contamination led to a drastic growth inhibition in the Cu10 and Cu30
256 treatments (Fig. 1). We thus chose to simplify the toxicodynamic model as follows, with an
257 infinite value for e :

$$\text{If } ci > NEC, \text{ then } \frac{d}{dt} l = 0 \quad (7)$$

258

259 In this situation, there were only two parameters to calibrate in the model: Ke and the
260 NEC. The parameter Ke was estimated at 1.19 and the NEC at 65 mg kg⁻¹ of copper (Table 1).
261 These parameter values were common to the three different ages.

262

263

264

265 **Table 1.** Estimated parameter values and confidence intervals (CI 95%) for the copper
266 fungicide.

Parameters	Value	CI (95%)
Ke	1.19	1.17 - 1.67
NEC copper (mg kg ⁻¹)	65.006	64.79 - 65.01
e	infinite	-

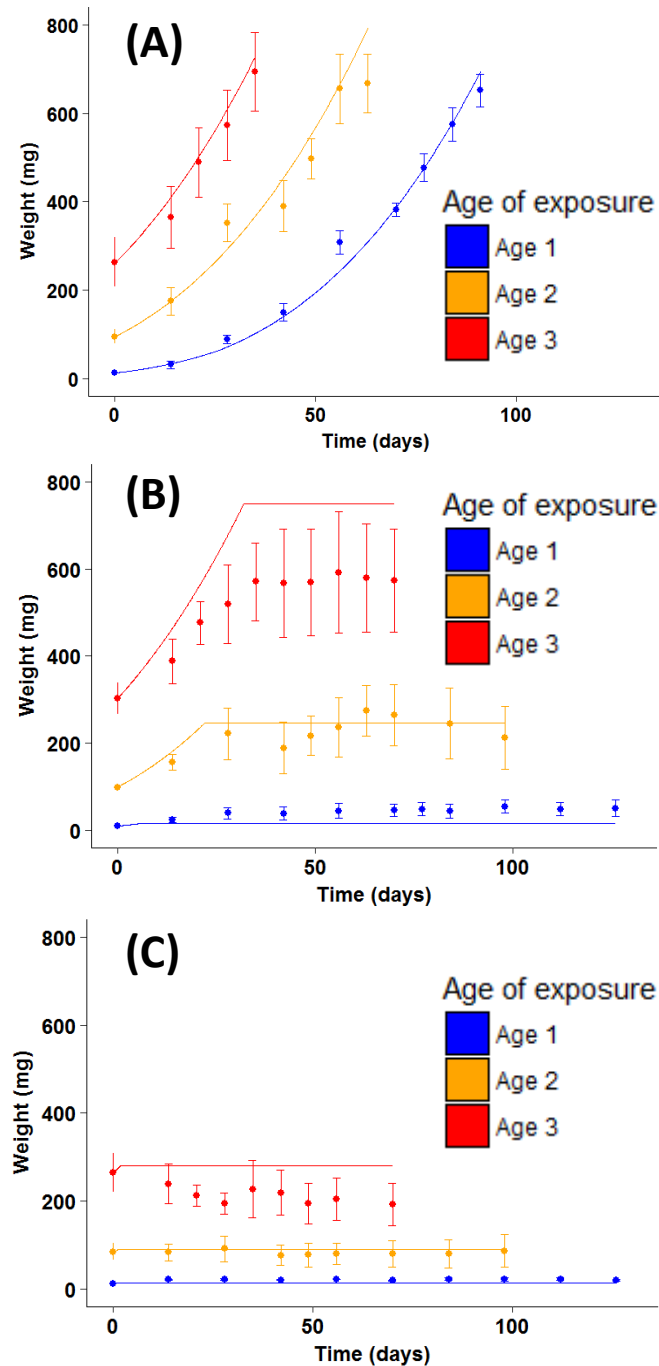
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268

269 Fig. 1 presents the growth data and the description by our model. The data were not
270 significantly different from the model description in 59% of the cases ($P > 0.05$ with
271 Student's t-tests). The difference was mainly due to over-estimation of the asymptotic mass of
272 individuals of age 3 exposed to Cu10 and Cu30 treatment, and under-estimation of the
273 asymptotic mass of individuals of age 1 exposed to the Cu10 treatment.

274 The copper fungicide applied at 25.8 mg kg⁻¹ of copper did not impact the growth for
275 the 3 different ages of exposure because the NEC was not exceeded (Fig. 1). The growth
276 pattern in this treatment thus corresponded to the growth pattern provided by the model in the
277 control. At 77.5 mg kg⁻¹ of copper, an inhibition of the growth was observed, appearing at
278 different times after the beginning of the exposure for the different ages: the effects appeared
279 immediately after the exposure for new-hatched individuals (Age 1) and after respectively 20
280 and 30 days after the exposure for individuals of Age 2 and 3. The model accounted for these
281 differences through the dilution by growth in the toxicokinetic model and provided a good
282 description of the data although it slightly overestimated the growth of the bigger juveniles

283 (Age 3). At 232.5 mg kg⁻¹ of copper, the growth was totally inhibited right after the beginning
284 of the exposure, whatever the age of the earthworms. Our model also accounted for this
285 absence of difference between ages.

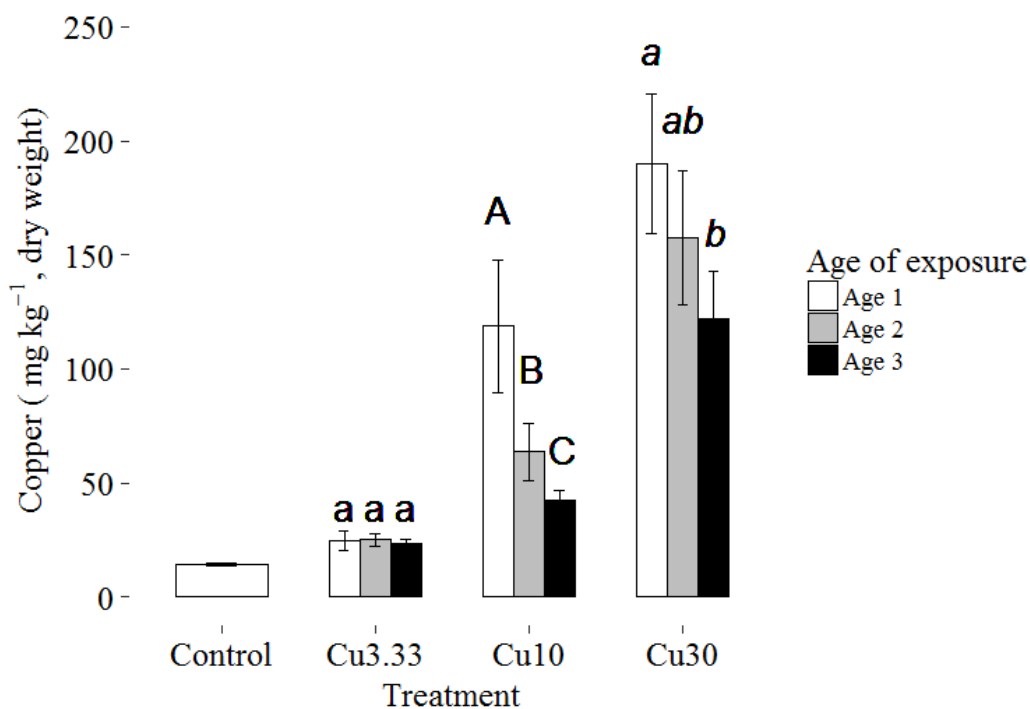


286
287 **Fig. 1** Growth pattern of *A. caliginosa* juveniles exposed at different ages to the Cuprafor
288 micro[®] fungicide at (A) 3.33 times the RD (Recommended Dose), corresponding to 25.8 mg
289 kg⁻¹ of copper, (B) 10 times the RD, corresponding to 77.5 mg kg⁻¹ of copper, and (C) 30

290 times the RD, corresponding to 232.5 mg kg⁻¹ of copper. Full lines represent the description
291 of the observations (n=7 ± SD) by the model.

292
293 The copper accumulation in earthworm significantly differed between treatments and
294 the control (Fig. 2). At 25.8 mg kg⁻¹ of copper (Cu3.33), the copper internal concentration at
295 the end of the experiment did not differ between individuals exposed at Age 1, 2, or 3. For the
296 individuals exposed in the Cu10 and Cu30 treatments, the copper concentration in tissues
297 significantly decreased with increase of the age of exposed earthworms corresponding to an
298 increase in copper accumulation with the time of exposure (Fig. 2).

299
300



301
302 **Fig. 2** Copper concentration in *A. caliginosa* individuals at the end of the growth experiment
303 in mg kg⁻¹ (dry weight). Individuals of Age 1 were exposed for on average 91 days to the
304 Cu3.33 (25.8 mg kg⁻¹ of copper) treatment and 126 days to Cu10 (77.5 mg kg⁻¹ of copper) and
305 Cu30 (232.5 mg kg⁻¹ of copper). Individuals of Age 2 were exposed for on average of 63 days

306 to the Cu3.33 treatment and 98 days for the Cu10 and Cu30 treatment. Individuals of Age 3
 307 were exposed for on average of 35 days to the Cu3.33 treatment and 70 days to the Cu10 and
 308 Cu30 treatment. Different letters mean significant differences between ages of exposure for
 309 each copper treatment.

310

311 **The Swing[®] Gold fungicide**

312 The effects of Swing[®] Gold on the growth appeared immediately after the start of
 313 exposure in the SG3 treatment (Fig. 3C). We thus assumed a very fast toxicokinetics and used
 314 directly the total DMX or EPX soil concentration as internal concentration (*ci*) in the effect
 315 model. Two parameters were thus calibrated: *e* and the NEC. The parameter *e* was estimated
 316 at 13.27 and 13.24 for the DMX and the EPX in the Swing[®] Gold formulation respectively
 317 (Table 2). The NEC was estimated at 0.387 mg kg⁻¹ (dry soil) and 0.128 mg kg⁻¹ of DMX and
 318 EPX in the Swing[®] Gold formulation respectively (Table 2). These parameter values were
 319 common to the three different ages.

320

321 Table 2. Estimated parameter values and confidence intervals (CI 95%) for the Swing[®] Gold
 322 fungicide.

Parameters	Value	CI (95%)
<i>Ke</i>	infinite	-
NEC Dimoxystrobin (mg kg ⁻¹)	0.387	0.375 - 0.402
NEC Epoxiconazole (mg kg ⁻¹)	0.128	0.123 - 0.143
<i>e</i> Dimoxystrobin	13.27	11.98 - 21.00
<i>e</i> Epoxiconazole	13.24	13.22 - 44.18

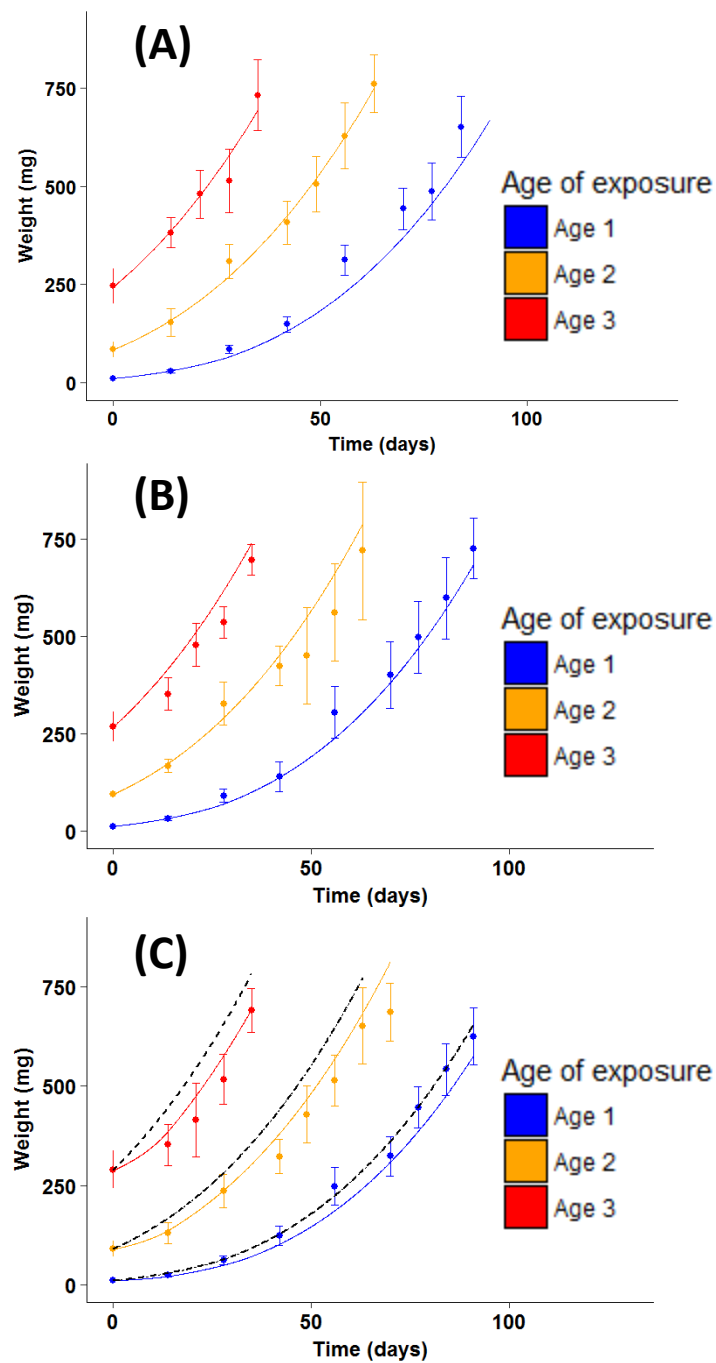
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324

325 Fig. 3 presents the growth data and the description by our model. The data were not
 326 significantly different from the model description in 81% of the cases ($P > 0.05$ with
 327 Student's t-tests). The growth of individuals exposed at 0.33 and 1 times the RD (SG0.33 and

328 SG1) was not affected because the NEC was not exceeded. In these treatments, the growth
329 pattern thus corresponded to the growth pattern provided by the model in the control (Fig. 3A
330 and 3B). At 3 times the RD (SG3), the growth was negatively affected just after the exposure
331 whatever the age of exposure (Fig. 3C), and during a period of 15 days corresponding to the
332 time during which the concentration exceeded the NEC.

333

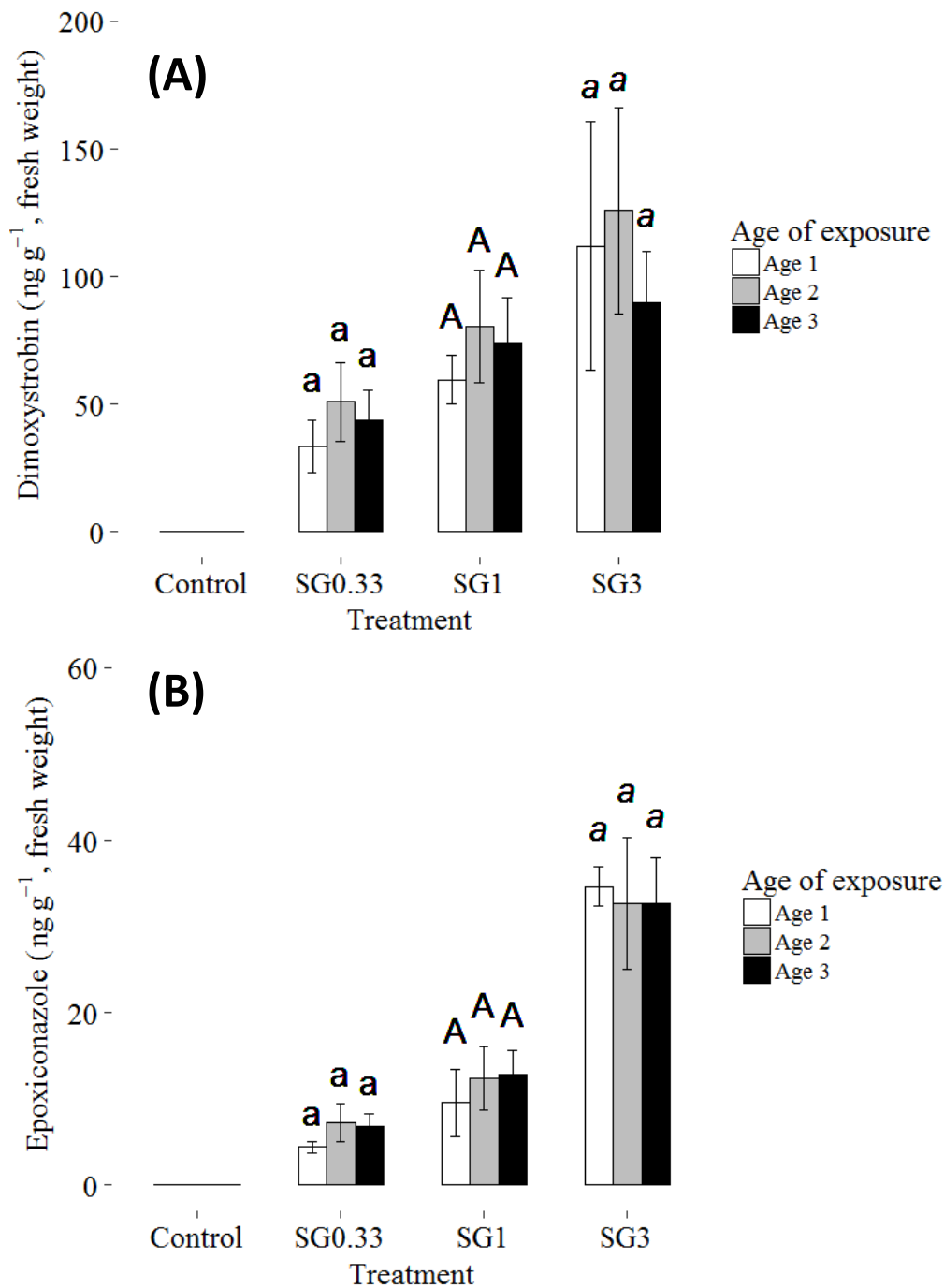


334

335 **Fig. 3** Growth pattern of *A. caliginosa* exposed at different ages to the Swing[®] Gold fungicide
336 at (A) 0.33 times the RD (Recommended Dose) corresponding to 5.2×10^{-2} mg kg⁻¹ of DMX
337 and 1.94×10^{-2} mg kg⁻¹ of EPX. (B) 1 time the RD corresponding to 1.55×10^{-1} mg kg⁻¹ of
338 DMX and 5.81×10^{-2} mg kg⁻¹ of EPX. (C) 3 times the RD corresponding to 4.62×10^{-1} mg
339 kg⁻¹ of DMX and 1.74×10^{-1} mg kg⁻¹ of EPX. Full lines represent the description of
340 observations ($n=7 \pm$ SD) by the model, and the dash lines represent the description of the
341 observations in the control treatment by the model.

342

343 The DMX and EPX accumulation in earthworm significantly differed between the
344 treatments and the control (Fig. 4). There was no significant difference in the accumulation of
345 DMX or EPX between the different ages of exposure corresponding to no difference
346 accumulation with the time of exposure (Fig. 4).



347

348 **Fig. 4** Concentration of dimoxystrobin (A) and epoxiconazole (B) in *A. caliginosa* individuals
 349 at the end of the growth experiment in ng g⁻¹ (fresh weight). Individuals of Age 1 were
 350 exposed for on average 91 days to the SG 0.33 and SG1 treatment and 95 days to the SG3
 351 treatment. Individuals of Age 2 were exposed for on average 63 days to the SG 0.33 and SG1
 352 treatment and 67 days to the SG3 treatment. Individuals of Age 3 were exposed an average 35
 353 days to the SG 0.33 and SG1 treatment and 38 days to the SG3 treatment. Different letters
 354 mean significant differences between ages of exposure for each Swing[®] Gold treatment.

355

356 **Discussion**

357 **Impact of the two tested fungicides on earthworm growth.**

358 We here showed that the growth pattern of one of the most representative species of
359 earthworms in cultivated fields was highly influenced by the presence of two fungicides at
360 environmentally relevant doses. Moreover, we highlighted that the magnitude of the effects
361 depend on the age of the individuals. Finally, we pointed out that the model proposed in the
362 supplementary material coupled with a TK-TD model can be very useful to understand
363 impacts and provide threshold value of the ecotoxicity of the tested fungicides.

364 The copper fungicide appeared highly harmful to earthworm growth beyond the NEC,
365 estimated at 65 mg kg⁻¹ of copper, corresponding to 8.4 times the RD of the fungicide. This
366 result is in accordance with the EC50 growth estimated at 81.8 mg kg⁻¹ of copper in a
367 previous study (Khalil et al. 1996). Others authors showed that copper affected the growth of
368 *E. fetida* from 8.92 mg Cu kg substrate⁻¹ (which was urine-free cattle manure) and that
369 earthworms exposed to 346.85 mg Cu kg substrate⁻¹ exhibited hardly any increase in weight
370 (Helling et al. 2000). It has also been showed that copper inhibited the growth of the
371 earthworm *Lumbricus rubellus* at a concentration of 370 mg kg⁻¹ of copper in a sandy soil
372 (Ma 1984). It thus appears that copper is harmful for earthworm growth but the threshold
373 concentration inducing impact is highly dependent on the soil characteristics and species
374 considered (Ma and Rao 1997, EU 2008). Finally, it is important to notice that the NEC value
375 estimated (i.e. 65 mg kg⁻¹) could be reached in agricultural systems because copper
376 accumulates in soils (Brun et al. 1998). This is the case in vineyards in which copper can
377 reach more than 100-200 mg kg⁻¹ and explain the very low density of earthworms in these
378 agroecosystems (Paoletti et al. 1998).

379 Harmfulness of the Swing[®] Gold fungicide on earthworm growth was estimated by a
380 NEC value of 2.5 times the RD. Moreover, DMX and EPX have estimated DT50 values (lab
381 at 20°C) of 210 and 226 days respectively (PPDB, 2018), suggesting that these compound
382 could accumulate and persist in the environment. The NEC values provided in this study are
383 valid only in the studied commercial formulation and we could not determine which of the
384 two substances caused the effect on growth. However, literature suggest that DMX is more
385 harmful than EPX based on LC50 values (Pelosi et al. 2016; PPDB, 2018).

386 Growth is a key component of the life history parameter that directly influences the
387 population dynamics in a way comparable to reproduction or survival. In the fields,
388 earthworms are active between 3 and 7 months per year, generally in spring and autumn
389 (Baker et al. 1992). In the present study, *A. caliginosa* individuals in the control soil took
390 three months to become adult in optimal conditions (fed *ad libitum*, soil moisture of 70% of
391 the WHC, temperature at 15°C, Bart et al. 2018). We can thus assume that there would be no
392 more than one or two new generations of *A. caliginosa* per year. And it is worthwhile to
393 underline that *A. caliginosa* grow and reproduce relatively fast (Bart et al. 2018, Bart et al.,
394 2019c) compared to other species of earthworms such as anecic species (e.g. *Lumbricus*
395 *terrestris*, Butt 1993; Pelosi et al. 2008. or *Octolasion cyaneum*, Butt 1993). A growth delay of
396 about ten percent, as we observed for Swing[®] Gold, could have a strong impact on the
397 population dynamics, with adults appearing significantly later in the year at a period which
398 could not be optimal for the reproduction. It could be even more problematic with compounds
399 such as copper that completely inhibit earthworm growth.

400

401

402

403

404 **Relevance of the toxicity analysis with TK-TD model.**

405

406 The two tested fungicides had very different toxicokinetics and toxicodynamics. For
407 the copper fungicide, we showed a drastic inhibition of growth and a slow kinetics whereas
408 the toxicokinetics for the Swing[®] Gold fungicide was very fast with moderate effects. These
409 conclusions came from the analysis of the data with our TK-TD model which indicated the
410 relevance of assuming either very fast kinetics or very strong effects. The conclusions
411 regarding the kinetics were confirmed with the measurements of the fungicide internal
412 concentrations at the end of the experiment.

413 We saw that the effects of the copper fungicide significantly depended on the age of
414 the exposed individuals and the exposure duration. Indeed, the effects appeared earlier for
415 small organisms (Age 1) compared to older organisms (Age 2 and 3) and that was accounting
416 for by the dilution by growth and the influence of weight on the kinetics parameters. Thus, the
417 difference between ages is fully explained by growth, with same parameters for kinetics and
418 effects. This also supports that the use of a one compartment-model was satisfactory here. In
419 some cases, for which, for instance, uptake rate depends on exposure concentrations because
420 of saturation of the uptake, a model with more compartments could be necessary (Steen
421 Redeker and Blust 2004). The difference in copper accumulation was certainly due to the
422 longer exposure of individuals of Age 1 and 2, because low elimination rates mean that the
423 longer the exposure the higher the accumulation. Still after more than 90 days of exposure, the
424 plateau for accumulated concentration was thus still not reached. For the Swing[®] Gold
425 fungicide, EPX and DMX accumulation in earthworms did not differ between the different
426 ages and times of exposure. This is consistent with rapid kinetics, which implies no
427 dependence between the accumulated concentration and the exposure duration. However,
428 further work on the accumulation in earthworm are required to validate our work. The model,

429 which accounts for the exposure throughout time, take into account that the two active
430 substances of the Swing[®] Gold fungicide degraded over time, and we were able to explain the
431 toxicodynamics. Indeed, the total concentration in soil became lower than the NEC after 15
432 days of exposure to the highest nominal concentration (SG3) and the growth was not affected
433 anymore afterwards.

434 In this study, we hypothesized that the physiological mode of action of the two tested
435 fungicides was an increase in the growth energy costs. Another possible effect on growth
436 could have occurred through a decrease in the feeding rate. From a modeling point of view, it
437 is tricky to assess which model would fit at best the observed data because they are very
438 similar for small concentrations. To make the difference between these two modes of action,
439 authors performed experiments in two feeding conditions, *ad libitum* and limited food
440 conditions (Péry et al. 2003). Here, we only used *ad libitum* conditions. For copper, some
441 elements in the literature support the assumption of an increase in growth cost. First, this was
442 the mode of action found for chironomids (Péry et al. 2003). Moreover, the increase in growth
443 energy costs could be linked to detoxification process. For example, it has been showed for
444 *Lumbricus rubellus* that the production of metallothionein (MT) proteins increased 5-fold in
445 soil contaminated with copper compared to a control soil (Stürzenbaum et al. 1998). The same
446 has been shown with *E. fetida* exposed to cadmium (Brulle et al. 2007). The MT(s) are
447 responsible for detoxification processes after an exposure to a metal contamination. The
448 energy could be redirected to the production of such proteins in response to the
449 contamination.

450

451 **Environmental implications.**

452

453 Earthworm growth appeared as a sensitive endpoint that should be taken into account
454 in the ecological risk assessment of pesticide. First, the threshold values are, as for the
455 reproduction (Neuhauser et al. 1985), lower than the LC50 value (based on survival).
456 Moreover, growth can have a strong impact on population dynamics that determines the
457 occurrence in the field and the related provided functions. The strength of our study relies on
458 the ability of the TK-TD model to fit, with the same parameters, the data obtained for three
459 different ages of exposure, despite apparent differences in the toxicodynamics. The NEC
460 values provided are common to all ages of exposure and do not depend on the time of
461 exposure as for ECx values. Finally, TK-TD models are interesting tools that can be used in
462 the regulatory risk assessment to assess bioaccumulation and effects of pesticides as it is
463 suggested for aquatic organisms in a recent EFSA report (EFSA, 2018).

464 **Supplementary material**

465 Figure of the experimental design. Description of the growth energy-based model. Chemical
466 analysis methods, and results.

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473

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