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

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Highlights

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

Abstract

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

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

Keywords: Ecotoxicology; Ecological risk assessment; Soil invertebrates; Lumbricidae; Pesticide

1. Introduction

During the last decades, the increasing demand for food products has led to changes in land use and management. Along with an increase in cultivated areas, the enhancement of yields has been promoted by the use of chemical inputs such as pesticides (Tilman et al., 2002). In temperate soils, earthworms represent a large proportion of the living biomass (Gobat et al., 2004). These primary consumers of organic matter are involved in soil structuring, and thus maintain soil health (Bertrand et al., 2015; Blouin et al., 2013). While earthworm densities generally range from 200 to 400 individuals m⁻² in permanent grasslands (Amossé et al., 2018; Beylich and Graefe, 2012; Edwards and Bohlen, 1996), they can be lower than 100 individuals m⁻² in conventional agricultural fields (Scullion et al., 2002). Mechanical tillage such as ploughing has a direct negative impact on earthworm populations (Chan, 2001; Mele and Carter, 1999). However, the impact of pesticides on earthworm populations under field conditions is much harder to assess and rarely documented. Using a concentration-response-based field experiment, Schnug et al. (2015) showed that a mixture of three biocides (i.e., the insecticide esfenvalerate, the fungicide picoxystrobin and the bactericide triclosan) affected earthworm abundance and juvenile proportions. These authors also highlighted that pesticide concentrations corresponded to the EC₅₀ for *E. fetida* reproduction (Schnug et al., 2013; Schnug et al., 2014) and had effects on earthworms in the field exceeding 50%, indicating that the effects observed in the field could be greater than those observed under laboratory conditions.

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

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

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

2. Materials and methods

2.1. Soil, animals, and pesticides

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

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

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

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

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

with mg ha⁻¹ = 10 000 000 mg Cu, area = 1 000 000 dm², a soil density of 1.29 kg dm⁻³, and considering that copper was mainly found in the top 0.5 dm of soil (Couto et al., 2015). The RD was thus calculated as 15.5 mg kg⁻¹ of dry soil (corresponding to 7.75 mg kg⁻¹ of copper). We used the following concentrations for all experiments: 3.33, 10 and 30 times the RD abbreviated as Cu3.33, Cu10 and Cu30, which correspond to 25.8, 77.5 and 232.5 mg kg⁻¹ of copper, respectively. These concentrations were expected to be sub-lethal, considering that the LC50 for *Eisenia fetida* has been estimated to be > 486.6 mg kg⁻¹ of copper (which corresponds to 63 times the RD) (PPDB, 2017). The second fungicide was Swing Gold® (BASF Agro SAS, dimoxystrobin 133 g L⁻¹, epoxiconazole 50 g L⁻¹), used to protect cereal crops in conventional farming. The French Recommended Dose (RD) for this product is 1.5 L ha⁻¹ on wheat (ANSES, 2017a). Using the same formula as for copper, the laboratory RD was calculated as 1.16 10⁻³ mL kg⁻¹ of dry soil, with the same soil density and considering that the active compounds of

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

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

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

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

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

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

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

2.4. Statistical analyses

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

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

3. Results

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

The effects of Cuprafor micro[®] on earthworm biomass, cocoon production, and cocoon weight are presented in Fig. 1. The hatching time and success of the produced cocoons are presented in Table 1, and the hatching dynamics (all replicates included) in Fig. S1.

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

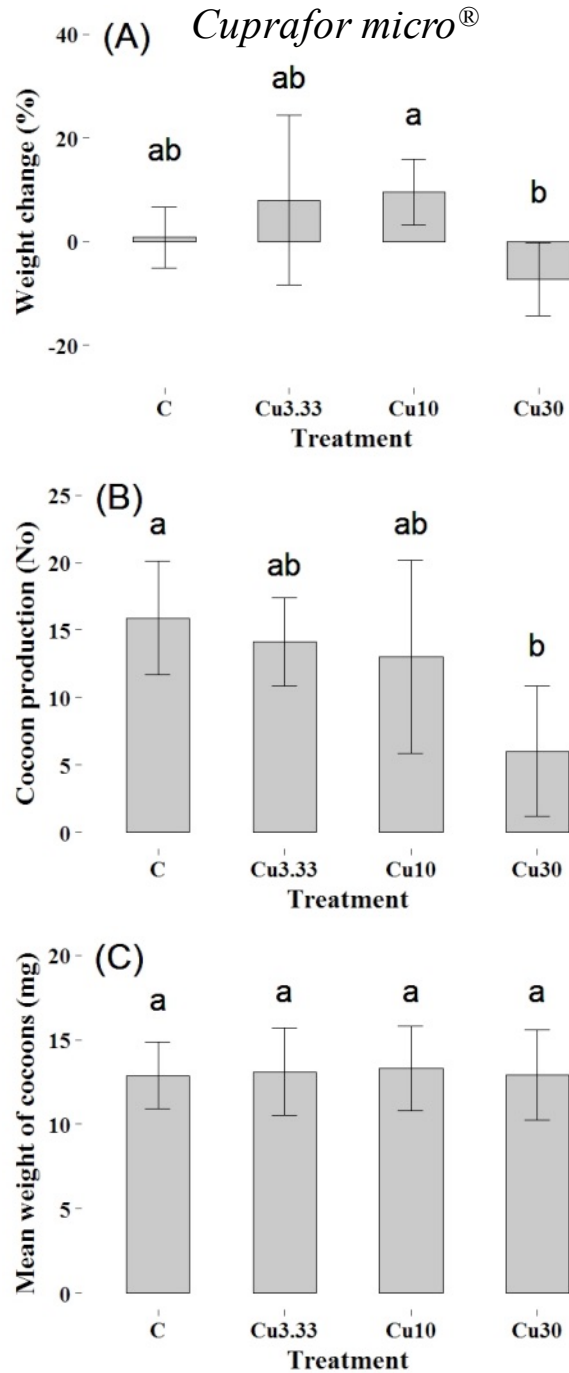


Fig. 1. (A) Weight change (% of difference between the beginning and the end of the experiment, 28 days), (B) cocoon production (number of cocoons per vessel produced by 4 individuals), and (C) mean weight of the cocoons produced by earthworms exposed to control soil or soils spiked with different concentrations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

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

Table 1. Mean hatching time (days) and mean hatching success (%) of cocoons produced by *A. caliginosa* adults exposed to Cuprafor micro^{®a}

	Treatment ^b			
	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

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

^b Different letters indicate significant differences among concentrations (p<0.05).

The effects of Swing Gold[®] on earthworm biomass, cocoon production, and cocoon weight are presented in Fig. 2. The hatching time and success of the produced cocoons are presented in Table 2, and the hatching dynamics (all replicates included) in Fig. S2.

At the end of the experiment, no mortality was recorded in the SG0.33 treatment. 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[®] 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[®] concentrations
248 did not influence cocoon weight (Fig. 2 C).

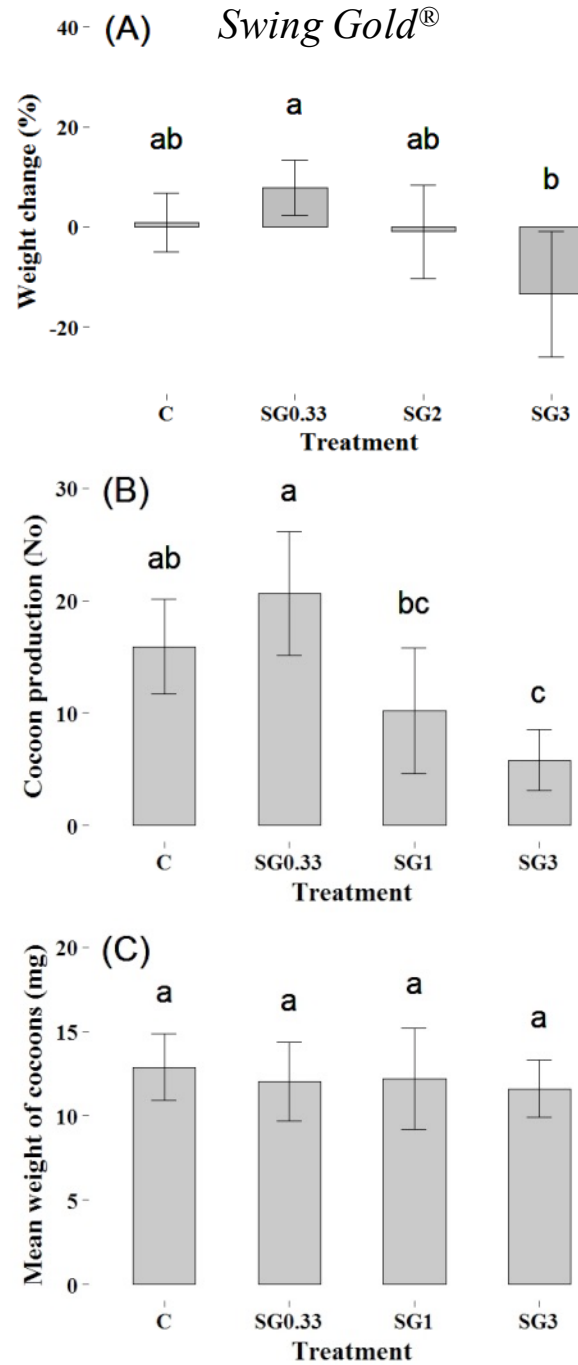


Fig. 2. (A) Weight change (% of the difference between the beginning and the end of the experiment, 28 days), (B) cocoon production (number of cocoons per vessel produced by 4 individuals), and (C) mean weight of the cocoons produced by earthworms exposed to control soil or soils spiked with different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

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

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

	Treatment ^b			
	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

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

^b Different letters indicate significant differences among the concentrations (p<0.05).

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

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

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

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

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

Table 3. Mean hatching time (days) and mean hatching success (%) of “naïve” *A. caliginosa* cocoons^a exposed to Cuprafor micro^{®b}.

	Treatment ^c			
	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

^a Cocoons were produced over a 7-day period in the breeding culture

^b Cocoons were incubated at 20°C in the control soil C or in soils spiked with different Cuprafor micro[®] concentrations (3.33, 10 and 30 times the RD, corresponding to 25.8, 77.5 and 232.5 mg kg⁻¹ of copper, abbreviated Cu3.3, Cu10 and Cu30, respectively).

^c Different letters indicate significant differences among the concentrations (p<0.05).

Table 4. Mean hatching time (days) and mean hatching success (%) of “naïve” *A. caliginosa* cocoons^a exposed to Swing Gold^{®b}.

	Treatment ^c			
	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

^a Cocoons were produced over a 7-day period in the breeding culture

^b Cocoons were incubated at 20°C in the control soil C or in soils spiked with different Swing Gold[®] concentrations (0.33, 1 and 3 times the RD, abbreviated SG0.33, SG1 and SG3, respectively).

^c Different letters indicate significant differences among the concentrations (p<0.05).

4. Discussion

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

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

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

A. caliginosa adults produced one cocoon per individual per week in the control treatment. This is in accordance with other studies (Lofs Holmin, 1982; Spurgeon et al., 2000), which found between 0.6 to 2.6 cocoons per individual per week at 15 °C in natural soils for *A. caliginosa*. Cocoon production sharply dropped at the two highest concentrations of both fungicides, although partially explained by the mortality rate in the SG3 treatment. Other studies with the same species reported EC₅₀ values for cocoon production between 68 and 186 mg Cu kg⁻¹ (Khalil et al., 1996b; Ma 1988). Similarly, Martin (1986) estimated a NOEC of 50 mg Cu kg⁻¹ for *Aporrectodea tuberculata*, which belongs to the *A. caliginosa* complex of

species (Sims and Gerard, 1999). Mirmonsef et al. (2017) found an EC₅₀ value of 220 mg kg⁻¹ for *Aporrectodea tuberculata*. In our experiment, copper toxicity was a little lower than previously reported because the addition of horse manure may have decreased copper bioavailability (Ma and Rao, 1997) for earthworms and thus alleviated its ecotoxicological effects. Copper toxicity highly depends on the copper form and the soil (EU 2008).

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

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

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

5. Conclusions

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

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

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supplementary material

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

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

Cuprafor micro [®]			
Abbreviation	Times RD	Commercial form (mg kg ⁻¹)	Copper (mg kg ⁻¹)
Cu3.33	3.33	52	25.8
Cu10	10	155	77.5
Cu30	30	465	232.5

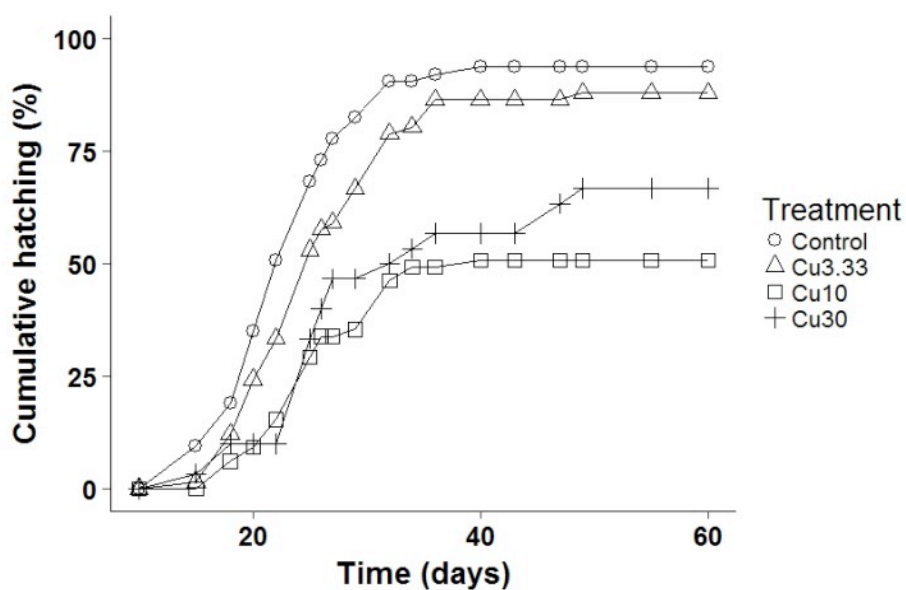


Fig. S1. Hatching dynamics of cocoons produced for a 28 days period in the control soil and in soils spiked with different Cuprafor micro[®] concentrations (3.33, 10 and 30 times the RD corresponding to 25.8, 77.5 and 232.5 mg kg⁻¹ of copper abbreviated Cu3.3, Cu10 and Cu30 respectively).

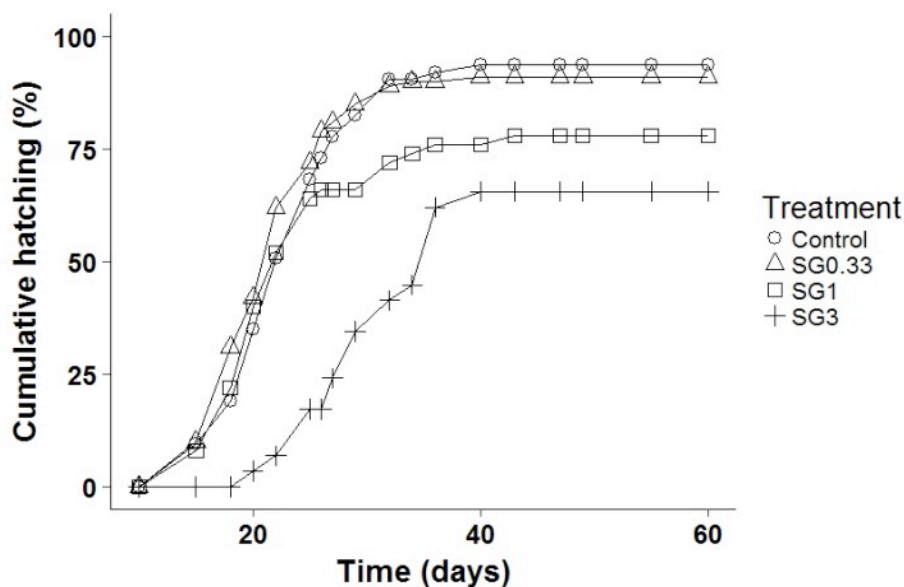


Fig. S2. Hatching dynamics of cocoons produced for a 28 days period in the control soil and in soils spiked with different Swing Gold[®] concentration (0.33, 1 and 3 times the RD abbreviated SG0.33, SG1 and SG3 respectively).

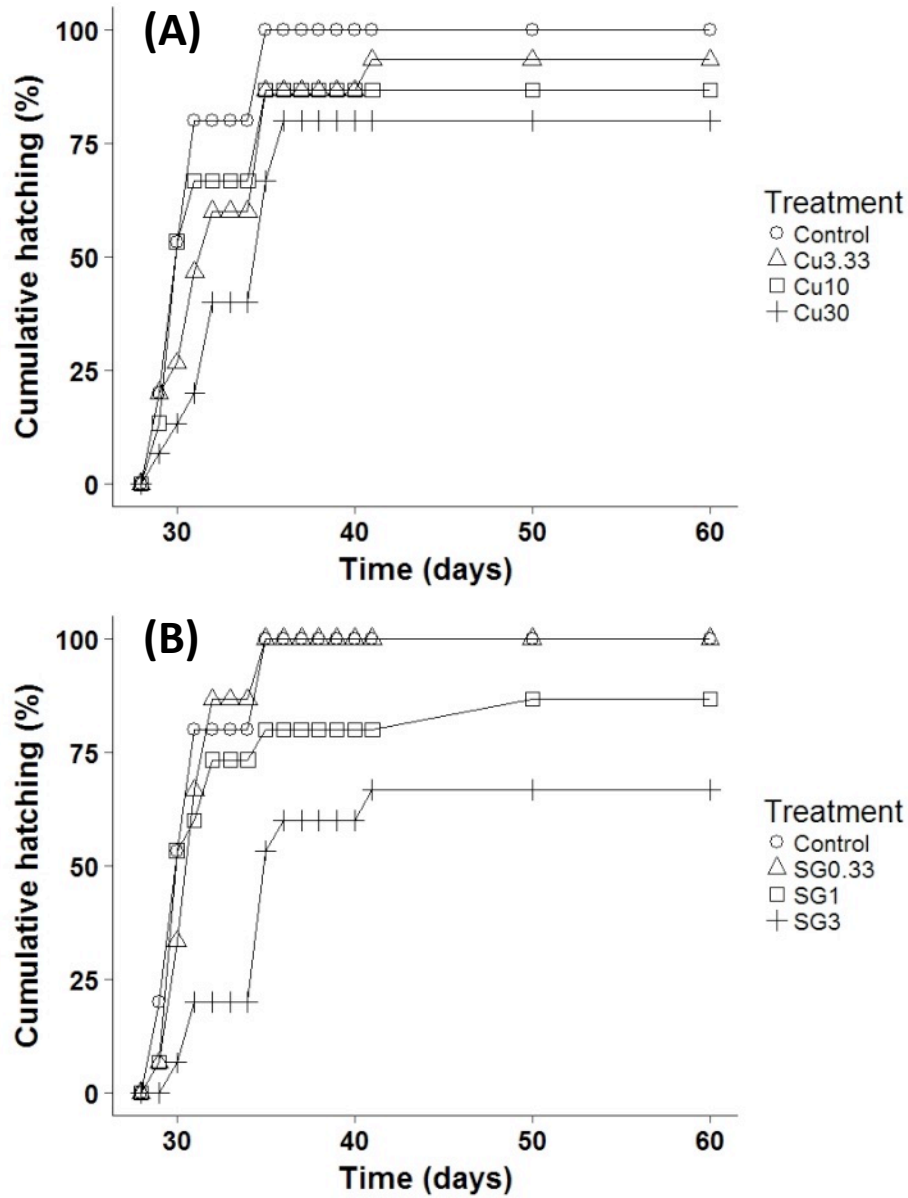


Fig. S3. Hatching dynamics of cocoons produced for a 7 days period in the breeding culture and incubated at 20°C in the control soil or in soils spiked with **(A)** different Cuprafor micro® concentrations (3.33, 10 and 30 times the RD corresponding to 25.8, 77.5 and 232.5 mg kg⁻¹ of copper abbreviated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