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Abstract

The representativeness of laboratory studies of the fate of pesticides in soil in field conditions is questionable. This study aimed at comparing the dissipation and bioavailability to earthworms of two fungicides, dimoxystrobin (DMX) and epoxiconazole (EPX), over 12 months under laboratory and field conditions. In both approaches, the fungicides were applied to the same loamy soil as a formulated mixture at several concentrations. We determined total DMX and EPX concentrations in the soil using exhaustive extraction, their environmental availability using mild extraction, and their bioavailability through internal concentrations in exposed earthworms. The initial fungicide application appeared as much better controlled in terms of dose and homogeneity in the laboratory than in the field. One year after application, a similar dissipation rate was observed between the laboratory and field experiments (ca 80% and 60% for DMX and EPX, respectively). Similarly, the ratio of available/total concentrations in soil displayed the same trend whatever the duration and the conditions (field or lab), EPX being more available than DMX. Finally, the environmental bioavailability of the two fungicides to earthworms was heterogeneous in the field, but, in the laboratory, the bioaccumulation was evidenced to be dose-dependent only for DMX. Our findings suggest that the actual fate of the two considered fungicides in the environment is consistent with the one determined in the laboratory, although the conditions differed (e.g., presence of vegetation, endogeic earthworm species). This study allowed better understanding of the fate of the two considered active substances in the soil and underlined the need for more research dedicated to the link between environmental and toxicological bioavailability.

Keywords: soil; ecotoxicology; pesticide; bioavailability; fate; exposure

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Introduction

About 2.5 million tons of pesticides with various modes of action are used yearly over vast areas worldwide (Zhang et al. 2011). Once applied, pesticides are subject to physical, chemical and biological dissipation processes including volatilization, adsorption, leaching and degradation. A fraction of applied pesticides remaining in the topsoil could be absorbed by living organisms, potentially generating ecotoxicological effects. This risk has been conceptualized through bioavailability, where the bioavailable fraction is the amount of a compound that is freely available and able to cross organisms' cell membrane from the soil where the organisms live at a given time, and thus enter the food chain (Semple et al. 2004). More practically, bioavailability can be described in three steps: (1) environmental availability (or soil offer) related to the potential of a compound to interact with an organism; (2) environmental bioavailability which is the uptake of the compound by the organism; and, finally (3) the toxicological bioavailability corresponding to the effect of the compound on the organism (Lanno et al. 2004; Harmsen 2007). Current methods used to assess the environmental availability and bioavailability include determining the concentration of the chemical in the soil, following a mild extraction and measuring the chemical concentration in the organism, respectively (ISO 17402 2008). Pesticide characteristics (e.g. polarity, solubility in water) influence the available concentrations in soils and the one measured in soil organisms. However, parameters such as particle distribution, organic matter content, their association into soil structure or climatic conditions (Chaplain et al. 2011), as well as the lifestyle of the exposed organisms (e.g. activity, feeding behaviour), also play a role. Pesticide concentrations in different environmental compartments can thus evolve in time and space, being subject to several factors interacting at various temporal and spatial scales (Harmsen 2007).

The fate of pesticides in soil under field conditions is often assessed through modelling, based on data from laboratory experiments that use homogenized soil and controlled incubation conditions. The representativeness and thus the relevance of such experiments is questionable due to soil heterogeneity (McDonald et al. 2006), vegetation cover (Zhao et al. 2003) and climatic variations (Chai et al. 2013) that occur under field conditions. Among the few studies (about 12 studies) performed under field and laboratory conditions with the same soils, pesticide persistence and/or sorption on soil constituents was found to be different. For instance, Ahmad et al. (2003) observed lower clopyralyd persistence in the laboratory than in the field due to differences in temperature, soil water content, shading and plant cover. In contrast, pesticide persistence was reported to be lower under field conditions in other studies (Herrero-Hernández et al. 2015; Karlsson et al. 2016). Herrero-Hernandez et al. (2015) mainly attributed this difference to the leaching of the pesticide to deeper soil. Karlsson et al. (2016) hypothesized and tested various reasons, such as extraction efficiency, losses from spray drift, interception and leaching to deeper horizons, but none of those processes could explain the discrepancy. The authors finally hypothesized (but not demonstrated) differences and dynamics in soil temperature, soil water content, plant uptake and stimulated degradation in the rhizosphere. Beulke et al. (2000) suggested that laboratory studies overestimated the persistence of pesticides by a factor of more than 1.25 in 44% of case-studies because of the above-mentioned parameters, as well as the occurrence of extra phenomena unlikely to take place in laboratory conditions (volatilization, wind erosion, photodegradation, leaching). The difference between field and laboratory may also vary depending on the studied pesticide: in Di et al. (1998), the persistence in topsoil was rather similar for chlorpyrifos, chlorthal dimethyl and promethryne, higher in the field than in the laboratory for fenamiphos, metalaxyl (because of leaching) and linuron but lower for metribuzin and propyzamide. In the same way, laboratory experiments using pure solutions of pesticides may lead to an underestimation of the pesticide sorption compared with field conditions, where formulated pesticides are applied (Cryer 2005). Overall, a tiered lab-to-field approach may be necessary to efficiently predict the fate of pesticides in the environment, whereas modelling is often based on laboratory results only (Beulke et al. 2000).

Pesticide risk assessment procedures include tests on non-target soil organisms such as collembolan, acari and earthworms. However, for soil invertebrates, little information is available on the extent to which data on pesticide effects from higher-tier tests using semi-field (e.g. terrestrial model ecosystems) and field conditions can be compared with laboratory data. In a review, Jänsch et al. (2006) found that, for 61 of the 71 pesticides for which data were available, this mismatch was due to different taxonomic composition between conditions, but also to a lack of information about the concentrations at which chronic toxicity could occur. Furthermore, most of the studies did not provide any information on pesticide availability, bioavailability or even fate in the soil

(Jänsch et al. 2006). In a more recent review, laboratory studies were considered to provide opportunities to limit the number of variables potentially affecting the outcome of bioaccumulation assessments, but the importance of tiered testing approaches was recognized (Hoke et al. 2015). The same review described the bioavailable fraction as "*the fraction of greatest importance when conducting terrestrial exposure studies*". They also stated that "*field studies may be necessary to benchmark the accuracy of these estimates of bioavailability*".

Among living soil organisms, earthworms are some of the most sensitive soil taxa to chemicals due to their soft bodies, feeding habit (e.g. soil feeder) and the huge quantity of soil they ingest (e.g. about several tons per hectare, Lavelle 1996). They are recognized as bioindicators of soil health, quality and functioning (Paoletti 1999). Moreover, they are involved in key soil processes such as organic matter degradation and soil structure dynamics (Lavelle 1996).

To our knowledge, the link between pesticide use, available concentrations and internal concentrations in earthworms has never been explored simultaneously under laboratory and field conditions over relatively long periods such as several months. The aim of this study was to evaluate over 12-month laboratory and field experiments, (i) dissipation (in terms of total contents), (ii) environmental availability and (iii) bioavailability to earthworms of two active substances present in a formulated mixture. The commercial formulation was applied at three doses in the laboratory and two doses under field conditions. The higher dose, selected to maximize the processes, was modulated in field and laboratory conditions according to the lethal concentration. In the laboratory, the effect of the earthworms (and the organic matter added for feeding) on pesticide environmental availability and the effect of the applied dose on environmental bioavailability were also examined. The selected commercial formulation of a fungicide, containing dimoxystrobin (DMX) and epoxiconazole (EPX), was chosen for its common use in Europe on cereal crops. According to the PPDB database (2020), which retranscribes information from European EFSA dossiers, these active substances differ notably in their persistence in the field with dissipation half-life (DT_{50}) of 23 and 120-days for DMX and EPX, respectively. The persistence of the two molecules differs to a lesser extent in the laboratory with DT_{50} of 210 and 354-days for DMX and EPX, respectively. Two common species of earthworms were selected for our laboratory and field experiments: *Aporrectodea caliginosa* (Savigny, 1826) and *Aporrectodea icterica* (Savigny, 1826), respectively.

Material and methods

Fungicides

Epoxiconazole (EPX, CAS 133855-98-8, (2*RS*,3*SR*)-1-[3-(2-chlorophenyl)-2,3-epoxy-2-(4-fluorophenyl)propyl]-1H-1,2,4-triazole) is a triazole fungicide acting as a sterol biosynthesis inhibitor with preventative and curative action. Dimoxystrobin (DMX, CAS 149961-52-4, (*E*)-2-(methoxyimino)-*N*-methyl-2-[a-(2,5-xylyloxy)-o-tolyl]acetamide) is a stobilurin fungicide acting as a respiration inhibitor with protectant, curative and translaminar action. According to the PPDB database (2020), both compounds are relatively hydrophobic and poorly soluble in water (log K_{ow} 3.59 and 3.3, water solubility 4.3 mg/L and 7.1 mg/L for DMX and EPX, respectively). They were used in the field and laboratory experiments in the formulated mixture Swing® Gold (BASF Agro SAS), which contains 133 g/L DMX and 50 g/L EPX.

Field experiment

The field experiment was set up in a meadow where earthworms were abundant (> 200 individuals/m^2), as recommended by the ISO standard method for earthworm field studies (ISO 11268-3 2014). The initial earthworm density and earthworm community were determined from ten sampling points randomly selected. The sampling of each sampling point and the earthworm identification methods are described in Amosssé et al. (2018). The most abundant species was *Allolobophora icterica* (mean of 180.6 ind/m^2), followed by *Lumbricus terrestris* (mean of 32.5 ind/m^2), *Aporrectodea giardi* (mean of 23.8 ind/m^2), *Lumbricus castaneus* (mean of 20.6 ind/m^2), *Aporrectodea caliginosa* (mean of 10.6 ind/m^2), *Allolobophora chlorotica* (mean of 6.9 ind/m^2), *Aporrectodea longa* (mean of 6.3 ind/m^2) and three others not exceeding a mean of 5 ind/m^2. This meadow,

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located in Versailles (France), had not received pesticides for more than 20 years. The main characterictics of the soil (Luvisol, FAO soil classification) were as follows: loam texture (29% sand, 48% silt and 23% clay), pH 7.5, soil bulk density 1.29, organic matter 32.6 g/kg, C/N 12.7, $CaCO_3$ 23.3 g/kg, water holding capacity WHC 0.41 g/g (see Bart et al. 2017 for more details). Four replicate plots (10 m x 10 m) of each fungicide treatment were randomly located and separated by 6 m buffer strips. After cutting the vegetation as short as possible, the Swing® Gold formulation was applied with manual sprayers, using 8 L of diluted solution per plot (Amossé et al. 2018). The application on the plots was performed at the recommended dose (replicates D1-1 to D1-4), ten-fold the recommended dose (D10-1 to D10-4), and control plots were not treated. The recommended dose corresponds to 199.5 and 75 g/ha of DMX and EPX, respectively. Considering that the active compounds of the Swing® Gold formulation are almost always found in the top 10 cm of soil (McDonald et al. 2013; Chabauty et al. 2016), the recommended dose D1 corresponded to 150 and 60 µg/kg dry soil of DMX and EPX, respectively (thus D10 to 1500 and 600 µg/kg dry soil of DMX and EPX, respectively). Soil (three cores per plot, 5 cm diameter and 10 cm depth, were mixed to obtain a composite sample submitted to analysis) and earthworms *Aporrectodea icterica* (three adults/sub-adults per plots) were sampled before application and 5, 26, 209 and 363 days after the application. This endogeic species of earthworm was chosen because it was the most abundant species found at the experimental site. To evaluate initial leaching of the active substances, extra soil samples were taken at 9 days after contamination at 0-10 cm, 10-20 cm and 20-30 cm in four of the plots (two D1 plots and two D10 plots).

Laboratory study

Laboratory studies were performed using the same soil as in the field experiment. The soil was collected from the top 0-20 cm one month before starting the experiment, air-dried, ground and sieved at 2 mm before being stored at 20°C. The recommended dose considered here was defined as previously, i.e. 150 and 60 µg/kg dry soil of DMX and EPX, respectively. The laboratory experiments were performed with Swing® Gold at one third the recommended dose (D0.33), the recommended dose (D1) and three-times this dose (D3); untreated soils (control) were also included. The D10 dose used in the field was not tested in the laboratory as Swing® Gold presented a lethal concentration (LC_{50}) for the studied earthworm corresponding to 6.3 times the recommended dose (Bart el al. 2017). As we planned to assess the internal concentration of living earthworm, we thus limited the highest dose to D3. Furthermore, we added the dose D0.33 under laboratory conditions to evaluate the fate of the fungicides under controlled conditions. For each tested concentration, the soil was spiked by mixing dry soil with aqueous dilutions of Swing® Gold in tap water and the soil moisture was adjusted concomitantly at 70% of the Water Holding Capacity (WHC). The equivalent of 400 g dry soil was placed in 1 L plastic vessels with a removable perforated cover for gas exchange. The moisture of each vessel was checked every 14 days by weighing vessels and adjusted if necessary. All the vessels were stored in a climate room (15±1 °C, 24 h darkness). To evaluate i) the persistence and the environmental availability of DMX and EPX in soil over time and ii) the link between environmental availability and bioavailability of DMX and EPX for earthworms, two groups of vessels were performed and four replicates were used for each group and modality.

The first group corresponded to the vessels containing only soil. It was sampled for analysis 0, 28, 56, 84, 247, 275, 303 and 366 days after spiking.

The second group included the vessels with earthworms. The chosen earthworm species was a dominant earthworm species in cultivated fields in temperate areas (Bart et al. 2018), *Aporrectodea caliginosa.* It is also an endogeic species, but relatively easy to breed in the lab (Bart et al. 2018), contrarily to *A. icterica*. Moreover, although *A. caliginosa* was present in the field site, the abundance was too low (i.e., mean of 10.6 individuals/m^2, compared to *A. icterica* at 180.6 individuals/m^2) to collect enough individuals for internal concentration measurements. Finally, *A. caliginosa* and *A. icterica* are ecologically similar, with similar length, pigmentation and habitat, both living in the first 20 cm of soil (Bouché, 1972). One earthworm (reared in the laboratory) was added per vessel and the equivalent of 3 g dry horse dung (adjusted to 70% of its WHC) was added by mixing with the soil every 14 days. The horse dung, necessary as a food source for the earthworms, was previously frozen and defrosted twice and then milled (< 1 mm) (Lowe and Butt, 2005). Earthworms were

exposed for 28, 56 or 84 days before being sampled for analysis. The earthworms exposed for 56 and 84 days were transferred to new vessels (prepared at day 0) every 28 days (i.e. one transfer for individuals exposed for 56 days and two transfers for individuals exposed for 84 days). This procedure was necessary to avoid soil compaction and excessive organic matter enrichment that could acidify the soil, which could lead to unsuitable conditions for earthworms (Bart et al. 2018). The soil from the second group of vessels was also sampled at days 28, 56 and 84.

Chemical analysis

Chemicals

The EPX and DMX used as analytical standards (Pestanal grade, ie 99.0% and 99.9%, respectively), hydroxypropyl-β-cyclodextrin (HPCD), Na_2SO_4, NaCl, $CaCl_2$, formic acid and acetic acid were purchased from Sigma-Aldrich. All organic solvents of analytical grade were purchased from Carlo-Erba, and LC-grade water was obtained through a Milli-Q system (Millipore).

Soil extraction

The soil sampled through three cores from the field experiment was mixed, homogenized, removing the main pieces of vegetation, and then sieved at 5 mm. For both field and laboratory experiments, an aliquot of sampled soil was immediately analysed to evaluate the environmental availability and a complementary sample was stored at -40°C to evaluate the total DMX and EPX contents.

To evaluate the environmental availability of DMX and EPX in soil, an extraction was performed according to Nélieu et al. (2016). Briefly, 4 g of wet soil was placed in a 50-mL polypropylene tube (Falcon BD) and 9 mL of 50 mM (70 g/L) hydroxypropyl-β-cyclodextrin aqueous solution was added. Tubes were shaken for 17 h on a reciprocating shaker at 17 rpm at ambient temperature and then centrifuged at $10,000g$ and 4 °C for 10 min (Beckman Coulter Allegra X-15R). The matrix effect was evaluated at less than 15 %. Analyses were performed within 24 h after preparation.

To evaluate the total content of DMX and EPX, freeze-dried soil was manually ground with a mortar. Triplicate soil subsamples of 5 g were placed in 50-mL polypropylene tubes (Falcon BD) and 10 mL of methanol was added to each tube. The tubes were shaken on an orbital shaker (10 min, 300 rpm) and sonicated for 20 min, before being centrifuged for 10 min at 1300 g and 20 °C. After collecting 7 mL of supernatant, the soil was again extracted with 10 mL of methanol with the same procedure. Then, 10 mL of supernatant was collected and mixed with the first extract. The D0.33, D1 and D3 samples were analysed without further preparation. For D10, an aliquot was diluted by a factor of 10 using a water/acetonitrile mixture (80:20, v/v) prior to analysis, to allow these samples to be within the calibration curve range. The samples containing low concentrations (less than 2 μg/kg, ie pre-application and controls) were further diluted by 200 mL of water then purified and concentrated by solid phase extraction on HR-XA cartridges (500 mg, Macherey-Nagel) preconditioned successively by 5 mL of methanol, acetonitrile and water. After percolation of the diluted extracts, cartridges were rinsed with 5 mL of water, dried under vacuum and eluted in 6 mL of a 95:5 (v/v) acetonitrile/formic acid mixture. Samples were then evaporated under a N_2 stream, dissolved in 3 mL of 8:2 (v/v) water/acetonitrile and analysed.

Earthworm extraction

After sampling, earthworms were relieved from their gut contents for 48 h before being frozen at -80°C. Total DMX and EPX measurements in earthworms were measured using a method based on that described by Nélieu et al. (2016). Frozen earthworms from the field experiment were cut and pieces were divided into three 2 mL microtubes (Sarstedt). These triplicate subsamples (ca 1 g/tube) were homogenized using a FastPrep®-24 (MP Biomedicals) in 0.5 mL of Milli-Q water with a ceramic sphere, by two 20 s cycles at 6 m/s. Homogenization was similar for each single earthworm issuing from laboratory studies (0.5-0.8 g/tube). The homogenate was

then transferred to a 15-mL polypropylene tube (Falcon BD), with three times 0.5 mL water used to rinse the 2 mL tube. DMX and EPX extractions were then performed by adding 4 mL acetonitrile in the tube, shaking on an orbital shaker (Ika KS 501, at 300 rpm for 10 min) and sonicating for 15 min. Then, 2 mL of water, 1.6 g Na_2SO_4 and 0.4 g NaCl were added to carry out a QuEChERS purification. The tube was vigorously shaken and centrifuged for 10 min at 10,900 g and 4 °C. The upper organic layer was collected, placed at least 2 h at −40 °C and centrifuged for 10 min at 10,900 g and 4 °C. A 3 mL fraction of the supernatant was dried through 2-g Na_2SO_4 addition and centrifugation (10 min, 10,900 g, 4 °C). A 1 mL aliquot of supernatant was then evaporated to dryness under a N_2 stream and redissolved in 0.5 mL chloroform. This extract was further purified on 500-mg Florisil cartridges (preconditioned successively by 2 mL of methanol, acetone and chloroform). Elution was performed by 5 mL of a 9:1 (v/v) chloroform/acetone mixture. After evaporation under a N_2 stream, the residue was dissolved in 1 mL of 8:2 (v/v) water/acetonitrile and analysed by liquid chromatography-mass spectrometry.

Analysis by liquid chromatography-mass spectrometry

Analyses were performed on an ultra-high performance liquid chromatograph (Acquity UPLC, Waters) coupled through an electrospray interface to a triple quadrupole mass spectrometer (TQD, Waters). The analytical conditions were as described by Nélieu et al. (2016), except the UPLC gradient (95/5 to 45/55 of water/acetonitrile, each containing 0.1% acetic acid) and the introduction of conditions to detect DMX (cone voltage 23 V, MRM transitions 327>205 at 10 eV for quantitation and 327>116 at 17 eV for confirmation). In soil, the LOD (limit of detection, according to a signal-to-noise ratio of 3) was 0.03 µg/kg and 0.05 µg/kg for DMX and EPX, respectively, and the LOQ (limit of quantification, validated by accuracy profile methodology) was 0.28 µg/kg and 0.22 µg/kg for DMX and EPX, respectively. In the earthworms, the LOQ (according to a signal-to-noise ratio of 7) was 0.02 µg/g for DMX and 0.05 µg/g for EPX.

Adsorption experiments

Adsorption experiments were performed according to OECD guideline 106 (OECD, 2000), using Swing® Gold and a mixture of DMX and EPX in pure solution (water with 2% acetonitrile). Duplicate dry soil samples of 1.5 g were equilibrated for 24 h in borosilicate tubes with 15 mL of $CaCl_2$ 10 mM solution, before spiking at concentrations ranging from 5.8 to 250 µg/L for DMX and from 2.3 to 100 µg/L for EPX. After 24-h of agitation, the tubes were centrifuged (5000 g, 10 min) and the supernatant analysed. Adsorption isotherms were described according to the Freundlich model as follows:

$$K_f = Qads / Ce^{1/n}$$

where Qads (mg/kg) is the amount of adsorbed fungicide in soil at equilibrium concentration, Ce (mg/L) is fungicide concentration in supernatant solution, and K_f and n are Freundlich empirical adsorption coefficients.

Data analysis

Data were represented by the mean ± standard deviation, or the slope ± confidence interval at $p = 0.05$ in the case of linear regressions. Fungicide dissipation in the field experiment was tested with the non-parametric Mann and Whitney test. For bioaccumulation factors in the laboratory study, non-parametric tests (Kruskal–Wallis test followed by a multiple comparison) were used. In the laboratory study, the dissipation kinetics were fitted according to the FOCUS recommendations (FOCUS 2006), using R (package kinfit), to select the best kinetic model according to the goodness of fit. To test the influence of presence/absence of earthworms and horse dung on the linear relationship between total and available concentrations of DMX end EPX, we used linear models and tested the interactions with the group "presence/absence".

Results and discussion

Dissipation of the two fungicides

Field experiment

Before their application, the concentrations of both fungicides DMX and EPX were below limit of detection in the soil where the experiment took place. They remained under the limit of quantification in the soil sampled in control plots all throughout the experiment.
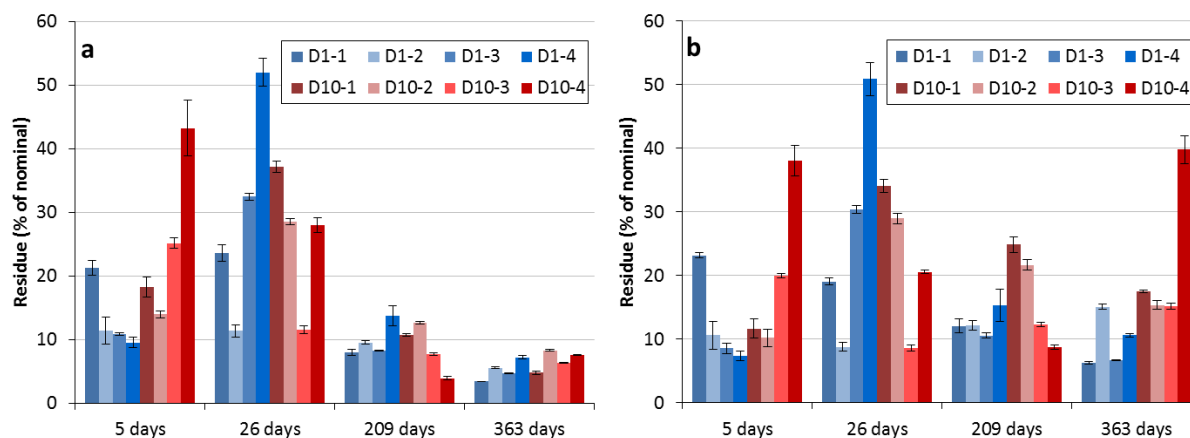

Fig. 1 Dimoxystrobin (a) and epoxiconazole (b) residues under field conditions in the 0-10 cm soil layer, in % of the nominal dose (mean and standard deviation of analytical measurements, n=3)

Five days after the application of Swing® Gold, the total DMX and EPX contents in the soil were found to be far below the applied concentrations (Figure 1). The maximum concentrations were ca 43% and 38% of the applied dose for DMX and EPX, respectively (in D10-4, see Figure 1). A minor part of this difference could have been due to leaching since we found 2.2 to 6.3% and 1.8 to 4.8% for DMX and EPX, respectively, between 10 and 30 cm deep (at day 9). However, the main reason for the initial low concentration in the 0-10 cm soil layer was certainly the vegetation cover that acted as a barrier limiting the fungicide from migrating into the soil. The foliage of a short cut pasture also intercepted as high as 90% of an herbicide deposit in another study (Ahmad et al. 2003). A large heterogeneity was observed between replicates, with minimal concentrations 2.2 to 5 times lower than the maximal concentration. For example, in D10 plots, the EPX concentration was estimated as 61 ± 8 µg/kg and 228 ± 14 µg/kg in D10-2 and D10-4 plots, respectively (nominal 600 µg/kg). Considering the four replicate plots, the mean percentages versus nominal concentrations appeared as lower at D1 than at D10 (figure S1), but the difference was not significant (Mann and Whitney test). At D1 the means (± standard deviation) corresponded to 13.3 ± 5.4% and 12.4 ± 7.3% of nominal DMX and EPX, respectively, whereas at D10 they were 25.2 ± 12.9% and 19.9 ± 12.8% for DMX and EPX, respectively. The heterogeneity could not be attributed to the analytical determination, as shown by the analytical standard deviations in Figure 1. Similarly, the discrepancy between nominal and observed fungicide concentrations could not be due to an analytical problem as extraction yields were estimated as 93-110% with a low matrix effect due to electrospray ionization. Furthermore, even with a maximal storage time of 4 months between soil sampling and analysis, the yields of the analytical method did not decrease. However, the heterogeneity of the measures could result from an uneven application of the fungicide on the plots. This uneven application could both result from (1) irregular spraying and (2) since the vegetation was not completely homogeneous on the experimental site, it consequently unevenly intercepted the fungicides. The collection and mixing of three soil cores did not seem to be able to counteract these factors.

Twenty six days after the application of Swing® Gold, the DMX and EPX concentrations in the soil increased significantly (Mann and Whitney test) in half of the plots (Figure 1): D1-3, D1-4, D10-1 and D10-2 for both

active substances and (slightly even if it was significant) D1-1 for DMX only. However, no significant change in concentration was observed in the plot D1-2 and the fungicides concentration in soil significantly decreased in D10-3 and D10-4 (and D1-1 for EPX only). The increases were interpreted as a transfer from the vegetation cover to the soil. However, the vegetation was not completely homogeneous in the experimental site, and this fact could explain the difference between the various replicates. This transfer reached up to a factor of 7, in D1-4 where EPX increased from 7.3 ± 0.8% at day 5 to 50.9 ± 2.6% at day 26. Considering all four replicate plots, the means increased between days 5 and 26, from approximately 12-25% to 23-30%, but the change was not significant due to the high inter-plot variability (Figure S1). The changes resulted at day 26 in residues varying between 11 and 52 % of the DMX nominal dose and in 8.6 and 51% of the EPX nominal dose. An important difference between the minimum and maximum values was also observed for the days 209 and 363, mostly for EPX. For instance at day 363, "only" a factor 2.4 differentiates the minimal and maximal DMX residue percentage (3.5% vs 8.3%), but this factor reaches up 6.4 and the case of DMX (6.2% vs 39.8%). This heterogeneity led to high inter-plot standard deviations (Figure S1). Six months (209 days) and 1 year (363 days) after Swing® Gold application, a decrease in DMX concentration was observed. This DMX dissipation was significant between days 26 and 363, but not between days 26 and 209 (Mann and Whitney test). However, if data were considered plot per plot, all DMX concentrations significantly decreased between days 26 and 209. There was no significant dissipation of EPX in any condition, and not even a tendency was observed for EPX at D10 (Figure S1). However, considering the EPX change plot per plot, a significant EPX dissipation after 26 days was observed in all plots except D1-2, D10-3 and D10-4, where EPX concentrations were stable or even increased over time. These apparent increases could be due to high field heterogeneity which caused high fungicide adsorption in localised spots. The variability in the field dissipation of pesticides was previously observed by other authors and attributed to spatial variation in soil and/or microbial properties (Bending et al. 2006).

The persistence of both active substances was higher in this experiment than expected from data reported in the literature. After 1 year, a value below LOQ and ca. 12% was expected for DMX and EPX respectively, according to the DT_{50} (PPDB 2020) in the field and assuming first order dissipation kinetics. However, after our 1 year experiment, we found approximately 13-48% and 20-53% of DMX and EPX remaining, respectively (considering as initial value the maximal concentration at 5 or 26 days).

Laboratory study and comparison with the field experiment
In contrast to the results obtained from the field experiment, the concentrations of the two active substances in the laboratory experiment evaluated 1 day after soil contamination were close to the nominal concentration: 101.9 ± 13.4 % and 98.1 ± 16.7 % for DMX and EPX, respectively. In addition, the heterogeneity between replicates remained low, with standard deviations rarely exceeding 5% (for $n = 4$). The absence of vegetal cover and the soil mixing, which homogenized its properties and the contamination, are the obvious factors explaining these results. Such factors were already underlined by various studies (Beulke et al. 2000; Wu et al. 2015; Karas et al. 2018; de Santo et al. 2019).

After 84 days of incubation under all the studied conditions in the laboratory, a similar fungicide dissipation level was observed, reaching ca 40% of the applied concentration (Figure S2). Overall, neither the initial concentration nor the presence of earthworm and horse dung had an influence on the dissipated fraction. The absence of a concentration effect on dissipation is not consistent with observations on other pesticides such as azoxystrobin, chlorpyrifos, tebuconazole or isoproturon (Herrero-Hernandez et al. 2015; Chai et al. 2013; Papadopoulou et al. 2016). For these active substances, previous studies found that the higher the concentration, the slower the dissipation. However, considering a maximal spiking of 1 mg/kg (thus limiting the impact on soil microbes of applied fungicides), the EPX dissipation was shown as not dependent on the dose (Neuwirthová et al. 2018).
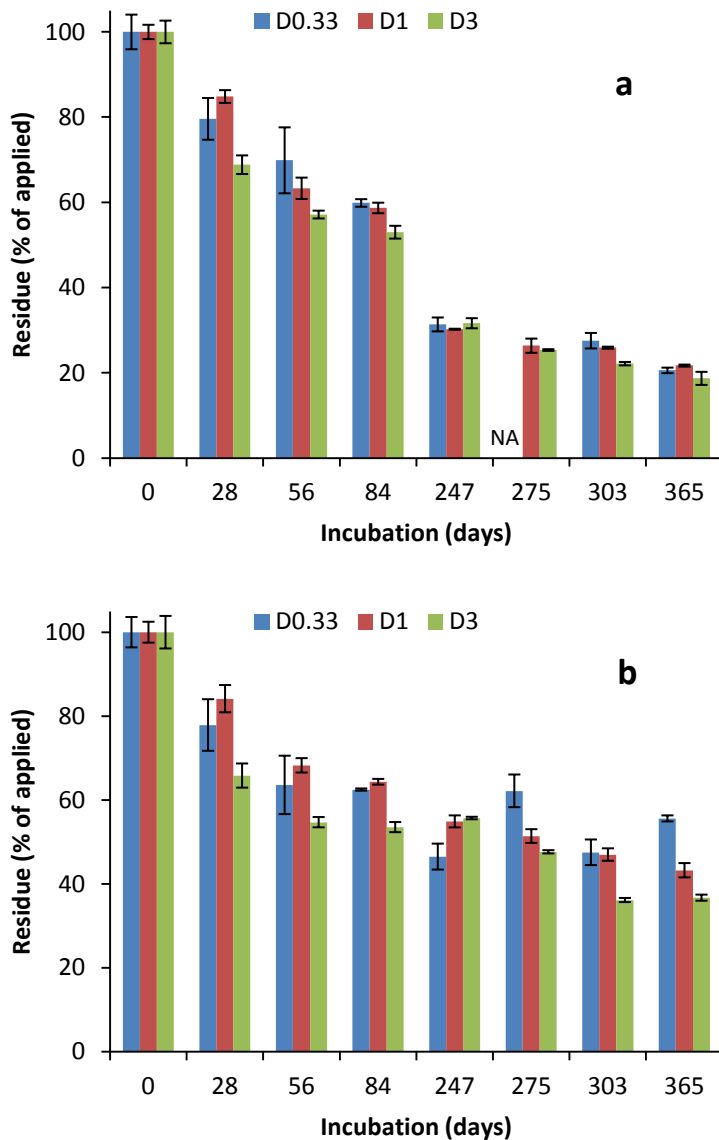
Fig. 2 Dimoxystrobin (a) and epoxiconazole (b) residues in soils, during laboratory studies without earthworms (mean and standard deviation of replicates, n=4; NA for not available)

In the present study, the dissipation kinetics of both active substances were quite similar over 84 days (Figures 2 and S2). However, their degradation behaviour was different when a longer incubation time was considered (Figure 2). DMX was degraded more than EPX after 1 year, reaching ca 20% of residues while ca 40% of EPX was still present. The half-lives were estimated as 94-135 and 153-301 days for DMX and EPX, respectively. After temperature and moisture normalisation according to FOCUS recommendations (FOCUS 2006), the DT_{50} shift to 108-156 and 177-348 days for DMX and EPX, respectively, i.e. on average 39 and 18% quicker than expected from laboratory DT_{50} data available in the PPDB database. This trend was in striking contrast to that observed in the field experiment where active substance degradation was slower than that based on PPDB data. It is worth noting that the kinetics observed in our laboratory and field experiments in the same soil were quite similar.

Evaluation of environmental availability

For all the studied conditions, regardless of time and conditions (laboratory or field), environmental availability was proportional to the total residues (Figure 3). Thus, although the fungicides degraded over time and conditions were not the same, an equivalent fraction of the tested pesticides remained available in the soil. The linear regressions explained 90.4% variability of EPX and 85.5% of DMX in field experiments, and 95.5 to 98.7% in laboratory experiments.
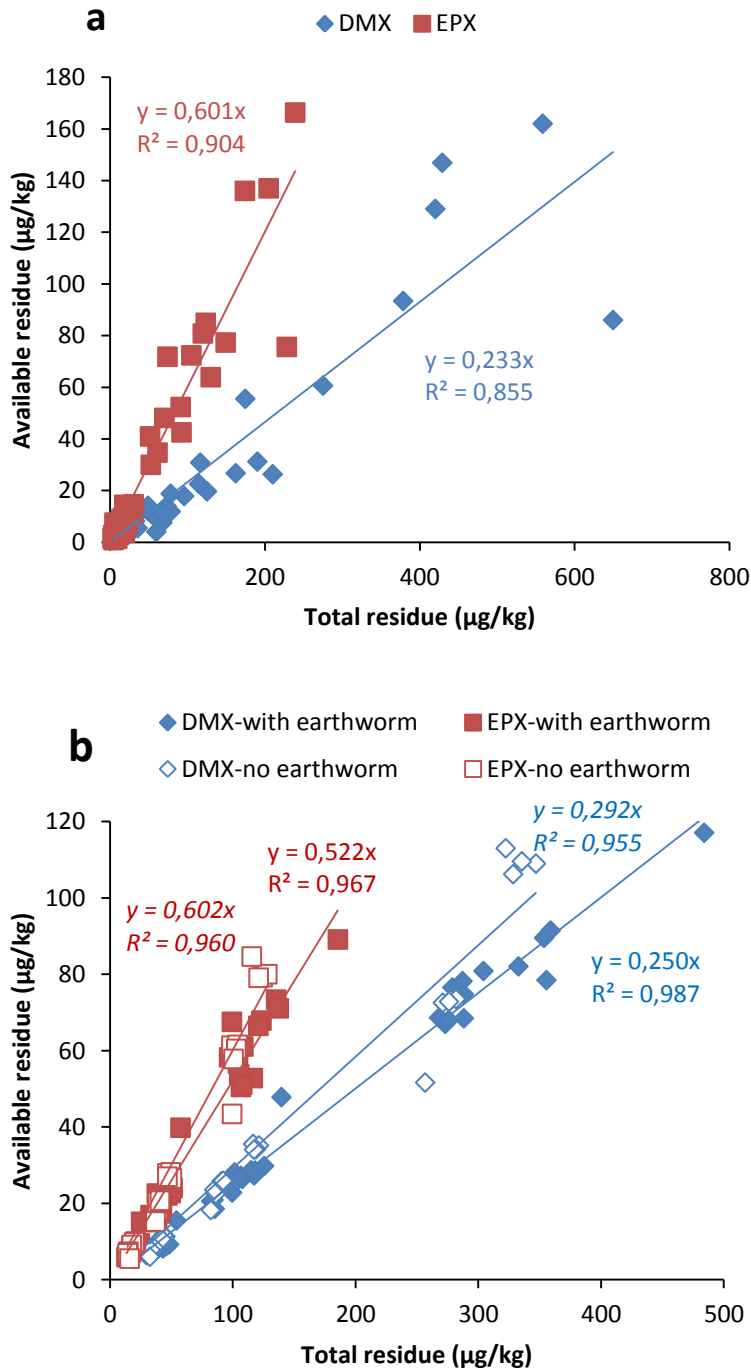

Fig. 3 Available fungicides in soil as a function of total residues: (a) field experiment, (b) laboratory study. DMX: Dimoxystrobin, EPX: Epoxiconazole

DMX was less available than EPX in both lab and field conditions. According to the slopes shown in Figure 3a, in the field experiment, 23.3 ± 2.4% of DMX was available versus 60.1 ± 4.6% of EPX. The results from the lab experiment also showed a slight effect of the presence of earthworms and horse dung, which reduced the compound availability (Figure 3b). DMX availability shifted from 29.2 ± 1.6% without earthworms and horse dung to 25.0 ± 0.6% when they were present. EPX availability decreased from 60.2 ± 3.1% to 52.2 ± 2.0%. The interaction test between variables of the linear model showed that this shift was only slightly significant for EPX ($p = 0.053$) but highly significant for DMX ($p < 0.001$). The decrease in availability when earthworms are present was previously observed (Bolan and Baskaran 1996; Shan et al. 2011). It was attributed to changes (1) in soil structure, by break down of some soil aggregates releasing silt and clay sorption sites and/or (2) in the nature of the soil organic matter increasing the abundance of functional groups able to engage interactions with the pollutants (Shan et al. 2011). This reduction could result from the impact of the horse dung that was necessary for earthworm maintenance during the experiment. This added organic matter probably participated in pesticide adsorption as was widely observed in other studies (Herrero-Hernandez et al. 2015; Gosh and Singh 2009). Indeed, we previously demonstrated that horse dung decreased the EPX availability (Nélieu et al. 2016).

Table 1: Freundlich adsorption coefficients for dimoxystrobin and epoxiconazole in pure and formulated solutions (K_f in $mg^{(1 - 1/n)}.L^{1/n}.kg^{-1}$)

	Dimoxystrobin	Epoxiconazole
Data in PPDB	K_f = 9.6 (range 0.58-18.62)	K_f = 12.18 (range 4.79-21.78)
	$1/n$ = 0.92 (range 0.894-0.942)	$1/n$ = 0.836 (range 0.766-0.910)
	K_{foc} = 486 (range 195.8-935.3)	K_{foc} = 1073 (range 280-2647)
Pure solution	K_f = 5.38 ± 1.07	K_f = 21.7 ± 1.1
	$1/n$ = 0.87 ± 0.01	$1/n$ = 0.85 ± 0.01
	K_{foc} = 285	K_{foc} = 115
Swing® Gold	K_f = 8.38 ± 1.21	K_f = 28.8 ± 1.2
	$1/n$ not significant (0.96 ± 0.05)	$1/n$ = 0.89 ± 0.03
	K_{foc} = 444	K_{foc} = 153

The large difference in availability between DMX and EPX, observed both under field and laboratory conditions, was surprising based on their physicochemical properties, in particular their close hydrophobicity (log K_{ow} 3.59 and 3.3, respectively) and Freundlich adsorption coefficients (K_f 9.6 and 12.18, Table 1). We hypothesized that this unexpectedly sizeable difference could result either from a specificity of our soil (in texture or organic matter content), or from the use of the fungicide as a formulated solution, as formulating agents may affect the sorption of the pesticides. The adsorption of the two fungicides in our soil was thus determined using a pure solution and the formulated Swing® Gold (Table 1). The Freundlich adsorption coefficients obtained for DMX were three to four-fold lower than those for EPX. According to these data, DMX should have been retained to a significantly lesser extent than EPX, but this contradicted our findings for environmental availability. Anecdotally, we also found that the presence of formulating agents increased the K_f by 56 and 33% for DMX and EPX, respectively (5.38 to 8.38 and 21.7 to 28.8 $mg^{(1 - 1/n)}.L^{1/n}.kg^{-1}$). Thus, a higher sorption was observed with the formulated fungicide, as previously observed for metalaxyl or tebuconazole (Pose-Juan et al. 2010; Čadková et al. 2013). When compared with the data of EU dossiers and published in the PPDB database, our values were globally within the same range for DMX (except the parameter $1/n$ with Swing® Gold), whereas for EPX the K_f was slightly higher and K_{foc} largely lower. To explain the difference in behaviour evaluated through environmental availability (DMX less available than EPX) and adsorption studies (DMX more available than EPX), two reasons can be hypothesized. First, a hysteresis phenomenon could occur, decreasing DMX desorption as previously observed for some other strobilurin fungicides (Reddy et al. 2013, Wu et al. 2016; Liu et al. 2018). The second hypothesis is based on potential interactions driving pesticides remobilisation from soil, independantly of their solubilisation. The HPCD used to study environmental availability is mostly efficient for establishing hydrophobic interactions and hydrogen bonds leading to inclusion in the hydrophobic cavity (Reid et al. 2000), whereas the CaCl$_2$ used for the adsorption study engages ionic interactions (Harmsen 2007). Thus,

our results may come from the interactions between soil constituents and the pesticide that relied more on ionic interactions for DMX than for EPX. This could occur because of the lateral chain in DMX, which presents polar groups and lone pairs (see structures, Figure S3). Indeed, Ghosh et al. (2009) suggested an interaction between the carbonyl group of azoxystrobin (also present in DMX) and the organic or mineral fraction of the soil.

Environmental bioavailability: bioaccumulation in earthworms

The fungicide residues found in the soil were compared with residues analysed in earthworms in the laboratory and field studies. Overall, the concentration in earthworms remained lower (DMX) or largely lower (EPX) than the total residues measured in the soil. The only exceptions were observed for DMX at the lowest tested dose, once during the field experiment (5 days after application in D1), and in most cases at D0.33 in the laboratory study. However, even in these cases, the bioaccumulation remained limited as the earthworm/soil ratio was still lower than 2 in any case.

During the field experiment, an early and transitory bioaccumulation of the tested active substances could have happened in some earthworms at D10, which induced the death of numerous anecic and endogeic earthworms (Amossé et al. 2018). This is in accordance with the LC_{50} of Swing® Gold for *A. caliginosa*, which is 6.3 times the recommended dose (Bart et al. 2017). However, the environmental bioavailability was measured only on earthworm individuals sampled alive. In the field experiment, no correlation was found between the fungicide concentration in earthworms and in soil, whether we considered the total or available fraction (Figure S4). This could be due to earthworm behaviour since the pesticide could limit their feeding activity and/or increase avoidance of the most polluted spots (Jager et al. 2005). However, after 1 month, *A. icterica* population (the sampled species) showed no significant change at D1 but decreased slightly at D10 (Amossé et al. 2018). Meanwhile, in the field, the *A. caliginosa* species (used in lab study) was absent from D10 plots 1 month after treatment (Amossé et al. 2018).
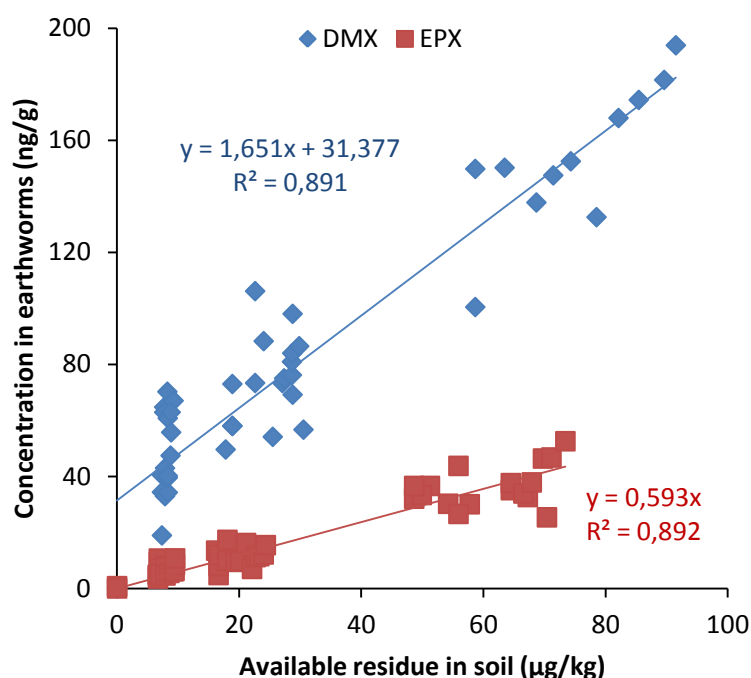

Fig. 4 Fungicide residues in earthworms as a function of available residues in the soil during the laboratory study at all exposure times. DMX: Dimoxystrobin, EPX: Epoxiconazole

In the laboratory study, a linear correlation was found between available residues in soil and concentrations in earthworms (Figure 4). No significant intercept was found in the equation obtained for EPX, traducing proportionality between EPX environmental availability and concentration in earthworms. The slope of 0.593 ± 0.034 was significantly lower than 1, showing that the extraction with HPCD was not as efficient as in our previous study mimicking the environmental bioavailability of EPX to earthworms (Nélieu et al. 2016). This could be due to differences in soil characteristics, tested commercial formulations (Opus® in Nélieu et al. 2016 versus Swing® Gold in this study), soil water content (90% WHC in Nélieu et al. 2016 vs 70% in this study) and/or because of differences in earthworm species (*A. icterica* in Nélieu et al. 2016 vs *A. caliginosa* here). The linear model was adequate for DMX and EPX, with R^2 regression coefficients of 0.891 and 0.892, respectively. The slope of the line in the DMX regression was higher than 1 (1.65 ± 0.17) but there was also a large intercept of 31.4 ± 6.9. To interpret this atypical equation, the notions of steady-state and bioaccumulation factor (BAF) were considered. As a steady-state classically occurs between 3 and 14 days (Svobodová et al. 2018; Chang et al. 2016; Jager et al. 2005), a steady state can be assumed in this study where the minimum exposure time was 28 days. This legitimised the calculation of a BAF, using the ratio of the concentration in earthworms / total concentration in soil for BAF_{tot} or concentration in earthworms / available concentration in soil for BAF_{avail} (Figure S5). The BAF were independent of exposure time, reinforcing the steady-state assumption. However, the two active substances clearly showed different BAF patterns. For EPX, the ratio was roughly independent of the dose, with an average of 0.32 ± 0.09 and 0.68 ± 0.24 for BAF_{tot} and BAF_{avail}, respectively. This quasi-independence is consistent with that observed for various chemicals (Lanno et al. 2004; Gaw et al. 2012). In contrast, the BAF_{avail} of DMX significantly decreased when the dose increased, with 5.47 ± 1.72 at D0.33, 3.08 ± 0.79 at D1, and 2.06 ± 0.25 at D3. These changes between doses were less striking for BAF_{tot}, but this factor was still significantly lower at D3. A dose effect on the ratio was previously observed for some insecticides and other chemicals in earthworms (Chang et al. 2016; Jager et al. 2005). Three reasons were proposed to explain this effect. First, there could have been a change in the availability of the fungicide in soil. However this was not applicable in our case as environmental availability was demonstrated to be not dose-dependent. Second, earthworm behaviour may have changed, with earthworms becoming rather inactive to limit bioaccumulation. However, as the BAF of the co-pollutant and thus co-ingested EPX was not dose-dependent, this does not appear to be the reason. Finally, a plausible factor for the observations with DMX in the present study was an increase in the efficiency of the earthworms to excrete or metabolise the chemical when they were exposed to higher doses. This hypothesis is supported by the change in metabolism observed by Chang et al. (2016), which evidenced that increasing the dose of a pesticide led to a higher excretion of its main metabolite.

Conclusions

Here we showed that total, available and bioavailable concentrations of two widely used fungicides (DMX and EPX) were highly heterogeneous under field conditions, which make difficult data interpretation. However, dissipation rates and environmental availabilities displayed similar trends between field and laboratory experiments, even if the experimental conditions largely differed, considering for instance the presence of vegetation and the earthworm species. Dimoxystrobin was less persistent, less available, but bioaccumulated more in earthworms than epoxiconazole. The two chemicals we tested also differed in their sorption to soil constituents and their sensitivity (regarding bioaccumulation factor in earthworms) to the applied dose, which was not expected from available data. This study of the different steps assessing chemical bioavailability in different situations, regarding their fate and interactions with living organisms, represent a step toward a better understanding of the behaviour of the two considered active substances in the environment. Further research should be devoted to determine the link between environmental and toxicological bioavailability of the two fungicides in laboratory and field experiments.

Conflict of interest: The authors declare that they have no conflict of interest.

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Supplementary material

Environ. Sci. Pollut. Res.

Soil dissipation and bioavailability to earthworms of two fungicides under laboratory and field conditions

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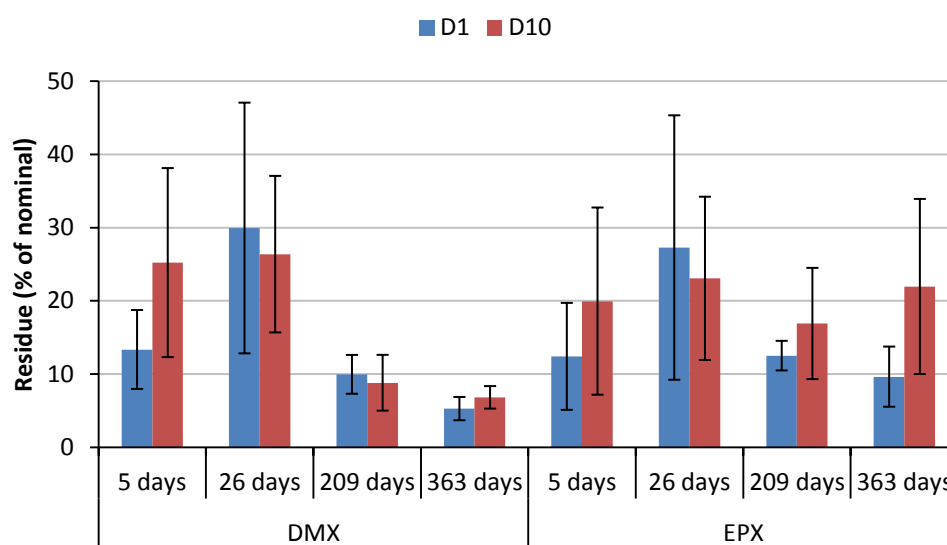
Figure S1. Dimoxystrobin (DMX) and epoxiconazole (EPX) residues in 0-10 cm soils, in the field, in % of nominal dose (mean and standard deviation of plots, n=4).
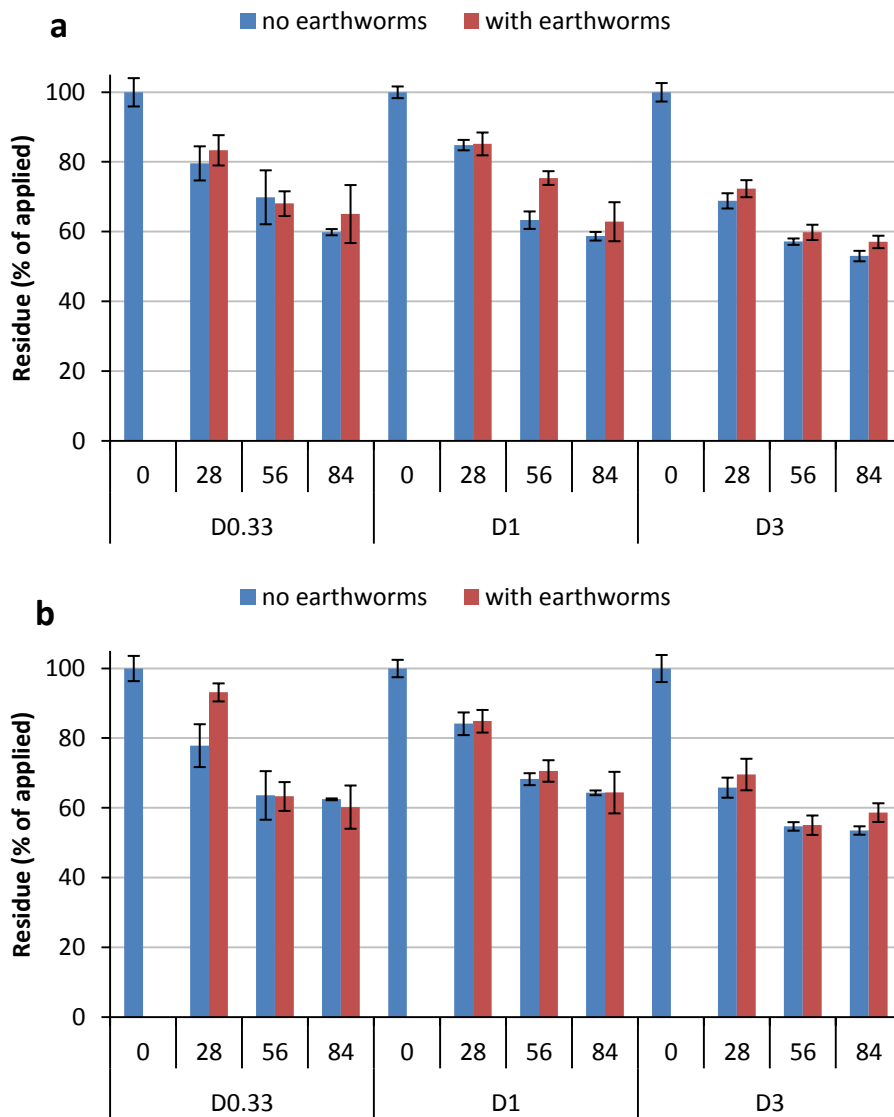
Figure S2. Dimoxystrobin (a) and epoxiconazole (b) residues in soils, during laboratory studies in the presence or not of earthworms (mean and standard deviation of replicates, n=4).
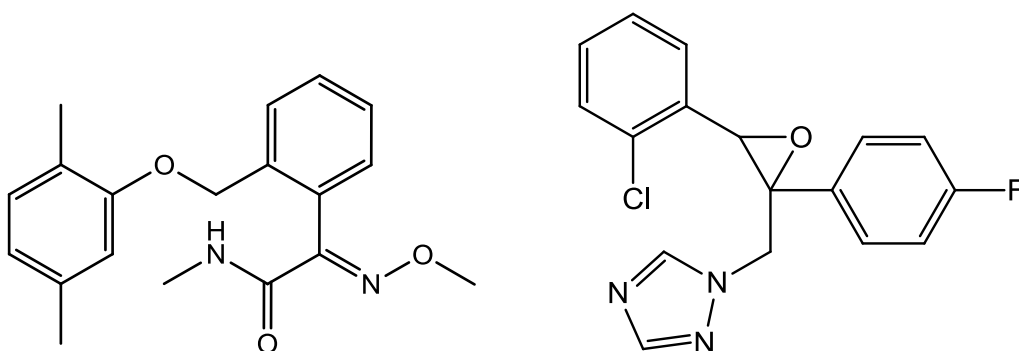

Figure S3. Chemical structures of dimoxystrobin (left) and epoxiconazole (right)
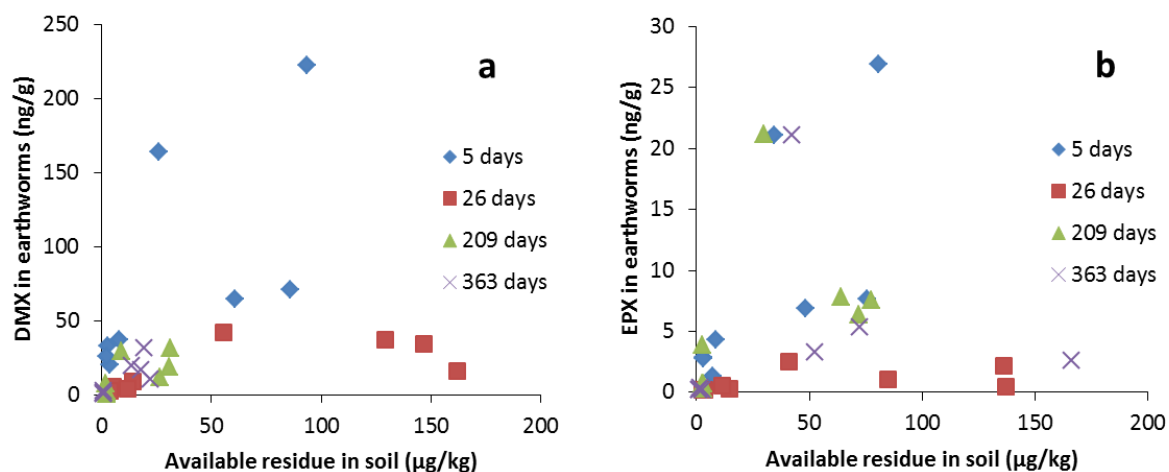
Figure S4. Pesticides residues in earthworms as a function of available residue in soil during field experiments: dimoxystrobin (a) and epoxiconazole (b).
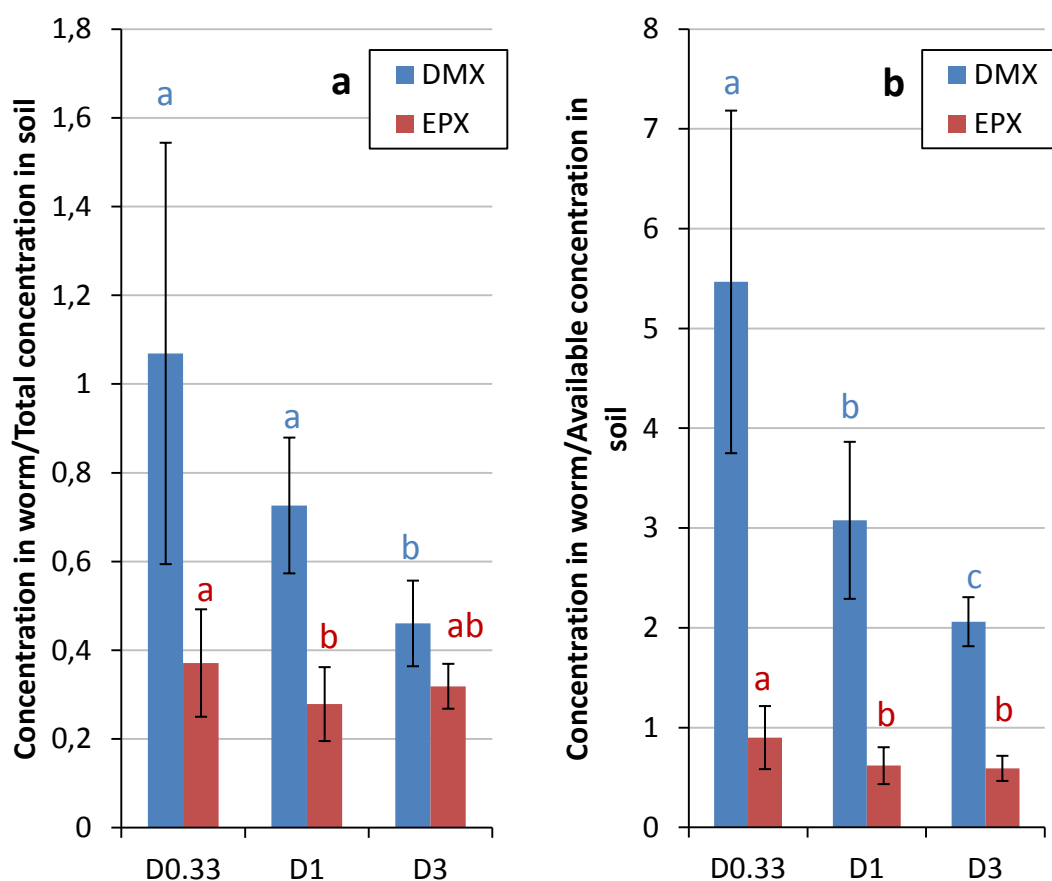

Figure S5. Ratio of concentration observed in earthworms (fresh weight) and soil (dry weight) during laboratory experiments: total concentration (a) and environmentally available concentration (b). DMX: Dimoxystrobin, EPX: Epoxiconazole.