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Differences in sensitivity between earthworms and enchytraeids exposed to two commercial fungicides

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Highlights

- Effects of two commercial formulations of fungicides were assessed on oligochaetes
- Negative effects were found on their survival, biomass, and avoidance
- No acute effects were measured at the field recommended dose
- Enchytraeids were less sensitive than earthworms to the tested fungicides

Abstract

The use of pesticides in crop fields may have negative effects on soil *Oligochaeta Annelida*, i.e., earthworms and enchytraeids, and thus affect soil quality. The aim of this study was to assess the effects of two commercial fungicide formulations on the earthworm *Aporrectodea caliginosa* and the enchytraeid *Enchytraeus albidus* in a natural soil. The fungicides were Cuprafor micro® (copper oxychloride), commonly used in organic farming, and Swing Gold® (epoxiconazole and dimoxystrobin), a synthetic fungicide widely used in conventional farming to protect cereal crops. Laboratory experiments were used to assess the survival, biomass loss and avoidance behaviour. No lethal effect was observed following exposure to the copper fungicide for 14 days, even at 5,000 mg kg⁻¹ of copper, i.e. 650 times the recommended dose (RD). However, a significant decrease in biomass was observed from 50 mg kg⁻¹ of copper (6.5 times the RD) for *A. caliginosa* and at 5,000 mg kg⁻¹ of copper (650 times the RD) for *E. albidus*. These sublethal effects suggest that a longer period of exposure would probably have led to lethal effects. The EC50 avoidance for the copper fungicide was estimated to be 51.2 mg kg⁻¹ of copper (6.7 times the RD) for *A. caliginosa*, and 393 mg kg⁻¹ of copper (51 times the RD) for *E. albidus*. For the Swing Gold® fungicide, the estimated LC50 was 7.0 10⁻³ mL kg⁻¹ (6.3 times the RD) for *A. caliginosa* and 12.7 10⁻³ mL kg⁻¹ (11.0 times the RD) for *E. albidus*. No effect on biomass or avoidance was observed at sublethal concentrations of this synthetic fungicide. It was concluded that enchytraeids were less sensitive than

earthworms to the two commercial fungicides in terms of mortality, biomass loss and avoidance behaviour. Therefore we discuss the different strategies possibly used by the two *Oligochaeta* species to cope with the presence of the pesticides were discussed, along with the potential consequences on the soil functions.

Keywords

Potworms, ecotoxicology, pesticides, risk assessment, mortality.

1. Introduction

Pesticide applications in agricultural systems decrease biodiversity and biological activity in cultivated soils (Bengtsson et al., 2005; Hole et al., 2005; Pelosi et al., 2014). The non-intentional effects of pesticides on soil organisms have to be understood and quantified in order to assess their possible consequences on soil functioning (Lavelle et al., 2006; Blouin et al., 2013; Bertrand et al., 2015). Earthworms are often used as soil biological indicators (Spurgeon et al., 2003). They represent the largest living biomass in terrestrial ecosystems (Edwards, 2004) and have positive impacts on soil structure and porosity, nutrient cycling, and organic matter fragmentation (Lavelle et al., 2004; Seeber et al., 2008; Bottinelli et al., 2010). Although enchytraeids are less studied, they have also been recognized as indicators of soil quality (Graefe and Beylich, 2002; Jänsch et al., 2005), chemical stress (Didden and Römbke, 2001) and agricultural management practices (Pelosi and Römbke, 2016). Like earthworms, enchytraeids play key functional roles in the soil but at a smaller scale (van Vliet et al., 1993; Marinissen and Didden, 1997). They are involved in the evolution of the soil structure (Didden, 1990; van Vliet et al., 1995). Moreover, they influence organic matter decomposition and nutrient cycling (i) directly by consuming large amounts of organic matter (Briones et al., 1998; Cole et al., 2000; Maraldo et al., 2011), and (ii) indirectly by creating favourable microhabitats for microbial activity (Kasprzak 1982; Bardgett 2005).

Interactions between earthworms and enchytraeids can increase organic matter consumption and decomposition rates as compared to the same organisms taken separately (Sandor and Schrader 2012). In a cultivated soil under tillage pressure, enchytraeid activity increased when earthworm activity decreased (Topoliantz et al. 2000). The authors concluded that enchytraeids played a key role for soil structure and porosity when earthworm density was low. Thus, the use of enchytraeids in addition to earthworms, for testing chemicals, may provide relevant and complementary information. This is unfortunately rarely documented.

In 2009, Marques et al. highlighted “a need for a careful assessment of the impacts of formulated products”. Indeed, most of the studies assessing the impact of pesticides on *Oligochaeta Annelida* had used only active substances or pesticides no longer authorized in Europe (Kobeticova et al., 2009; Pelosi et al., 2014). The effects of active substances can substantially differ from those of commercial formulations because formulations can contain more than one active substance as well as adjuvants also likely to have adverse ecotoxicological effects (De Silva et al., 2010; Lajmanovich et al., 2011; Piola et al., 2013).

The aim of the present study was to assess the impact of two commercial pesticide formulations on the survival, biomass and avoidance behaviour of two species of terrestrial oligochaetes: an earthworm species, *Aporrectodea caliginosa* and an enchytraeid species, *Enchytraeus albidus*. Experimental studies were conducted under laboratory conditions using a natural soil and different pesticide concentrations. Commercial pesticides formulations were given preference over pure molecules to be as close as possible to the field conditions. The two pesticides are widely used in cultivated fields in Europe. The first one was Cuprafor micro®, a copper fungicide composed of 50% copper oxychloride, used in organic farming to prevent spore germination. The second one was Swing Gold®, a synthetic fungicide used to protect cereal crops in conventional farming. It is composed of two active substances, dimoxystrobin (DMX) and epoxiconazole (EPX) recognized as dangerous for the environment as well as carcinogenic, mutagenic and reprotoxic (PPDB, 2016). *Eisenia* sp. is currently recommended as the model earthworm species in standardized tests (OECD 1984). However, it is not found in arable lands and is less sensitive to pesticides and metabolites than species found in cultivated fields (Pelosi et al., 2013). That is why we chose *Aporrectodea caliginosa*, a dominant earthworm species in cultivated fields in temperate areas (Bouché 1972; Perez-Losada et al., 2009; Decaëns et al., 2011), for this study. As knowledge about enchytraeids is relatively poor and species found in fields are difficult to breed, we used *Enchytraeus albidus*, the species recommended in standardized tests (OECD 220, 2004; Römbke 2003).

2. Material and methods

2.1. Soil, pesticides, animals

The same soil was used for all experiments. We chose a natural soil rather than an artificial soil to be closer to field conditions. It was a Luvisol (FAO soil classification), sampled from a fallow in Versailles (48°48' N, 2°5' E) where no chemicals had been applied for more

than 20 years. It was collected from the top 0-20 cm, air-dried and passed through a 2-mm sieve. Its main physical and chemical properties were as follows: pH 7.5, organic matter 32.6 g kg⁻¹, C/N 12.7, 29 % sand, 48 % silt, and 23% clay (Table 1). The dry soil was stored at 20°C for a maximum of 50 days before the experiments.

The French RD for Cuprafor micro® (Quimicas del Valles, 50% copper oxychloride) is 10 kg ha⁻¹ on potato and in vineyards (E-phy, 2016a). The French RD for Swing Gold® (Bayer Agro SAS, dimoxystrobin 133 g L⁻¹, epoxiconazole 50 g L⁻¹) is 1.5 L ha⁻¹ on wheat (E-phy, 2016b). Considering that the active compounds of this fungicide are almost always found in the top 10 cm of soil (McDonald et al., 2013; Chabauty et al., 2016) the RD for Swing Gold® was calculated as 1.16 10⁻³ mL kg⁻¹ of dry soil, with a soil density of 1.29. Considering that copper is mainly found in the top 5 cm of soil, the Cuprafor micro® RD was calculated as 15.5 mg kg⁻¹ of dry soil. Different concentrations of the two fungicides were tested separately. The natural dry soil was spiked with aqueous solutions of the fungicides, and the soil water holding capacity was adjusted concomitantly.

Mature individuals of *Aporrectodea caliginosa* s.s (Savigny, 1826) were collected by hand-sorting from an agricultural field in Estrées-Mons (49°52' N 3°01' E). They were maintained in the soil used for the experiments at 15 ± 1°C, 24h darkness for at least 7 days before the experiments. Mature individuals of *Enchytraeus albidus* (Henle, 1837) were from a laboratory culture (INRA Versailles-Grignon, France) where they were kept at 15 ± 1°C, 24h darkness in the soil used for the experiments. They were fed once a week with rolled oats.

2.2. Ecotoxicological tests

2.2.1. Acute toxicity tests

The active compound of the Cuprafor micro® formulation is copper oxychloride, whose LC50 for *E.fetida* was found to be > 489.6 mg kg⁻¹ for 14 days of exposure (PPDB, 2016). This data was used to define a range of concentrations as follows: 5, 50, 500, and 5,000 mg kg⁻¹ of copper corresponding to 0.65, 6.5, 65 and 650 times the RD. In the Swing Gold® commercial formulation, DMX is the main active compound (see section 2.1). It also has more potential harmful effects on the studied animals than EPX (For *E. fetida*: the DMX LC50 is 23.65 mg kg⁻¹ and the EPX LC50 is >500 mg kg⁻¹). Moreover, the DMX LC50 for *E. fetida* in a formulation such as Swing Gold® (i.e dimoxystrobin 133 g L⁻¹, epoxiconazole 50 g L⁻¹) was found to be 3.35 mg kg⁻¹ (Agritox 2016). This data was used to define a range of concentrations based on the DMX concentration in Swing Gold®, as follows: 0.01, 0.1, 1, 10 and 50 mg kg⁻¹ of DMX,

corresponding to 0.065, 0.65, 6.5, 65, and 324 times the RD of Swing Gold®. These concentrations are detailed in Table 2, with simplified abbreviations for the different concentrations used in the acute toxicity test, i.e. C1, C2, C3 and C4 for the copper fungicide, and SGA1, SGA2, SGA3, SGA4 and SGA5 for Swing Gold®.

Acute toxicity tests on earthworms were adapted from the ISO 11268-1 (2012). Three replicates were used for each concentration of the two fungicides. For each replicate, 500 g of dry soil and 10 g of horse dung were mixed and placed in a 1 L plastic vessel with a removable perforated cover for gas exchanges. Horse dung used as a food source was frozen and defrosted twice and then milled (<1 mm) before use (Lowe and Butt 2005). Previous tests had revealed the need to supply food to *A. caliginosa* individuals at that density to avoid weight loss in the control treatment after 14 days (personal observation). The soil moisture was adjusted to 65% of the soil water holding capacity using tap water. Five individuals were introduced into each plastic vessel that were stored in a climate room ($15 \pm 1^\circ\text{C}$, 24h darkness) for 14 days. To evaluate earthworm biomass loss during the experiment, individuals were first weighed before being introduced into the vessels, and then after 14 days, at the end of the experiment. Before being weighed, earthworms were individually placed in petri dishes on damp filter paper for 48 hours in the dark at $15 \pm 1^\circ\text{C}$ to void gut contents (Hartenstein et al., 1981). After 14 days, dead earthworms were counted, and the LC50 was calculated (see section 2.3). The health and activity status (active, dead, in quiescence) of each earthworm was visually evaluated at the end of the experiment. Quiescence is a state of reduced metabolic activity during which earthworms remain coiled up in a chamber (Satchell 1967) (see the picture on Fig. 2 and Fig. 3).

Acute toxicity tests on enchytraeids were adapted from the ISO 16387 (2014). Based on the results obtained with Swing Gold® (see section 3.1.2), a second test was performed with lower concentrations (10, 25, and 50 times the RD) to precisely estimate the LC50. Four replicates were used for each treatment of the two fungicides. For each replicate, 30 g of dry soil, and 40 mg of rolled oats as a food source, were mixed and placed in a 75 mL plastic vessel with a removable perforated cover for gas exchanges. Ten individuals were introduced into each plastic vessel. The soil moisture was also adjusted to 65% of the soil water holding capacity using tap water. The vessels were stored in a climate room ($15 \pm 1^\circ\text{C}$, 24h darkness) for 14 days. After 14 days, dead individuals were counted using a binocular microscope and the LC50 was calculated (see section 2.3). To evaluate enchytraeid biomass before pesticide application and after 14 days of exposure, each individual was photographed under a binocular microscope. The volume was then estimated by measuring the length and diameter of each individual using ImageJ software (Schneider et al., 2012). Diameters were measured at two

different positions (anterior and posterior sections) and averaged for each individual. Enchytraeids were considered as cylinders to estimate their volumes, and fresh biomass values were indirectly estimated from their volume multiplied by a factor of 1.051 (Phillipson et al., 1979).

2.2.2 Avoidance behaviour tests

The range of concentrations of each fungicide was determined according to the results of the acute toxicity tests so as to apply sublethal concentrations. The Cuprafor micro® concentrations were the same as those used for the acute toxicity test because no mortality was observed in this test; consequently, abbreviations remained the same (Table 2: C1, C2, C3 and C4). For the Swing Gold®, concentrations were adjusted at 1, 2, 5, and 10 times the RD; abbreviations were SGB1, SGB2, SGB3 and SGB4 (Table 2).

The avoidance behaviour test on earthworms was adapted from the ISO 17512 (2009), using the test procedure with two-section vessels. Five replicates were performed for each concentration of each fungicide and 700 g of dry soil were placed in each plastic vessels (the same vessels as for acute toxicity tests, see section 2.2.1). A removable piece of plastic-coated cardboard was placed in each vessel to divide it into two equal sections. Then, 350 g of the control soil were placed in one-half of the vessel and 350 g of the spiked (i.e. contaminated) soil were inserted into the other half. The plastic divider was removed, and then ten earthworms were placed in the centre of the vessel on the two neighbouring soil types. Soil moisture was adjusted to 65% of the soil water holding capacity. The vessels were stored in a climate room ($15 \pm 1^\circ\text{C}$, 24h darkness) for 48 hours. At the end of the test, the divider was placed again to separate the control soil from the spiked soil, and the number of individuals on each side was counted.

The test procedure for *E. albidus* was the same as for earthworms. The only difference was the amount of soil in the vessels. We used the same vessels as those used for the enchytraeid acute toxicity tests (see section 2.2.1). In each replicate, we inserted 50 g of dry soil, 25 g of the control soil on one side and 25 g of the spiked soil on the other side.

2.3. Statistical analysis

The Rextox model was used to calculate (i) the concentration that was lethal for 50 % of the individuals (LC50), and (ii) the effective concentration that induce 50 % of avoidance (EC50 avoidance) (Vindimian, 2016).

All the other data were analysed with the statistical software R Core Team (2015). For all the statistical tests, differences were considered significant if $p < 0.05$. When normality and homoscedasticity conditions were satisfied, ANOVA and Fisher's least significant difference (LSD) post hoc tests were performed to analyse biomass losses and the ratios of active, quiescent, or dead earthworms between the different tested concentrations of both substances. When normality and homoscedasticity conditions were not satisfied, non-parametric Wilcoxon-Mann-Whitney tests were used.

In the avoidance test, correlations were tested using Spearman's method to determine potential dose-response relationships. Moreover, Kruskal-Wallis tests and post hoc multiple comparison tests were performed to analyse the avoidance response between concentrations tested. The results were graphed in terms of average net-response (NR) expressed as percentage and calculated as follows (Amorim et al., 2005; ISO 17512, 2009): $NR = \left\{ \frac{C-T}{N} \right\} * 100$, where C is the number of earthworms or enchytraeids observed in the control soil; T is the number of earthworms or enchytraeids observed in the treated soil; N is the total number of earthworms or enchytraeids per replicate.

According to the ISO 17512 (2009), the habitat function of the soil was considered as limiting if on average more than 80% of the individuals were found in the control soil. When the avoidance rate was below 25%, the results were considered as a non-response to the polluted soil (Kobeticova et al., 2009).

3. Results

3.1 Acute toxicity tests

3.1.1 Cuprafor micro®

No mortality was observed for enchytraeids and earthworms in the control treatment or following exposure to any of the different concentrations of Cuprafor micro®. Therefore it was not possible to determine an LC50 value for Cuprafor micro® after 14 days of exposure. For earthworms, significant biomass losses of 13.3%, 15.2% and 34.1% were calculated at 50, 500, and 5,000 mg kg⁻¹ of copper (6.5, 65, and 650 times the RD, respectively) compared to the control (Fig. 1). For enchytraeids, a significant biomass loss of 59.3% was recorded at 500 mg kg⁻¹ of copper (650 times the RD) but no significant difference was observed at lower concentrations (Fig. 1).

At the end of the experiment, the number of earthworms in quiescence was significantly higher at 500 and 5,000 mg kg⁻¹ of copper (65 and 650 times the RD, the two highest concentrations) than in the other three treatments (Fig. 2). At these concentrations, 86 % and 66% of the earthworms were found to be in quiescence after 14 days of exposure, respectively. After gut voiding, 20% of the earthworms from the C4 treatment (5,000 mg kg⁻¹ of copper or 650 times the RD) displayed autotomy i.e., separation of a body part (data not shown).

3.1.2 Swing Gold®

No mortality was observed for earthworms in the control, SGA1, and SGA2 treatments. A mortality rate of 53% of the individuals was recorded in the SGA3 treatment, corresponding to 6.5 times the RD. 100% of the individuals died in the SGA4 and SGA5 treatments, corresponding to 65 and 324 times the RD. The LC50 (Table 3) for *A. caliginosa* exposed to Swing Gold® for 14 days was estimated to be 7.0 10⁻³ mL kg⁻¹ dry soil (confidence interval = [5.8 x 10⁻³ - 9.6 x 10⁻³]) of the commercial formulation or 6.3 times the RD (confidence interval = [4.9 - 8.5]), corresponding to 0.97 mg kg⁻¹ dry soil of DMX (confidence interval = [0.80 - 1.29]) and 0.37 mg kg⁻¹ dry soil of EPX (confidence interval = [0.29 - 0.47]). For the surviving earthworms, no significant difference in biomass loss was observed between the different treatments (7.8%, 11.3%, 9.5%, and 10% of biomass loss for the control, 0.065, 0.65, and 6.5 times the RD respectively). At the end of the experiment, no significant difference in the ratio of quiescence of the surviving earthworms was observed between the treatments (Fig. 3).

No mortality was observed for enchytraeids in the control, SGA1, SGA2 and SGA3 treatments. However, 100% mortality occurred in the SGA4 and SGA5 treatments, corresponding to 65 and 324 times the RD. Due to this marked threshold effect, it was not possible to accurately estimate an LC50 value from this first experiment. In the second experiment, 30% of mortality occurred at 10 times the RD and 100% mortality occurred at 25 and 50 times the RD. According to these results, the LC50 (Table 3) for *E. albidus* exposed to Swing Gold® for 14 days was estimated to be 12.7 x 10⁻³ mL kg⁻¹ (confidence interval = [12.0 x 10⁻³ - 14.2 x 10⁻³]) of the commercial formulation or 11.0 times the RD (confidence interval = [10.3 - 12.2]), corresponding to 1.69 mg kg⁻¹ of DMX (confidence interval = [1.598 - 1.893]) and 0.63 mg kg⁻¹ of EPX (confidence interval = [0.287 - 0.471]). For the surviving enchytraeids (in the control and in 0.065, 0.65 and 6.5 times the RD), no significant differences in biomass loss were observed between the different treatments (2.5%, 9.5% and 10% of biomass gain for the control, 0.065 and 0.65 times the RD, respectively, and 12% of biomass loss for 6.5 times the RD).

3.2 Avoidance behaviour tests

A significant positive correlation was found between the Cuprafor micro® concentration and earthworm avoidance responses (Fig. 4 A). Based on the 80% effect criterion (ISO 17512, 2009), avoidance was significant in the C3 and C4 treatments (500 and 5,000 mg kg⁻¹ of copper, or 65 and 650 times the RD, respectively). The EC50 avoidance value for *A. caliginosa* (Table 3) was estimated to be 51.2 mg kg⁻¹ of copper (confidence interval = [46.0 – 77.5]) corresponding to 6.7 times the RD (confidence interval = [6.0 – 10.1]) and 102 mg kg⁻¹ of Cuprafor micro® (confidence interval = [92 - 155]).

A significant positive correlation was found between the Cuprafor micro® concentration and enchytraeid avoidance responses (Fig. 4 B). Based on the 80% effect criterion (ISO 17512, 2009), avoidance was significant in the C4 treatments (5,000 mg kg⁻¹ of copper or 650 times the RD). The EC50 avoidance value for *E. albidus* (Table 3) was estimated to be 393 mg kg⁻¹ of copper (confidence interval = [206 – 557]) corresponding to 51 times the RD (confidence interval = [27 – 72]) and 786 mg kg⁻¹ of Cuprafor micro® (confidence interval = [412 - 1114]).

For the Swing Gold® formulation, the threshold value of 80% was not reached at any of the tested concentrations (1, 2, 5, and 10 times the RD) and no significant dose-response relationship was observed for earthworms or enchytraeids (Fig. 5). Therefore, it was not possible to determine an EC50 avoidance value for Swing Gold®.

4. Discussion

4.1 The copper fungicide

We found no lethal effect of the copper fungicide on both species of terrestrial *Oligochaeta*. The high soil organic matter content (i.e. meadow soil, see Table 1) and the addition of horse manure may have decreased copper bioavailability (Ma and Rao, 1997) for earthworms and thus alleviate its ecotoxicological effects. However, the high rate of quiescence of earthworms, probably allowing them to avoid lethal direct effects by soil ingestion, and the biomass loss at the end of the experiment evidenced the adverse effects of the copper fungicide. Longer exposure would have probably led to lethal effects. Indeed, Khalil et al. (1996a) found LC50 values for *A. caliginosa* of 1 425 and 640 mg kg⁻¹ copper sulfate after 14 and 56 days of

exposure respectively in a natural soil. Copper could thus have harmful long-term effects that could lead to death, depending on the concentrations and the length of exposure. In accordance with our results, Paoletti et al. (1998) showed that *A. caliginosa* biomass and abundance were negatively correlated to copper concentration in a vineyard soil. Furthermore, Eijsackers et al. (2005) showed that “vineyard soil conditions resulted in lower growth rates than in grassland soils”. This lower growth rate could delay earthworm maturation and thereby negatively affect reproduction rates. These authors concluded that copper oxychloride may hinder the population development and dispersal. Similarly, several authors highlighted the negative effects of copper fungicides on earthworm growth (Khalil et al., 1996b; Reinecke et al., 2002) and cocoon production (Helling et al., 2000) from 100 mg kg⁻¹ of copper. These concentrations, as those tested in our study, could be reached in agricultural systems because copper accumulates in soils (Brun et al., 1998).

For enchytraeids, as for earthworms, we found sublethal effects (i.e. biomass loss at the highest concentration) that could have led to lethal effects over a longer period of exposure. Copper was also found to induce other sublethal effects. For instance, Konečný et al. (2014) found that soils from a copper smelter (containing concentrations ranging between 37 and 8,980 mg kg⁻¹ of copper) had negative effects on *Enchytraeus crypticus* reproduction with an estimated EC50 of 351 mg kg⁻¹ of copper in the soil. In our study, the biomass loss of enchytraeids in the control treatment may have been due to an insufficient food supply for 14 days of experiment, although the supply was based on the recommendation given in the ISO 16387 (2014).

Moreover, our results highlighted a great ability of the tested animals to avoid the soil contaminated with the copper fungicide (with EC50 avoidance values of 51.2 mg kg⁻¹ and 393 mg kg⁻¹ of copper for earthworms and enchytraeids, respectively). For earthworm, our results are in accordance with other studies showing avoidance behaviour of *A. caliginosa* and other earthworm species when exposed to copper and other trace elements in soils (Eijsackers et al., 2005; Lukkari and Haimi, 2005). In our study, when earthworms could not escape from the contaminated zones, earthworms displayed another resistance mechanism by entering into quiescence. Quiescence is a period during which development is suspended, as a result of adverse environmental conditions (Diaz Cosin et al., 2006), in our case by contamination by a copper fungicide. For the two highest copper concentrations (500 and 5000 mg kg⁻¹ of copper), we observed 86% and 66% of earthworms in quiescence, respectively. This can probably explain the biomass loss. The autotomy observed among earthworms at the highest copper

concentration could be a detoxification process aimed at eliminating the copper from their body. Maleri et al. (2008) showed that *A. caliginosa* could concentrate trace elements (i.e. Cr, Mn and Ni) in their posterior region. The authors explained that they probably did so to get rid of pollutants by autotomy. These two processes, i.e. quiescence and autotomy, can be considered as survival strategies of *A. caliginosa* exposed to high copper concentrations, and probably to other trace elements in soils. For enchytraeids, Amorim et al. (2008) found 80% of avoidance response in a standard sandy loam LUFA 2.2 soil (Speyer, Germany) contaminated with 320 mg kg⁻¹ CuCl₂. We found an EC50 avoidance value of 393 mg kg⁻¹ of copper, corresponding to 51 times the RD of Cuprafor micro®. This difference could be explained by the soil used in both experiments since the bioavailability of some pollutants including trace elements has been found to be higher in standardized soil than in natural soil (e.g., Smit and Van Gestel 1996). Amorim et al. (2012), in a natural soil, found an EC50 avoidance value of 475 mg kg⁻¹ of CuCl₂ salt for *E. albidus*, which is close to our result (i.e. 393 mg kg⁻¹). Thus, as already demonstrated by some authors, the choice of the soil in ecotoxicological tests is crucial (Amorim et al., 2005).

4.2 The Swing Gold® fungicide

The responses of the tested annelids to the synthetic fungicide Swing Gold®, used in conventional farming, were qualitatively very different from those obtained with the copper fungicide. First of all, neither species was able to avoid the fungicide, even at 10 times the RD. These results suggest that both species could undergo potential lethal and sublethal (growth, reproduction) effects in the field. Moreover, mortality rates allowed us to estimate LC50 values, i.e., 6.3 and 11.0 times the RD for earthworms and enchytraeids, respectively. No data were available about enchytraeid sensitivity to the two active compounds of the Swing Gold® formulation, i.e., DMX and EPX. However, the earthworm species *E. fetida* presented LC50 values > 500 mg kg⁻¹ dry soil for EPX (PPDB, 2016) and 23.65 mg kg⁻¹ dry soil for DMX (PPDB, 2016). We did not find any LC50 value for EPX in a formulation such as Swing Gold® (i.e., 133 g L⁻¹ of dimoxystrobin and 50 g L⁻¹ of epoxiconazole). However, for DMX in such a commercial formulation, the LC50 value of *E. fetida* was 3.35 mg kg⁻¹ dry soil (Agritox, 2016), more than three times higher than the LC50 value found in this study for *A. caliginosa* (0.97 mg kg⁻¹ dry soil for DMX). Although the conditions of the tests were not the same, it appeared that *A. caliginosa* was more sensitive than *E. fetida* to the tested pesticide. This is in accordance with previous studies showing lower sensitivity of *E. fetida* to pesticides as compared to species found in mineral soils (Pelosi et al., 2013). Moreover, according to regulatory test data (Agritox,

2016), DMX is seven times more harmful to *E. fetida* when tested in a formulation with an LC50 value of 3.35 mg kg⁻¹ dry soil, than when it is used as a pure molecule (LC50 of 23.65 mg kg⁻¹ dry soil). This substantial difference is probably due to the mixture of the two active substances as well as the adjuvants whose composition is unknown. Pelosi et al. (2016) investigated the effects of a commercial formulation of EPX on *Aporrectodea icterica* in a natural loamy soil. The authors found an LC50 of 45.5 mg kg⁻¹ dry soil, which is more than 10 times lower than the LC50 of *E. fetida* in standard condition tests with pure EPX. They suggested that this difference could be due to the use of a commercial formulation instead of a pure molecule, and/or to the lower sensitivity of *E. fetida* as compared to *A. icterica*. Thereby, along with other authors (Da Silva et al., 2010; Lajmanovich et al. 2011), Pelosi et al. (2016) underlined the importance of (i) comparing the toxicity of commercial formulations with that of pure molecules, and (ii) working on species that can be found in arable lands in addition to *E. fetida*.

4.3 Difference in sensitivity between earthworms and enchytraeids, and potential consequences on the services annelids can provide in crop fields.

This study showed that the LC50 value of earthworms exposed to for Swing Gold® was almost half as much as that of enchytraeids (6.3 and 11.0 times the RD for earthworms and enchytraeids, respectively). When exposed to the copper fungicide, earthworms lost biomass and avoided the polluted soil at a lower concentration than enchytraeids. Considering these results, enchytraeids were less sensitive than earthworms to the tested fungicides. These results could depend on the studied *Oligochaeta* species. Nevertheless, our findings are in accordance with other studies that suggest a difference in sensitivity to chemicals between earthworms and enchytraeids (Bezchlebova et al., 2007). For instance, Leitão et al. (2014) assessed the impact of azoxystrobin, chlorothalonil and ethoprophos on the reproduction of an earthworm (*Eisenia fetida*), a collembolan (*Folsomia candida*) and an enchytraeid species (*Enchytraeus crypticus*): enchytraeids were the least sensitive species. Similarly, Kobetičová et al. (2010) found that *Eisenia fetida* presented a greater ability to avoid a contaminated soil than *Enchytraeus crypticus*. Under field conditions, McColl (1984) found no effect of a nematicide on enchytraeids while earthworm numbers depressed. The difference in sensitivity between these two *Oligochaeta Annelida* families could have important consequences on the soil functioning. Actually, as enchytraeids provide key roles in soils, close to those of earthworms, so that they could perform functions related to the evolution of the soil structure and organic matter that

earthworms can no longer perform under pesticide pressure (Topoliantz et al., 2000). We support the need to further investigate this difference in sensitivity to assess the ecotoxicity of pesticide applications in the field and understand the impact on soil functioning and ecosystem services.

5. Conclusion

Acute toxicity and avoidance tests provided complementary information that allowed us to compare the sensitivity of earthworms and enchytraeids to two commonly used pesticides. The acute toxicity test assessed lethal (i.e., acute) effects of pesticides and a potential effect on biomass. As underlined by Römbke (2003), the avoidance test is relevant for a first evaluation of pesticide toxicity over a short period of time i.e. 48 hours. It can provide useful information not only on differences between annelid behavioural patterns in the field but also on their ability to avoid potential lethal and sublethal effects. This could provide clues to understand why some species are not found in the pesticide-treated fields and determine the potential consequences for soil functioning (e.g. Pelosi et al., 2014).

Our study also highlighted that considering the two groups of terrestrial *Oligochaeta*, i.e., earthworms and enchytraeids, is of prime importance when assessing the effects of pesticides. While earthworms might escape from or die in pesticide-treated areas, enchytraeids could provide functions such as organic matter decomposition that earthworms can no longer provide. We also underlined once again that it is quite relevant to study commercial pesticide formulations to assess the potential negative effects of adjuvants and substance mixtures. In the same way, and to address the challenge of putting more ecology into ecotoxicology (Beketov and Liess, 2012; Chapman, 2002), it is recommended to work on species that live in mineral soils where pesticides are applied. Moreover, not only do we have to better investigate the sublethal effects (e.g., on reproduction and growth) of pesticides to understand their impacts at the population level, but we also need to study the effects of low concentrations such as field recommended doses on the behaviour of soil organisms. This could pave the way for a better assessment of the consequences of pesticide use on the “things that matter” in protecting the environment, i.e., ecosystem services (Forbes and Callow, 2012).

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References

- Amorim, M. J. B., J. Rombke, A. Scheffczyk and A. Soares, 2005. Effect of different soil types on the enchytraeids *Enchytraeus albidus* and *Enchytraeus luxuriosus* using the herbicide Phenmedipham. *Chemosphere*. 61, 1102-1114.
- Amorim, M. J. B., S. Novais, J. Rombke and A. Soares, 2008. *Enchytraeus albidus* (*Enchytraeidae*): A test organism in a standardised avoidance test? Effects of different chemical substances. *Environ. Int.* 34, 363-371.
- Amorim, M. J. B. and J. J. Scott-Fordsmand, 2012. Toxicity of copper nanoparticles and CuCl₂ salt to *Enchytraeus albidus* worms: Survival, reproduction and avoidance responses. *Environ. Pollut.* 164, 164-168.
- Bardgett, R.D., 2005. *The Biology of Soil*. Oxford University Press Inc., New York.
- Beketov M.A., Liess M., 2012. Ecotoxicology and macroecology--time for integration. *Environ. Pollut.* 162, 247-254.
- Bengtsson, J., J. Ahnstrom and A. C. Weibull, 2005. The effects of organic agriculture on biodiversity and abundance: a meta-analysis. *J. Appl. Ecol.* 42, 261-269.
- Bertrand, M., S. Barot, M. Blouin, J. Whalen, T. de Oliveira and J. Roger-Estrade, 2015. Earthworm services for cropping systems. A review. *Agron. Sustainable Dev.* 35, 553-567.
- Bezchlebova, J., J. Cernohlavkova, J. Lana, I. Sochova, K. Kobeticova and J. Hofman, 2007. Effects of toxaphene on soil organisms. *Ecotoxicol. Environ. Saf.* 68, 326-334.
- Blouin, M., M. E. Hodson, E. A. Delgado, G. Baker, L. Brussaard, K. R. Butt, J. Dai, L. Dendooven, G. Peres, J. E. Tondoh, D. Cluzeau and J. J. Brun, 2013. A review of earthworm impact on soil function and ecosystem services. *Eur. J. Soil Sci.* 64, 161-182.
- Bottinelli, N., T. Henry-des-Tureaux, V. Hallaire, J. Mathieu, Y. Benard, T. D. Tran and P. Jouquet, 2010. Earthworms accelerate soil porosity turnover under watering conditions. *Geoderma*. 156, 43-47.
- Bouché, M. B., 1972. *Lombriciens de France. Ecologie et Systématique*. INRA Ann. Zool. Ecol. Anim. Publication, France.

- Briones, M. J. I., J. Carreira and P. Ineson, 1998. *Cognettia sphagnetorum* (Enchytraeidae) and nutrient cycling in organic soils: a microcosm experiment. *Appl. Soil Ecol.* 9, 289-294.
- Brun, L. A., J. Maillet, J. Richarte, P. Herrmann, J.C. Remy, 1998. Relationships between extractable copper, soil properties and copper uptake by plants in vineyards soils. *Environ. Pollut.* 102, 151–161.
- Chabauty, F., V. Pot, M. Bourdat-Deschamps, N. Bernet, C. Labat and P. Benoit, 2016. Transport of organic contaminants in subsoil horizons and effects of dissolved organic matter related to organic waste recycling practices. *Environ. Sci. Pollut. Res. Int.* 23 (7): 6907-6918.
- Chapman P.M., 2002. Integrating toxicology and ecology: putting the "eco" into ecotoxicology. *Marine Poll. Bull.* 44, 7-15.
- Cole, L., R. D. Bardgett and P. Ineson, 2000. Enchytraeid worms (*Oligochaeta*) enhance mineralization of carbon in organic upland soils. *Eur. J. Soil Sci.* 51, 185-192.
- De Silva P.M.C.S, Pathiratne A., van Gestel C.A.M, 2010. Toxicity of chlorpyrifos, carbofuran, mancozeb and their formulations to the tropical earthworm *Perionyx excavatus*. *Appl. Soil Ecol.* 44, 56–60
- Decaëns, T., P. Margerie, J. Renault, F. Bureau, M. Aubert and M. Hedde, 2011. Niche overlap and species assemblage dynamics in an ageing pasture gradient in north-western France. *Acta Oecol.* 37, 212-219.
- De Silva P. M. C. S., Pathiratne A, van Gestel C. A. M., 2010. Toxicity of chlorpyrifos, carbofuran, mancozeb and their formulations to the tropical earthworm *Perionyx excavatus*. *Appl. Soil Ecol.* 44, 56–60.
- Diaz Cosin, D. J., M. P. Ruiz, M. Ramajo and M. Gutierrez, 2006. Is the aestivation of the earthworm *Hormogaster elisae* a paradiapause? *Invertebr Biol.* 125, 250-255.
- Didden W. A. M., 1990. Involvement of *Enchytraeidae* (*Oligochaeta*) in soil structure evolution in agricultural fields. *Biol. Fertil. Soils.* 9, 152- 158.
- Didden, W. and J. Rombke, 2001. Enchytraeids as indicator organisms for chemical stress in terrestrial ecosystems. *Ecotoxicol. Environ. Saf.* 50, 25-43.
- Edwards C.A., 2004. *Earthworm ecology*, CRC Press, Boca Raton.
- Eijsackers, H., P. Beneke, M. Maboeta, J. P. Louw and A. J. Reinecke, 2005. "The implications of copper fungicide usage in vineyards for earthworm activity and resulting sustainable soil quality." *Ecotoxicol. Environ. Saf.* 62, 99-111.

- Forbes V.E., Calow P., 2012. Promises and problems for the new paradigm for risk assessment and an alternative approach involving predictive systems models. *Environ. Toxicol. Chem.* 31, 2663-2671.
- Graefe, U. and A. Beylich, 2002: The German long-term soil monitoring program and its implications for the knowledge of *Enchytraeidae*. *Newslet. Enchytraeidae* 7, 91–93.
- Hartenstein F, Hartenstein E, Hartenstein R., 1981. Gut load and transit time in the earthworm *Eisenia foetida*. *Pedobiologia* 22, 5–20.
- Hole, D. G., A. J. Perkins, J. D. Wilson, I. H. Alexander, P. V. Grice and A. D. Evans, 2005. Does organic farming benefit biodiversity? *Biol. Conserv.* 122, 113-130.
- Helling, B., S. A. Reinecke and A. J. Reinecke, 2000. Effects of the fungicide copper oxychloride on the growth and reproduction of *Eisenia fetida* (*Oligochaeta*). *Ecotoxicol. Environ. Saf.* 46, 108-116.
- ISO (International Organisation for Standardization), 2009. Qualité du sol - Essai d'évitement pour contrôler la qualité des sols et les effets des produits chimiques sur le comportement. Partie 1: Essai avec des vers de terre (*Eisenia fetida* et *Eisenia andrei*). No. 17512-1. Geneva.
- ISO (International Organisation for Standardization), 2012. Soil quality - Effects of pollutants on earthworms - Part 1: Determination of acute toxicity to *Eisenia fetida*/*Eisenia andrei*. No. 11268-1. Geneva.
- ISO (International Organisation for Standardization), 2014. Soil quality - Effects of contaminants on *Enchytraeidae* (*Enchytraeus sp.*) - Determination of effects on reproduction. No. 16387. Geneva.
- Jänsch, S., M. Garcia and J. Rombke, 2005: Acute and chronic isopod testing using tropical *Porcellionides pruinosus* and three model pesticides. *Eur. J. Soil Biol.* 41, 143-152.
- Kasprzak, K., 1982. Review of enchytraeid (*Oligochaeta*, *Enchytraeidae*) community structure and function in agricultural ecosystems. *Pedobiologia.* 23, 217-232.
- Khalil, M. A., H. M. Abdel-Lateif, B. M. Bayoumi, N. M. van Straalen and C. A. M. van Gestel, 1996a. Effects of metals and metal mixtures on survival and cocoon production of the earthworm *Aporrectodea caliginosa*. *Pedobiologia.* 40, 548-556.
- Khalil, M. A., H. M. Abdel-Lateif, B. M. Bayoumi and N. M. van Straalen, 1996b. Analysis of separate and combined effects of heavy metals on the growth of *Aporrectodea caliginosa* (*Oligochaeta*; *Annelida*), using the toxic unit approach. *Appl. Soil Ecol.* 4, 213-219.
- Kobeticova, K., J. Hofman and I. Holoubek, 2009. Avoidance response of *Enchytraeus albidus* in relation to carbendazim ageing. *Environ Pollut.* 157, 704-706.

- Kobeticova, K., J. Hofman and I. Holoubek, 2010. Ecotoxicity of wastes in avoidance tests with *Enchytraeus albidus*, *Enchytraeus crypticus* and *Eisenia fetida* (*Oligochaeta*). *Waste Manage.* 30, 558-564.
- Konečný, L., V. Ettlér, S. M. Kristiansen, M. J. B. Amorim, B. Křibek, M. Mihaljevič, O. Šebek, I. Nyambe and J. J. Scott-Fordsmand, 2014. Response of *Enchytraeus crypticus* worms to high metal levels in tropical soils polluted by copper smelting. *J. Geochem. Explor.* 144, 427-432.
- Lajmanovich R, Attademo A, Peltzer P, Junges C, Cabagna M, 2011. Toxicity of four herbicide formulations with glyphosate on *Rhinella arenarum* (*Anura: Bufonidae*) tadpoles: B-esterases and glutathione– S-transferase inhibitors. *Arch. Environ. Contam. Toxicol.* 60, 681–689
- Lavelle, P., Charpentier, F., Gilot, C., Rossi, J.P., Derouard, L., Pashanasi, B., André J., Ponge J.F and Bernier N., 2004. Effects of earthworms on soil organic matter and nutrient dynamics at a landscape scale over decades. In: *Earthworm Ecology* (ed C.A. Edwards). CRC Press, Boca Raton, FL.
- Lavelle, P., T. Decaens, M. Aubert, S. Barot, M. Blouin, F. Bureau, P. Margerie, P. Mora and J. P. Rossi, 2006. Soil invertebrates and ecosystem services. *Eur. J. Soil Biol.* 42, 3-15.
- Leitão, S., M. J. Cerejeira, P. J. Van den Brink and J. P. Sousa, 2014. Effects of azoxystrobin, chlorothalonil, and ethoprophos on the reproduction of three terrestrial invertebrates using a natural Mediterranean soil. *Appl. Soil Ecol.* 76, 124-131.
- Lowe, C. N. and K. R. Butt, 2005. Culture techniques for soil dwelling earthworms: A review. *Pedobiologia.* 49, 401-413.
- Lukkari, T. and J. Haimi, 2005. Avoidance of Cu- and Zn-contaminated soil by three ecologically different earthworm species. *Ecotoxicol. Environ. Saf.* 62, 35-41.
- Ma, L.Q., Rao, G.N., 1997. Chemical fractionation of cadmium, copper, nickel, and zinc in contaminated soils. *J. Environ. Qual.* 26, 259-264.
- Maraldo, K., B. Christensen and M. Holmstrup, 2011. The excretion of ammonium by enchytraeids (*Cognettia sphagnetorum*). *Soil Biol. Biochem.* 43, 991-996.
- Maleri, R. A., A. J. Reinecke and S. A. Reinecke, 2008. Metal uptake of two ecophysiologicaly different earthworms (*Eisenia fetida* and *Aporrectodea caliginosa*) exposed to ultramafic soils. *Appl. Soil Ecol.* 38, 42-50.
- Marinissen, J. C. Y. and W. A. M. Didden, 1997. Influence of the Enchytraeid worm *Buchholzia appendiculata* on aggregate formation and organic matter decomposition. *Soil Biol. Biochem.* 29, 387-390.

- Marques C, Pereira R, Goncalves F, 2009. Using earthworm avoidance behaviour to assess the toxicity of formulated herbicides and their active ingredients on natural soils. *J. Soils Sediments*. 9, 137–147.
- McDonald, J., L. Gaston, T. Elbana, K. Andres and E. Crandfield, 2013. Dimoxystrobin sorption and degradation in sandy loam soil: impact of different landscape positions. *Soil Sci*. 178, 662-670.
- OECD, 1984. Guideline for the testing of chemicals. No. 207. Earthworm, acute toxicity tests. (Organization for Economic Cooperation and Development). Paris, France.
- OECD 220, 2004: Guidelines for testing of chemicals – Enchytraeid reproduction test. OECD (Organization for Economic Cooperation and Development). Paris, France.
- Perez-Losada, M., M. Ricoy, J. C. Marshall and J. Dominguez, 2009. Phylogenetic assessment of the earthworm *Aporrectodea caliginosa* species complex (Oligochaeta: Lumbricidae) based on mitochondrial and nuclear DNA sequences. *Mol. Phylogenet. Evol.* 52(2), 293-302.
- Paoletti, M. G., D. Sommaggio, M. R. Favretto, G. Petruzzelli, B. Pezzarossa and M. Barbafieri, 1998. Earthworms as useful bioindicators of agroecosystem sustainability in orchards and vineyards with different inputs. *Appl. Soil Ecol.* 10, 137-150.
- Pelosi, C., S. Joimel and D. Makowski, 2013. Searching for a more sensitive earthworm species to be used in pesticide homologation tests - A meta-analysis. *Chemosphere*. 90, 895-900.
- Pelosi, C., S. Barot, Y. Capowicz, M. Hedde and F. Vandenbulcke, 2014. Pesticides and earthworms. A review. *Agron. Sustainable Dev.* 34, 199-228.
- Pelosi, C., M. Lebrun, L. Beaumelle, N. Cheviron, G. Delarue and S. Nelieu, 2016. Sublethal effects of epoxiconazole on the earthworm *Aporrectodea icterica*. *Environ. Sci. Pollut. Res.* 23(4), 3053-3061.
- Pelosi C., J. Römbke, 2016. Are *Enchytraeidae* (*Oligochaeta*, *Annelida*) good indicators of agricultural management practices? *Soil Biol. Biochem.* 100, 255-263.
- Phillipson, J., R. Abel, J. Steel and S. R. J. Woodell, 1979. Enchytraeid numbers, biomass, respiratory metabolism in a beech woodland-Wytham woods, Oxford. *Oecologia*. 43, 173-193.
- Piola L, Fuchs J, Oneto ML, Basack S, Kesten E, Casabé N, 2013. Comparative toxicity of two glyphosate-based formulations to *Eisenia andrei* under laboratory conditions. *Chemosphere*. 91, 545–551

- Reinecke, S. A., B. Helling and A. J. Reinecke, 2002. Lysosomal response of earthworm (*Eisenia fetida*) coelomocytes to the fungicide copper oxychloride and relation to life-cycle parameters. *Environ. Toxicol. Chem.* 21, 1026-1031.
- Rombke, J., 2003. Ecotoxicological laboratory tests with enchytraeids: A review. *Pedobiologia.* 47, 607-616.
- Sandor, M. and S. Schrader, 2012. Interaction of earthworms and enchytraeids in organically amended soil. *North-West J. Zool.* 8, 46-56.
- Satchell J. E., 1967. Lumbricidae. In: *Soil Biology*. Burgess A and Raw F, eds., pp. 259–322. Academic Press, London.
- Schneider, C. A., W. S. Rasband and K. W. Eliceiri, 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods.* 9, 671-675.
- Seeber, J., G. U. H. Seeber, R. Langel, S. Scheu and E. Meyer, 2008. The effect of macro-invertebrates and plant litter of different quality on the release of N from litter to plant on alpine pastureland. *Biol. Fertil. Soils.* 44, 783-790.
- Smit, C. E., Van Gestel, C. A. M., 1996. Comparison of the toxicity of zinc for the springtail *Folsomia candida* in artificially contaminated and polluted field soils. *Appl. Soil Ecol.* 3, 127–136.
- Spurgeon, D. J., J. M. Weeks and C. A. M. Van Gestel, 2003. A summary of eleven years progress in earthworm ecotoxicology. *Pedobiologia.* 47, 588-606.
- Topoliantz, S., J. F. Ponge and P. Viaux, 2000. Earthworm and enchytraeid activity under different arable farming systems, as exemplified by biogenic structures. *Plant Soil.* 225, 39-51.
- van Vliet, P. C. J., L. T. West, P. F. Hendrix and D. C. Coleman, 1993. The influence of *enchytraeidae (oligochaeta)* on the soil porosity of small microcosms. *Geoderma.* 56, 287-299.
- van Vliet, P.C.J., Beare M.H., Coleman D.C., 1995. Population dynamics and functional roles of *Enchytraeidae (Oligochaeta)* in hardwood forest and agricultural ecosystems. In: Collins, H.P., Robertson, G.P., Klug, M.J. (Eds.), *The significance and regulation of soil biodiversity*. Kluwer Academic Publishers, Dordrecht, pp. 237–245.

Web references

- Agritox, 2016. <http://www.agritox.anses.fr/php/sa.php?sa=1519>
- E-phy, 2016a. <http://e-phy.agriculture.gouv.fr/spe/9400346-10024578.htm>
- E-phy, 2016b. <http://e-phy.agriculture.gouv.fr/spe/2090171-1559.htm>

Vindimian E., 2016. http://www.normalesup.org/~vindimian/fr_index.html

PPDB, 2016. <http://sitem.herts.ac.uk/aeru/ppdb/en/>

Table 1. Main characteristics of the studied soil

Characteristics	Soil
Clay (< 2 μm , g kg^{-1})	226.0
Fine silt (2-20 μm , g kg^{-1})	174.1
Coarse silt (20-50 μm , g kg^{-1})	298.9
Fine sand (50-200 μm , g kg^{-1})	239.1
Coarse sand (200-2000 μm , g kg^{-1})	47.9
CaCO ₃ total (g kg^{-1})	23.3
Organic matter (g kg^{-1})	32.6
P ₂ O ₅ (g kg^{-1})	0.1
Organic carbon (g kg^{-1})	18.9
Total nitrogen (N) (g kg^{-1})	1.5
C/N	12.7
pH	7.5
Total Cu (mg kg^{-1})	25.2

Fig.1. Mean percentage of biomass loss for *A. caliginosa* (black bars, n = 3, ± SE) and *E. albidus* (grey bars, n = 4, ± SE) exposed to a control soil (without pesticides) and the same soil spiked with different Cuprafor micro® concentrations (0.65, 6.5, 65 and 650 times the RD or 5, 50, 500, and 5,000 mg kg⁻¹ of copper for C1, C2, C3 and C4 respectively). Different letters indicate significant differences between the tested concentrations. One statistical analysis per species (ANOVA: p < 0.05). RD: Recommended Dose.

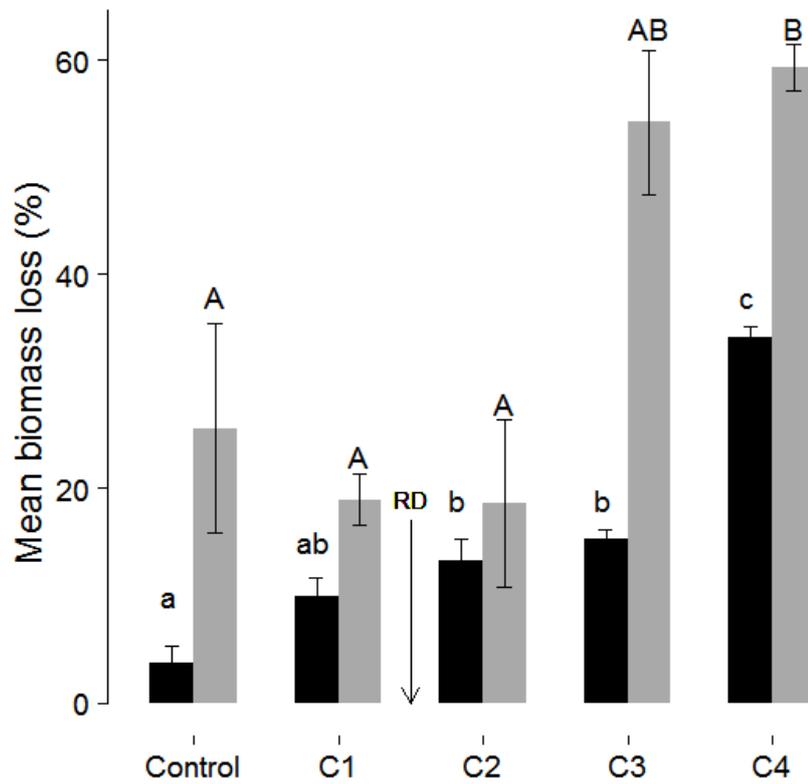


Fig.2. Ratio of quiescent *A. caliginosa* individuals at the end of the experiment (14 days) exposed to soils spiked with different Cuprafor micro® concentrations (0.65, 6.5, 65 and 650 times the RD or 5, 50, 500, and 5,000 mg kg⁻¹ of copper for C1, C2, C3 and C4 respectively) (n = 3, ± SE). Different letters indicate significant differences between concentrations according to Kruskal-Wallis tests (p < 0.05). RD: Recommended Dose. Photo: *A. caliginosa* in quiescence.

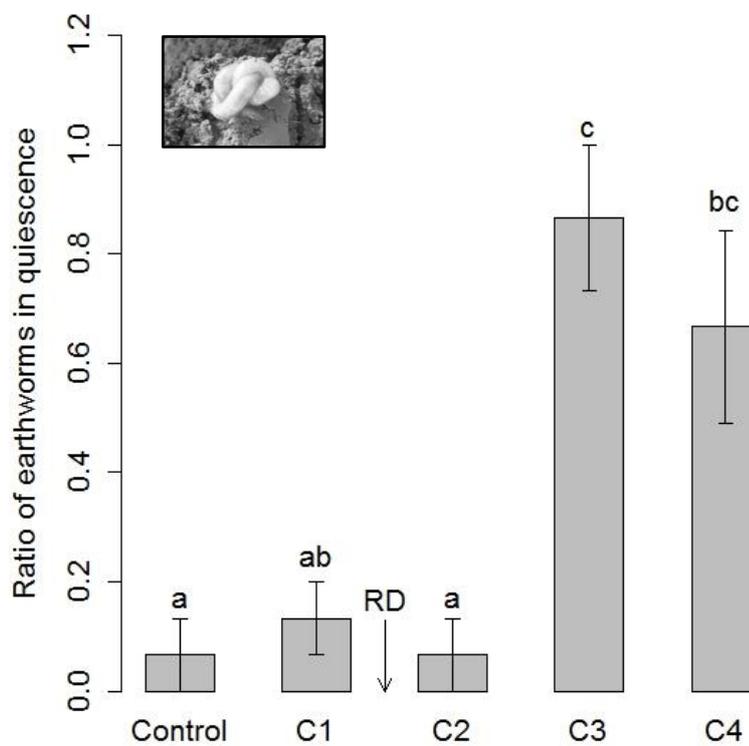


Fig.3. Ratio of quiescent *A. caliginosa* individuals at the end of the experiment (14 days) exposed to soils spiked with different Swing Gold® concentrations (0.065, 0.65, 6.5, 65 and 324 times the RD or 1×10^{-2} , 1×10^{-1} , 1, 10, and 50 mg k^{-1} of DMX or 3.76×10^{-3} , 3.76×10^{-2} , 3.76×10^{-1} , 3.76, and 18.8 of EPX for SGA1, SGA2, SGA3, SGA4 and SGA5 respectively) ($n = 3, \pm \text{SE}$). Different letters indicate significant differences between concentrations according to Kruskal-Wallis tests ($p < 0.05$). Black crosses indicate 100% mortality. RD: Recommended Dose. Photo: *A. caliginosa* in quiescence.

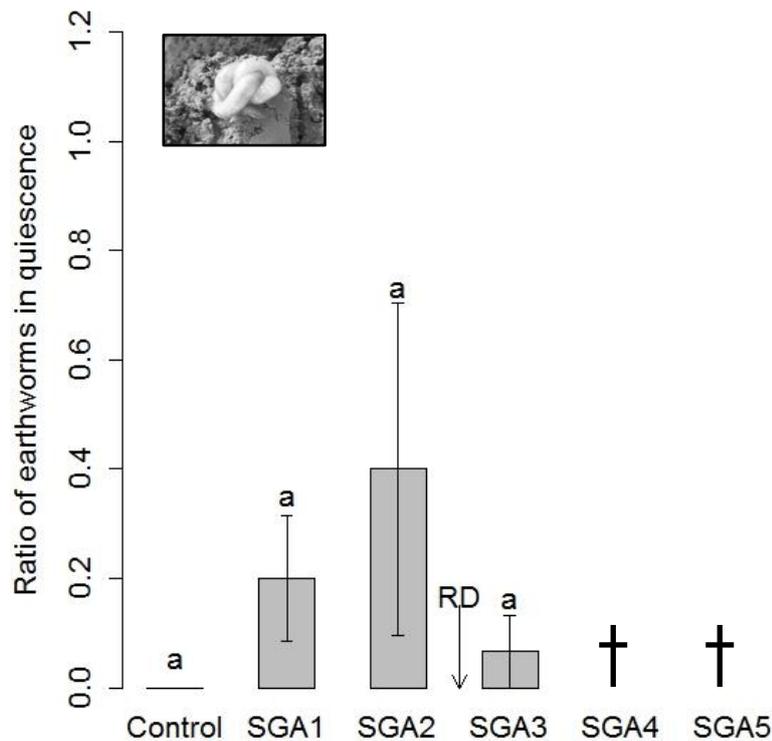


Fig.4. Mean avoidance net-response ($n = 5$, \pm SE) of (A) *A. caliginosa* and (B) *E. albidus* exposed to soils spiked with different Cuprafor micro® concentrations (0.65, 6.5, 65 and 650 times the RD or 5, 50, 500, and 5,000 mg kg⁻¹ of copper for C1, C2, C3 and C4 respectively). The dashed line indicates the trigger value of 80% of the individuals. Different letters indicate significant differences in avoidance net-response between the concentrations (Kruskal-Wallis tests, $p < 0.05$). r indicates the rho correlation coefficient (Spearman correlation tests, * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$). RD: Recommended Dose.

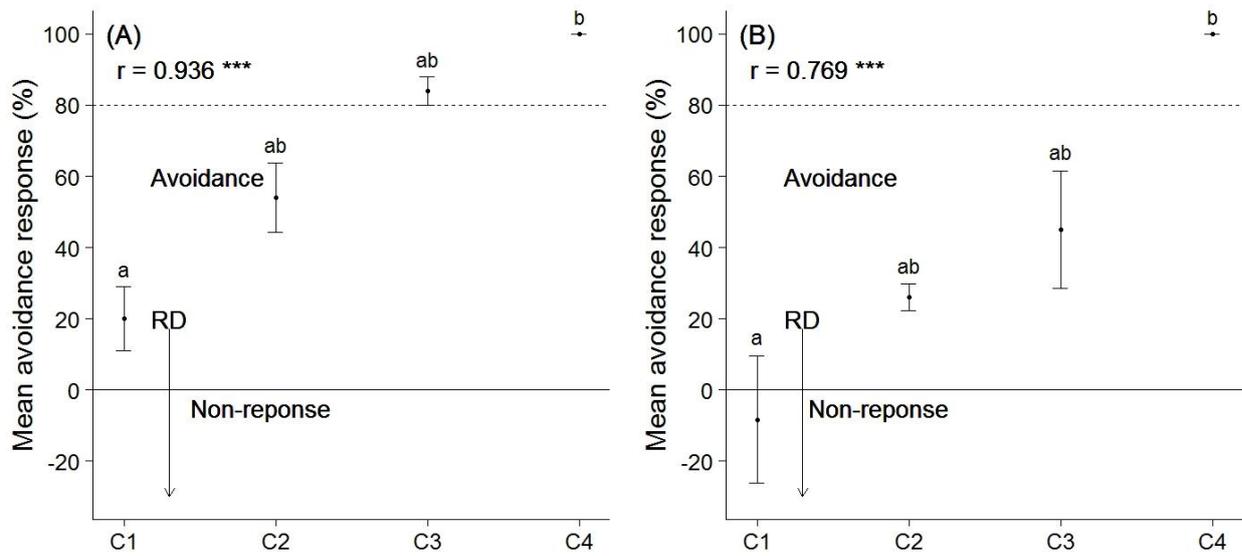


Fig.5. Mean avoidance net-response (n = 5, ± SE) of (A) *A. caliginosa* and (B) *E. albidus* exposed to soils spiked with different Swing Gold® (1, 2, 5 and 10 times the RD or 1.55×10^{-1} , 3.10×10^{-1} , 7.75×10^{-1} , and 1.55 mg k^{-1} of DMX or 5.81×10^{-2} , 1.16×10^{-1} , 2.91×10^{-1} , and 5.81×10^{-1} of EPX for SGB1, SGB2, SGB3 and SGB4 respectively). The dashed line indicates the trigger value of 80% of the individuals. Different letters indicate significant differences in avoidance net-response between the concentrations (Kruskal-Wallis tests, $p < 0.05$). r indicates the rho correlation coefficient (Spearman correlation tests, ns = no significant). RD: Recommended Dose.

