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### ► To cite this version:

Sylvain Bart, Alexandre Barraud, Joël Amossé, Alexandre R.R. Pery, Christian Mougin, et al.. Effects of two common fungicides on the reproduction of *Aporrectodea caliginosa* in natural soil. *Ecotoxicology and Environmental Safety*, 2019, 181, pp.518-524. 10.1016/j.ecoenv.2019.06.049 . hal-02244969

**HAL Id: hal-02244969**

**<https://hal.science/hal-02244969>**

Submitted on 1 Sep 2020

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1 **Effects of two common fungicides on the reproduction of *Aporrectodea caliginosa* in**  
2 **natural soil**

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12 **Highlights**

- 13 • The effects of two commercial fungicides were tested on earthworm reproduction
- 14 • Adult individuals and “naïve” cocoons were exposed
- 15 • Negative effects were found on cocoon production, hatching time, hatching success
- 16 • “Naïve” cocoons were less affected than cocoons produced by exposed adults

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28 **Abstract**

29 The use of pesticides in agroecosystems can have negative effects on earthworms, which play  
30 key roles in soil functioning such as organic matter decomposition. The aim of this study was  
31 to assess the effects of two fungicides (Cuprafor micro<sup>®</sup>, composed of copper oxychloride, and  
32 Swing Gold<sup>®</sup>, composed of epoxiconazole (EPX) and dimoxystrobin (DMX)) on earthworm  
33 reproduction by exposing adults and cocoons. First, adult *Aporrectodea caliginosa* individuals  
34 were exposed for 28 days to 3.33, 10 and 30 times the recommended dose (RD) of Cuprafor  
35 micro<sup>®</sup> corresponding to 25.8, 77.5 and 232.5 mg kg<sup>-1</sup> dry soil of copper, respectively, and 0.33,  
36 1 and 3 times the RD of Swing Gold<sup>®</sup> (corresponding to 5.2 x 10<sup>-2</sup> mg DMX kg<sup>-1</sup> + 1.94 x 10<sup>-2</sup>  
37 mg EPX kg<sup>-1</sup>, 1.55 x 10<sup>-1</sup> mg DMX kg<sup>-1</sup> + 5.81 x 10<sup>-2</sup> mg EPX kg<sup>-1</sup> and 4.62 x 10<sup>-1</sup> mg DMX  
38 kg<sup>-1</sup> + 1.74 x 10<sup>-1</sup> mg EPX kg<sup>-1</sup> respectively), in addition to a control soil with no fungicide  
39 treatment. Cocoon variables (production, weight, hatching success, hatching time) were  
40 monitored. Second, “naïve” cocoons produced by uncontaminated earthworms were exposed  
41 to soils contaminated by the same concentrations of the two fungicides, and we assessed  
42 hatching success and hatching time. In the first experiment, cocoon production was halved at  
43 the highest copper concentration (232.5 mg Cu kg<sup>-1</sup> of dry soil) as compared to the control.  
44 Cocoons took 5 more days to hatch, and the hatching success decreased by 35% as compared  
45 to the control. In the Swing Gold<sup>®</sup> treatments, cocoon production was reduced by 63% at 3  
46 times the RD, and the hatching success significantly decreased by 16% at the RD. In the second  
47 experiment, only the hatching success of cocoons was impacted by Swing Gold<sup>®</sup> at 3 times the  
48 RD (30% less hatching). It is concluded that the cocoon stock in the soil is crucial for the  
49 renewal of populations in the field. The most sensitive endpoint was the hatching success of the

50 cocoons produced by exposed adults. This endpoint and the effects observed on the “naïve”  
51 cocoons could be taken into account in pesticide risk assessment.

52

53 **Keywords:** Ecotoxicology; Ecological risk assessment; Soil invertebrates; Lumbricidae;  
54 Pesticide

## 55 **1. Introduction**

56 During the last decades, the increasing demand for food products has led to changes in  
57 land use and management. Along with an increase in cultivated areas, the enhancement of yields  
58 has been promoted by the use of chemical inputs such as pesticides (Tilman et al., 2002). In  
59 temperate soils, earthworms represent a large proportion of the living biomass (Gobat et al.,  
60 2004). These primary consumers of organic matter are involved in soil structuring, and thus  
61 maintain soil health (Bertrand et al., 2015; Blouin et al., 2013). While earthworm densities  
62 generally range from 200 to 400 individuals m<sup>-2</sup> in permanent grasslands (Amossé et al., 2018;  
63 Beylich and Graefe, 2012; Edwards and Bohlen, 1996), they can be lower than 100 individuals  
64 m<sup>-2</sup> in conventional agricultural fields (Scullion et al., 2002). Mechanical tillage such as  
65 ploughing has a direct negative impact on earthworm populations (Chan, 2001; Mele and  
66 Carter, 1999). However, the impact of pesticides on earthworm populations under field  
67 conditions is much harder to assess and rarely documented. Using a concentration-response-  
68 based field experiment, Schnug et al. (2015) showed that a mixture of three biocides (i.e., the  
69 insecticide esfenvalerate, the fungicide picoxystrobin and the bactericide triclosan) affected  
70 earthworm abundance and juvenile proportions. These authors also highlighted that pesticide  
71 concentrations corresponded to the EC<sub>50</sub> for *E. fetida* reproduction (Schnug et al., 2013; Schnug  
72 et al., 2014) and had effects on earthworms in the field exceeding 50%, indicating that the  
73 effects observed in the field could be greater than those observed under laboratory conditions.

74 This result showed that some endpoints such as reproduction were not sufficiently and correctly  
75 covered in laboratory experiments.

76

77 The reproduction test proposed in the ISO guideline with the *Eisenia fetida* species (ISO  
78 11268-2, 2012; OECD 222, 2016) currently allows assessing the number of juveniles produced  
79 by adults exposed to chemicals. But the procedure suffers from at least two weaknesses. First,  
80 this test procedure does not allow for specifically assessing the impacts of chemicals on cocoon  
81 production and then on their viability, which is a knowledge gap if we seek to understand the  
82 impact of pesticides on the earthworm reproduction process. Secondly, the cocoons produced  
83 before the chemical applications, which end up in contact with them, could be affected. No  
84 assessment on these “naïve” cocoons is currently performed. Nevertheless, the soil harbours a  
85 cocoon stock that is necessary for the populations to be renewed (Bouché, 1972; Boström and  
86 Lofs Holmin, 1996) in case of disturbances (e.g., drought, freezing temperatures, chemical  
87 stress) that directly affect adult and juvenile individuals. Consequently, not only is it relevant  
88 to assess the impacts of pesticides on cocoon production and hatching success by exposing adult  
89 earthworms, but it is also equally relevant to study the viability of cocoons by exposing them  
90 directly to chemicals. Finally, the tests proposed by the ISO guidelines are performed with  
91 *Eisenia fetida*, which is not representative of agricultural fields and is less sensitive than other  
92 soil-dwelling species to pesticides (Pelosi et al., 2013). The relevance of using species other  
93 than *E. fetida* in pesticide risk assessment is also increasingly supported in the scientific  
94 literature (Lowe and Butt, 2007; Spurgeon et al., 2003). *Aporrectodea caliginosa* is one of the  
95 increasingly advocated species in ecotoxicity tests. It is highly representative of temperate  
96 agricultural fields (Bart et al., 2018; Decaëns et al., 2011; Perez-Losada et al., 2009) and  
97 according to Pelosi et al. (2013), it is three times more sensitive to pesticides than *E. fetida*.

98 In this context, our study aimed to assess the impacts of two commercial formulations  
99 of fungicides (Cuprafor micro<sup>®</sup>, composed of copper oxychloride, and Swing Gold<sup>®</sup>, composed  
100 of epoxiconazole and dimoxystrobin) on *A. caliginosa* reproduction. We selected copper  
101 because it can negatively impact earthworm biomass and growth (Bart et al., 2017; Eijsackers  
102 et al., 2005; Khalil et al., 1996a) and accumulates in the soil, potentially leading to high  
103 concentrations (Brun et al., 1998). We selected Swing Gold<sup>®</sup> because its active substances are  
104 recognized as dangerous for the environment, and dimoxystrobin is considered as reprotoxic.  
105 Both fungicides are widely used in cultivated fields in Europe. Finally, the choice to use  
106 commercial formulations was given preference over pure molecules so as to be as close as  
107 possible to field conditions, and this generates relevant data for risk assessment. We assessed  
108 the impact of the two fungicides on *A. caliginosa* reproduction by exposing adult earthworms  
109 to contaminated soils, and also by exposing “naïve” cocoons. Thereby, we tested the assumption  
110 that the hatching success and time to hatch of cocoons produced by exposed adults could be  
111 more affected by pesticides than those of “naïve” cocoons because “naïve” cocoons were  
112 produced by unexposed adults and could better tolerate unfavorable environmental conditions  
113 (e.g., drought, freezing temperatures, chemical stress).

114

## 115 **2. Materials and methods**

### 116 *2.1. Soil, animals, and pesticides*

117 A loamy soil (FAO soil classification) was used in all experiments. It was collected from  
118 a permanent meadow in Versailles (48°48' N, 2°5' E) where any synthetics and metals  
119 compounds had been applied for more than 20 years. The soil was collected from the top 0-20  
120 cm, air-dried and crushed at 2 mm. Its main physico-chemical characteristics were: pH 7.5,

121 organic matter 32.6 g kg<sup>-1</sup>, C/N 12.7, 29% sand, 48% silt, 23% clay, and total Cu 25.2 mg kg<sup>-1</sup>  
122 (see Bart et al., 2017 for more details).

123 *A. caliginosa* individuals came from an agricultural field in Estrées-Mons, France  
124 (49°52' N 3°01'E). They were mature, with a fully developed clitellum, and their weight ranged  
125 from 600 to 1 000 mg. They were bred by incorporating horse dung to the above-mentioned  
126 soil at 15°C for 14 days; the full breeding procedure is presented in Bart et al. (2018).

127 We studied two fungicide formulations. The first was Cuprafor micro<sup>®</sup> (Quimicas del  
128 Valles, 50% copper oxychloride), used to prevent fungal and bacterial germination in  
129 conventional and organic farming. The French Recommended Dose (RD) for this product is 10  
130 kg ha<sup>-1</sup> on potato and in vineyards (ANSES, 2017b). The laboratory RD was calculated with  
131 the following formula:

$$132 \quad [\text{mg kg}^{-1}] = \frac{[\text{mg ha}^{-1}]}{\text{area (dm}^2\text{) x depth (dm) x soil density (kg dm}^{-3}\text{)}}$$

133 with mg ha<sup>-1</sup> = 10 000 000 mg Cu, area = 1 000 000 dm<sup>2</sup>, a soil density of 1.29 kg dm<sup>-3</sup>, and  
134 considering that copper was mainly found in the top 0.5 dm of soil (Couto et al., 2015). The  
135 RD was thus calculated as 15.5 mg kg<sup>-1</sup> of dry soil (corresponding to 7.75 mg kg<sup>-1</sup> of copper).  
136 We used the following concentrations for all experiments: 3.33, 10 and 30 times the RD  
137 abbreviated as Cu3.33, Cu10 and Cu30, which correspond to 25.8, 77.5 and 232.5 mg kg<sup>-1</sup> of  
138 copper, respectively. These concentrations were expected to be sub-lethal, considering that the  
139 LC50 for *Eisenia fetida* has been estimated to be > 486.6 mg kg<sup>-1</sup> of copper (which corresponds  
140 to 63 times the RD) (PPDB, 2017). The second fungicide was Swing Gold<sup>®</sup> (BASF Agro SAS,  
141 dimoxystrobin 133 g L<sup>-1</sup>, epoxiconazole 50 g L<sup>-1</sup>), used to protect cereal crops in conventional  
142 farming. The French Recommended Dose (RD) for this product is 1.5 L ha<sup>-1</sup> on wheat (ANSES,  
143 2017a). Using the same formula as for copper, the laboratory RD was calculated as 1.16 10<sup>-3</sup>  
144 mL kg<sup>-1</sup> of dry soil, with the same soil density and considering that the active compounds of

145 this fungicide were mainly found in the top 1 dm of soil (Chabauty et al., 2016; McDonald et  
146 al., 2013). We used the following concentrations for all experiments: 0.33, 1 and 3 times the  
147 RD, abbreviated as SG0.33, SG1 and SG3, respectively. These concentrations were expected  
148 to be sub-lethal, considering that the 14-day LC50 was estimated to be 6.3 times the RD for *A.*  
149 *caliginosa* (Bart et al., 2017).

150 The dry soil was spiked with aqueous solutions of fungicides, and the soil moisture was  
151 adjusted concomitantly at 70% of the water-holding capacity (WHC) (corresponding to 28% of  
152 water content) in all experiments. Only tap water was used for the control treatments.

153

## 154 2.2. Cocoon production, hatching success and time to hatch through adult exposure

155 Five replicates were performed for each concentration and the control. For each  
156 replicate, 500 g of soil (dry mass) and 24 g of horse dung were mixed and placed in a 1-L plastic  
157 vessel (15 x 10 x 7 cm) with a removable perforated cover for gas exchanges. Horse dung used  
158 as a food source was frozen - defrosted twice and then milled (< 1 mm) before use (Lowe and  
159 Butt, 2005). The food amount corresponded to 6 g ind<sup>-1</sup> month<sup>-1</sup>, which is enough to maintain  
160 *A. caliginosa* weight. This food amount was previously determined and close to that proposed  
161 by Lowe and Butt (2005). Four adult individuals with a visible clitellum were weighed and  
162 introduced in each vessel and stored in a climate room (15±1 °C, 24 h darkness, Bart et al.,  
163 2018) for 28 days. The soil moisture (i.e., 70% of WHC) was checked weekly by monitoring  
164 the mass of the vessels throughout the experiment and adjusted with tap water if necessary. At  
165 the end of the exposure period, individuals were removed, counted, and weighed. The  
166 difference between the individual weights at the beginning and at the end of the experiment was  
167 used to calculate the percentage of adult weight change. The wet sieving method was used to  
168 retrieve produced cocoons, and results were expressed as numbers of cocoons per vessel. The



169 cocoons were then weighed and incubated at  $20^{\circ} \pm 0.5$  C in petri dishes in aqueous tap water  
170 solution (i.e., semi-immersed). The cocoons were monitored every two days over a 60-day  
171 period. After that period, we considered that the unhatched cocoons would never hatch. Mean  
172 hatching time and hatching success were calculated for each replicate.

173

### 174 2.3. Cocoon hatching success and time to hatch through “naïve” cocoon exposure

175 More than 120 cocoons were produced over a period of 7 days in the *A. caliginosa*  
176 breeding culture. In order to get a homogenous cocoon cohort, we discarded the 5% of cocoons  
177 that were too big or too small. The sinks of 24-well microtiter plates were filled with 2 g of soil  
178 (dry mass). All the plates had a removable perforated cover for gas exchanges. Five replicates  
179 were set up for each fungicide and concentration tested, and for the control. Three cocoons  
180 randomly selected among the cocoon cohort were introduced in each sink. The cocoons were  
181 gently buried in the soil. The plates were incubated at  $20 \pm 0.5$  °C, and the soil moisture (i.e.,  
182 70% WHC) was checked weekly by monitoring the mass of the plates, and adjusted with tap  
183 water if necessary. The hatching cocoons were checked every 2 days, and the experiment was  
184 stopped after 60 days of incubation, considering that the remaining cocoons would never hatch  
185 beyond that delay. The mean hatching time and hatching success per replicate were calculated  
186 for each modality.

187

### 188 2.4. Statistical analyses

189 All data were analysed with the statistical software program R Core Team (2015).  
190 Differences were considered significant if the p-value  $< 0.05$ . When the homogeneity of  
191 variance (Bartlett-test, Snedecor and Cochran, 1989) and the normality of residuals (Shapiro  
192 test) were respected, ANOVA and Fisher's least significant difference (LSD) post-hoc tests

193 were performed to analyse the differences among treatments. When normality and  
194 homoscedasticity conditions were not satisfied, the non-parametric Kruskal-Wallis test was  
195 used.

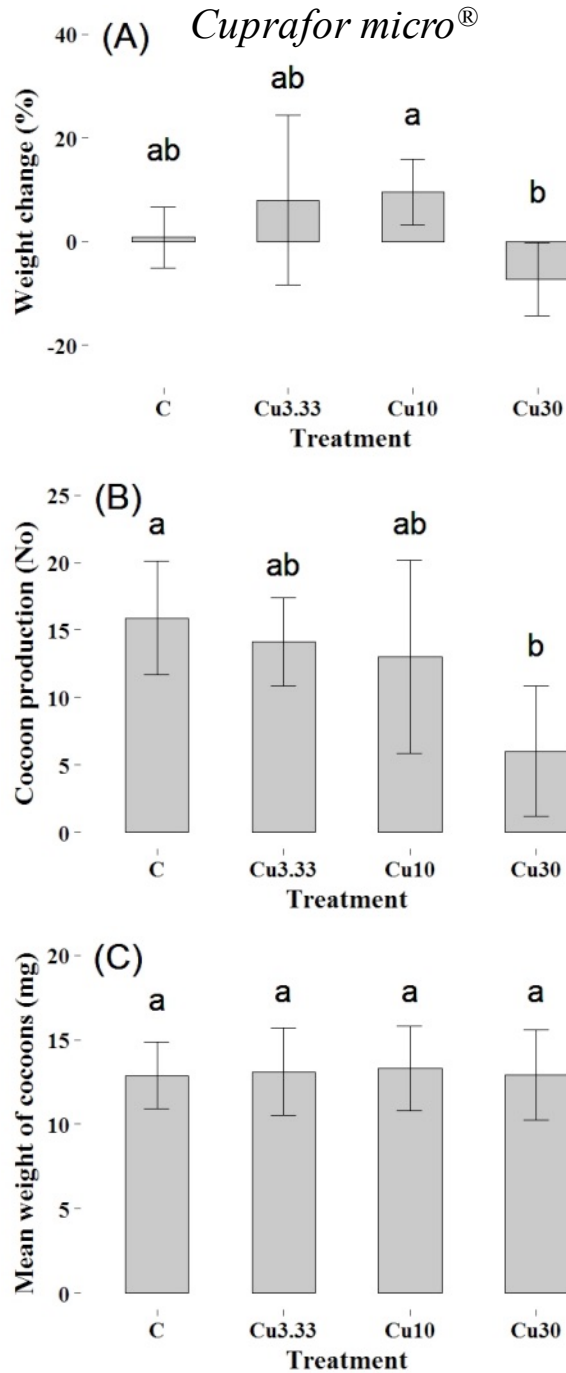
196

### 197 **3. Results**

#### 198 *3.1. Cocoon production, hatching success and time to hatch through adult exposure*

199 The effects of Cuprafor micro<sup>®</sup> on earthworm biomass, cocoon production, and cocoon  
200 weight are presented in Fig. 1. The hatching time and success of the produced cocoons are  
201 presented in Table 1, and the hatching dynamics (all replicates included) in Fig. S1.

202 At the end of the experiment, a mortality rate of 5% was recorded in the Cu3.33 and the  
203 control treatments. No mortality occurred in the Cu10 and Cu30 treatments. No significant  
204 difference in adult weight change was recorded between the control and Cuprafor micro<sup>®</sup>  
205 treatments. However, adult weight change was significantly lower in the Cu30 treatment (-  
206 7.3%) than in the Cu10 treatment (+9.6 %, Fig. 1 A). Earthworms produced 15.9 cocoons (mean  
207 number of cocoons per vessel) on average in the control treatment. Cocoon production was  
208 significantly two times lower in the Cu30 treatment (corresponding to 30 times the RD or 232.5  
209 mg kg<sup>-1</sup> of copper) than in the control (Fig. 1 B). We did not find any significant effect of the  
210 copper fungicide on the mean weight of the produced cocoons (Fig. 1 C).



211

212 **Fig. 1.** (A) Weight change (% of difference between the beginning and the end of the  
 213 experiment, 28 days), (B) cocoon production (number of cocoons per vessel produced by 4  
 214 individuals), and (C) mean weight of the cocoons produced by earthworms exposed to control  
 215 soil or soils spiked with different concentrations of Cuprafor micro<sup>®</sup> (3.33, 10 and 30 times the  
 216 RD, corresponding to 25.8, 77.5 and 232.5 mg kg<sup>-1</sup> of copper, abbreviated Cu3.3, Cu10 and  
 217 Cu30, respectively). n=5 ± SD; different letters indicate significant differences among the  
 218 concentrations (p<0.05).

219 The hatching dynamics of the cocoons (all replicates included) in the Cu10 and Cu30  
 220 treatments differed from the dynamics of the control treatment (Fig. S1). More specifically, the  
 221 mean hatching time of the cocoons produced in the Cu30 treatment was significantly longer (5  
 222 more days) than in the control treatment (Table 1). Moreover, the hatching success was  
 223 significantly two times lower in the Cu10 treatment than in the control (Table 1). Although the  
 224 mean hatching success was 61% and 94% in the Cu30 and control treatments, respectively, the  
 225 difference was not significant due to high variability in the Cu30 treatment: only one cocoon  
 226 was produced in one replicate (N=5) and did not hatch (hatching success of 0%), while 2  
 227 cocoons were produced in another replicate and hatched (hatching success of 100%).

228

229 **Table 1.** Mean hatching time (days) and mean hatching success (%) of cocoons produced by *A.*  
 230 *caliginosa* adults exposed to Cuprafor micro<sup>®a</sup>

	Treatment <sup>b</sup>			
	C	Cu3.33	Cu10	Cu30
Hatching time (days)	23.5 ± 1.7 A	25.6 ± 1.8 AB	26.8 ± 3.5 AB	28.5 ± 2.4 B
Hatching success (%)	94.4 ± 5.3 A	88.5 ± 13.7 A	45.9 ± 18.9 B	61.0 ± 38.5 AB

231 <sup>a</sup> Cocoons were produced over a 28-day period in a control soil C and in soils spiked with  
 232 different Cuprafor micro<sup>®</sup> concentrations (3.33, 10 and 30 times the RD, corresponding to 25.8,  
 233 77.5 and 232.5 mg kg<sup>-1</sup> of copper abbreviated Cu3.3, Cu10 and Cu30, respectively). C = Control  
 234 treatment.

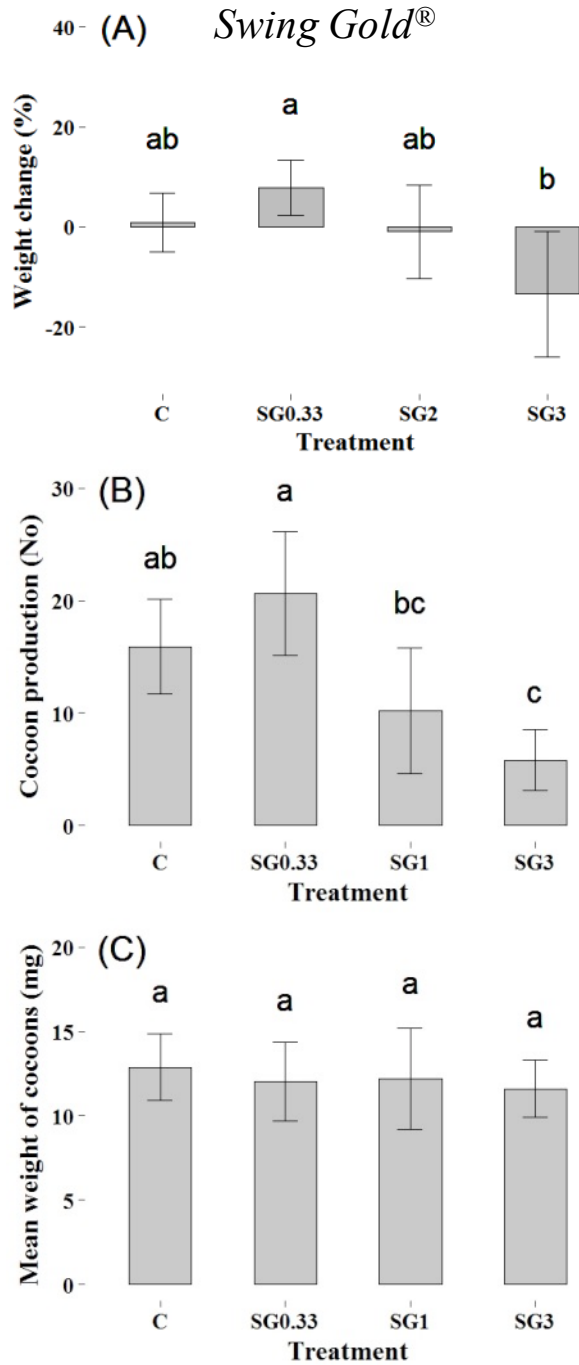
235 <sup>b</sup> Different letters indicate significant differences among concentrations (p<0.05).

236  
 237

238 The effects of Swing Gold<sup>®</sup> on earthworm biomass, cocoon production, and cocoon  
 239 weight are presented in Fig. 2. The hatching time and success of the produced cocoons are  
 240 presented in Table 2, and the hatching dynamics (all replicates included) in Fig. S2.

241 At the end of the experiment, no mortality was recorded in the SG0.33 treatment.  
 242 Mortality rates of 5% were found in the SG1 and control treatments, and of 20% in the SG3

243 treatment. No significant difference in adult weight change was recorded between the control  
244 and the Swing Gold<sup>®</sup> treatments. However, adult weight was significantly lower in the SG3  
245 treatment (-13.4%) than in the SG0.33 treatment (+7.9%, Fig. 2 A). Earthworms produced  
246 significantly three times less cocoons at 3 times the recommended dose (SG3) as compared to  
247 the control soil (Fig. 2 B). As for the copper fungicide, the different Swing Gold<sup>®</sup> concentrations  
248 did not influence cocoon weight (Fig. 2 C).



249

250 **Fig. 2.** (A) Weight change (% of the difference between the beginning and the end of the  
 251 experiment, 28 days), (B) cocoon production (number of cocoons per vessel produced by 4  
 252 individuals), and (C) mean weight of the cocoons produced by earthworms exposed to control  
 253 soil or soils spiked with different *Swing Gold*<sup>®</sup> concentrations (0.33, 1 and 3 times the RD,  
 254 abbreviated SG0.33, SG1 and SG3, respectively).  $n=5 \pm SD$ ; different letters indicate significant  
 255 differences among the concentrations ( $p<0.05$ ).

256

257 The hatching dynamics of the cocoons (all replicates included) in the SG1 and SG3  
 258 treatments differed from the dynamics of the control treatment (Fig. S2). More specifically, the  
 259 Swing Gold® fungicide negatively affected the hatching time of cocoons: they took 5 more days  
 260 to hatch in the SG3 treatment than in the other treatments (Table 2). The hatching success was  
 261 16% and 36% lower for the cocoons produced by individuals exposed to the SG1 and SG3  
 262 treatments, respectively, as compared to the control treatment (Table 2).

263

264 **Table 2.** Mean hatching time (days) and mean hatching success (%) of the cocoons produced  
 265 by *A. caliginosa* adults exposed to Swing Gold®<sup>a</sup>.

	Treatment <sup>b</sup>			
	C	SG0.33	SG1	SG3
Hatching time (days)	23.5 ± 1.7 A	22.2 ± 1.4 A	22.9 ± 1.7 A	28.8 ± 3.4 B
Hatching success (%)	94.4 ± 5.3 A	91.1 ± 15.6 AB	78.9 ± 6.8 B	60.3 ± 14.4 C

266 <sup>a</sup> Cocoons were produced over a 28-day period in a control soil C and in soils spiked with  
 267 different Swing Gold® concentrations (0.33, 1 and 3 times the RD, abbreviated SG0.33, SG1  
 268 and SG3, respectively).

269 <sup>b</sup> Different letters indicate significant differences among the concentrations (p<0.05).

270

271

272 *3.2. Cocoon hatching success and time to hatch through “naïve” cocoon exposure*

273 The effects of Cuprafor micro® on the hatching time and success of “naïve” cocoons are  
 274 presented in Table 3, and the hatching dynamics (all replicates included) are presented in Fig  
 275 S3 A.

276 The hatching dynamics (all replicates included) of “naïve” cocoons produced in a non-  
 277 contaminated soil and then exposed to Cu3.33 treatment, Cu10 treatment, or to a control soil  
 278 were similar (Fig. S3 A). The dynamics in the Cu30 treatment was slower and stopped at around  
 279 80% of the hatching success, versus 100% in the control treatment. However, there was no  
 280 significant effect on the hatching time and success, whatever the concentration tested (Table 3).

281

282 The effects of Swing Gold® on the hatching time and success of “naïve” cocoons are  
283 presented in Table 4, and the hatching dynamics (all replicates included) are presented in Fig  
284 S3 B.

285 The hatching dynamics (all replicates included) of “naïve” cocoons produced in a non-  
286 contaminated soil and then exposed to SG0.33 or SG1 treatment were close to the dynamics of  
287 the control (Fig. S3 B). The hatching dynamics of the cocoons exposed to the SG3 treatment  
288 was slower than the dynamics of the control treatment (Fig. S3 B), and the hatching success  
289 was significantly affected, with only 67% of hatching success (Table 4).

290

291 **Table 3.** Mean hatching time (days) and mean hatching success (%) of “naïve” *A. caliginosa*  
292 cocoons<sup>a</sup> exposed to Cuprafor micro<sup>®b</sup>.

	Treatment <sup>c</sup>			
	C	Cu3.33	Cu10	Cu30
Hatching time (days)	31.1 ± 1.1 A	32.4 ± 1.0 A	32.7 ± 3.6 A	33.1 ± 1.5 A
Hatching success (%)	100 ± 0.0 A	93.3 ± 14.9 A	86.7 ± 18.3 A	80 ± 18.25 A

293 <sup>a</sup> Cocoons were produced over a 7-day period in the breeding culture

294 <sup>b</sup> Cocoons were incubated at 20°C in the control soil C or in soils spiked with different Cuprafor  
295 micro<sup>®</sup> concentrations (3.33, 10 and 30 times the RD, corresponding to 25.8, 77.5 and 232.5  
296 mg kg<sup>-1</sup> of copper, abbreviated Cu3.3, Cu10 and Cu30, respectively).

297 <sup>c</sup> Different letters indicate significant differences among the concentrations (p<0.05).

298

299

300

301

302 **Table 4.** Mean hatching time (days) and mean hatching success (%) of “naïve” *A. caliginosa*  
303 cocoons<sup>a</sup> exposed to Swing Gold<sup>®b</sup>.

	Treatment <sup>c</sup>			
	C	SG0.33	SG1	SG3



Hatching time (days)	31.1 ± 1.1 A	31.3 ± 1.4 A	32.6 ± 3.0 A	34.4 ± 2.6 A
Hatching success (%)	100 ± 0.0 A	100 ± 0.0 A	86.7 ± 29.8 A	66.7 ± 0.0 B

---

304 <sup>a</sup> Cocoons were produced over a 7-day period in the breeding culture

305 <sup>b</sup> Cocoons were incubated at 20°C in the control soil C or in soils spiked with different Swing  
306 Gold<sup>®</sup> concentrations (0.33, 1 and 3 times the RD, abbreviated SG0.33, SG1 and SG3,  
307 respectively).

308 <sup>c</sup> Different letters indicate significant differences among the concentrations (p<0.05).

309

#### 310 **4. Discussion**

311       The impacts of pesticides on earthworm reproduction are difficult to assess under  
312 natural conditions because several factors can influence on it at the same time (e.g., agro-  
313 pedoclimatic factors). In this study, we used an ecologically relevant earthworm species, a  
314 natural soil, and commercial formulations of pesticides so as to be closer to field conditions.  
315 The results revealed negative effects of two fungicides on *A. caliginosa* reproduction at  
316 concentrations not far from those applied in the field. For the Swing Gold<sup>®</sup> fungicide, effects  
317 were observed starting from the RD. Concerning the active substances, Silva et al. (2019) found  
318 a median concentration of 0.02 mg kg<sup>-1</sup> of epoxiconazole and a maximum of 0.16 mg kg<sup>-1</sup> in  
319 agricultural soil, which is in the range of the RD (0.058 mg kg<sup>-1</sup> of epoxiconazole, Table S1).  
320 Furthermore, according to the EFSA Scientific Report 46, 1-82 (2005), the no-observed-effect  
321 concentration (NOEC) of dimoxystrobin for *E. fetida* reproduction in a formulation with the  
322 same properties as Swing Gold<sup>®</sup> (i.e., 133 g L<sup>-1</sup> of dimoxystrobin and 50 g L<sup>-1</sup> of epoxiconazole)  
323 was estimated to be <0.0887 mg kg<sup>-1</sup> of dimoxystrobin, which is equivalent to half the RD in  
324 our experimental conditions (i.e., 10 cm depth and 1.29 kg dm<sup>-3</sup> soil density). Finally, as regards  
325 epoxiconazole, the NOEC for *E. fetida* reproduction was estimated to be 0.084 mg kg<sup>-1</sup> (PPDB,  
326 2017). We carried out our experiments with the endogeic species *A. caliginosa*, which inhabits  
327 agroecosystems and will consequently come in contact with this pesticide. Our results strongly

328 support the conclusion that the Swing Gold<sup>®</sup> formulation is potentially harmful for earthworm  
329 reproduction in the field. Concerning the copper fungicide, the effects were observed from 10  
330 times the RD, corresponding to 77.5 mg kg<sup>-1</sup> of Cu, which added up to the soil background Cu  
331 concentration, leading to around 102.5 mg kg<sup>-1</sup>. This value is a bit far from the average  
332 maximum Cu concentration in vineyard soils in Europe, i.e., 49.26 mg kg<sup>-1</sup> of Cu (Ballabio et  
333 al., 2018), but could be reached in the field because copper accumulates treatment after  
334 treatment.

335

336 In our experimental conditions (i.e., soil characteristics, 28 % water content, and  
337 temperature), the individuals of the control group did not lose weight, indicating that the amount  
338 of food was sufficient for individuals. The pesticide concentrations were sublethal for *A.*  
339 *caliginosa*. Even if the Swing Gold<sup>®</sup> treatment was applied at three times the recommended  
340 dose, it killed 20% of earthworms. This result is in accordance with Bart et al. (2017), who  
341 estimated an LC50 at 6.3 times the RD for *A. caliginosa*. Furthermore, a recent field experiment  
342 showed that this fungicide applied at 10 times the recommended dose in the field dramatically  
343 affected earthworm density, especially *A. caliginosa* density (Amossé et al., 2018).

344 *A. caliginosa* adults produced one cocoon per individual per week in the control  
345 treatment. This is in accordance with other studies (Lofs Holmin, 1982; Spurgeon et al., 2000),  
346 which found between 0.6 to 2.6 cocoons per individual per week at 15 °C in natural soils for *A.*  
347 *caliginosa*. Cocoon production sharply dropped at the two highest concentrations of both  
348 fungicides, although partially explained by the mortality rate in the SG3 treatment. Other  
349 studies with the same species reported EC<sub>50</sub> values for cocoon production between 68 and 186  
350 mg Cu kg<sup>-1</sup> (Khalil et al., 1996b; Ma 1988). Similarly, Martin (1986) estimated a NOEC of 50  
351 mg Cu kg<sup>-1</sup> for *Aporrectodea tuberculata*, which belongs to the *A. caliginosa* complex of

352 species (Sims and Gerard, 1999). Mirmonsef et al. (2017) found an EC<sub>50</sub> value of 220 mg kg<sup>-1</sup>  
353 for *Aporrectodea tuberculata*. In our experiment, copper toxicity was a little lower than  
354 previously reported because the addition of horse manure may have decreased copper  
355 bioavailability (Ma and Rao, 1997) for earthworms and thus alleviated its ecotoxicological  
356 effects. Copper toxicity highly depends on the copper form and the soil (EU 2008).

357         The weight of the cocoons produced by adults exposed to fungicides or chemicals as an  
358 ecotoxicological endpoint is not documented in the scientific literature. However, it can reveal  
359 key information on the reproductive strategy of *A. caliginosa*. Neither fungicide impacted the  
360 weight of the cocoons produced by the exposed adults, even at the highest concentrations. When  
361 a cocoon is produced, the vitellus allows for the development of the embryo (Bouché, 1972). A  
362 hypothesis could be that when *A. caliginosa* are exposed to fungicides, their strategy could be  
363 to produce fewer cocoons but with the required amount of vitellus to ensure the embryo  
364 development, but further experiments would be required to investigate that point. The cocoons  
365 produced by exposed adults took longer to hatch and had a lower hatching success, even at  
366 the recommended dose of Swing Gold<sup>®</sup>. Previous studies showed that the sperm quality and  
367 quantity of earthworms, including *A. caliginosa*, can be affected by pesticides (Espinoza-  
368 Navarro and Bustos-Obregon, 2004; Espinoza-Navarro and Bustos-Obregon, 2005; Mosleh et  
369 al., 2003; Venter and Reinecke, 1985) and might explain our results.

370         In the second experiment, in which “naive” cocoons were exposed to the two fungicides,  
371 we found a mean incubation period of 31 days in the control treatment. This number is  
372 underestimated because cocoons were produced over the 7-day period at 15°C before the  
373 beginning of the incubation, so that some cocoons had already started incubating. The observed  
374 incubation period is in accordance with Holmstrup et al. (1991), who found a cocoon incubation  
375 period in petri dishes on a moist filter paper of 36 days at 20 °C for cocoons of *A. caliginosa*.

376 Several authors recorded a hatching success of more than 90% in a natural soil (Boström and  
377 Lofs Holmin, 1996) or in petri dishes (Holmstrup, 2001; Jensen and Holmstrup, 1997) at 15°C.  
378 This is in accordance with our results in the control treatment, where 100% of the cocoons  
379 hatched. To our knowledge, the monitoring of “naive” cocoons incubated in a contaminated  
380 soil has never been documented in the scientific literature. Nevertheless, cocoons from “naive”  
381 earthworms can be exposed to pesticides after being produced, although this is less probable  
382 than the previous situation (cocoons produced by exposed earthworms). These “naive” cocoons  
383 appeared to be less affected by the fungicides than the cocoons produced by the exposed adults,  
384 with only significant effects for the Swing Gold® fungicide at 3 times the RD. These results  
385 mean that the ecotoxicological effects are mainly related to the quality of the cocoons rather  
386 than the environment in which they are deposited. Nevertheless, the effects observed with the  
387 Swing Gold® fungicide indicate that this endpoint should be monitored in pesticide risk  
388 assessment since the cocoon stock is crucial for the renewal of populations in the field. This  
389 result was significant for the hatching success in the SG3 treatment. In addition, the cocoons  
390 produced by adults exposed to pesticides under field conditions are generally laid in the same  
391 contaminated soil. Thus, the impacts on reproduction could be greater than our observations  
392 appear to indicate.

393

## 394 **5. Conclusions**

395 In risk assessment procedures, the reproduction test with *Eisenia fetida* directly assesses  
396 the impact of chemicals on the number of produced juveniles. This does not provide information  
397 about the mechanisms whereby pesticides affect earthworm reproduction. In this study, we took  
398 one step further by taking the cocoon stage (i.e., weight, hatching success and time) into account  
399 after cocoon production, and by separately exposing “naïve” cocoons to contaminated soils so

400 as to allow for a better understanding of the impact of pesticides on earthworm reproduction.  
401 Our results showed that earthworm reproduction could be affected through adult fertility  
402 (cocoon production and viability of the produced cocoons), but also through the cocoon stock  
403 in the soil. These results highlight that there is a need to assess the impact of pesticides on  
404 earthworm reproduction through both adult exposure and cocoons.

405

## 406 **Acknowledgements**

407 The authors thank Véronique Etiévant and Jean Pierre Pétraud for their help and  
408 technical assistance. This work is supported by the "IDI 2015" project funded by the IDEX  
409 Paris-Saclay, ANR-11-IDEX- 0003-02. The authors thank the anonymous reviewers for their  
410 careful reading and their helpful suggestions, which contributed to improving the quality of the  
411 manuscript.

412

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## supplementary material

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573 **Table S1.** Concentrations of fungicides tested as commercial forms and active compounds.

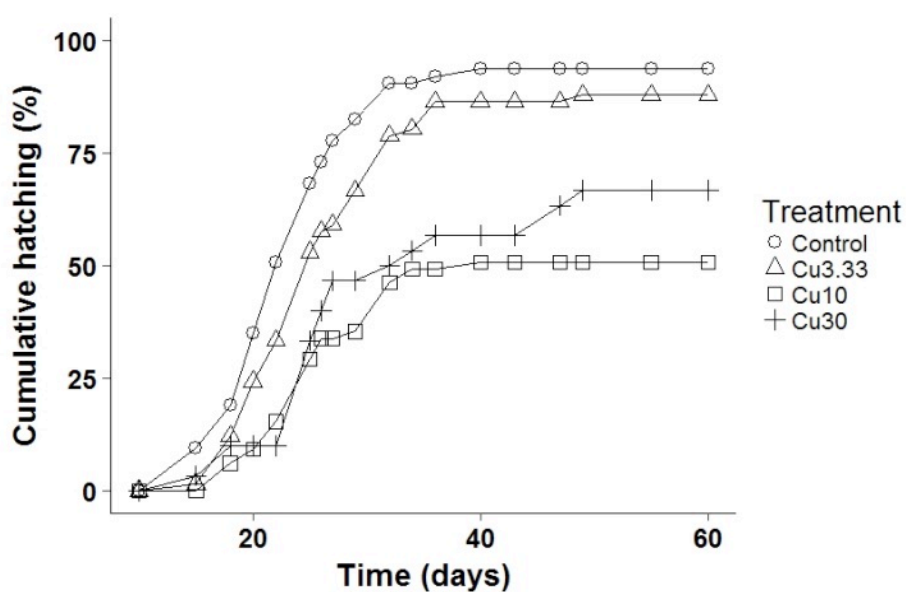
Swing Gold <sup>®</sup>				
Abbreviation	Times RD	Commercial form (mL kg <sup>-1</sup> )	Dimoxystrobin (mg kg <sup>-1</sup> )	Epoxiconazole (mg kg <sup>-1</sup> )
SG0.33	0.33	0.39 x 10 <sup>-3</sup>	5.2 x 10 <sup>-2</sup>	1.94 x 10 <sup>-2</sup>
SG1	1	1.16 x 10 <sup>-3</sup>	1.55 x 10 <sup>-1</sup>	5.81 x 10 <sup>-2</sup>
SG3	3	3.48 x 10 <sup>-3</sup>	4.62 x 10 <sup>-1</sup>	1.74 x 10 <sup>-1</sup>

Cuprafor micro <sup>®</sup>			
Abbreviation	Times RD	Commercial form (mg kg <sup>-1</sup> )	Copper (mg kg <sup>-1</sup> )
Cu3.33	3.33	52	25.8
Cu10	10	155	77.5
Cu30	30	465	232.5

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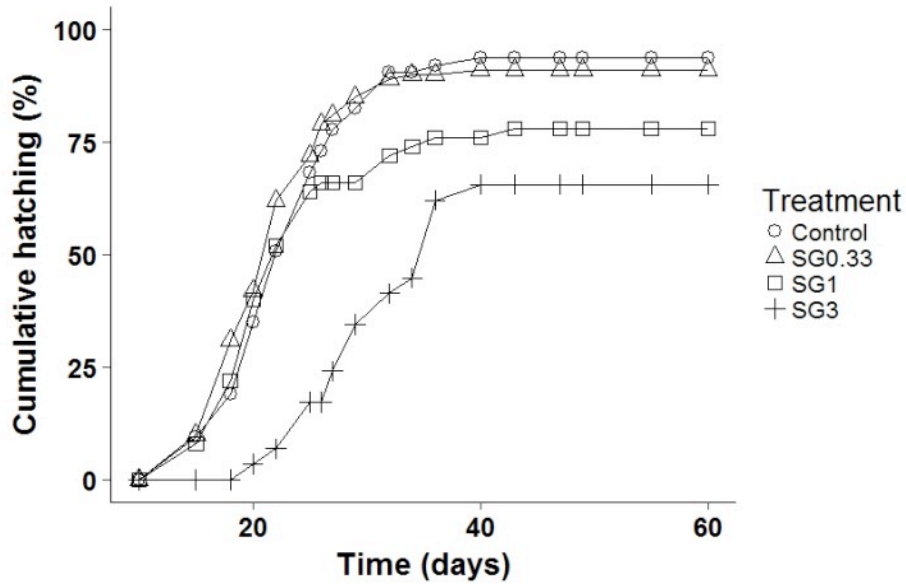
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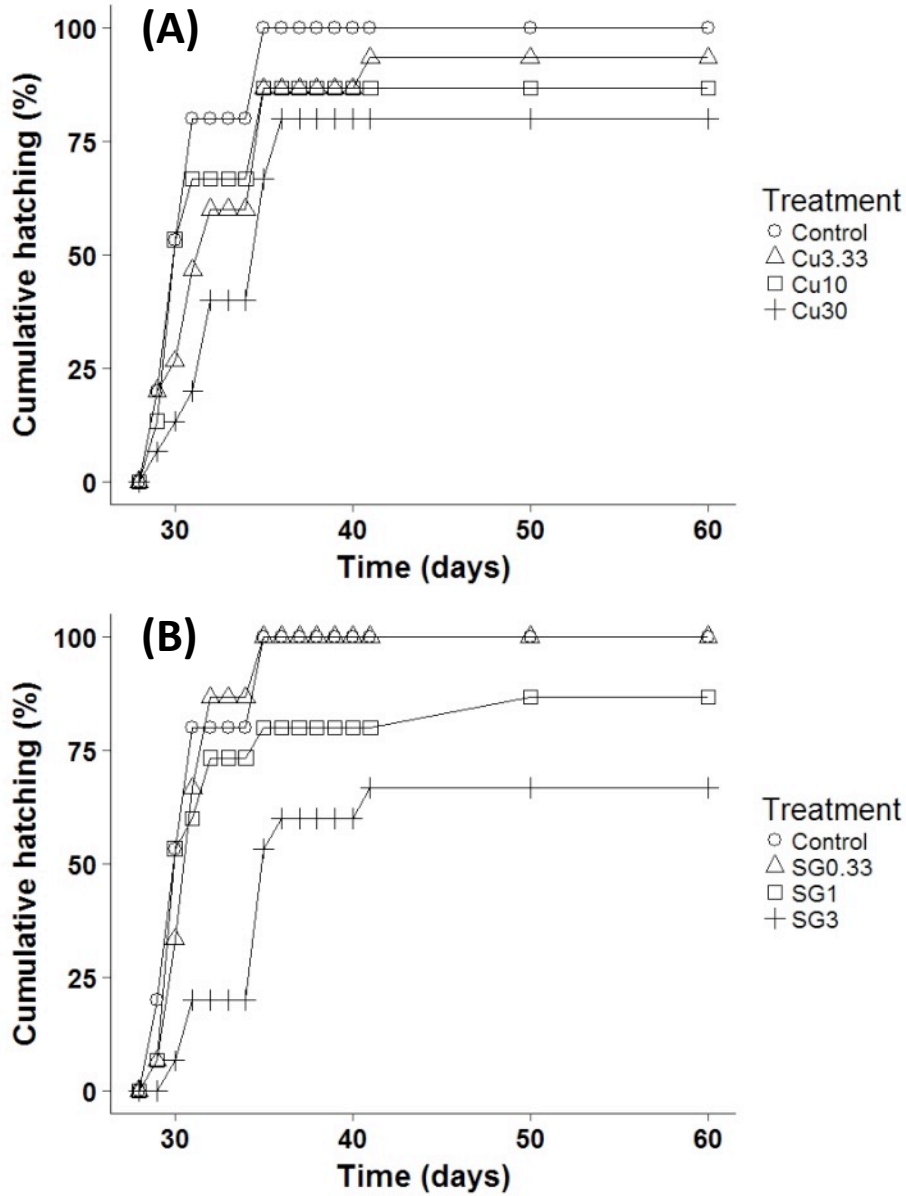
578 **Fig. S1.** Hatching dynamics of cocoons produced for a 28 days period in the control soil and in  
579 soils spiked with different Cuprafor micro<sup>®</sup> concentrations (3.33, 10 and 30 times the RD  
580 corresponding to 25.8, 77.5 and 232.5 mg kg<sup>-1</sup> of copper abbreviated Cu3.3, Cu10 and Cu30  
581 respectively).



582

583 **Fig. S2.** Hatching dynamics of cocoons produced for a 28 days period in the control soil and in  
584 soils spiked with different Swing Gold<sup>®</sup> concentration (0.33, 1 and 3 times the RD abbreviated  
585 SG0.33, SG1 and SG3 respectively).

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589

590 **Fig. S3.** Hatching dynamics of cocoons produced for a 7 days period in the breeding culture  
 591 and incubated at 20°C in the control soil or in soils spiked with **(A)** different Cuprafor micro<sup>®</sup>  
 592 concentrations (3.33, 10 and 30 times the RD corresponding to 25.8, 77.5 and 232.5 mg kg<sup>-1</sup> of  
 593 copper abbreviated Cu3.3, Cu10 and Cu30 respectively) or **(B)** different Swing Gold<sup>®</sup>  
 594 concentrations (0.33, 1 and 3 times the RD abbreviated SG0.33, SG1 and SG3 respectively).

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596